NIH Public Access

Author Manuscript

Oncogene. Author manuscript; available in PMC 2010 April 6.

Published in final edited form as:

Oncogene. 2008 January 10; 27(3): 300–307. doi:10.1038/sj.onc.1210654.

Ski can negatively regulates macrophage differentiation through its interaction with PU.1

N Ueki, **L Zhang**, and **MJ Haymann**

Department of Molecular Genetics and Microbiology, Stony Brook University, Stony Brook, NY, USA

Abstract

In the hematopoietic cell system, the oncoprotein Ski dramatically affects growth and differentiation programs, in some cases leading to malignant leukemia. However, little is known about the interaction partners or signaling pathways involved in the Ski-mediated block of differentiation in hematopoietic cells. Here we show that Ski interacts with PU.1, a lineage-specific transcription factor essential for terminal myeloid differentiation, and thereby represses PU.1-dependent transcriptional activation. Consistent with this, Ski inhibits the biological function of PU.1 to promote myeloid cells to differentiate into macrophage colony-stimulating factor receptor (M-CSFR)-positive macrophages. Using a Ski mutant deficient in PU.1 binding, we demonstrate that Ski–PU.1 interaction is critical for Ski's ability to repress PU.1-dependent transcription and block macrophage differentiation. Furthermore, we provide evidence that Ski-mediated repression of PU.1 is due to Ski's ability to recruit histone deacetylase 3 to PU.1 bound to DNA. Since inactivation of PU.1 is closely related to the development of myeloid leukemia and Ski strongly inhibits PU.1 function, we propose that aberrant Ski expression in certain types of myeloid cell lineages might contribute to leukemogenesis.

Keywords

ski oncoprotein; PU.1; HDAC3; hematopoiesis; leukemogenesis; transcriptional repression

Introduction

Ski was originally identified as the oncoprotein from the avian Sloan Kettering retroviruses (Stavnezer *et al.*, 1981). Analyses of human tumors have accumulated evidence that Ski is overexpressed in certain types of tumors such as leukemias, melanomas and esophageal carcinomas (Reed et al., 2001; Fukuchi *et al.*, 2004; Kronenwett *et al.*, 2005; Ritter *et al.*, 2006). Ski has been also shown to influence the growth and differentiation of hematopoietic cells (Larsen *et al.*, 1992, 1993; Beug *et al.*, 1995; Dahl *et al.*, 1998). These previous studies demonstrated that Ski could transform hematopoietic multipotent progenitors and cause highly malignant leukemia. In addition, Ski induces immortalization of primary multipotential progenitor cells from avian bone marrow. In all cases, it appears that Ski is acting by blocking or delaying the ability of the cells to differentiate into mature hematopoietic cells. Such effects of Ski exhibit a very high degree of specificity for particular hematopoietic lineages and their stage of differentiation. However, the molecular mechanisms underlying this Ski-mediated block of differentiation are not well understood.

© 2008 Nature Publishing Group All rights reserved

Correspondence: Dr MJ Hayman, Department of Molecular Genetics and Microbiology, Stony Brook University, Stony Brook, NY 11794-5222, USA. mhayman@ms.cc.sunysb.edu.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Ski has been shown to be involved in certain distinct signaling pathways including nuclear receptors (Dahl *et al.*, 1998; Nomura *et al.*, 1999; Ueki and Hayman, 2003b; Ritter *et al.*, 2006), transforming growth factor-β (TGF-β) (Akiyoshi *et al.*, 1999; Luo *et al.*, 1999; Sun *et al.*, 1999; Xu *et al.*, 2000; Ueki and Hayman, 2003a) and tumor suppressors (Nomura *et al.*, 1999; Tokitou *et al.*, 1999; Khan *et al.*, 2001) where Ski can act as a transcriptional corepressor. This is in part due to its direct and indirect interactions with histone deacetylase (HDAC) complexes including nuclear receptor-core-pressor/silencing mediator for retinoid and thyroid hormone receptors (N-CoR/SMRT) and Sin3A core-pressors (Nomura *et al.*, 1999; Jepsen and Rosenfeld, 2002). Ski has been proposed to recruit such corepressor complexes to certain DNA-binding proteins (Nomura *et al.*, 1999; Liu *et al.*, 2001). In addition, we recently identified GATA1, a lineage-specific transcription factor essential for erythropoiesis (Ohneda and Yamamoto, 2002), as a target of Ski (Ueki *et al.*, 2004). The mechanism by which Ski blocks GATA1 function is unique, as Ski prevents GATA1 DNA binding. These divergent roles of Ski are thought to reflect Ski's ability to associate with multiple protein partners that have disparate functions. Little is known, however, about interaction partners or signaling pathways involved in the Ski-mediated block of differentiation in the different hematopoietic cells.

Previous studies identified a point mutation in Ski (L110P) which severely affects its interaction with N-CoR, Smad2/3 and GATA1, but not with Sin3A, Smad4, retinoic acid receptor and vitamin D receptor (Ueki and Hayman, 2003a, b; Ueki *et al.*, 2004; Ritter *et al.*, 2006). This mutation substantially affects the repression activity of Ski on nuclear receptors and GATA1 but not TGF-β signaling. We have been using this mutant form of Ski as a molecular tool to investigate the mechanisms of Ski-mediated transcriptional repression.

We previously reported that Ski regulates GATA1 function (Ueki *et al.*, 2004). Since GATA1 and PU.1, a myeloid- and B-cell-specific E26 transformation-specific (ETS) transcription factor (Dahl and Simon, 2003), are known to antagonize each other's activity for specification of the hematopoietic cell system (Rekhtman *et al.*, 1999; Zhang *et al.*, 2000), we investigated the possibility of a functional link between Ski and PU.1. In this report, we show that Ski blocks PU.1-induced transcriptional activation by mediating interaction between PU.1 and HDAC3. Using a human myeloid cell line U937 as a model system, we were able to show PU.1 as a novel target of Ski. Our results provide a possible mechanism by which Ski contributes to myeloid leukemia through antagonizing PU.1 function.

