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Ikaros SUMOylation: Switching Out of Repression

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Ikaros plays a key role in lymphocyte development and homeostasis by both potentiating and repressing gene expression. Here we show that Ikaros interacts with components of the SUMO pathway and is SUMOylated in vivo. Two SUMOylation sites are identified on Ikaros whose simultaneous modification results in a loss of Ikaros' repression function. Ikaros SUMOylation disrupts its participation in both histone deacetylase (HDAC)-dependent and HDAC-independent repression but does not influence its nuclear localization into pericentromeric heterochromatin. These studies reveal a new dynamic way by which Ikaros-mediated gene repression is controlled by SUMOylation.

Ikaros is a member of a family of Krüppel-like zinc finger DNA-binding factors that includes Aiolos, Helios, and Eos (9, 15, 18, 23, 33, 37). The *Ikaros* gene is composed of seven translated exons (from two to eight) from which eight identified isoforms can be generated through alternative splicing (Ik-1 to -8) (22, 32). All Ikaros isoforms contain a C-terminal zinc finger dimerization domain encoded by exon 8 that mediates interactions with itself and other family members (45). They differ in their composition of N-terminal DNA-binding zinc fingers. On the basis of DNA-binding activity, Ikaros isoforms can be subdivided into two groups; in the first group are isoforms with two to four zinc fingers that can bind DNA, and in the second group are isoforms with fewer than two zinc fingers that do not bind DNA. The latter group of Ikaros isoforms maintains the ability to dimerize and exert a dominant-negative effect on the DNA-binding group of isoforms (32, 45).

Genetic studies have established that Ikaros proteins play critical roles during development and homeostasis of the immune system (8, 47, 48). Ikaros is required in the hematopoietic stem cell and its multipotent progeny to promote cell fate decisions along the lymphoid pathway (5, 7, 36). In differentiating and mature lymphocytes, Ikaros functions as a tumor suppressor by negatively regulating proliferation and by providing homeostasis to this developmental pathway (1, 48). Reduction of Ikaros DNA-binding activity causes the rapid development of T-cell leukemias and lymphomas (48). Ikaros-deficient T cells display an augmented response to activation signals, whereas Ikaros-overexpressing cells arrest at the transition from G_1 to S phase (1, 13). Ikaros' ability to control the cell cycle is regulated by phosphorylation at its C-terminal region, a modification that interferes with its DNA-binding activity and facilitates cell cycle progression (13).

Ikaros can function as an unconventional potentiator of gene expression during T-cell development, possibly by recruiting the Swi/Snf chromatin-remodeling complex to appropriate lineage-specific gene targets like CD8 (3, 17, 28). Ikaros can also function as a transcriptional repressor (29). Ikaros-dependent repression relies, in some cases, upon its association with histone deacetylase(HDAC)-containing complexes (NuRD and Sin3) (29) and in others upon its interaction with the corepressor CtBP for HDAC-independent mechanisms (26). Ikaros interacts with the NuRD complex ATPase Mi-2β and with Sin3 through both its N-terminal and C-terminal regions (24, 29). Ikaros-CtBP interaction relies on a PEDLS motif (amino acids 34 to 38) located at the N-terminal region of Ikaros (26).

SUMOylation is a posttranslational modification that involves conjugation of the small ubiquitin-related modifier (SUMO) protein (30, 31, 42). SUMOylation of proteins proceeds via a multienzymatic pathway that is mechanistically similar to ubiquitination but uses a SUMO-specific enzymatic machinery: the E1 SUMO-activating enzyme formed by the heterodimer Aos1/Uba2, the E2 SUMO-conjugating enzyme Ubc9, and the E3 ligases, which promote SUMO transfer from Ubc9 to specific protein substrates (25, 30, 31, 42). The biological implications of protein SUMOylation are broad, reflecting the biological activities of the substrates. Unlike ubiquitination, SUMOylation does not mediate protein degradation and in some cases induces protein stability (6, 41). For example, SUMOylation of the inhibitor of NF-κB (IκBα) antagonizes its ubiquitin-dependent degradation (6). SUMOylation has been shown to affect the integrity of nuclear bodies and polycomb group bodies, possibly by regulating the localization of promyelocytic leukemia/SP100 and polycomb proteins to these nuclear domains (21, 35, 41, 42, 44). SUMOylation has also been reported to control the activity of a number of transcription factors through mechanisms not dependent on nuclear localization (10, 12, 34, 46).

