KLF8 (Krüppel-like factor 8) is a member of the Krüppel transcription factor family that binds CACCC elements in DNA and activates or represses their target genes in a context-dependent manner. Here we present sumoylation as a novel mechanism that regulates KLF8 post-translationally. We found that KLF8 can be covalently modified by small ubiquitin-like modifier (SUMO)-1, SUMO-2, and SUMO-3 in vivo. We showed that KLF8 interacted with the PIAS family of SUMO E3 ligases PIAS1, PIASy, and PIASxα but not with E2 SUMO-conjugating enzyme Ubc9. Furthermore, we demonstrated that the E2 and E3 ligases enhanced the sumoylation of KLF8. In addition, site-directed mutagenesis identified lysine 67 as the major sumoylation site on KLF8. Lysine 67 to arginine mutation strongly enhanced activity of KLF8 as a repressor or activator to its physiological target promoters and as an inducer of the G1 cell cycle progression. Taken together, our results demonstrated that sumoylation of KLF8 negatively regulates its transcriptional activity and cellular functions.

Post-translational modifications of proteins, such as phosphorylation, acetylation, methylation, ubiquitination, and sumoylation, play crucial roles in many cellular processes due to their ability to cause rapid changes in the conformation and functions of preexisting proteins. Small ubiquitin-like modifier (SUMO)2-1, -2, and -3 are ubiquitin-like proteins that can be covalently attached to a large number of proteins through the formation of isopeptide bonds between the C terminus of mature SUMOs and the ε-amino group of a lysine in the acceptor proteins (1, 2). The conjugated forms of SUMO-2 and SUMO-3 differ from one another only by three N-terminal residues. They form a distinct subfamily known as SUMO-2/3 and are 50% identical in sequence to SUMO-1 (2, 3). Sumoylation of proteins proceeds via a multi-enzymatic pathway that shares similarity with the ubiquitin-conjugation system but uses a SUMO-specific enzymatic machinery (4).

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Like ubiquitin, SUMOs are expressed in inactive precursors that have to be processed by SUMO-specific proteases to expose a C-terminal double glycine motif that is required for SUMO conjugation. The processed form of SUMO is specifically activated in an ATP-dependent manner by an E1-activating enzyme consisting of an SAE1 (AOS1)-SAE2 (UBA2) heterodimer. Activated SUMO is transferred to Ubc9, the E2-conjugating enzyme, and is subsequently attached to the ε-amino group of a specific lysine in the target protein (4, 5). In Saccharomyces cerevisiae, sumoylation involves an E3 ligase (e.g. Siz1 and Siz2) for ligation of SUMO to its substrates. In contrast, E3 enzyme is not required for the addition of SUMO to its target proteins in mammals, although specific E3 ligases can promote the conjugation of SUMO from the E2 to its target protein (6–8). To date, three types of SUMO E3 ligases, the nucleoporel RanBP2 (4, 9), the PIAS proteins (10), and the polycomb group protein Pc2 (11), have been described. The PIAS proteins were initially described as protein inhibitors of activated STATs, and subsequent studies have shown that the PIAS proteins influence the activity of many transcription factors (12–15). PIAS proteins are characterized by a central cysteine-rich SPRING (Siz/PIAS RING) domain, which is exclusively found in SUMO E3s and is required for the sumoylating activity (16). This domain shares sequence homology with the RING motif of ubiquitin ligases. Mammals have at least five PIAS proteins: PIAS1, PIAS3, PIASxα, PIASxβ, and PIASy (10). The conjugation of proteins with SUMO is reversible through the activity of a number of SUMO-specific isopeptidase enzymes that participate in both processing of immature SUMO and removal of SUMO conjugates from substrates, allowing for a dynamic regulation of SUMO-dependent processes (5, 7).

Unlike ubiquitination, sumoylation does not target a protein for degradation but rather affects its stability, localization, or activity with crucial effects for many cellular processes (17). The functional consequences of sumoylation vary depending on the target proteins. A consensus SUMO acceptor site, consisting of the sequence ΨKXE, where Ψ is a large hydrophobic amino acid, X is any amino acid, and K is the site of SUMO conjugation, has been identified, although nonconsensus SUMO acceptor sites have also been found in vivo (18, 19). Sumoylation mediates divergent effects on many transcription factors and can regulate the transcriptional activity either negatively or positively (20, 21).