Results

Ski inhibits PU.1-mediated transcription in an HDAC-dependent manner

We previously reported that Ski regulates GATA1 function by interfering with its DNA binding (Ueki *et al.*, 2004). Since GATA1 and PU.1 are known to antagonize each other's activity for specification of the hematopoietic cell system (Rekhtman *et al.*, 1999; Zhang *et al.*, 2000), we investigated the possibility of a functional link between Ski and PU.1. Transient transfection of PU.1 in QT6 cells resulted in strong activation of luciferase reporter construct containing multimerized PU.1-binding sites (Figure 1a). Notably, co-transfection of wild-type Ski (wt-Ski) revealed a significant repressive effect on the PU.1-dependent transcription (Figure 1a, left). In contrast, co-transfection of a point mutant of Ski (L110P), mutant Ski (mt-Ski) that does not interact with PU.1 (Figure 2a), had practically no effect compared to that of wt-Ski (Figure 1a, right). This Ski-mediated transcriptional repression was increased in a dose-dependent manner by wt-Ski, while mt-Ski never gave rise to repression (Figure 1a). Similar results were also obtained from another luciferase reporter construct containing a promoter sequence from the human macrophage colony-stimulating factor receptor (M-CSFR) gene (Figure 1b).

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Ski can repress certain transcription factors through distinct mechanisms such as recruitment of corepressor complexes and interference of DNA binding. To investigate the mechanism of Ski-mediated repression on PU.1 activity, we examined if Ski affects the PU.1-dependent transactivation when PU.1 is tethered to DNA by a heterologous DNA-binding domain. Full-length PU.1 was fused to the GAL4 DNA-binding domain and co-transfected with a luciferase reporter construct regulated by five upstream GAL4-binding sites. The GAL4-PU.1 fusion protein transactivated the reporter over eightfold above the activity induced by the GAL4 DNA-binding domain alone (Figure 1c, vector). Similar to the results shown above, wt-Ski efficiently inhibited the ability of GAL4-PU.1 fusion protein to transactivate this reporter (Figure 1c, wt-Ski). Again, mt-Ski showed significantly reduced effect compared to that by wt-Ski (Figure 1c, mt-Ski). Ski was able to repress the activity of the GAL4-PU.1 fusion protein that binds DNA independently of the PU.1 DNA-binding domain. This would indicate that Ski's ability to block PU.1 activity does not involve interference with PU.1's DNA binding. Consistent with this interpretation, Ski did not prevent PU.1 DNA binding in electrophoretic mobility shift assay (data not shown).

Ski was shown to form repressor complexes including Sin3A-HDAC1 and N-CoR/SMRT (Nomura *et al.*, 1999). To test if the Ski-mediated repression of PU.1 activity is HDAC-dependent, we used trichostatin A (TSA) as an inhibitor of HDACs (Figure 1d). Interestingly, the repression activity by Ski was significantly reverted by the addition of TSA (Figure 1d, TSA, wtSki). This result would suggest that the repression of PU.1 by Ski is at least in part HDAC dependent.

To identify which HDAC is responsible for the Ski's ability to repress PU.1-dependent transcription, we performed a similar luciferase reporter assays using short-hairpin RNA constructs to knockdown either HDAC1 or HDAC3 (Figure 1e). While knocking-down of HDAC1 had no effect (Figure 1e, left, wt-Ski), HDAC3 knockdown significantly reversed the Ski's repression activity on PU.1 (Figure 1e, right, wt-Ski). These results strongly suggest that HDAC3 is required for the repression of PU.1 activity by Ski.

Ski forms a complex including PU.1 and HDAC3

Ski can act as a transcriptional corepressor by directly interacting with several transcription factors. To determine if Ski's ability to inhibit PU.1 activity is mediated by protein–protein interaction, co-immunoprecipitation assays were performed using COS-1 cell lysates expressing Ski (wt or mt-Ski) and PU.1. wt-Ski clearly bound to PU.1, whereas the mt-Ski was defective to interact with PU.1 (Figure 2a, lanes 3 and 6, respectively). These results indicate that Ski can interact with PU.1 and that the L110P mutation significantly impairs Ski's ability to associate with PU.1.

Having shown that repression of PU.1 activity by Ski is dependent on PU.1–Ski interaction and HDAC activity, we hypothesized a model whereby Ski recruits a HDAC complex to PU.1 leading to transcriptional repression. Since our results described above suggested a link between Ski and HDAC3, we speculated that the complex might associate with PU.1. To test if HDAC3 is recruited to PU.1 through interaction with Ski, we performed immunoprecipitation assays using COS-1 cell lysates expressing HDAC3 alone, HDAC3 plus PU.1, HDAC3, PU.1 plus wt-Ski, or HDAC3, PU.1 plus mt-Ski (Figure 2b). In the absence of wt-Ski, PU.1 was undetectable when the complex was immunoprecipitated by antibodies against Myc-tagged HDAC3 (Figure 2b, lane 6). In contrast, co-expression of wt-Ski enabled PU.1 to now be detected in the HDAC3 immune complex (Figure 2b, lane 9), whereas mt-Ski expression was unable to do so (Figure 2b, lane 12). Interestingly, while wt-Ski was readily co-precipitated by HDAC3 (Figure 2b, lane 9), the recovery of mt-Ski was substantially reduced (Figure 2b, lane 12). In favor of our model, these results indicate that Ski can form a complex including HDAC3 and PU.1 through its multiple interactions.

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

Next, to extend this analysis and confirm if Ski, PU.1 and HDAC3 form a complex in a physiological context, similar co-immunoprecipitation assays were performed using lysates from the myeloid cell line U937. Consistent with the results above, endogenous PU.1 and HDAC3 could be detected in the endogenous Ski immune complex (Figure 2c, PU.1 and HDAC3, respectively). In favor of our model, these results indicate that Ski can form a complex with HDAC3 and PU.1 through its specific interactions with these two proteins. To further verify if Ski–PU.1 interaction is direct, co-purification assays were performed using bacterially co-expressed GST-Ski (22–186) and PU.1 (Figure 2d). PU.1 clearly bound to GST-Ski (22–186) (Figure 2d, left, lane 2), strongly supporting the notion that Ski–PU.1 interaction is direct.

Ski blocks morphological change of U937 cells macrophage differentiation induced by TPA

We next investigated if Ski can affect PU.1's biological activity. PU.1 is required for the expression of genes associated with terminal myeloid differentiation (Simon *et al.*, 1996). Several proteins including C/EBP*α* (Reddy *et al.*, 2002) and AML1-ETO (Vangala *et al.*, 2003) have been shown to block this late-stage macrophage differentiation by inactivating PU. 1 function. Treatment of the myeloid cell line U937 with the macrophage differentiating agent 12-*O*-tetradecanoylphorbol-13-acetate (TPA) have been reported to induce the expression of a number of macrophage-specific genes, including *M-CSFR* (Behre *et al.*, 1999), in a PU.1-dependent manner.

