

Transcriptional Activity of Erythroid Kruppel-like Factor (EKLF/KLF1) Modulated by PIAS3 (Protein Inhibitor of Activated STAT3)*

Received for publication, September 7, 2014, and in revised form, February 22, 2015. Published, JBC Papers in Press, February 24, 2015, DOI 10.1074/jbc.M114.610246

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Background: EKLF is a transcription factor that is critical for red cell proliferation and differentiation.

Results: EKLF interactions with the PIAS family of proteins repress or superactivate expression of its target genes.

Conclusion: PIAS3 interaction modulates EKLF activity in a promoter-dependent and SUMO-independent manner.

Significance: Our studies reveal a novel group of coregulators that affect differentiation of red blood cells.

Erythroid Kruppel-like factor (EKLF or KLF1) is a transcription factor crucial for red cell development that is directly involved in regulation of a large number of erythroid genes. EKLF serves mostly as an activator of expression of these genes; however, it can act also as a repressor. Here, we present evidence that EKLF interacts with proteins from the PIAS (protein inhibitor of activated STAT) family that convey repressive activity to EKLF in the absence of sumoylation. Our studies identify PIAS3 as a transcriptional corepressor of EKLF for at least a subset of its target genes during erythropoiesis (e.g. β -globin, α -hemoglobin stabilizing protein). We demonstrate an interaction between EKLF and PIAS proteins confirmed by *in vivo* coimmunoprecipitation assays with both exogenous and endogenous proteins. We identified an LXXLL signature motif located near the N terminus of PIAS proteins that, although not involved in the EKLF-PIAS3 interaction, is required for the transrepression activity. Knockdown of endogenous PIAS3 accelerates differentiation of both murine erythroleukemia cells, as well as fetal liver cells, whereas an increase in PIAS3 levels inhibits this increase. Using chromatin immunoprecipitation assays, we show that PIAS3 preferentially occupies the β -globin promoter in undifferentiated murine erythroleukemia cells. Together these results demonstrate that an interaction between EKLF and PIAS3 provides a novel mode of regulation of EKLF activity in the absence of sumoylation and furthermore shows an important involvement of PIAS proteins in erythropoiesis.

EKLF or KLF1 is a transcription factor that plays an essential role during erythropoiesis (1, 2). Its expression and activity is restricted to the hematopoietic organs in a strictly controlled manner. EKLF plays crucial functions during all important stages of erythropoiesis through a wide variety of mechanisms, which include gene activation and repression, regulation of

chromatin configuration, transcriptional initiation, and elongation, as well as localization of gene loci to transcription factories in the nucleus (reviewed in Ref. 3).

EKLF consists of two domains: an N-terminal proline-rich transactivation domain and a C-terminal highly conserved zinc finger DNA-binding domain. The EKLF binding site motif (5'-CCMCRCCCN-3') is found in the regulatory regions of many erythroid genes. EKLF activates genes essential for all categories of red blood cell functions including the β -globin, transmembrane, and cytoskeletal protein genes. Heme biosynthesis and blood group antigen activation is also dependent on EKLF. At the later stages of terminal maturation of erythrocytes, EKLF activates genes controlling enucleation and cell cycle exit (reviewed in Ref. 4). EKLF function is modulated by post-transcriptional modifications, which leads to various downstream effects, including altered interactions with other proteins (reviewed in Ref. 3; Refs. 5 and 6).

Although EKLF has been primarily characterized as a transcriptional activator, it can also act as a repressor. It interacts with corepressors Sin3A and components of the NuRD complex (7, 8). The mechanisms by which EKLF mediates transcriptional repression are incompletely understood, but one possibility is that its interaction with or sumoylation by PIAS family proteins might play an important role.

Mammalian PIAS family proteins were identified in yeast two-hybrid screens and initially characterized as negative regulators of STAT-mediated signaling (9). This family of proteins consists of four members: PIAS1, PIAS3, PIASx, and PIASy. PIAS proteins have a SUMO E3 ligase activity and are implicated in regulation of many transcription factors by promoting protein sumoylation (10–12). Recent evidence suggests that the effect of PIAS proteins on substrate function can also be uncoupled from their SUMO ligase activity; hence, the role of PIAS proteins extends beyond promoting protein sumoylation (12). The PIAS family of proteins can exert regulation of transcription through several mechanisms including blocking the DNA binding activity of transcription factors, recruitment of transcriptional corepressors, coactivators, and sequestration of transcription factors in distinct subcellular or subnuclear compartments (13). PIAS1 can also serve as an epigenetic regulator

* This work was supported, in whole or in part, by National Institutes of Health Grant R01 DK46865 from the U.S. Public Health Service (to J. J. B.). National Science Centre Grant 2013/09/B/NZ1/01879 (to M.S.).

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that controls repression of the myeloerythroid program (14) and as an epigenetic modifier critical for T-cell differentiation (15).

In this study we have focused on interactions between EKLF and PIAS family proteins and their outcome for red blood cell development. EKLF coordinates expression of numerous genes during both proliferation and differentiation stages. The timing of their expression is particularly crucial, because some of them have to be appropriately repressed. Currently, the information about transcriptional repression mediated by EKLF is still unsatisfactory.

Here, we continue our experiments where we established that PIAS1 serves as a E3 ligase in the process of EKLF sumoylation that was linked to repression during megakaryopoiesis (8). Presently we examine the SUMO-independent negative regulation by PIAS on a subset of EKLF target genes during erythropoiesis. Specifically, we focus our experiments on regulation of β -globin gene expression. Using gain and loss of function experiments, we show an inverse correlation between the amount of PIAS3 in cells and their ability to undergo differentiation and efficient hemoglobinization. We find that PIAS3 represses EKLF activity at the β -globin promoter in the proliferating, undifferentiated stage of erythroid cells that later, in the course of differentiation, is released. Our results support the interaction between EKLF and PIAS3 as a novel mode of regulation of erythroid lineage gene expression.

EXPERIMENTAL PROCEDURES

Cells and Reagents—293T, Cos 7, and MEL² cells were cultured in Dulbecco's modified Eagle's medium, and K562 cells were maintained in RPMI medium. All media were supplemented with 10% fetal calf serum and penicillin/streptomycin, and all cultures were incubated at 37 °C in 5% CO₂. For erythroid differentiation, MEL cells were treated with 1.8% DMSO for 6 days.

Stable DS19MEL cells, which express the reverse tetracycline-controlled transactivator (rtTA) (16), were cotransfected with plasmid pTRE2 (containing Flag-PIAS3-WT or Flag-PIAS3 Δ LXXLL) and with pBabe-puro in a 10:1 ratio using Lipofectamine LTX Plus reagent (Life Technologies) according to the manufacturer's protocol. Transfected cells were maintained in medium containing 800 μ g/ml G418 and 0.25 μ g/ml puromycin. Cells expressing hemoglobin were detected by benzidine staining as previously described (17).

Cell Transfection, Protein Extraction, Immunoprecipitation, and Western Immunoblotting—293T cells were transiently transfected with the expression plasmid as indicated using FuGENE 6 (Promega) reagent for 36 h. Cells were lysed with immunoprecipitation buffer containing: 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 5 mM EDTA, 10 μ g/ml leupeptin, 5 mM sodium fluoride, 1 mM sodium vanadate, supplemented with complete protease inhibitor mixture (Roche Applied Science). Equivalent amounts of total proteins were incubated with anti-Flag M2 (Sigma), anti-PIAS3 monoclonal

antibodies C-12 (sc-46682), or anti-EKLF monoclonal antibodies (6B3) overnight at 4 °C, followed by incubation with protein G-Sepharose for 2 h at 4 °C. Immunoprecipitates were washed four times with lysis buffer and then subjected to Western blot analyses utilizing: anti-Flag M2-HRP antibody (catalog no. A8592; Sigma); anti-EKLF 7B2, 4B9, and 6B3 antibodies (mouse monoclonal antibodies made in this laboratory) (18–20); anti-EKLF goat polyclonal antibody F-20 (catalog no. sc-27194; Santa Cruz Biotechnology); anti-PIAS3 E-3 antibody (catalog no. sc-48339; Santa Cruz Biotechnology); and anti-Hsp90 H-114 antibody (sc-7947) (Santa Cruz Biotechnology). Horseradish peroxidase-conjugated anti-rabbit (Jackson Labs) and anti-mouse antibodies (Pierce) were used as secondary antibodies.