Members of the Sp/Krüppel-like factor family of zinc finger-containing transcription factors are important components of transcriptional machinery in eukaryotic cells (22, 23). Krüppel-like factors (KLFs) are characterized by a distinctive DNA-binding domain at the C terminus that consists of three Krüppel-like Cys2-His2 (C2/H2) zinc fingers. Outside of this domain, there is little homology among the known KLF members (23). Zinc finger domains in KLFs bind related CACCC- and GC-boxes, although the binding preferences are different (24). Seventeen KLFs (KLF1–KLF17) have been identified in the human genome (23, 25). KLFs have been shown to play diverse cellular functions, including cell proliferation, apoptosis, differentiation, development, and oncogenesis, by regulating the expression of a large number of genes (23, 25).

KLF8 (also called ZNF741 or BKLFS) was initially isolated from K562 cells (a human chronic myelogenous leukemia cell line) and identified as a ubiquitously expressed Krüppel-like transcription factor. This protein

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2 The abbreviations used are: SUMO, small ubiquitin-like modifier; E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase; STAT, signal transducers and activators of transcription; KLF, Krüppel-like factor; HA, hemagglutinin; GFP, green fluorescent protein; RFP, red fluorescence protein; ChIP, chromatin immunoprecipitation; BOP, biotinylated oligonucleotide precipitation; IP, immunoprecipitation; BrdUrd, bromodeoxyuridine; CebP, E1a C-terminal-binding protein.
Sumoylation of KLF8

represses gene expression by associating with the E1a C-terminal-binding protein (CtBP) co-repressors. However, the abolition of interaction between KLF8 and CtBP does not release the repressive properties of KLF8, suggesting that KLF8 may recruit other co-repressors to regulate transcription (26). Our recent studies identified KLF8 as a specific downstream transactivation factor of focal adhesion kinase in the regulation of cell cycle progression by activating the cyclin D1 promoter (27). Consistent with cyclin D1 up-regulation, KLF8 expression was found to have a stimulatory effect on cell proliferation when transfected into NIH 3T3 cells. KLF8 could repress or activate reporter genes in different cell lines, suggesting that KLF8 may exert dual functions dependent on the specific promoters and the cellular environments. Therefore, it is important to clarify the molecular mechanisms underlying the regulation of KLF8 for understanding its biological roles.

Here we report that KLF8 is a target for modification of SUMOs. We found that PIAS1, PIASy, and PIASα were SUMO E3 ligases physically interact with KLF8 and enhance its sumoylation. We identified a single lysine residue as the major sumoylation site of KLF8. We showed that sumoylation of KLF8 did not affect its subcellular localization but modulated its transcriptional activity and its regulation of the cell cycle. These results suggested sumoylation of KLF8 as a novel regulatory mechanism in the control of KLF8-mediated transcription and cellular functions.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—The rabbit polyclonal anti-hemagglutinin (anti-HA) antibody (Y11), mouse monoclonal anti-Myc antibody (9E10), and anti-SUMO-1 antibody (FL-101) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). α-HA antibody-conjugated agarose was from Sigma.