U937 cells retrovirally infected with empty vector, wt-Ski and mt-Ski were established. Upon TPA treatment, the vector control cells differentiated into macrophages showing the typical morphological changes such as an expanded highly vacuolated cytoplasm (Figure 3a, vector). In contrast, wt-Ski expression substantially reduced the number of cells with this more differentiated macrophage morphology (Figure 3a, wt-Ski). However, mt-Ski-expressing cells showed the morphological changes similar to those of the vector control (Figure 3a, mt-Ski). The inhibitory effect of wt-Ski on macrophage differentiation of U937 cells were also analysed by reverse transcription–polymerase chain reaction (RT–PCR) to monitor induction of typical differentiation markers such as M-CSFR and CD11b, both of which are primarily dependent on PU.1. Consistent with the block of morphological changes observed in wt-Ski-expressing cells, the levels of M-CSFR and CD11b were clearly downregulated by wt-Ski (Figure 3b). These results indicate that wt-Ski can block macrophage differentiation of U937 cells by TPA.

Ski overexpression antagonizes PU.1-dependent M-CSFR upregulation induced by TPA

Transcription of *M-CSFR* is regulated by PU.1 via binding to PU.1 consensus sites of the gene (Zhang *et al*., 1994). Therefore, we checked the level of endogenous *M-CSFR* protein expression during the differentiation process induced by TPA (Figure 4a). Consistent with the morphological changes and RT–PCR analysis, M-CSFR level was highly upregulated in the vector control cells (Figure 4a, lane 4). In contrast, wt-Ski expression substantially reduced M-CSFR production (Figure 4a, lane 5). Again, mt-Ski-expressing cells produced M-CSFR at a similar level to the vector control (Figure 4a, lane 6). We also monitored the levels of Ski and PU.1 expression during the differentiation process. The levels of both endogenous and exogenous Ski protein appeared to be downregulated following addition of TPA; however, both retrovirally transduced wt-Ski and mt-Ski are still expressed in the cells (Figure 4a, lanes 5 and 6, Ski). The level of endogenous Ski protein was significantly lower in the vector control cells induced to differentiate (Figure 4a, lane 4, Ski). Despite a greater expression level than that of wt-Ski, mt-Ski was defective in its ability to repress M-CSFR production. In contrast, the levels of endogenous PU.1 protein were upregulated in all cells followed by TPA treatment (Figure 4a, PU.1), being even higher in wt-Ski-expressing cells than that in the vector control, thus excluding the possibility that wt-Ski reduces PU.1 protein expression and thereby inhibits PU.1 function. Taken together, these results indicate that Ski blocks TPA-induced macrophage differentiation of U937 cells through repression of PU.1 function, and the L110P mutation

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

practically eliminates Ski's inhibitory activity. The data also indicate that Ski is normally down-regulated during TPA-induced macrophage differentiation of U937 cells and repression of PU.1 function by sustained Ski expression counteracts this differentiation. The interaction between endogenous PU.1 and exogenously expressed Ski in TPA-treated U937 cells was confirmed by immunoprecipitation assay (Figure 4b), consistent with the notion that Ski directly affects PU.1 function in these cells.

Ski recruits HDAC3 to the M-CSFR promoter occupied by PU.1 in living cells

To assess if Ski can recruit HDAC3 to PU.1 bound to DNA in living cells, we performed a chromatin immunoprecipitation (ChIP) assay using the U937 cells expressing either vector control or Ski. We monitored the recruitment of PU.1, HDAC3 and Ski to the *M-CSFR* promoter region which contains a potential PU.1-binding element (Figure 5a). PU.1 specifically bound to the promoter in both cell populations, however Ski expression significantly increased the recruitment of HDAC3 to this promoter in comparison to that of the vector control (Figure 5a, lanes 1 and 2). This Ski-dependent effect was confirmed by the presence of Ski protein in the complex present on the promoter (Figure 5a, lane 2). This result further supports our hypothesis that Ski recruits HDAC3 to the M-CSFR promoter occupied by PU.1.

Discussion

In this study, we demonstrated that Ski blocks PU.1-induced transcriptional activation by mediating interaction between PU.1 and HDAC3. The results reported here propose a mechanism by which Ski antagonizes the action of PU.1 (Figure 5b). By associating with PU.1 on DNA, Ski recruits HDAC3 to PU.1-occupied promoters, thereby blocking PU.1-mediated transcriptional activation and consequently its ability to promote U937 cells to differentiate into M-CSFR-positive macrophages. This mechanism of repression by Ski is distinct from that of Ski-mediated inhibition of GATA1 where Ski prevents GATA1 DNA binding.

PU.1 is essential for proper development of the hematopoietic cell system (Scott *et al.*, 1994; McKercher *et al.*, 1996). PU.1-deficient mice lack mature myeloid cells. Aberrant expression of PU.1 (overexpression and graded reduction) significantly affects the hematopoietic lineage fate decisions and can promote erythroleukemia and myeloid leukemia in mice (Moreau-Gachelin *et al.*, 1996; Rosenbauer *et al.*, 2004; Metcalf *et al.*, 2006). Given that Ski can strongly influence the biological role of PU.1, Ski might also be involved in the cell fate determination and leukemogenesis. Recently, we and others reported that Ski expression is highly upregulated in bone marrow samples from patients with monosomy 7 or deletion 7q acute myeloid leukemia (AML) and CD34+ cells from patients with chronic myelogenous leukemia (CML) (Kronenwett *et al.*, 2005; Ritter *et al.*, 2006). The effect of Ski on PU.1 might render Ski a more potent inhibitor of cell differentiation in cooperation with its known repression activity in retinoic acid signaling in myeloid cells (Dahl *et al.*, 1998; Ritter *et al.*, 2006).

Certain lineage-specific transcription factors such as PU.1, GATA1 and C/EBPα can antagonize each other's activity (Rekhtman *et al.*, 1999; Zhang *et al.*, 1999, 2000; Reddy *et al.*, 2002). Most importantly, graded changes in each factor's concentration could result in committing a hematopoietic progenitor cell fate (Dahl and Simon, 2003; Rosenbauer *et al.*, 2004). Our findings have supported general important roles of Ski in the hematopoietic cell system by inhibiting the function of critical transcription factors including PU.1 and GATA1. It is possible that Ski contributes to the fine tuning of these lineage-determining factors for proper hematopoiesis and its aberrant expression results in malignant transformation in leukemia.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Recently, Suzuki *et al.* (2003) reported that MeCP2-mediated transcriptional repression of PU.1 involves Sin3A and HDAC1 in a TSA-sensitive manner. Since mt-Ski which can bind to Sin3A (Ueki and Hayman, 2003b) was defective in PU.1 repression, the repression by Ski seems to be Sin3A independent. However, it is still not clear whether Ski-mediated repression of PU.1 involves these repressor complexes or distinct complexes including N-CoR and HDAC3.