Transient Transfection and Dual Luciferase Assays—K562 cells seeded in 12-well plates transfected with DMRIE-C (liposome reagent; Life Technologies) were lysed and assayed for luciferase activities with a dual luciferase system (Promega), 36 h post-transfection. Plasmid construct pRLTK was included as a normalization control for transfection efficiency. Luminescence was quantified with a luminometer (Promega). Results are indicative of at least three experiments performed in triplicate.

Site-directed Mutagenesis—Deletion mutants of Flag-tagged PIAS3 were constructed using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. Mutations were confirmed by DNA sequencing. The primer sequences utilized for site-directed mutagenesis to generate the constructs are available upon request.

Electrophoretic Mobility Shift Assay—A double-stranded, synthetic oligonucleotide spanning the EKLF-binding site from the mammalian β -globin promoter (21) and whole cell extracts from Cos7 cells transfected with pSG5-EKLF or pCMV-PIAS3 were used for EMSA as described previously (18, 22).

Immunofluorescence Studies and Analysis of Subcellular Localization—K562 cells were used to examine the expression and subcellular localization of the EKLF-GFP fusion along with Flag-tagged PIAS3 WT or PIAS3 Δ LXXLL constructs. Cells were cotransfected using Lipofectamine LTX with Plus reagent (Life Technologies). After 36 h, cells were cytospun onto slides and fixed for 10 min at room temperature in 4% formaldehyde (in PBS) and permeabilized in 0.1% Triton X-100 for 15 min at room temperature. Nonspecific sites were then blocked by incubation with PBS containing 3% goat serum for 30 min at room temperature. Cells were then incubated with anti-Flag M2 antibodies F3165 (Sigma) overnight at 4 °C followed by secondary Cy3 goat anti-mouse antibodies (catalog no. 115-165-062; Jackson ImmunoResearch Laboratories, Inc.) for 1 h at room temperature in the dark. The slides were then mounted in Vectashield (Vector Laboratories, Inc.) supplemented with DAPI. GFP, Cy3, and DAPI cells were visualized on a Leica SP5 DM confocal microscope to identify cell nuclei.

RNA Interference—Lentiviral vectors containing shRNAs targeting mouse PIAS3 mRNA were purchased from Sigma: NM_018812 protein inhibitor of activated STAT3 MISSION shRNA Plasmid DNA; TRC numbers #1 TRCN0000081998, #5 TRCN0000082002, #6 TRCN0000321624, and #9 TRCN0000321693. Lentiviral supernatants were produced in

² The abbreviations used are: MEL, murine erythroleukemia; ZnF, zinc finger; AHSP, α -hemoglobin stabilizing protein; HDAC, histone deacetylase; TSA, trichostatin A.

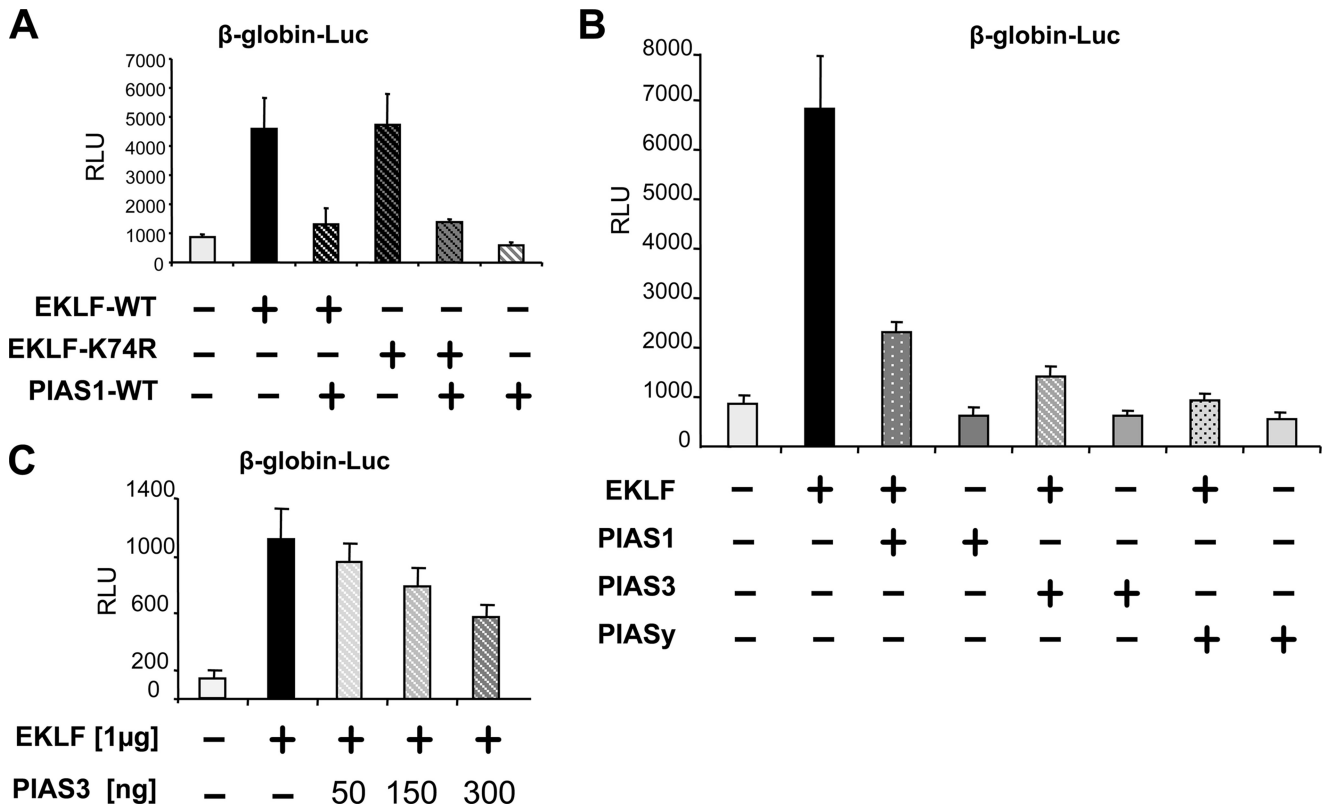


FIGURE 1. PIAS family members inhibit transcriptional activity of EKLF on the β-globin gene in a SUMO-independent and dose-dependent manner. K562 cells were cotransfected with plasmids expressing the luciferase reporter gene under the control of β-globin promoter together with plasmids expressing EKLF and PIAS proteins as indicated. A plasmid expressing *Renilla* luciferase was included as a control for normalization of transfection efficiency. Analyses were performed in triplicate. **A**, comparison of transcriptional activity of EKLF-WT and EKLF-K74R with or without the addition of PIAS3. **B**, inhibition of EKLF transcriptional activity in the presence of different PIAS proteins, as indicated. **C**, cells were transfected with increasing amounts of PIAS3-WT revealing a dose-dependent repressive effect of PIAS3 on transcriptional activity of EKLF. RLU, relative luminescence units.

Phoenix cells by transient transfection with FuGENE 6 (Promega). After 24 h, the medium was replaced by DMEM supplemented with 4 mM sodium butyrate for an additional 24 h, and the cells were incubated at 32 °C. Lentiviral particles were collected every 24 h for 2 days and filtered through 0.45-μm filters. Transduction of 25 × 10⁴ MEL cells was carried out by spinoculation (1000 × g) for 120 min in the presence of 8 μg/ml Polybrene (Sigma) at 30 °C. After 48 h, the infected MEL cells were subjected to a second round of infection and subsequently to selection by addition of 1 μg/ml puromycin (Sigma). After 4 days, samples were prepared for mRNA and protein analysis. For control experiments, cells were infected either with empty viral vector, vector expressing a scrambled shRNA, or pLKO.3G (Addgene), which contains an enhanced GFP gene. After selection with puromycin for 4 days, the infected cells were resuspended in fresh medium containing 1.8% DMSO for differentiation. Cells were collected at the indicated times for benzidine staining.