Plasmid DNA Construction—Mammalian expression plasmids pH3, pHAN, and pKH3-KLF8 have been described previously (27, 28). The KLF8 mutants K67R, K67R-DLmut, K217R, and K67R/K217R were generated with PCR-directed mutagenesis. pLEGFP-KLF8 and pLEGFP-K67R were produced by ligating PCR-generated inserts into the Xhol-HindIII sites of pLEGFP-C1 (Clontech). The mouse SUMO-1 construct was kindly provided by Dr. Jun-Ying Yuan (29) and inserted into pHAN vector or pLERFP-C1 derived from pLEGFP-C1 where GFP had been replaced by RFP, a kind gift from Dr. Reinhard Fischer (30). pKH0-SUMO-1 vector encoding tag-free SUMO-1 was constructed by inserting the SUMO-1 expression protein (CtBP) co-repressors. However, the abolition of interaction between KLF8 and CtBP does not release the repressive properties of KLF8, suggesting that KLF8 may recruit other co-repressors to regulate transcription (26). Our recent studies identified KLF8 as a specific downstream transactivation factor of focal adhesion kinase in the regulation of cell cycle progression by activating the cyclin D1 promoter (27). Consistent with cyclin D1 up-regulation, KLF8 expression was found to have a stimulatory effect on cell proliferation when transfected into NIH 3T3 cells. KLF8 could repress or activate reporter genes in different cell lines, suggesting that KLF8 may exert dual functions dependent on the specific promoters and the cellular environments. Therefore, it is important to clarify the molecular mechanisms underlying the regulation of KLF8 for understanding its biological roles.

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sumoylation motifs that are conserved among KLF8 protein in mammals (Fig. 1A). Lysines 67 and 217 of human KLF8 are in the sumoylation consensus tetrapeptide IKIE and VKVD, respectively, which could act as target sites for SUMO modification (4, 6). To test whether KLF8 can be modified by SUMO, 293T cells were transfected with expression vector encoding HA-tagged KLF8 in the absence or presence of Myc-tagged SUMO-1 expression plasmid. Cell lysates were prepared under conditions preserving SUMO modification and then precipitated with anti-HA-agarose conjugates and antibodies against Myc or anti-HA antibodies (Fig. 1B). In the cells co-transfected with HA-KLF8 and Myc-SUMO-1, anti-HA antibody detected an additional slower migrating band (Fig. 1B, bottom, lanes 3 and 4). This band, however, was hardly detected in the cells expressing HA-KLF8 alone (Fig. 1B, bottom, lane 1) and not detected in the cells expressing Myc-SUMO-1 alone (Fig. 1B, bottom, lane 2). Co-IP confirmed that this slower migrating band consisted of both HA-KLF8 and Myc-SUMO-1, since it could be detected in the precipitates by both HA and Myc antibodies (Fig. 1B, top, lanes 2 and 3). These results suggested that the HA-KLF8 was sumoylated by the Myc-SUMO-1. Co-expression of HA-KLF8 and RFP-SUMO-1 further suggested the sumoylation of KLF8, since a band with even slower motility (due to the larger size of RFP compared with the Myc tag) was detected in the cell lysates and in the anti-HA precipitates (Fig. 1B, bottom, lane 5).

We next tested whether KLF8 can be modified by different SUMO members by cotransfection of 293T cell with plasmids encoding HA-tagged KLF8 and Myc-tagged SUMO-1, SUMO-2, or SUMO-3. Fig. 1C shows that KLF8 can be modified by all of the three SUMOs tested, although its sumoylation by SUMO-1 was much stronger than those by SUMO-2 or SUMO-3. In this experiment, a weaker modified band above KLF8 was detected in the absence of cotransfected SUMOs (lane 1), suggesting that the transfected KLF8 may be modified by endogenous SUMO-1. This was confirmed by Western blotting with anti-SUMO-1 antibody (data not shown). Taken together, these results demonstrate that KLF8 is a target for SUMO modification.

Enhancement of Sumoylation of KLF8 by Co-expression of Ub9 and PIAS Proteins—Sumoylation is a dynamic process that is controlled by SUMOylases and deSUMOylases with the opposing enzyme activities (4, 6). Sumoylation of many of target proteins is stimulated by the PIAS family of E3 ligases (6, 19). Thus, we examined whether sumoylation of KLF8 can be increased by E2 conjugase Ub9 or E3 PIASs by co-transfection of 293T cells with plasmids encoding HA-