Our results show that Ski regulates transcriptional activator function of PU.1 by recruiting a HDAC3 complex through its interaction with PU.1, leading to a block in the myeloid differentiation program. These findings, along with previously reported data, contribute to our understanding of how Ski acts in the normal hematopoietic cell system and malignant transformation in leukemia.

Materials and methods

Tissue culture, transfection and plasmids

QT6, COS-1, HEK293 and U937 cells were maintained in Dullbecco's modified Eagle's medium or RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, penicillin G (100 U/ml) and streptomycin (100 μg/ml). For QT6 cells, medium was additionally supplemented with 1% chicken serum (Sigma, St Louis, MO, USA). Expression plasmids were introduced into the cells by using FuGENE 6 as described by the manufacturer (Roche Applied Science, Indianapolis, IN, USA). Retrovirally transduced U937 cells expressing vector control, wild-type Ski (wt-Ski) and mutant (mt-Ski) were established and maintained in the presence of blasticidin (5 μg/ml, ICN, Aurora, OH, USA) as described previously (Ueki and Hayman, 2003b). For macrophage differentiation, U937 cells were treated with vehicle (ethanol) or TPA (16 nM, Sigma) for 48 h followed by cytospin and Diff-Quik staining (Dade Behring, Deerfield, IL, USA). Myc-PU.1 and pM-PU.1 were constructed by inserting human PU.1 cDNA fragment amplified by PCR using primer pair (5′-TTT GAA TTC ATG GAA GGG TTT CC-3′ and 5′-TTT CTC GAG TCA GTG GGG CGG GTG-3′) into EcoRI/XhoI sites of pcDNA3-6Myc and pM (Clontech, Mountain View, CA, USA), respectively. Myc-HDAC3 was constructed by cloning mouse HDAC3 cDNA fragment amplified by PCR using primer pair (5′-TTT GAA TTC ATG GCC AAG ACC GTG GCG TAT TTC TAC G-3′ and 5′-TTT CTC GAG TCA ACT TTC CTT GTC GTT GTC ATG G-3′) into *Eco*RI/*Xho*I sites of pcDNA3-6Myc. All cloning procedures were verified by sequencing.

Luciferase reporter assays

QT6 or HEK293 cells on 24-well plates were transfected with PU×3-TK81-Luc (Ano *et al.*, 2004), pFR-Luc (Stratagene, La Jolla, CA, USA) or pGL3-M-CSFR-Luc (Dahl *et al.*, 2007) luciferase reporter construct (50 ng/well), together with effector plasmids (DEB-PU.1 (Ano *et al.*, 2004), pCMV-T7-wtcSki, pCMV-T7-mt-cSki, pM or pM-PU.1) as indicated in the figure legend. The total amount of transfected DNA was equalized with pEGFP-C1 (vector, Clontech). Luciferase activity was measured 24 h after transfection according to the manufacture's instructions (Promega, Madison, WI, USA) and normalized to the total protein amount. When indicated, TSA (100 nM) was added to the medium 6 h after transfection. For short-hairpin RNA interference against HDAC1 or HDAC3, pKD-HDAC1-v4 or pKD-HDAC3-v1 (Upstate, Charlottesville, VA, USA) was pre-introduced into HEK293 cells 24 h before the luciferase reporter and effector plasmids transfection. The data are presented as means±standard deviations of duplicated assays as representatives of at least three independent experiments.

NIH-PA Author Manuscript

Immunoprecipitations and western blotting

Cells were lysed in Nonidet P-40 buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM ethylenediaminetetraacetate (EDTA), 0.5% Nonidet P-40 and 10% glycerol) containing phosphatase and protease inhibitors. After sonication and clarification, extracts were precleared with protein G-Sepharose (GE Healthcare, Piscataway, NJ, USA) for 1 h at 4°C on a rotating wheel. Protein complexes were incubated with appropriate antibodies or normal mouse immunoglobulin G (IgG, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Immunocomplexes were recovered using protein G-Sepharose for 8 h at 4°C on a rotating wheel, followed by washing four times with Nonidet P-40 buffer. Recovered proteins were separated on sodium dodecyl sulfate–polyacrylamide electrophoresis (SDS–PAGE) gels and transferred onto nitrocellulose membranes (Protoran, Schleicher & Schüll, Keene, NH, USA). Antibodies used for immunoprecipitation and western blotting were anti-T7 (Novagen, Madison, WI, USA), anti-Myc (9E10, Santa Cruz Biotechnology), anti-PU.1 (T-21, Santa Cruz Biotechnology), anti-PU.1 (9G7, Cell Signaling Technology, Danvers, MA, USA), anti-M-CSFR (C-20, Santa Cruz Biotechnology), anti-Ski (G8, Cascade Bioscience, Winchester, MA, USA), anti-Ski (H-329, Santa Cruz Biotechnology), anti-HDAC3 (3G6, Upstate) and anti-α-Tubulin (Sigma). Proteins were detected with the appropriate secondary antibodies by chemiluminescence (ECL kit, GE Healthcare).

GST-fusion protein-binding assays

PU.1 cDNA was introduced into a modified pET-28a(+) vector utilizing P15A replication origin to allow co-expression of His-tagged PU.1 and GST (pGEX-KG) or GST-Ski (pGEX-Ski-22-186) in the same bacteria. His-tagged PU.1 and GST or GST-Ski were co-expressed in DH5α host strain and purified as described (Dahl *et al.*, 1998). For each copurification, bacterial lysate containing 1 μg of GST fusion protein was applied to Glutathione Sepharose 4B (GE Healthcare), washed extensively in phosphate-buffered saline (PBS) containing 0.5% Triton X-100. Purified proteins were eluted in SDS sample buffer, separated by SDS–PAGE, and either stained with Coomassie Brilliant Blue (CBB) or transferred onto a nitrocellulose membranes followed by western blotting.

RT–PCR

Total RNA was isolated by using the RNeasy minikit (Qiagen, Valencia, CA, USA). cDNA was synthesized with Thermo-Script reverse transcriptase (Invitrogen) by using oligo(dT) primer. The PCR conditions were as follows: 95°C for 3 min and 32 cycles of 30 s at 94°C, 30 s at 60°C and 40 s at 72°C, followed by an extension time of 5 min at 72°C. The primer pairs used were as follows: human *M-CSFR* gene, 5′-CGG TGC AGA GCC TGC TGA CTG T-3′ and 5′-ACA GGC TCC CAG AAG GTT GAC G -3′; human CD11b gene, 5′-AGT GAG AAA TCC CGC CAA GAG C-3′ and 5′-GTC AGG TCT ACC AGT CCA TCC A-3′ and β-actin gene, 5′-CCA GGC TGT GCT ATC CCT GTA-3′ and 5′-TGA TCT CCT TCT GCA TCC TGT C-3′. The PCR products were separated by 2% agarose gel electrophoresis and visualized by ethidium bromide staining.