There are many reports of SUMOylation negatively regulating the activity of transcriptional activators (34). For example, SUMOylation confers repression activity on Elk-1 by promoting its interactions with HDAC-2 (51). ERK-mediated phosphorylation of Elk-1 induces its deSUMOylation and causes its switch to a transcriptional activator (50). In a fashion similar to that of Elk-1, SUMOylation of the coactivator p300 promotes recruitment of HDAC-6 and induces transcriptional repression

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of some promoters (11). The components of the SUMO pathway were also reported to negatively regulate transcription. The SUMO moiety or the E2-conjugating enzyme Ubc9, when tethered to the Gal4 DNA-binding domain, represses transcription (39, 43). Nonetheless, SUMOylation was also reported to increase the transcriptional activity of p53 and Tcf4 (14, 38, 49).

In this report, we show that Ikaros protein is SUMOylated in vivo and that this modification negatively impacts its properties as a transcriptional repressor. Two SUMOylation sites were identified within the Ikaros N-terminal repression domain, at positions K58 and K240. Mutation of these residues prevents Ikaros SUMOylation and unexpectedly increases Ikaros activity as a repressor. In line with this finding, deSUMOylases increase Ikaros-mediated repression, whereas E3 SUMO ligases reverse this effect. Ikaros SUMOylation interferes with its ability to repress transcription not by changing its nuclear localization to pericentromeric heterochromatin but by disrupting its interactions with HDAC-dependent and -independent corepressors, but not with other nuclear regulators. These studies provide us with a new example of how gene repression by Ikaros and its corepressors can be regulated by SUMO.

MATERIALS AND METHODS

Plasmids and reagents. The CDM8-Ik-1, CMV2-FlagIk-1, and pMX-GFP-IRES-Ik-1 expression and reporter plasmids used for repression and activation studies were previously described (13, 26). The reagents used in the yeast two-hybrid screen were described previously (24). SUMO1GG fused to green fluorescent protein (GFP-SUMO1), GFP-SUMO1GA, and Flag-tagged PIASxα and PIASxβ were generously provided by J. Palvimo. Flag-tagged PIAS1 and -3 were obtained from K. Shuai. Myc-tagged Senp1 was provided by E. Yeh, and GFP-Axam and a mutant form of Axam were provided by A. Kikuchi. Hemagglutinin (HA)-SUMO1, SUMO2, and SUMO3 were provided by R. Hay. Flag-SUMO1 and Flag-SUMO2 were provided by Gregory David and Rudolf Grosschedl. pcDNA3 HA-ubiquitin and mutant forms of ubiquitin were provided by T. Kamitani. Ikaros antibodies 4E9, 8H2, and IKD14 have been previously described (24, 45). Anti-SUMO2/3 was provided by H Saitoh. Anti-GMP1 (anti-SUMO1) was purchased from Zymed, anti-GFP was from Clontech and Santa Cruz Biotechnology, anti-HA was from Santa Cruz Biotechnology, and anti-Flag(M2) antibody and Flag(M2) peptide were from Sigma.

Yeast two-hybrid screen. A thymocyte library generated in the pJG vector was screened with Aiolos (amino acids 60 to 554), full-length Ikaros (amino acids 1 to 518), and their subdomains Y4, Y6, and Y9 (24) fused to the LexA DNA-binding domain (expressed from vector pEG202). The screen was carried out as described in reference 24, with an EGY48 yeast strain expressing the fusion protein baits.