![Figure 1](image1.png)  
**FIGURE 1.** Identification of KLF8 as a SUMO substrate. A schematic representation of KLF8 (detailed in Fig. 3A) and alignment of KLF8 sequences from different species surrounding the putative sumoylation site (arrows). PVDSL is a proposed motif that mediates KLF8 interaction with the CBP co-repressor protein. This motif was disrupted in the K67R-DLmut mutant used in Figs. 5 and 6. NLS, predicted nuclear localization signal sequences flanking the three zinc fingers of KLF8. Identical residues are shaded gray, and the sumoylation consensus motif is in boldface letters. The numbers indicate the position of the lysine in the proteins. B and C, 293T cells were transiently co-transfected with expression vectors coding for HA-tagged KLF8 or HA vector alone (lane 2 in B) and Myc-tagged or RFP-tagged SUMO-1, SUMO-2, or SUMO-3, as indicated. Lysates were prepared from transfected cells, immunoprecipitated with anti-HA antibody, and blotted with either anti-Myc antibody (top) or anti-HA antibody (middle). Whole cell lysates were also blotted using anti-HA antibody (bottom). The full-length HA-KLF8 and SUMO-conjugated HA-KLF8 are marked with arrows.

![Figure 2](image2.png)  
**FIGURE 2.** Stimulation of KLF8 sumoylation by Ub9, PIAS1, PIASy, and PIASx through physical association. A, 293T cells were transfected with a plasmid encoding HA-tagged KLF8 alone (lane 1) or together with plasmids encoding a tag-free SUMO-1 in combination with a vector control, Myc-tagged Ub9, PIAS1, PIASy, PIASx, or HA-tagged SENP1 (lanes 2–7), as indicated. Whole cell lysates (WCL) were blotted (IB) using anti-HA or anti-Myc, as indicated. The full-length HA-KLF8 and SUMO-conjugated HA-KLF8, as well as Myc-tagged Ub9, PIAS1, PIASy, or PIASx, are marked with arrows. A nonspecific band recognized by anti-Myc antibody is marked with an asterisk. B, 293T cells were co-transfected with pKH3 vector control or pKH3-KLF8 encoding HA-KLF8 and plasmids encoding Myc-tagged PIAS1, PIASy, or PIASx, as indicated. Lysates were prepared and immunoprecipitated by anti-HA-conjugated agarose beads. The immunoprecipitates or whole cell lysates were blotted with anti-Myc or anti-HA as indicated.
tagged-KLF8, SUMO-1, and Myc-tagged Ubc9 or PIAS members. Fig. 2A shows that KLF8 sumoylation was increased by Ubc9 and to a greater extent by PIAS1, PIASy, or PIASx/H9251. Co-transfection of a deSUMOylase SENP1 led to a reduction of KLF8 sumoylation (compare lanes 2 and 7). Similar experiments showed that another potential sumoylation E3 ligase Pc2 did not exert the stimulation effect on KLF8 sumoylation (data not shown). Furthermore, we found that KLF8 can associate with the PIAS family members PIAS1, PIASy, and PIASx/H9251 as measured by coimmunoprecipitations (Fig. 2B), although we could not detect its interaction with Ubc9 (data not shown). These results suggested that PIAS1, PIASy, and PIASx function as E3 ligases for KLF8 sumoylation.

Identification of Lys67 as the Major Sumoylation Site on KLF8—To determine the regions of KLF8 important for its sumoylation and to map the target lysine residues, we first examined a series of KLF8 truncation mutants (Fig. 3A) for their modification by SUMO-1. Fig. 3B shows that, whereas deletion of the N-terminal 56 residues (ΔN56; lane 3) did not affect KLF8 sumoylation, removal of residues 1–100 (ΔN100; lane 4) completely abrogated it. Stepwise deletion of C-terminal sequences up to residue 167, including the zinc finger DNA binding domains (1–262, 1–240, and 1–166; lanes 5, 6, and 8) did not affect KLF8 sumoylation. These results suggested that the target sites were located within residues 56–100 and that DNA binding is not required for KLF8 sumoylation.