Chromatin Immunoprecipitation—ChIP assays were performed as previously described (19). Briefly, MEL cells were pretreated with 1.8% DMSO for 5 days to induce differentiation followed by cross-linking with 1.0% formaldehyde. Chromatin was sonicated using a Sonics Vibra Cell 500 for ten 40-s pulses at 21% amplitude with 1-min intervals. The antibodies, against mouse IgG (catalog no. 557273; BD Pharmingen), EKLF (catalog no. 61233; Active Motif), and PIAS3 (E-3) (catalog no.

sc-48339; Santa Cruz Biotechnology) were prebound to Magnetic Protein G Dynabeads (catalog no. 10004D; Life Technologies), and immunoprecipitations were performed with lysates (200 μg) containing fragmented DNA overnight at 4 °C. Precipitated genomic DNA was amplified by PCR with the following primers that span the mouse β-globin major proximal promoter: forward βmaj, GACAAACATTATTAGAGGGAGTACCC; and reverse βmaj, AGGTGCACCATGATGTATGTTTATGG.

RESULTS

Members of PIAS Family Proteins Modulate EKLF Transcriptional Activity—EKLF undergoes several post-translational modifications that include phosphorylation, acetylation, and ubiquitination, and we have shown that EKLF is also sumoylated at a single lysine residue (8). During the course of our experiments, we established that PIAS1 is able to efficiently sumoylate EKLF. However, we also observed that the presence of PIAS1 repressed EKLF transcriptional activity in a SUMO-independent manner. For our present studies, we addressed the biological significance of this observation by performing luciferase reporter gene assays in the human erythroid cell line K562, which expresses little to no endogenous EKLF (23, 24). Cotransfection of K562 cells with the β-globin promoter-luciferase reporter and either WT EKLF or sumoylation-deficient EKLF (EKLF-K74R) expression plasmids resulted in activation of the reporter (Fig. 1A). However, addition of PIAS1 resulted in

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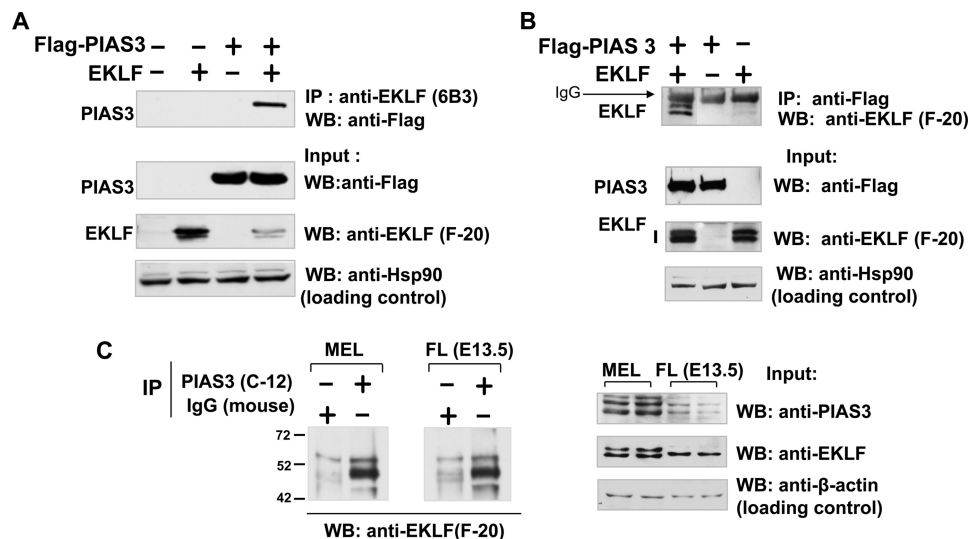


FIGURE 2. EKLF interacts with PIAS3. 293T cells were cotransfected with constructs encoding EKLF and Flag-tagged PIAS3. *A* and *B*, lysates were subjected to immunoprecipitation (IP) with anti-EKLF antibody (6B3), blotted, and probed with anti-Flag antibody (*A*) or with M2-agarose blotted, and probed with anti-EKLF antibody (F-20) (*B*). Whole cell extracts (input) were probed with anti-EKLF (F-20), anti-Flag, or anti-Hsp90 (loading control) antibodies. *C*, interaction between endogenous EKLF and PIAS3 proteins. Protein extracts from MEL cells or E13.5 fetal liver (FL) cells were subjected to immunoprecipitation with monoclonal anti-PIAS3 antibodies (C-12) or mouse IgG (negative control), blotted, and probed with anti-EKLF antibody (F-20). Whole cell extracts (input) were probed with anti-EKLF (F-20), anti-Flag, or anti-β-actin (loading control) antibodies. WB, Western blotting.

strong inhibition of activation by either EKLF or the EKLF-K74R mutant (Fig. 1A). This suggests that PIAS1 repression of EKLF activity is independent of its activity as an E3 ligase. This observation was further confirmed by testing other PIAS family members as well, because previously we observed that PIAS3 and PIASy do not function as E3 ligases for EKLF sumoylation (8). All three tested PIAS members exhibited a strong repressive activity (Fig. 1B), and this occurs irrespective of their ability to serve as an EKLF SUMO E3 ligase. This repression is dose-dependent as demonstrated for PIAS3 (Fig. 1C), and we used PIAS3 for subsequent experiments to be sure that the repressive and E3 ligase activity of PIAS proteins toward EKLF remains uncoupled.

EKLF Directly Interacts with PIAS3—Next, we examined the interaction between exogenous Flag-tagged PIAS3 and EKLF proteins in 293T cells (EKLF null cells) using cotransfection/coimmunoprecipitation assays. First, we used anti-EKLF antibodies (6B3), and the precipitated proteins were analyzed by Western blot with antibodies against Flag (Fig. 2A). Flag-PIAS3 was only detected in 293T cells that were cotransfected with both proteins. Second, we observed a similar result by utilizing anti-Flag antibodies for immunoprecipitation and anti-EKLF antibodies to detect precipitated proteins (Fig. 2B). These results demonstrate that EKLF and PIAS3 do in fact interact in cells. The endogenous interaction was confirmed by immunoprecipitation assays using MEL and E13.5 fetal liver cells, where EKLF and PIAS3 are endogenously expressed. In both cases the anti-PIAS3 antibody coprecipitates EKLF (Fig. 2C).

We then addressed the importance of the individual domains of both EKLF and PIAS3 for their interaction using coimmunoprecipitation assays in 293T cells. We first analyzed the role of the proline-rich transactivation domain and the zinc finger (ZnF; tagged with GFP) DNA-binding domain from EKLF. After immunoprecipitation of PIAS3 with anti-Flag antibody, we find that both the proline-rich (Fig. 3A) and ZnF (Fig. 3B)

domains are able to interact, indicating that regions across the entire EKLF protein are involved in the interaction with PIAS3. Full-length EKLF was used as a positive control (Fig. 3A).

PIAS proteins consist of multiple domains: SAP (SAF-A/B, Acinus, and PIAS), PINIT, RING finger-like, acidic with the SIM (SUMO interacting motif), and C-terminal serine/threonine rich domains, as depicted in Fig. 3 (10). It is known that regions of PIAS that are involved in interactions with different transcription factors vary considerably with no obvious hot spots for interactions (13). Therefore, we tested different combinations of domain deletions to determine which domains are involved in interactions with EKLF. The results of several immunoprecipitation assays conducted by cotransfection of EKLF and PIAS3 deletion mutants in 293T cells allowed us to conclude that the middle region of PIAS3, spanning the PINIT and RING domains, contributes to the binding to EKLF (Fig. 3C and data not shown).