ChIP assays

Preparation of crosslinked chromatin (2×10^7 U937 cells induced by 16 nM TPA for 24 h followed by 0.4% formaldehyde treatment for 10 min at room temperature), sonication to 300–800 base pairs fragments and immunoprecipitation were performed as described in the protocol (ChIP assay kit, Upstate). Antibodies used were as follows: anti-PU.1 (T-21), anti-HDAC3, anti-T7 and normal rabbit/mouse IgG cocktail (1:1, Santa Cruz Biotechnology). The DNA samples associated with immunocomplexes were isolated and used as templates for the PCR. The PCR condition was 95°C for 3 min and 36 cycles of 30 s at 94°C, 30 s at 52°C and 30s at 72°C, followed by an extension time of 5 min at 72°C. The primer pair used were 5′-CCT TGA

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

AGA TGG CTT TAG AAG G-3′ and 5′-GAG CTC TCA GCT ACT AGC TCC G-3′, yielding a 281 base pairs fragment of M-CSFR promoter (Zhang *et al.*, 1994). The PCR products were separated by 2% agarose gel electrophoresis and visualized by ethidium bromide staining.

Acknowledgments

We thank J Ghysdael and R Dahl for valuable reagents; K Donnelly for supporting our research project and the members of the Hayman laboratory for helpful discussions and criticisms on the manuscript. This work was supported by grant CA42573 from the National Institutes of Health (to MJH).

Abbreviations

ChIP	chromatin immunoprecipitation
EMSA	electrophoretic mobility shift assay
HDAC	histone deacetylase
M-CSFR	macrophage colony-stimulating factor receptor
TGF-β	transforming growth factor-β
TPA	12-O-tetradecanoylphorbol-13-acetate
TSA	trichostatin A

References

Akiyoshi S, Inoue H, Hanai J, Kusanagi K, Nemoto N, Miyazono K, et al. c-Ski acts as a transcriptional co-repressor in transforming growth factor-beta signaling through interaction with Smads. J Biol Chem 1999;274:35269–35277. [PubMed: 10575014]

Ano S, Pereira R, Pironin M, Lesault I, Milley C, Lebigot I, et al. Erythroblast transformation by FLI-1 depends upon its specific DNA binding and transcriptional activation properties. J Biol Chem 2004;279:2993–3002. [PubMed: 14570912]

Behre G, Whitmarsh AJ, Coghlan MP, Hoang T, Carpenter CL, Zhang DE, et al. c-Jun is a JNK-independent coactivator of the PU.1 transcription factor. J Biol Chem 1999;274:4939–4946. [PubMed: 9988737]

Beug H, Dahl R, Steinlein P, Meyer S, Deiner EM, Hayman MJ. *In vitro* growth of factor-dependent multipotential hematopoietic cells is induced by the nuclear oncoprotein v-Ski. Oncogene 1995;11:59–72. [PubMed: 7624132]

Dahl R, Iyer SR, Owens KS, Cuylear DD, Simon MC. The transcriptional repressor GFI-1 antagonizes PU.1 activity through protein–protein interaction. J Biol Chem 2007;282:6473–6483. [PubMed: 17197705]

Dahl R, Kieslinger M, Beug H, Hayman MJ. Transformation of hematopoietic cells by the Ski oncoprotein involves repression of retinoic acid receptor signaling. Proc Natl Acad Sci USA 1998;95:11187–11192. [PubMed: 9736711]

Dahl R, Simon MC. The importance of PU.1 concentration in hematopoietic lineage commitment and maturation. Blood Cells Mol Dis 2003;31:229–233. [PubMed: 12972030]

Fukuchi M, Nakajima M, Fukai Y, Miyazaki T, Masuda N, Sohda M, et al. Increased expression of c-Ski as a co-repressor in transforming growth factor-beta signaling correlates with progression of esophageal squamous cell carcinoma. Int J Cancer 2004;108:818–824. [PubMed: 14712482]

Jepsen K, Rosenfeld MG. Biological roles and mechanistic actions of co-repressor complexes. J Cell Sci 2002;115:689–698. [PubMed: 11865025]

Khan MM, Nomura T, Kim H, Kaul SC, Wadhwa R, Shinagawa T, et al. Role of PML and PML-RARalpha in Mad-mediated transcriptional repression. Mol Cell 2001;7:1233–1243. [PubMed: 11430826]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Kronenwett R, Butterweck U, Steidl U, Kliszewski S, Neumann F, Bork S, et al. Distinct molecular phenotype of malignant CD34(+) hematopoietic stem and progenitor cells in chronic myelogenous leukemia. Oncogene 2005;24:5313–5324. [PubMed: 15806158]

Larsen J, Beug H, Hayman MJ. The v-ski oncogene cooperates with the v-sea oncogene in erythroid transformation by blocking erythroid differentiation. Oncogene 1992;7:1903–1911. [PubMed: 1408132]

Larsen J, Meyer S, Steinlein P, Beug H, Hayman MJ. Transformation of chicken bone marrow cells by the v-ski oncogene. Oncogene 1993;8:3221–3228. [PubMed: 7504231]

Liu X, Sun Y, Weinberg RA, Lodish HF. Ski/Sno and TGF-beta signaling. Cytokine Growth Factor Rev 2001;12:1–8. [PubMed: 11312113]

Luo K, Stroschein SL, Wang W, Chen D, Martens E, Zhou S, et al. The Ski oncoprotein interacts with the Smad proteins to repress TGFbeta signaling. Genes Dev 1999;13:2196–2206. [PubMed: 10485843]

McKercher SR, Torbett BE, Anderson KL, Henkel GW, Vestal DJ, Baribault H, et al. Targeted disruption of the PU.1 gene results in multiple hematopoietic abnormalities. EMBO J 1996;15:5647–5658. [PubMed: 8896458]

Metcalf D, Dakic A, Mifsud S, Di Rago L, Wu L, Nutt S. Inactivation of PU.1 in adult mice leads to the development of myeloid leukemia. Proc Natl Acad Sci USA 2006;103:1486–1491. [PubMed: 16432184]