Because KLF8 contains two lysines (67 and 217) in consensus motifs for sumoylation (see Fig. 1A), the above results suggested Lys67 as the possible target residue. To verify this, we created KLF8 mutants designed as K67R, K217R, and K67R/K217R, which change either Lys67 or Lys217 or both to Arg, respectively. Analysis of the mutants showed that K67R and K67R/K217R mutants were not sumoylated, whereas K217R mutant was modified similarly as the wild type KLF8 (Fig. 3C). Furthermore, mutation of Lys67 to Arg also abolished KLF8 sumoylation by SUMO-2 or SUMO-3 (Fig. 3D). These results confirmed that Lys67 is the major sumoylation site of KLF8.

Effect of KLF8 Sumoylation on its Nuclear Localization—Sumoylation has been shown to regulate subcellular localization of target proteins (6). To determine whether KLF8 sumoylation can affect its localization, we created GFP fusion proteins containing KLF8 and K67R mutant and examined their intracellular distribution in the presence and absence of SUMO-1. Fig. 4A shows that both wild type KLF8 and the K67R mutant are localized exclusively in the nuclei. Coexpression of RFP-tagged SUMO-1 and Myc-tagged PIAS1 with GFP-KLF8 or GFP-K67R showed similar co-localization of SUMO-1 with both KLF8 and K67R mutant in nuclear granules (Fig. 4B). Together, these results suggest that sumoylation of KLF8 did not alter its subcellular localization.
Regulation of KLF8 Transcriptional Activity by Sumoylation—Sumoylation has been shown to alter the activity of quite a few transcription factors, including another KLF family member KLF3 (20, 40). Although synthetic promoter reporters, including the GAL4 fusion reporter system, are useful tools for the evaluation of transcription factor activity, these reporters sometimes give discrepant results, as indicated in the studies for both KLF8 and KLF3 (26, 40). Therefore, it is important to use direct in vivo target promoters, if available, to evaluate specific transcription factors.

KLF8 has been shown to function as a transcriptional repressor or activator (26, 27). We previously found that KLF8 could activate cyclin D1 promoter by directly binding to a GT-box using promoter reporter assays and in vitro gel shift assays (27). Here we confirmed the direct interaction in vivo between KLF8 and cyclin D1 promoter by ChIP.
The K67R mutant activated the cyclin D1 promoter more \( \text{e} \) enhanced activity was either decreased when SUMO-1 was co-expressed (Fig. 5, compare columns 3 and 2) but facilitated by SENP1 (Fig. 5, compare columns 4 and 2). Surprisingly, the K67R mutant repressed the KLF4 promoter even more potently (Fig. 5, compare column 5 with columns 4 and 2), whereas the K67R-DLmut mutant lost \( \sim 50\% \) of its repressor activity (Fig. 5, compare column 6 with columns 2 and 1). Taken together, these results suggest that sumoylation is a mechanism that restricts the activity of KLF8 as both a transcriptional activator and repressor.

\textbf{DISCUSSION}

KLF8 is a newly identified Krüppel-like transcription factor that is expressed ubiquitously in many cell types. Recent studies suggested that KLF8 functions as either a transcriptional repressor or activator, depending on the context of its target promoters, although molecular mechanisms underlying transcriptional regulation by KLF8 are currently unclear (26, 27). Our previous work linked the transcriptional activity of KLF8 to its role in promoting the cell cycle progression at G1 phase (27). Many transcription factor proteins undergo post-translational modifications that regulate their stability, subcellular localization, protein interactions, and so on, which eventually lead to changes in their transcriptional activity and associated cellular functions. The post-translational modifications often include phosphorylations and modifications by ubiquitin family proteins, such as ubiquitin (ubiquitination), SUMO (sumoylation), and Ned8 (neddylation). In this study, we identified KLF8 as a new target of sumoylation and mapped the major sumoylation site at Lys67. Furthermore, we showed that sumoylation decreased transcriptional activity of KLF8 in the regulation of the transcription of its physiological targets (i.e. cyclin D1 and KLF4). Finally, we demonstrated that sumoylation of KLF8 reduced its mediated cell cycle progression at G1 phase.