Promoter-dependent Effect of PIAS3 on Activation of EKLF Target Genes—EKLF is a crucial transcription factor activating expression of many erythroid genes throughout all stages of erythropoiesis. Therefore, we next examined several erythroid genes and tested whether their activation is affected by the presence of PIAS proteins in a similar manner as seen with β-globin gene expression. We utilized luciferase reporter gene assays in K562 cells. We studied a number of luciferase reporters driven by promoters of EKLF target genes. Based on the results, we were able to divide EKLF targets into two groups: a group whose activation is repressed by the presence of PIAS3 and a second group that is superactivated by PIAS3. We classify β-globin and α-hemoglobin stabilizing protein (AHSP) promoters into the first group, because the levels of activation of these reporters are dramatically reduced upon addition of PIAS protein (Fig. 4A). The second group includes p21, p18, and basic Krüppel-like factor 1b (BKLF(1b)) promoters, because they display superactivation over the single effect caused by

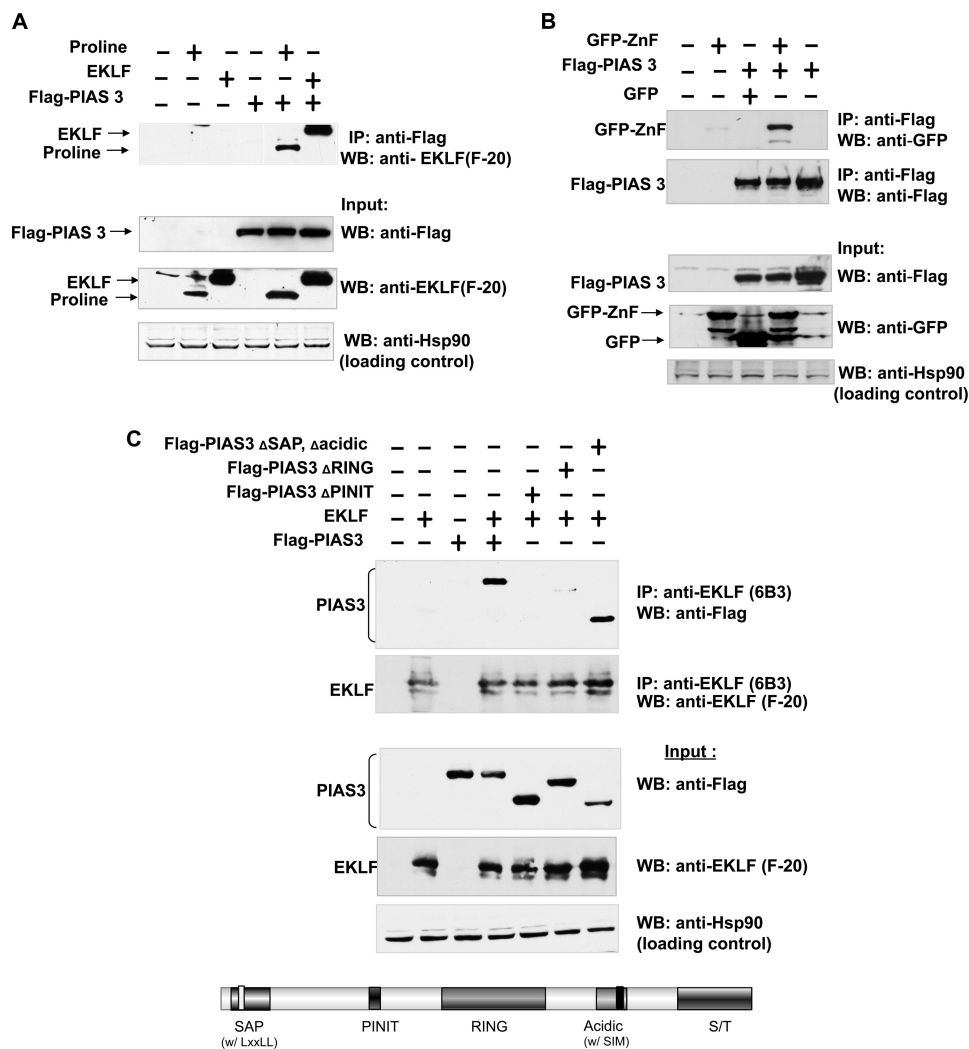


FIGURE 3. The EKLF transactivation proline and ZnF DNA-binding domains interact with multiple domains of PIAS3. *A*, 293T cells were cotransfected with constructs encoding full-length EKLF or its proline domain and Flag-tagged PIAS3. Lysates were subjected to immunoprecipitation (IP) with M2-agarose and probed with anti-EKLK polyclonal antibody (F-20). *B*, 293T cells were cotransfected with constructs encoding a GFP-EKLK zinc finger fusion protein (GFP-ZnF) or GFP alone and Flag-tagged PIAS3. Lysates were subjected to immunoprecipitation with M2-agarose and probed with anti-GFP antibodies. *C*, 293T cells were cotransfected with constructs encoding EKLF and Flag-tagged PIAS3-WT or Flag-PIAS3 with deleted domains as indicated. Lysates were subjected to immunoprecipitation with anti-EKLK monoclonal antibody (6B3) and probed with M2-HRP. In all cases, whole cell extracts (input) were probed with anti-EKLK antibody, anti-Flag antibody, or anti-Hsp90 antibodies (loading control). The bottom panel represents a schematic of domains of the PIAS family of proteins (10). WB, Western blotting.

either of EKLF or PIAS3 alone (Fig. 4B). These data show that not all EKLF target promoters behave equivalently in the presence of the PIAS3 protein. Given the fact that the β -globin promoter is the most well characterized EKLF activation target, we focused on the mechanism of its repression by PIAS3 for the rest of our analyses.

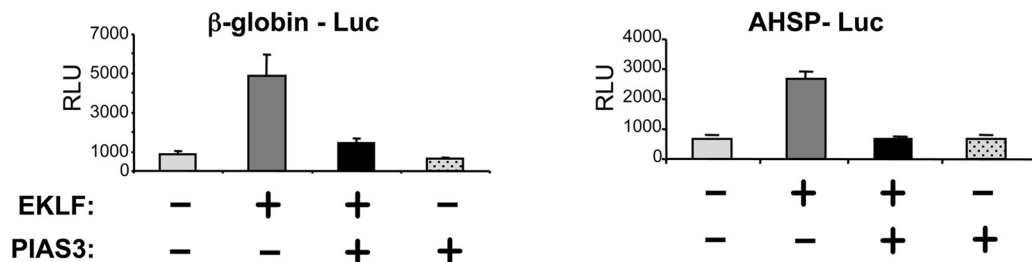
PIAS3 Does Not Inhibit the DNA Binding Activity of EKLF—After establishing that PIAS3 directly interacts with EKLF, we became interested in exploring its mechanism of repression. We first determined whether PIAS3 blocks the ability of EKLF to bind to DNA, as observed with the STAT3 protein (9). Protein extracts containing overexpressed PIAS3 or EKLF after transfection of Cos7 cells were generated, and complexes between EKLF and radiolabeled double-stranded DNA oligonucleotide comprising the EKLF binding site from β -globin promoter were resolved by an *in vitro* gel shift assay (Fig. 5). Specificity of the formed protein-DNA complexes was con-

firmed using anti-EKLK antibodies, which prevent complex formation. Using these conditions, we tested whether the addition of increasing amounts of extract containing PIAS3 would disrupt the complex formed between EKLF and its cognate DNA-binding site. However, the presence of PIAS3 has no effect on the ability of EKLF to bind DNA (Fig. 5), suggesting that PIAS3 repression of EKLF activation does not involve interference with the DNA binding activity of EKLF. We did not detect any EKLF-PIAS3 supershift in these assays, possibly because the EKLF-PIAS3 complex is not stable under these conditions, similar to PIASy-STAT1 (25).

The N-terminal LXXLL Motif of PIAS3 Is Required for the Inhibitory Activity of PIAS3—PIAS proteins contain a conserved LXXLL motif located in the N-terminal SAP domain (Fig. 6A) that is required for a variety of interactions with other proteins. A number of nuclear receptor coactivators also contain a similar LXXLL motif (26, 27). Furthermore this signature

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A: PIAS3 inhibition



B: PIAS3 superactivation

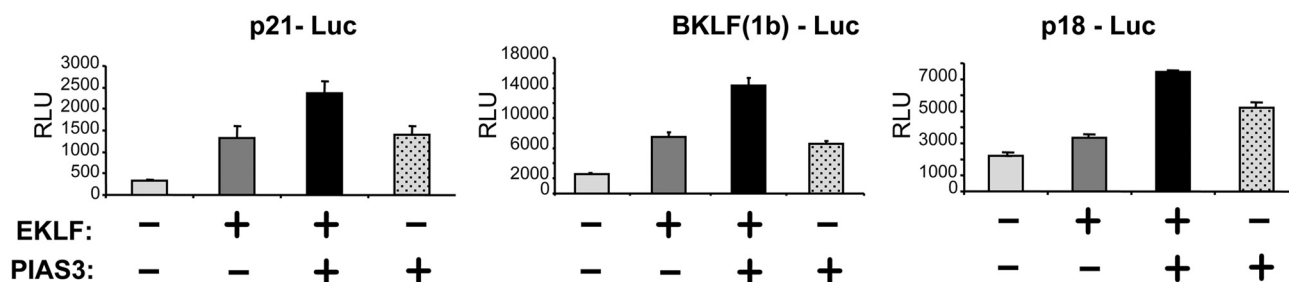


FIGURE 4. **PIAS3 modulates transcriptional activity of EKLF in a promoter-dependent manner.** K562 cells were cotransfected with plasmids expressing the luciferase reporter gene under the control of a variety of EKLF target gene promoters and with plasmids expressing EKLF and/or PIAS proteins as indicated. A plasmid expressing *Renilla* luciferase was included as a control for normalization of transfection efficiency. Analyses were performed in triplicate. **A**, PIAS3 represses transcriptional activity of EKLF at the β -globin and AHSP promoters. **B**, PIAS3 enhances transcriptional activity of EKLF at the p21, basic Krüppel-like factor (1b erythroid-specific, *BKLF(1b)*), and p18 promoters. *RLU*, relative luminescence units.