Moreau-Gachelin F, Wendling F, Molina T, Denis N, Titeux M, Grimber G, et al. Spi-1/PU.1 transgenic mice develop multistep erythroleukemias. Mol Cell Biol 1996;16:2453–2463. [PubMed: 8628313]

Nomura T, Khan MM, Kaul SC, Dong HD, Wadhwa R, Colmenares C, et al. Ski is a component of the histone deacetylase complex required for transcriptional repression by Mad and thyroid hormone receptor. Genes Dev 1999;13:412–423. [PubMed: 10049357]

Ohneda K, Yamamoto M. Roles of hematopoietic transcription factors GATA-1 and GATA-2 in the development of red blood cell lineage. Acta Haematol 2002;108:237–245. [PubMed: 12432220]

Reddy VA, Iwama A, Iotzova G, Schulz M, Elsasser A, Vangala RK, et al. Granulocyte inducer C/EBPalpha inactivates the myeloid master regulator PU.1: possible role in lineage commitment decisions. Blood 2002;100:483–490. [PubMed: 12091339]

Reed JA, Bales E, Xu W, Okan NA, Bandyopadhyay D, Medrano EE. Cytoplasmic localization of the oncogenic protein Ski in human cutaneous melanomas *in vivo*: functional implications for transforming growth factor beta signaling. Cancer Res 2001;61:8074–8078. [PubMed: 11719430]

Rekhtman N, Radparvar F, Evans T, Skoultchi AI. Direct interaction of hematopoietic transcription factors PU.1 and GATA-1: functional antagonism in erythroid cells. Genes Dev 1999;13:1398–1411. [PubMed: 10364157]

Ritter M, Kattmann D, Teichler S, Hartmann O, Samuelsson MK, Burchert A, et al. Inhibition of retinoic acid receptor signaling by Ski in acute myeloid leukemia. Leukemia 2006;20:437–443. [PubMed: 16424870]

Rosenbauer F, Wagner K, Kutok JL, Iwasaki H, Le Beau MM, Okuno Y, et al. Acute myeloid leukemia induced by graded reduction of a lineage-specific transcription factor, PU.1. Nat Genet 2004;36:624–630. [PubMed: 15146183]

Scott EW, Simon MC, Anastasi J, Singh H. Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages. Science 1994;265:1573–1577. [PubMed: 8079170]

Simon MC, Olson M, Scott E, Hack A, Su G, Singh H. Terminal myeloid gene expression and differentiation requires the transcription factor PU.1. Curr Top Microbiol Immunol 1996;211:113–119. [PubMed: 8585941]

Stavnezer E, Gerhard DS, Binari RC, Balazs I. Generation of transforming viruses in cultures of chicken fibroblasts infected with an avian leukosis virus. J Virol 1981;39:920–934. [PubMed: 6169846]

Sun Y, Liu X, Eaton EN, Lane WS, Lodish HF, Weinberg RA. Interaction of the Ski oncoprotein with Smad3 regulates TGF-beta signaling. Mol Cell 1999;4:499–509. [PubMed: 10549282]

Suzuki M, Yamada T, Kihara-Negishi F, Sakurai T, Oikawa T. Direct association between PU.1 and MeCP2 that recruits mSin3AHDAC complex for PU.1-mediated transcriptional repression. Oncogene 2003;22:8688–8698. [PubMed: 14647463]

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

Tokitou F, Nomura T, Khan MM, Kaul SC, Wadhwa R, Yasukawa T, et al. Viral ski inhibits retinoblastoma protein (Rb)-mediated transcriptional repression in a dominant negative fashion. J Biol Chem 1999;274:4485–4488. [PubMed: 9988677]

Ueki N, Hayman MJ. Direct interaction of Ski with either Smad3 or Smad4 is necessary and sufficient for Ski-mediated repression of transforming growth factor-beta signaling. J Biol Chem 2003a; 278:32489–32492. [PubMed: 12857746]

Ueki N, Hayman MJ. Signal-dependent N-CoR requirement for repression by the Ski oncoprotein. J Biol Chem 2003b;278:24858–24864. [PubMed: 12716897]

Ueki N, Zhang L, Hayman MJ. Ski negatively regulates erythroid differentiation through its interaction with GATA1. Mol Cell Biol 2004;24:10118–10125. [PubMed: 15542823]

Vangala RK, Heiss-Neumann MS, Rangatia JS, Singh SM, Schoch C, Tenen DG, et al. The myeloid master regulator transcription factor PU.1 is inactivated by AML1-ETO in t(8;21) myeloid leukemia. Blood 2003;101:270–277. [PubMed: 12393465]

Xu W, Angelis K, Danielpour D, Haddad MM, Bischof O, Campisi J, et al. Ski acts as a co-repressor with Smad2 and Smad3 to regulate the response to type beta transforming growth factor. Proc Natl Acad Sci USA 2000;97:5924–5929. [PubMed: 10811875]

Zhang DE, Hetherington CJ, Chen HM, Tenen DG. The macrophage transcription factor PU.1 directs tissue-specific expression of the macrophage colony-stimulating factor receptor. Mol Cell Biol 1994;14:373–381. [PubMed: 8264604]

Zhang P, Behre G, Pan J, Iwama A, Wara-Aswapati N, Radomska HS, et al. Negative cross-talk between hematopoietic regulators: GATA proteins repress PU.1. Proc Natl Acad Sci USA 1999;96:8705–8710. [PubMed: 10411939]

Zhang P, Zhang X, Iwama A, Yu C, Smith KA, Mueller BU, et al. PU.1 inhibits GATA-1 function and erythroid differentiation by blocking GATA-1 DNA binding. Blood 2000;96:2641–2648. [PubMed: 11023493]