Sumoylation has been shown to be a major regulatory modification for many proteins important for regulated gene expression, including promoter-specific transcription factors (20). Although sumoylation can both negatively and positively change the activity of target transcription

\textbf{FIGURE 6. Effect of sumoylation of KLF8 on its regulation of the cell cycle.} A. NIH3T3 cells expressing inducible KLF8 (27) were transfected with phAN-SUMO-1. The percentage of BrdUrd(+) cells at 12 h after serum stimulation under uninduced (U) and induced (I) conditions was examined. B. NIH3T3 cells were transfected with KLF8 or its mutants as indicated. The percentage of BrdUrd(+) cells at 12 h after serum stimulation was analyzed. The results show the mean \( \pm S.E. \) for at least three independent experiments.

\text{Sumoylation Limits KLF8 Activity to Regulate the Cell Cycle Progression.}—Based on the above results, we predicted that sumoylation might affect the cellular functions of KLF8. We previously showed that induced expression of ectopic KLF8 in 3T3 cells promoted the G1 phase progression of the cell cycle (27). To test the effect of sumoylation on cell cycle progression, we first overexpressed SUMO-1 in the inducible cell lines. Whereas in the absence of SUMO-1 overexpression, induction of KLF8 expression in the cells resulted in an \( \sim 2 \) times higher rate of new DNA synthesis as expected, as examined by BrdUrd incorporation assays (Fig. 6A, compare the left two columns), overexpression of SUMO-1 strikingly slowed down the BrdUrd intake rate (Fig. 6A, compare the two columns I), regardless of the equal induction of KLF8 expression as monitored by Western blotting (data not shown). In contrast, the K67R mutant was 2 times stronger than wild type KLF8 in promoting new DNA synthesis (Fig. 6B, compare columns 3 with columns 2 and 1). The K67R-DLmut mutant, however, showed slightly reduced activity (Fig. 6B, compare columns 4 and 3). Overall, these results suggested that sumoylation of KLF8 defines an extent to which KLF8 regulates the cell cycle (and probably other cellular events) by activating and/or repressing the transcription of its target genes.
Sumoylation of KLF8

factors, in most cases, it exerts an inhibitory effect (7, 20, 21, 42). For example, a SUMO-1 binding-deficient c-Jun mutant shows an increased transactivation potential on an AP-1-containing promoter (43); SUMO-1 covalently attached to c-Myb inhibits the transactivation function of c-Myb (44); sumoylation of p53 limits its transcriptional output (45); and overexpression of SUMO leads to the inhibition of NF-κB-dependent transcription (46). Consistently, we found that the sumoylation-deficient KLF8K67R mutant showed increased activity as both a transactivator on cyclin D1 promoter and a repressor on KLF4 promoter (Fig. 6), suggesting that sumoylation of KLF8 limits its transcriptional activity on both its activation and repression targets, which in turn decreases KLF8-mediated cellular functions. Indeed, our results demonstrated that overexpression of SUMO-1 decreased the BrdUrd intake rate by KLF8, and conversely the KLF8K67R mutant gained significantly higher activity in promoting BrdUrd incorporation (Fig. 6).

Recently, another KLF subfamily member, KLF3, has been reported to be sumoylated (40). Interestingly, the sumoylation-defective mutation turns KLF3 from a transcriptional repressor into a potent transactivator on the same synthetic promoter reporter, although the same KLF3 mutant showed little difference from wild type KLF3 in regulating its native target γ-globin promoter (40). Similarly, we observed that mutation of the sumoylation site Lys67 in the context of the N-terminal KLF8 (amino acids 1–262) fused to Ga4 DNA binding domain converted it from a transcription repressor to an activator too, although we did not see the same effect in the full-length KLF8K67R mutant on a synthetic promoter reporter (data not shown). Importantly, a previous report showed a differential requirement for interaction with CtBP co-repressor by the GAL4-fused KLF8 fragment and full-length KLF8 in transcriptional repression of the synthetic promoter reporters (26). These results strongly indicate that sumoylation regulates transcription factors in a highly target promoter–dependent manner and that one should exercise caution in extrapolating results derived from using synthetic promoter reporters to physiological target promoters.

How sumoylation changes the activity of KLF8 is not known yet. As described above, several consequences could potentially result from the sumoylation. These include altered KLF8 protein stability, subcellular localization, DNA binding ability, phosphorylation, protein interactions, and so on.