is also implicated in repression, because the motif in PIASy imposes transrepression activity on STAT1 (25). Hence we asked whether the LXXLL motif of PIAS3 plays a role in regulation of EKLF activity, perhaps as a nuclear corepressor. We generated a PIAS3 mutant where the LXXLL motif is deleted. Using this mutant, we observe a complete loss of the inhibitory effect of PIAS3 on EKLF transactivation utilizing the β -globin luciferase reporter assay (Fig. 6B). Because we had shown that the N-terminal part of PIAS3 (*i.e.* the region that contains the LXXLL motif) is not required for interactions with EKLF, we confirmed that the lack of repression by the mutant is not due to loss of its interaction. Western blot analysis of cotransfected/immunoprecipitated 293T cell extracts revealed that the LXXLL-deficient PIAS3 protein is still able to interact with EKLF (Fig. 6C). Thus we conclude that a loss of PIAS3 inhibitory activity after deletion of the LXXLL motif is not accounted for by loss of its interaction with EKLF.

One explanation for the contribution of the PIAS3 LXXLL motif to inhibition of EKLF activity could be through its recruitment of corepressors such as histone deacetylases (HDACs) (28–30). We addressed this possibility by testing the effects of HDAC inhibition (with trichostatin A (TSA)) on the ability of PIAS3 to repress EKLF activation of the β -globin luciferase reporter. We find that the presence of TSA does not alter the inhibitory effect of PIAS3 on EKLF activity (Fig. 6D). We conclude that the mechanism of repression by PIAS3 does not rely on either the recruitment of a corepressor HDAC or on a repressive complex containing class I or II HDACs that are sensitive to TSA. We then tested the possible involvement of the class III sirtuin family of NAD⁺-dependent deacetylases. These deacetylases are not affected by TSA, and it is known that

the LXXLL motif of FOXO1 mediates Sirt1-dependent transcriptional activity (31). Again we performed luciferase assays with the β -globin promoter to test whether Sirt1 is implicated in modulation of activity of EKLF. We cotransfected K562 cells with plasmids expressing EKLF, PIAS3, and either Sirt1 or a dominant negative mutant of Sirt1 containing an H355A mutation that lacks deacetylase activity (32). Neither the presence of Sirt1 nor the Sirt1 H355A mutant affects PIAS3-dependent repression of EKLF activity (data not shown), thus ruling out its involvement.

It is known that PIAS proteins can sequester other proteins (for example, transcription factors) into nuclear foci or nuclear periphery, hence regulating their activity (13, 33, 34). We utilized an immunofluorescence assay to examine whether lack of the LXXLL motif in PIAS3 might alter the distribution of EKLF in the nucleus and thus explain its inhibitory action. We cotransfected K562 cells with GFP-EKLF and either Flag-PIAS3-WT or Flag-PIAS3 Δ LXXLL, which we detected by anti-Flag and secondary Cy3-labeled anti-mouse antibodies. We observe that LXXLL-deficient PIAS3 is sequestered to nuclear speckles, whereas PIAS3-WT is more uniformly distributed in nuclei (Fig. 6E, *left panel*). However, the distribution of EKLF is not changed and remains indistinguishable in either case similar to the control cells transfected only with GFP-EKLF (Fig. 6E, *left panel*). In parallel, we concurrently monitored the transcriptional activity of GFP-EKLF to confirm that its activity is still repressed by Flag-PIAS3-WT but not with Flag-PIAS3 Δ LXXLL (Fig. 6E, *right panel*). The presence of both PIAS3 and EKLF in the nucleus corroborates our results, showing that PIAS3 serves as a repressor of EKLF transcriptional activity; however, the nuclear distribution of EKLF is unaffected

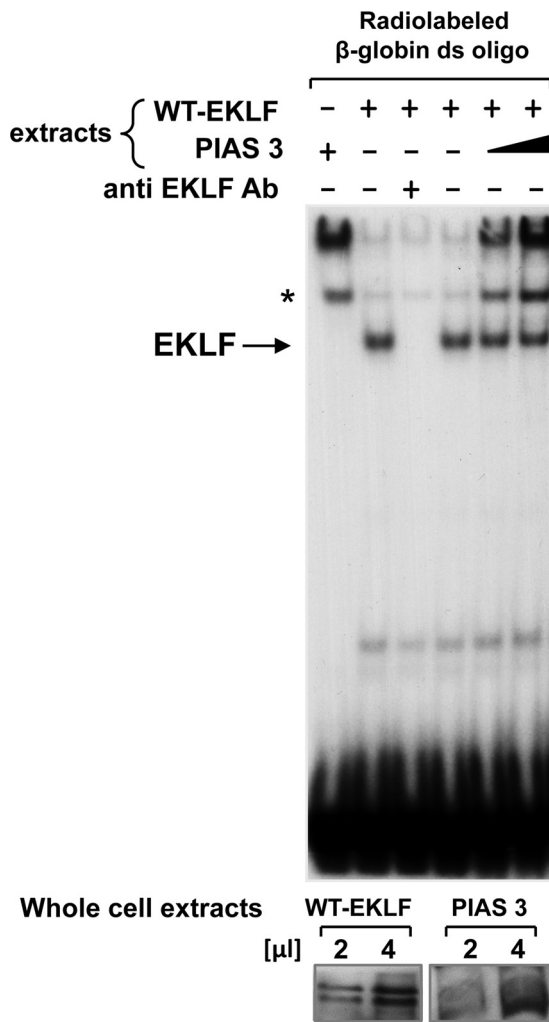


FIGURE 5. PIAS3 does not block EKLF binding to the β -globin promoter *in vitro*. Gel shift assays comparing complexes formed between EKLF and radiolabeled double-stranded oligonucleotide (*ds oligo*) comprising the EKLF binding site from the β -globin promoter (21) were performed in the absence or presence of increasing amount of PIAS3. Whole cell extracts were prepared from Cos7 cells transfected with WT-EKLF or PIAS3. Anti-EKLF antibody 4B9 was included to disrupt the complex, as indicated (18). *, nonspecific band. *Bottom panel*, EKLF or PIAS3 Western blots of whole cell extracts used for the gel shift analysis were probed with anti-EKLF monoclonal antibody (7B2) and anti-PIAS3 monoclonal antibody (E3), respectively.

by the LXXLL-dependent reorganization of nuclear PIAS3 (Fig. 6E and data not shown). Taken together, these data demonstrate that the LXXLL regulatory motif present at the N terminus of PIAS3, although not involved in PIAS3-EKLF interactions, is required for the HDAC/sirtuin-independent repressive activity of PIAS3 on EKLF-mediated gene activation.

Correlation between PIAS3 Levels and Differentiation of MEL and Fetal Liver Cells—Given our observations that overexpression of PIAS3 decreases EKLF transcriptional activity in reporter gene assays, we wanted to determine whether there is any functional role for this interaction in red blood cell development. EKLF is known to transactivate the β -globin promoter and up-regulate β -globin gene expression during erythroid differentiation, where its levels increase (reviewed in Ref. 4). On the other hand, PIAS3 levels decrease during terminal erythroid differentiation, as measured by RNA levels (35, 36) and protein analyses of primary cells from humans and mice

(Fig. 7). This inverse expression pattern suggests a functional antagonism between EKLF and PIAS3 during erythroid differentiation.