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

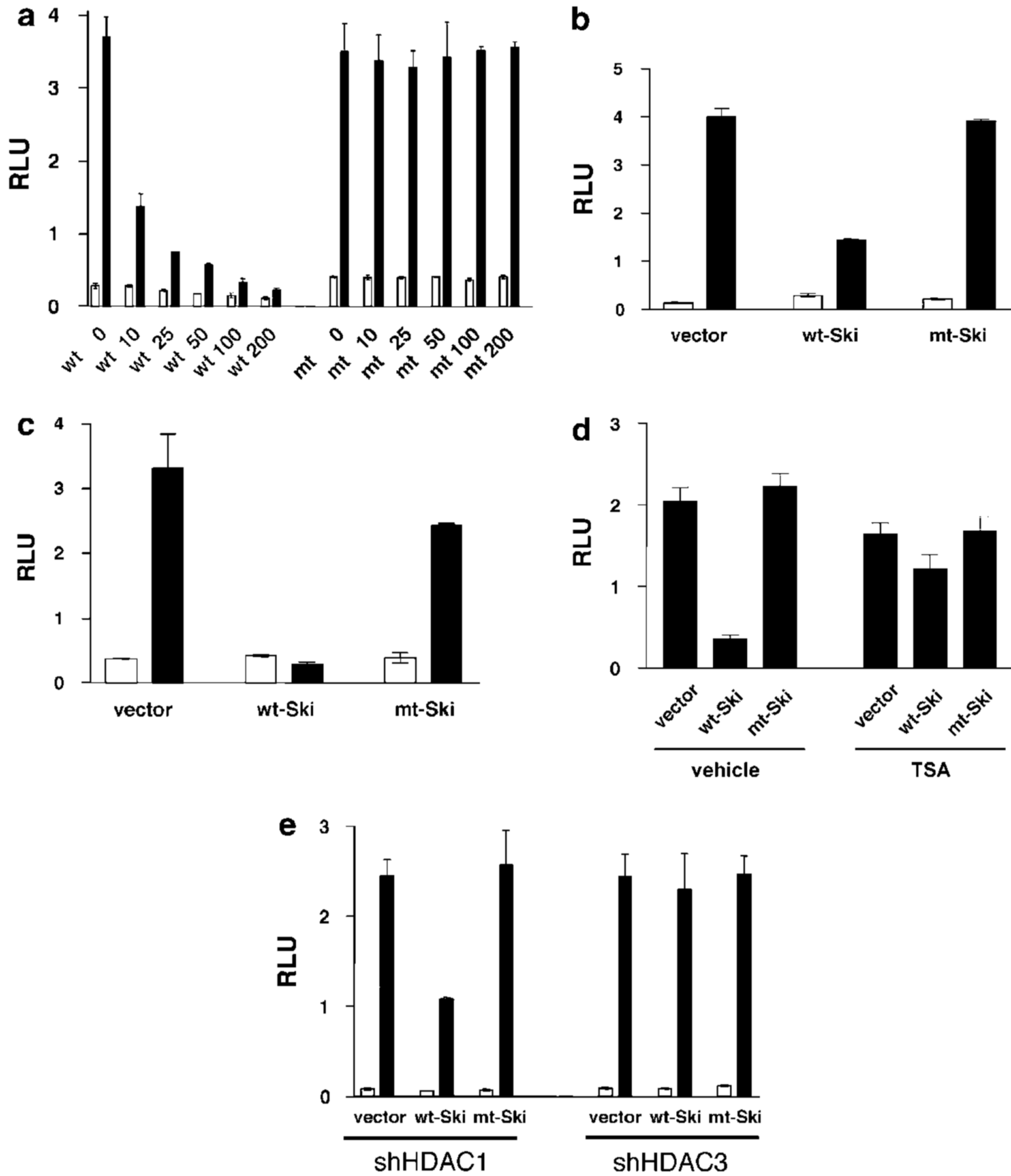

Figure 1.

PU.1-mediated transactivation is significantly repressed by Ski. (**a**) QT6 cells were transfected with PU × 3-TK81-Luc luciferase reporter construct (50 ng/well), together with (black bars) or without (white bars) PU.1 (100 ng/well) plus various amounts (ng/well) of effector plasmid (wt, wt-Ski or mt, mt-Ski). RLU, relative luciferase units. (**b**) Transfection was carried out as described in (**a**) using pGL3-M-CSFR-Luc luciferase reporter construct and each effector plasmid (50 ng/well). (**c**) QT6 cells were transfected with a reporter construct containing five multimerized GAL4-binding sites upstream of luciferase gene (pFR-Luc, 50 ng/well), together with 100 ng/well of expression plasmid pM (GAL4 DNA-binding domain only (white bars)) or pM-PU.1 (GAL4-PU.1 fusion protein (black bars)) plus 200 ng/well of effector plasmid

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

(vector, wt-Ski or mt-Ski). (**d**) Transfection was carried out as described in (**a**) using 50 ng/well of each effector plasmid in the absence (vehicle) or presence (TSA) of 100 nM TSA. (**e**) HEK293 cells were transfected as described in (**a**) using 50 ng/well of each effector plasmid and 250 ng/well of pKD-HDAC1-v4 (shHDAC1) or pKD-HDAC3-v1 (shHDAC3). TSA, trichostatin A; HDAC, histone deacetylase.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

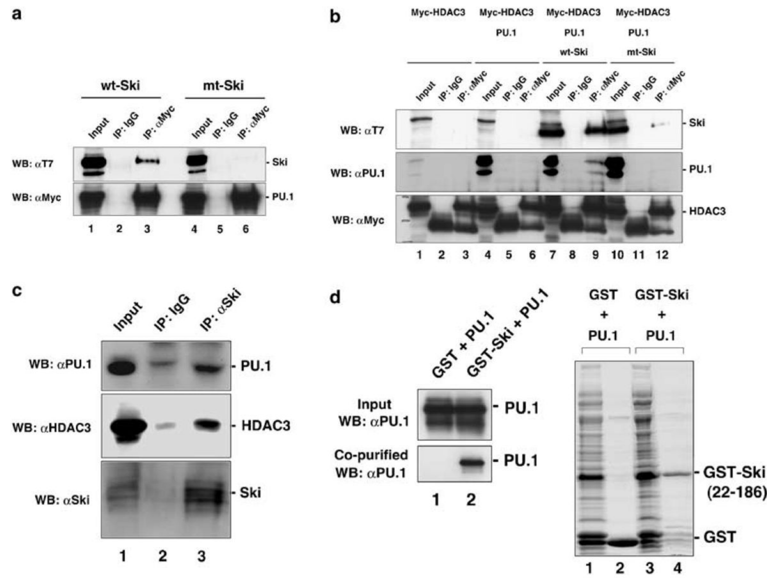

Figure 2.