Previous studies suggested that sumoylation and ubiquitination share some lysine residues in some target proteins and that sumoylation of the proteins inhibits ubiquitination at the same sites and consequently prevents ubiquitin-mediated degradation of the proteins (6, 46–48). KLF5 has recently been identified as a target of ubiquitin-mediated degradation (49). However, we did not observe any significant difference in the steady-state levels of the sumoylation site mutant K67R and the wild type KLF8, suggesting that sumoylation is unlikely to be involved in the regulation of KLF8 stability. Likewise, our data showed no apparent difference in the nuclear localization between K67R mutant and wild type KLF8, suggesting that sumoylation of KLF8 did not affect its subcellular localization, although sumoylation of several other proteins has been reported to regulate their subcellular localizations (6, 19). We did not observe significant change in DNA binding ability of the KLF8K67R mutant, although further mutation of the CtBP contact motif brought about a slight decrease in the binding to the KLF4 promoter but not the cyclin D1 promoter (Fig. 5C), indicating that sumoylation did not affect KLF8 binding to its target promoters. This result was consistent with the report indicating that mutation of the sumoylation sites on KLF3 did not affect its DNA binding activity (40). Phosphorylation has been shown to promote activity of other Sp1/KLF family transcription factors (50). Phosphorylation of the c-Jun oncoprotein, which is required for its transcriptional activity and stability, is inversely correlated with its sumoylation (43). Whether or not KLF8 is also regulated by phosphorylation and there is a cross-talk between its phosphorylation and sumoylation is not known. This will be an interesting area of future investigations.

Co-repressor proteins, including co-activators and co-repressors, are important interacting partners of transcription factors in the regulation of target gene promoters. Previous studies implicated that binding of the CtBP co-repressor to the PVDSL motif of KLF8 (residues 86–90, see Figs. 1A and 3A) was required for the repressor function of KLF8 in a GAL4 fusion reporter system (26). In support of this, our results showed that disruption of KLF8-CtBP interaction as in the K67R-DLmut mutant dramatically reduced the repressor activity of the sumoylation-deficient KLF8K67R mutant on the KLF4 promoter (Fig. 5E). We also found that the KLF8K67R mutant showed similar binding to CtBP as the wild type KLF8, and mutation of the CtBP contact motif did not affect sumoylation of KLF8 (data not shown), suggesting that sumoylation and CtBP recruitment are independent events in the regulation of repressor activity of KLF8. Interestingly, we noticed that mutation of the CtBP binding motif on the full-length KLF8 did not fully abolish the repressor activity of KLF8 on the KLF4 promoter (Fig. 5E) or had no effect on the repressor activity of KLF8 on synthetic promoter reporter (26). These results suggested that in addition to CtBP, KLF8 could potentially recruit other co-repressor proteins in the regulation, depending on the context of its repression targets. One potential candidate would be the histone deacetylase-1 co-repressor, since it has been shown to interact with zinc finger domains of several other members of the Sp/KLF family proteins (47, 51, 52). It would be interesting to examine whether KLF8 interaction with histone deacetylase-1 would be an alternative mechanism for its repressive role. Our previous work (27) and this study (Fig. 5) showed that KLF8 could directly bind and activate cyclin D1 promoter, suggesting that KLF8 could recruit an unknown co-activator protein(s) to exert its activator function. Interestingly, KLF5 has been shown to interact with both HDAC-1 co-repressor and p300 co-activator (53). Obviously, identification of the KLF8 co-activator(s) is another intriguing goal of future work.

A data base search suggested the presence of conserved sumoylation motifs in a number of other KLFs, including KLF1, -2, -4, -5, -12, and -15. We thus propose that sumoylation may be a potentially common and important mechanism for the post-translational regulation of KLF family transcription factors. We also propose that KLF8 protein is normally maintained in a hyposumoylated state in which a fraction of the proteins are sumoylated; this state could be switched either way to a hypersumoylated or desumoylated state, depending upon special requirement of the cell for KLF8 function under some specific physiological or pathological conditions.

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