MEL cells provide a complete means to test this notion, because these cells are stalled at a proerythroblast stage at steady state; however, induction with hexamethylene bisacetamide or DMSO induces terminal erythroid differentiation, leading to a limited number of cell divisions and onset of hemoglobin expression. Because EKLF and PIAS3 are both present in the uninduced cell (Ref. 20 and Fig. 2C), we hypothesize that EKLF is not able to transactivate the β -globin promoter because the high levels of PIAS3 protein interferes with its activity. One would predict that a decrease in PIAS3 level would increase EKLF activity and β -globin expression. We performed PIAS3 knockdown studies in MEL cells by infection of lentiviruses containing a shRNA against PIAS3 or a scrambled shRNA (as a negative control) and monitored the level of knockdown in the stably selected cell lines by immunoblotting with anti-PIAS3 antibodies. Two shRNAs against PIAS3 decrease its protein levels (Fig. 8A).

These two MEL cell lines were then induced to differentiation with DMSO. The progress of differentiation (hemoglobinization) was monitored by a benzidine assay, which stains globins blue. We observed that cells in which the level of PIAS3 protein is significantly reduced differentiate faster than the control as judged by the percentage of blue cells (Fig. 8B). However, the difference between them is most prominent at the beginning of the differentiation and with each passing day the difference diminishes (Fig. 8B), perhaps suggesting that the normal differentiation process relies in part on degradation of endogenous PIAS3 or alterations in cellular/nuclear localization (37). We performed a similar analysis in expanding primary fetal liver cells (38) by infecting them with lentivirus encoding shRNAs against PIAS3. In this case, erythropoietin and stem cell factor were added to differentiate the cells toward mature erythrocytes. The results are very similar to those from MEL cells (Fig. 8C); that is, the fetal liver cells infected with shRNA against PIAS3 differentiate precociously, although this effect again is diminished with each subsequent day of differentiation (Fig. 8C).

Taken together, these data suggest that the physiological reduction of PIAS3 protein levels during differentiation limits its ability to inhibit the transcriptional activity of EKLF, leading to a more efficient activation of its target genes. Supporting this idea, knockdown of PIAS3 in MEL and fetal liver erythroid cells enables accelerated maturation through a more robust expression of EKLF target genes required for erythroid differentiation.

To further strengthen this notion, we performed the reciprocal experiment and overexpressed PIAS3 within MEL cells. We generated two stable MEL cell lines with doxycycline-inducible expression of either PIAS3-WT or a LXXLL motif-deficient mutant of PIAS3 that loses its transrepressive activity toward EKLF. We first induced expression of particular forms of PIAS3 and then initiated MEL cell differentiation with DMSO and monitored hemoglobinization by a benzidine assay (Fig. 8D). The parental MEL cell line served as an internal control for this experiment. We observed that overexpression of the wild type of PIAS3 dramatically slows down the hemoglo-

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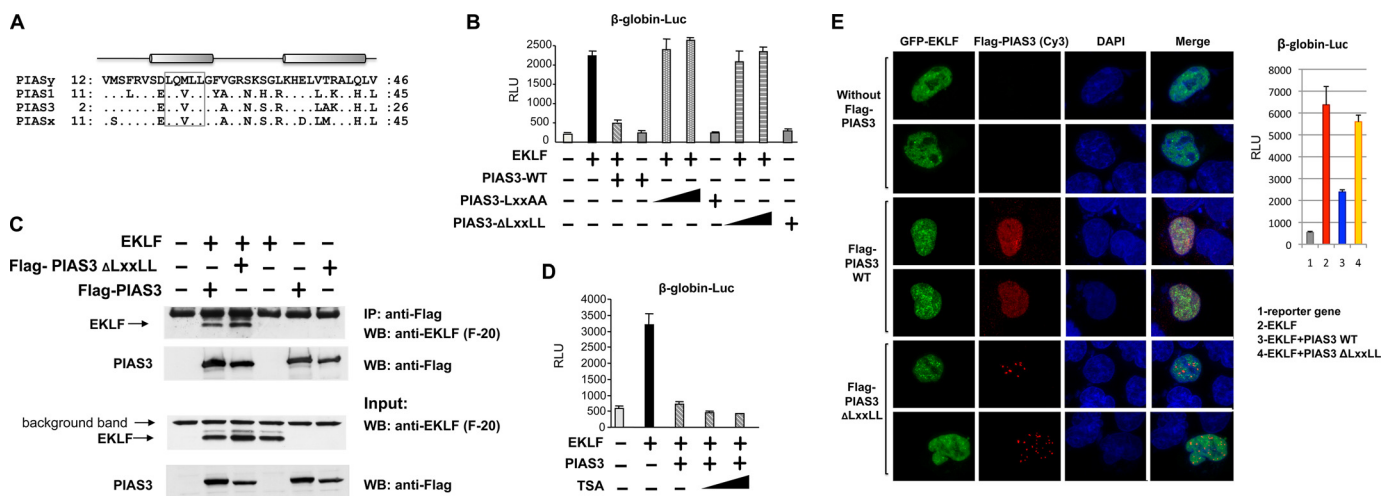


FIGURE 6. The LXXLL motif located in the SAP domain of PIAS3 is involved in inhibition of transcriptional activity of EKLF but not in direct interactions with EKLF. *A*, an alignment of amino acid sequences of proteins from mouse PIAS family spanning the LXXLL consensus motif. Amphipathic α -helices within the SAP box sequence are indicated by the cylinders in the linear diagram at the top (51). *B*, comparison of transcriptional activity of EKLF-WT in the presence of PIAS3-WT or LXXLL-deficient or LXXAA mutant PIAS3. K562 cells were cotransfected with plasmids expressing the luciferase reporter gene under control of a β -globin promoter together with plasmids expressing the indicated protein. A plasmid expressing *Renilla* luciferase was included as a control for normalization of transfection efficiency. Analyses were performed in triplicate. *C*, 293T cells were cotransfected with constructs encoding EKLF and Flag-tagged WT-PIAS3 or Flag-PIAS3 Δ LXXLL. Lysates were subjected to immunoprecipitation (IP) with M2-agarose and probed with anti-EKLF polyclonal antibodies (F-20). Whole cell extracts (input) were probed with anti-EKLF Ab, anti-Flag Ab (background band serves as a loading control). *D*, no effect of TSA on PIAS3 repressed EKLF. K562 cells were cotransfected with plasmids expressing the luciferase reporter gene under control of the β -globin promoter together with plasmids expressing EKLF with or without PIAS3-WT. After a 24-h transfection, increasing concentrations of TSA were added as indicated. A plasmid expressing *Renilla* luciferase was included as a control for normalization of transfection efficiency. Analyses were performed in triplicate. *E*, lack of LXXLL motif changes PIAS3 distribution within the nucleus of K562 cells. Localization of GFP-EKLF and Flag-PIAS3-WT or Flag-PIAS3 Δ LXXLL was within K562 cells. K562 cells were cotransfected with plasmids expressing GFP-EKLF and Flag-PIAS3-WT or Flag-PIAS3 Δ LXXLL together with a plasmid expressing the luciferase reporter gene under control of a β -globin promoter. After incubation for 36 h, cells were cytopun, fixed, permeabilized, and incubated with anti-Flag (M2) antibodies followed by anti-mouse Cy3 antibodies to visualize PIAS3 proteins. Slides were mounted with DAPI to visualize nuclei. *Inset*, in parallel, the same K562 cells were also subjected to luciferase assays to confirm the release of EKLF repression in the presence of Flag-PIAS3 Δ LXXLL. RLU, relative luminescence units; WB, Western blotting.