Cell lines, transfections, and infections. 293T, NIH 3T3, and U2 OS cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (HyClone). Ikaros-null T-cell line NA1 was derived from the thymuses of Ikaros-null mice (8) and maintained in RPMI medium supplemented with 10% fetal bovine serum. Transfections of 293T cells were carried out by the HEPES-buffered saline–-$CaPO_4$ precipitation method. NIH 3T3 and U2 OS cells were transfected with the Polyfect transfection reagent (Gibco BRL) at the manufacturer's recommendation. For infections, retroviruses were generated after transfecting the packaging cell line Phoenix with the pMX-GFP-IRES plasmid or the Ikaros-expressing derivatives (13). At 48 h after transfection, viral supernatants were recovered and used to infect NA1 cells by spinning them at 1,800 rpm for 45 min in a Beckman GS-6KR centrifuge (as described in infection protocols at http://www.stanford.edu/group/nolan). At 24 h after infection, GFP-positive cells were sorted and extracts were prepared and subjected to Western blotting.

Immunofluorescence studies. NIH 3T3 cells were transfected with Ikaros with or without GFP-SUMO1 or with GFP-SUMO1 and PIASxα. At 24 h after transfection, cells were split into chamber slides and left to grow for an additional 12 h. Cells were then fixed and permeabilized with 70% ethanol for 30 min at room temperature; this was followed by extensive washing and by blocking of unspecific staining. Cells were then stained with Ikaros antibodies as described previously (28).

Extract preparation, immunoprecipitation, and Western analysis. For thymocyte extracts, thymuses were surgically removed from wild-type mice; cell suspensions were prepared and split in two. One half was lysed with buffer C (20 mM HEPES [pH 7.9], 25% glycerol, 0.42 M NaCl, 0.2 mM EDTA, 1.5 mM $MgCl_2$, protease inhibitors) containing 1% NP-40, and the other half was lysed with buffer C containing 1% sodium dodecyl sulfate (SDS) and 1 mM *N*-ethylmaleimide (NEM). The latter buffer was also used to generate NA1 cell extracts. Other whole-cell extracts and nuclear extracts from transfected or untransfected cells were prepared as previously described (24, 29) in the presence or absence of NEM. For immunoprecipitations, the extracts were precleared with protein G-agarose beads (Roche). The precleared extracts were incubated with the antibody of interest or the relevant isotype control in the presence of 30 μl of protein G-agarose beads and rotated for 4 h to overnight. Beads were then collected, washed at least four times with lysis buffer, and resuspended in SDS sample buffer. For the anti-Flag immunoprecipitations, anti-Flag(M2) agarose beads and M2 peptide were used to immunoprecipitate and elute complexes from beads, respectively. Eluents were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene difluoride membranes, probed with the relevant antibody, and examined by autoradiography by Enhance Chemical Luminescence.

Transcriptional studies. For the repression studies, Gal4-Ikaros fusions (0.5 to 1 μg), the 5XGal4 thymidine kinase (tk) promoter driving chloramphenicol acetyltransferase (CAT) (10 μg), and the growth hormone (GH) control (pXGH5; 0.5 μg) were transfected into NIH 3T3 or U2 OS cells as indicated. Cells were harvested 36 h after transfection and split into two samples. One half was processed for the CAT assay as previously described (45), and the other half was used to generate proteins and test their level by Western blotting. GH assays were performed as recommended by the manufacturer (Nichols Institute). CAT values were normalized to the GH levels.

For the activation assays, 1 μg of the reporter 4XIKBS2 (Ikaros binding site) tkCAT, 1 μg of Ikaros expression vector, and 0.5 μg of the GH plasmid were used. Cells were harvested after 36 h and processed as described above for the repression experiments. All of the transcription assays were performed at least three times in duplicate or triplicate.

RESULTS

Ikaros is SUMOylated in lymphocytes. To identify Ikaros family interactors, a thymocyte library was used in a yeast two-hybrid screen with the Aiolos and Ikaros proteins as baits as described previously (24). Three different types of cDNA were isolated multiple times, which encoded Aiolos and Ikaros interactors belonging to the SUMO pathway (Fig. 1A). One was SUMO1, the second was the component of the E1-SUMO activating enzyme Uba2, and the third was the E2-conjugating enzyme Ubc9. These proteins exhibited stronger interactions with Ikaros in the yeast two-hybrid system (Fig. 1A). Delineation of the Ikaros-Ubc9 interaction interface revealed their association through the N-terminal (Y6, exons 2 to 7 [amino acids 1 to 282], and Y9, exons 2 to 4 [amino acids 1 to 141]) but not the C-terminal region (Y4, amino acids 364 to 518 of exon 8) of the Ikaros protein (Fig. 1A and B).