Ski interacts with both PU.1 and HDAC3, allowing HDAC3 recruitment to PU.1. (**a**) Cell extracts expressing Myc-tagged PU.1 plus wt-Ski or mt-Ski were immunoprecipitated by PU. 1, followed by western blotting (WB) with anti-T7 (Ski, top panel) and anti-Myc (PU.1, bottom panel) antibody. Equivalent amount of each protein is presented in the input control (10%, lanes 1, 4). (**b**) Cell extracts expressing proteins as indicated were immunoprecipitated by HDAC3 followed by WB with anti-T7 (Ski, top panel), anti-PU.1 (middle panel) and anti-Myc (HDAC3, bottom panel) antibody. Equivalent amount of each protein is presented in the input control (10%, lanes 1, 4, 7, 10). (**c**) U937 cell extract was immunoprecipitated by immunoglobulin G (IgG) control (lane 2) or anti-Ski (H-329, lane 3) antibodies followed by WB with anti-PU.1 (9G3, PU.1, top panel), anti-HDAC3 (HDAC3, middle panel),and anti-Ski (G8, Ski, bottom panel) antibodies. Input, 10% of the total cell extract used for the immunoprecipitation (lane 1). (**d**) Bacterially expressed PU.1 and GST or GST-Ski (22–186) were co-purified using Glutathione Sepharose followed by WB with anti-PU.1 (T-21) antibody (left panels). Input, 10% of the total extract applied for the co-purification. GST and GST-Ski (22–186) proteins (total extract, lanes 1, 3; purified, lanes 2, 4) were detected by CBB staining (right panel). HDAC, histone deacetylase; CBB, Coomassie Brilliant Blue.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

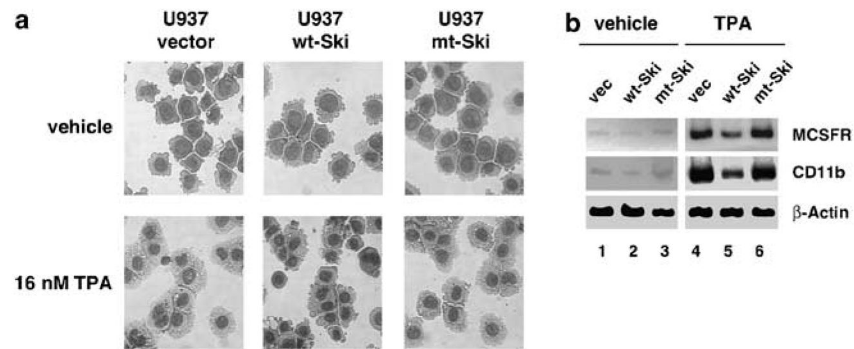

Figure 3.

Macrophage differentiation of U937 cells is blocked by Ski expression. (**a**) U937 cells expressing vector control, wt-Ski or mt-Ski were treated with vehicle (ethanol, top panels) or 16 nM TPA (bottom panels) for 48 h, followed by morphologic analysis. (**b**) reverse transcription–polymerase chain reaction analysis of TPA-induced terminal macrophage differentiation of U937 cells shown in (**a**). TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

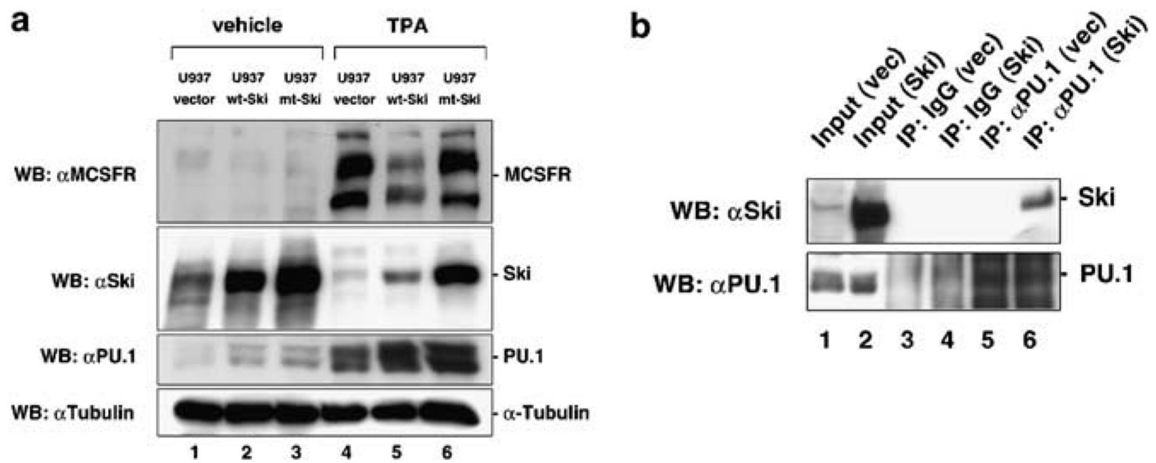

Figure 4.

Ski overexpression inhibits M-CSFR production in U937 cells induced by TPA treatment. (**a**) Level of M-CSFR production (top panel) without (lanes 1–3) or with (lanes 4–6) TPA treatment (48 h). The levels of Ski and PU.1 protein expression during differentiation are also shown. An *α*-Tubulin blot is presented as a loading control (bottom panel). (**b**) Cell extracts from U937 cells expressing vector control (vec) or wt-Ski (Ski) treated with 16 nM TPA for 24 h were immunoprecipitated by anti-PU.1 or IgG control antibody, followed by WB with anti-Ski (Ski, top panel) and anti-PU.1 (PU.1, bottom panel) antibody. Equivalent amount of each protein is presented in the input control (10% lane 1, 2). IgG, immunoglobulin G; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; M-CSFR, macrophage colony-stimulating factor receptor.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

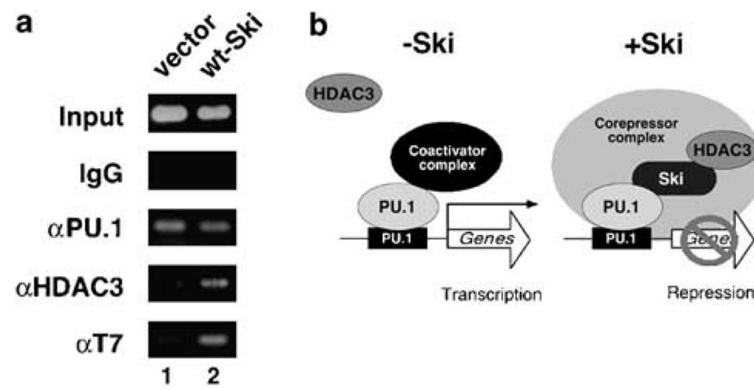

Figure 5.

Ski can recruit HDAC3 to the *M-CSFR* promoter occupied by PU.1 in living cells. (**a**) ChIP assays were performed using cell extracts from U937 cells expressing vector control or Ski treated with 16 nM TPA for 24 h. Input, 1% of chromatin lysate subjected to immunoprecipitation. IgG, normal IgG as a negative control. (**b**) Model for a mechanism of Ski-mediated repression of PU.1. See text for details. HDAC, histone deacetylase; M-CSFR, macrophage colony-stimulating factor receptor; ChIP, chromatin immunoprecipitation; IgG, immunoglobulin G.