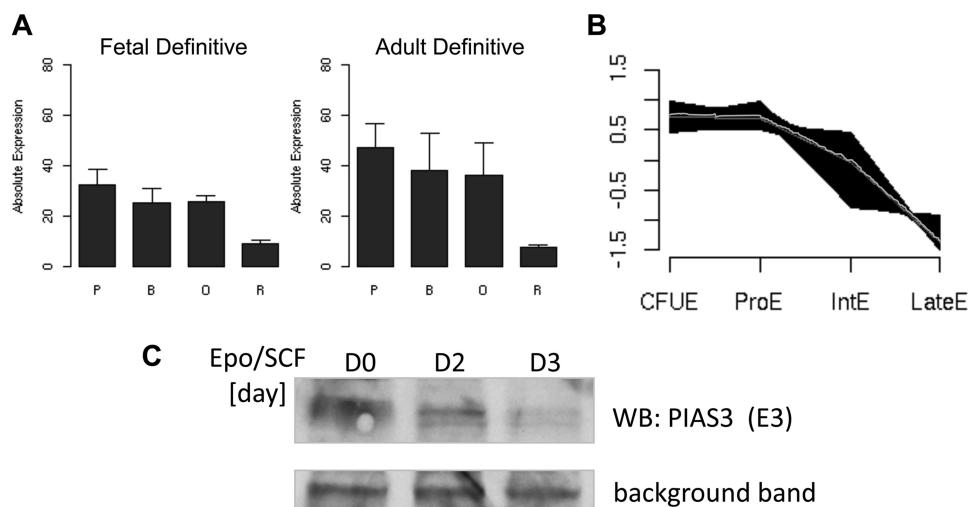


FIGURE 7. The levels of PIAS3 mRNA and protein are reduced during differentiation of erythroid cells. *A*, data from mouse Erythron Database (36). Column P, proerythroblasts; column B, basophilic erythroblasts; column O, orthochromatic erythroblasts; column R, reticulocytes. *B*, data from human Erythroblast Maturation Database (35). CFUE, colony-forming unit-erythroid erythroblasts; ProE, pro-erythroblasts erythroblasts; IntE, intermediate erythroblasts; LateE, late erythroblasts. *C*, expanding primary fetal liver cells (38) were differentiated in the presence of stem cell factor (SCF) and erythropoietin (Epo) for 3 days. Protein extracts were resolved and probed with anti-PIAS3 antibody, with a background band serving as a loading control. WB, Western blotting.

binization process. On the other hand, overexpression of PIAS3/ Δ LXXLL mutant considerably accelerates not only globin production but also the blue staining of the cells. These results clearly suggested that the level of PIAS3-WT in the cells negatively affects their ability to mature. Furthermore, it confirms that the LXXLL motif of PIAS3 is indispensable for repressive control of the transcriptional activity of EKLF.

Next, we investigated PIAS3 protein occupancy at the β -globin promoter. For this test, we compared MEL cells before and after initiating differentiation with DMSO. Based on the qPCR analysis centered on the EKLF-binding site on the β -globin promoter, we find that the promoter is occupied by PIAS3 to a higher level in undifferentiated MEL cells (Fig. 8E).

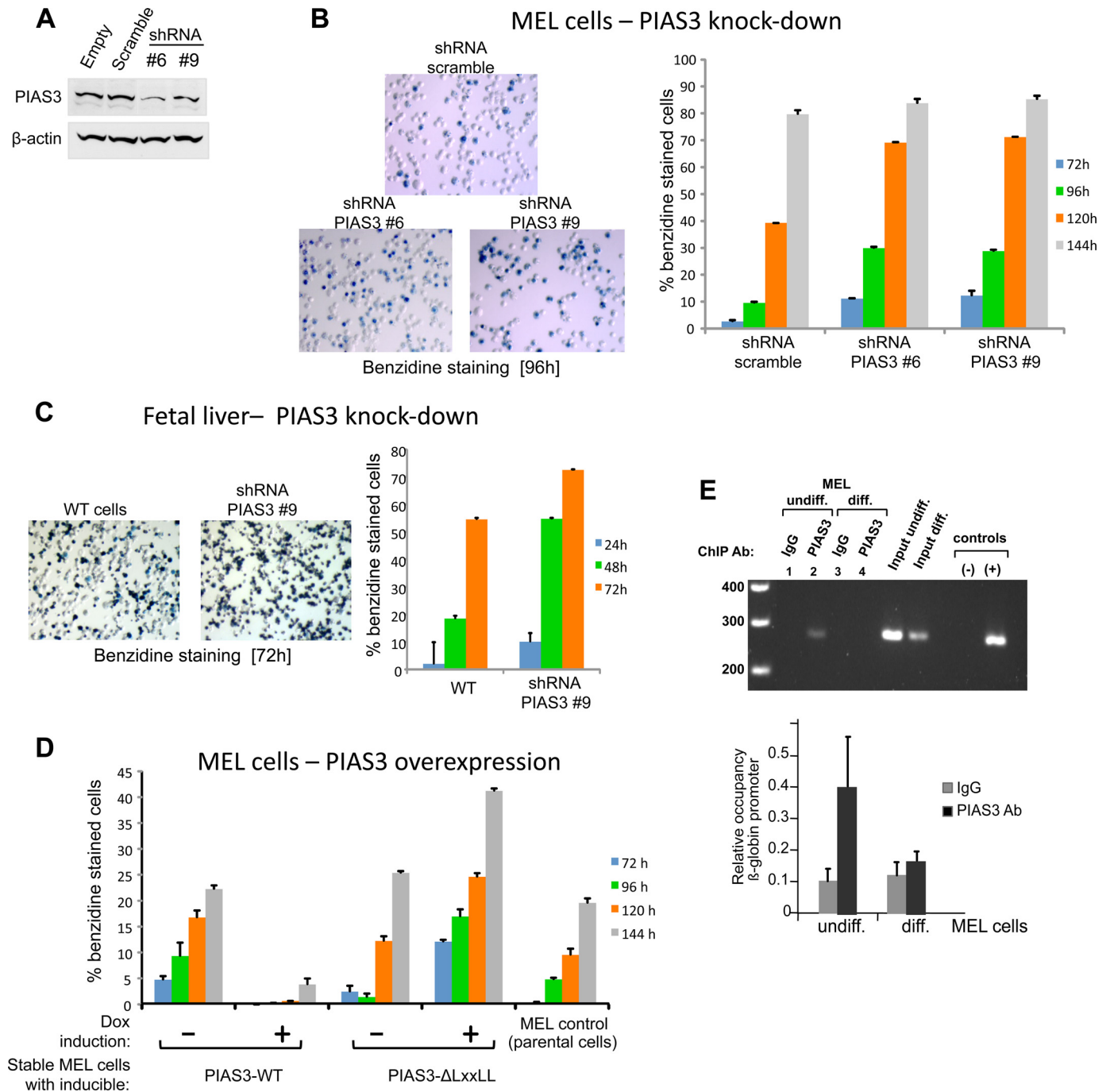


FIGURE 8. Correlation between PIAS3 level and differentiation of MEL cells and fetal liver cells. A–C, effects of PIAS3 knockdown on red cell differentiation. A, protein extracts isolated from MEL cells infected with lentivirus carrying shRNAs directed against PIAS3 (shRNA #6 and #9) or scrambled shRNA or empty vector as negative controls were subjected to separation by SDS-PAGE, blotted, and probed with antibodies against PIAS3, revealing the level of knockdown of the protein. B, MEL cells, infected as indicated, were treated with DMSO to initiate terminal differentiation, and hemoglobinization was monitored by benzidine staining 72, 96, 120, or 144 h after treatment (right panel). Visualization of stained cells at 96 h from the same experiment is shown in the left panel. C, uninfected (WT) or infected (shRNA #9) expanding primary fetal liver cells (38) were induced to differentiate with stem cell factor and erythropoietin and monitored for hemoglobinization by benzidine staining 24, 48, or 72 h after treatment (right panel). Visualization of stained cells at 72 h from the same experiment is shown in the left panel. D, independent stable MEL cell lines containing inducible PIAS3-WT or PIAS3- Δ LxxLL were treated with DMSO to initiate terminal differentiation. The graphs show the level of hemoglobinization as monitored by benzidine staining 72, 96, 120, or 144 h after DMSO treatment in cells that had been untreated or treated (– or + as indicated) with doxycycline (Dox) to induce PIAS expression. The parental MEL cell served as a standard for comparison. E, occupancy of PIAS3 on β -globin promoter before and after differentiation of MEL cells. ChIP was performed using anti-PIAS3 or mouse IgG (negative control) antibodies with uninduced MEL cells or with cells that had been induced to terminally differentiated by treatment with DMSO for 96 h. Input DNA served as a positive control (+) for β -globin promoter amplification from each sample and water as a negative (–) control. The graph presents quantified qPCR data for PIAS3 association at the β -globin promoter before and after DMSO differentiation of MEL cells. The data are the averages of two experiments each in triplicate for each point. *diff.*, differentiated; *undiff.*, undifferentiated.

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Collectively, these results suggest that the presence of PIAS3 at the β -globin promoter in undifferentiated cells interferes with the ability of EKLF to activate it and perhaps prevents premature β -globin expression prior to differentiation, after which it is no longer associated with the β -globin promoter and no longer inhibits.

DISCUSSION

EKLF is an essential transcription factor involved in red blood cell development. It regulates expression of a number of target genes throughout all stages of erythropoiesis. EKLF serves as both an activator and a repressor that exerts its functions, in part, by interacting with a number of cofactors such as histone acetyltransferases and deacetylases, as well as chromatin remodelers. In this study, we identified a novel group of cofactors, the PIAS family of proteins, which directly interact with EKLF, thus affecting its transcriptional activity. Previously, we showed that one member of the family, PIAS1, serves as an E3 ligase during EKLF sumoylation and plays an *in vivo* role in megakaryopoiesis (8). In this mode of function, PIAS proteins are thought to act as adapter proteins that enhance the interactions between the SUMO conjugating enzyme Ubc9 and the substrate proteins just to promote the efficiency of sumoylation (12). The integrity of RING finger domain/structure of PIAS1 is essential to facilitate sumoylation of EKLF that generates the interface for interactions with the repressor containing Mi-2 β involved in inhibition of megakaryopoiesis (8).

In contrast, our present studies reveal that a number of PIAS proteins can act in a SUMO-independent manner and either enhance or attenuate EKLF transcriptional activity in red blood cells. The latter effect of PIAS protein relies on interactions with a different domain, the SAP domain, instead of the RING. The SAP domain may modify DNA binding activity by altering its subnuclear localization and proximity to target genes (10).

It has not been clear why some types of cells that express high level of EKLF, for instance megakaryocyte-erythroid progenitors (39), primitive erythrocytes, early definitive cells, or uninduced MEL cells (20), do not activate certain highly induced EKLF targets, e.g. β -globin. That is, what mechanism prevents their premature expression, and what blocks their activation until a precisely defined moment? Interestingly, we found an inverse correlation between the amount of PIAS3 in cells and their ability to differentiate and undergo hemoglobinization. shRNA-mediated knockdown of PIAS accelerated β -globin expression and cell differentiation, whereas overexpression slowed it down. Correlating well with this are the observations that PIAS3 levels decrease during normal erythroid differentiation. We suggest the EKLF-PIAS3 interaction may, at least in part, be playing a global regulatory role and can help explain the erythropoietic lineage cell expression pattern.

Our CHIP data support this idea, because PIAS3 occupies the β -globin promoter in the same region that contains the EKLF binding element only in undifferentiated MEL cells. After induction and hemoglobinization, PIAS3 is displaced from the β -globin promoter, suggesting that PIAS3 binding to the chromatin at the promoter region may block premature β -globin expression. Overexpression of PIAS3 inhibits MEL cell differentiation, and silencing of PIAS3 enhances the process of hemoglobinization. This

observation is similar to the proposed role for PIAS3 during osteoclastogenesis (40, 41), in which the overexpression of PIAS3 in bone marrow-derived monocyte/macrophage lineage cells attenuates osteoclast formation and down-regulates the expression of NFATc1 (nuclear factor of activated T-cells cytoplasmic-1) and OSCAR (osteoclast-associated receptor), which are important modulators in osteoclastogenesis. At the same time, silencing of PIAS3 by RNA interference in osteoclast precursors enhances osteoclast formation, as well as gene expression of NFATc1 and OSCAR (40). In both processes, PIAS3 emerges as an important modulator of differentiation.

We suggest that individual PIAS proteins play different roles during hematopoiesis that are sumoylation-dependent and -independent. These differing interactions with EKLF are critical and relate to bipotential decisions emanating from the megakaryocyte/erythroid progenitor (39), where EKLF interaction with and sumoylation by PIAS1 leads to its repressive function in megakaryocytes and thus a decrease in their levels (8). However, EKLF interaction with PIAS3, a protein whose levels we have shown decrease during erythropoiesis, plays a role in preventing aberrant onset of EKLF-directed transcription at selected promoters.

The transcriptional consequences of interactions between EKLF and PIAS3 at different promoters are not equivalent. For β -globin and AHSP promoters, it is a dose-dependent repression caused by the presence of PIAS3. On the other hand, EKLF activation of the BKLf(1b), p21 and p18 promoters is enhanced by PIAS3. We are not certain whether this superactivation is a direct or indirect effect, because we were not able to demonstrate occupancy of PIAS3 on these promoters (not shown); it is possible that any PIAS binding sites may not be near the EKLF-binding promoter sites that we queried. Such differential outputs by EKLF have been observed before; for example, EKLF-TAF9 interactions are important for β -globin, but not for AHSP, promoter activation (42). Part of the TAF9 requirement may be related to whether initiator motifs and downstream promoter elements are present (42). The β -globin promoter contains these elements, whereas AHSP does not. Taken together, this suggests that initiator motifs and downstream promoter elements are not implicated in EKLF-PIAS3 interactions because we observe the same pattern for both the β -globin and AHSP promoters. In addition, there are not any obvious sequence motifs nor erythroid transcription factor (Gata, Scl/Tal1, and Ldb) binding data that might serve to differentiate PIAS responsiveness. As a result, the precise mechanism(s) remain to be uncovered.

The negative effects of PIAS3 gain of function are manifested only with the wild type but not with a mutant of PIAS3 that lacks an LXXLL motif; this, together with reporter gene analysis, suggest that LXXLL motif of PIAS3 is linked to its erythroid repressive effects. The LXXLL motif was originally identified in several nuclear receptor coactivators (26, 27). It forms amphipathic α -helices that interact with the ligand-binding domain of nuclear receptors (43). Based on a review of the literature, the LXXLL motif can act either as a protein interaction module or as a functional repressor module depending on the binding partners (44). In the first case, the LXXLL motif of PIAS3 interacts with the p65 subunit of NF- κ B, competing with

the LXXLL motif of another cofactor, CBP, for binding to p65. The final transcriptional output depends on concentration of particular cofactors in cells (44). This suggests that PIAS3 exerts its effects through interfering with the interaction between p65 and CBP. Overexpression of PIAS3 collides with binding of the CBP to p65 and renders transcriptional repression (44). The second mode of LXXLL action is observed when PIASy represses transcriptional activity of STAT1 (25) or androgen receptor (45). In both cases, the LXXLL motif is not involved in protein interaction with transcription factors but is absolutely required for the repressive activity of PIASy. The authors proposed that PIASy may act as an adaptor protein to recruit other corepressors to inhibit Stat1 or androgen receptor-mediated gene activation; thus the LXXLL motif would be involved in the assembly of the PIASy-containing corepressor complex (25). It is known that the LXXLL region from HIRA interacts with corepressor HDAC2 (28) and the LXXLL from FOXO1 with sirtuins (31).

Interestingly, neither of these two modes of LXXLL involvement in repression fits the case for EKLF. The LXXLL motif is not engaged in PIAS3 binding to EKLF nor does it recruit any corepressors from histone deacetylase classes that condense DNA structure and prevent transcription. However, the LXXLL motif resides in the N-terminal SAP domain, so it may be informative to consider the possible role of this domain for the present studies. The SAP domain is highly conserved among all members of PIAS family and evolutionarily conserved in proteins ranging from yeast to humans. It is shared by other chromatin-binding proteins, such as SAF-A and SAF-B (13, 46). The SAP consists of 35 amino acid residues that generate two amphipathic helices with LXXLL motif located in the first of them (46). The SAP domain does not tolerate inserts between helices, and any mutations in these helices or in the intervening region result in a complete loss of the DNA binding activity (47). Thus PIAS proteins might function by localizing transcription factors, via the SAP domain, to scaffold or matrix attachment regions, which have been proposed to stimulate transcription by forming active domains of chromatin (48, 49). PIAS lacking the LXXLL motif loses these features. Of potential relevance to the present studies, the N-terminal SAP domain of PIASy mediates its association with the nuclear matrix and is sufficient to repress the transcriptional activity of PITX2 (50). It will be of interest to address whether EKLF association with such scaffold or matrix regions plays a role in its activity. The binding of PIAS family members to AT-rich DNA in regions of active transcription and the potential to form nuclear scaffolds for attachment of regulatory proteins provide a basis for understanding the multifunctional nature of PIAS (51).

Acknowledgments—We thank Dr. Merlin Nithya Gnanapragasam for isolation of expanding primary fetal liver cells and Alec Stranahan for construction of PIAS3 mutant plasmids. We also thank Drs. Saghi Ghaffari, James G. Omichinski, Merlin Crossley, and Andrew Perkins for providing reagents and discussion. Confocal laser scanning microscopy was performed at the Mount Sinai School of Medicine Microscopy Shared Resource Facility. The Quantitative RT-PCR facility is also supported by MSSM.

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