Protein SUMOylation occurs on the lysine of a ψKxE protein motif, where ψ represents the hydrophobic residue isoleucine (I), leucine (L), or valine (V) and x is any amino acid. Analysis of the Ikaros protein sequence revealed four putative SUMOylation sites, at positions K58 and K240, located in the N-terminal half of the protein, and K425 and K459, located in the C-terminal region (Fig. 1B). Of these, the first three represent perfect consensus motifs for SUMOylation. The Ikaros SUMOylation motifs containing K58 and K240 are highly conserved across species from skates to humans (16). These two motifs were also found to be conserved in Aiolos at positions K63 and K256. The other two Ikaros family members, Helios

A

	Myc	Mxi-1	Aiolos	Ikaros	Y4	Y9	Y6
Sumo1	-	-	+	++++	nd	nd	nd
E1 (Uba2)	-	-	+	++	nd	nd	nd
E2 (Ubc9)	-	-	++	++++	-	++	++++

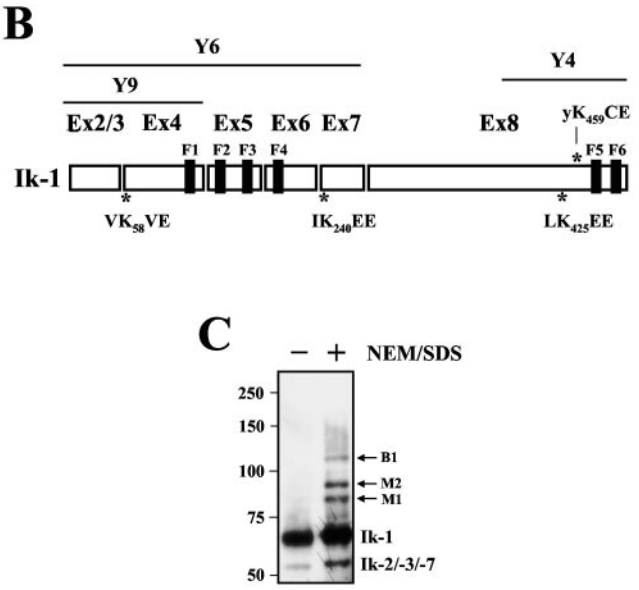

FIG. 1. Ikaros interacts with components of the SUMO pathway and is SUMOylated in primary lymphocytes. (A) SUMO1 and the SUMO enzymes E1 (Uba2 component) and E2 (Ubc9) were isolated in a yeast two-hybrid screen with Aiolos and Ikaros. These were found to interact specifically with both Ikaros and Aiolos but not with control baits like Myc and Mxi-1. The Ikaros-Ubc9 interacting domain was located at the N-terminal half of Ikaros (Y6), which harbors two putative Ikaros SUMOylation sites. nd, not determined. (B) Schematic representation of full-length Ikaros isoform 1 (Ik-1) showing the location of potential SUMOylation sites (I/V/L)KXE (asterisks). The modifiable lysine in each putative SUMOylation site is indicated by number. Horizontal rectangles represent translated exons (Ex), while vertical rectangles identify Ikaros zinc fingers (F1 to -6). The Ikaros N-terminal (Y6 and Y9) and C-terminal (Y4) subdomains used in the yeast two-hybrid assay are also shown. (C) Whole-cell extracts from murine thymocytes were generated in the presence (+) or absence (−) of 1% SDS and the SUMOylation inhibitor NEM (NEM/SDS). Proteins were separated by SDS–7% PAGE and subjected to Western blot analysis for Ikaros proteins. Ikaros isoforms 1, 2, 3, and 7 are shown. The three presumed SUMOylated Ikaros protein species are shown by arrows (M1, M2, and B1; see nomenclature explanation in Results). Positions of approximate molecular weight markers are shown on the left.

and Eos, had only one SUMOylation motif at their N termini, corresponding to Ikaros K58.

Given the potential of Ikaros for SUMOylation, we tested whether the protein was modified in vivo in primary T cells. Nuclear extracts, prepared from murine thymocytes in the presence or absence of 1% SDS and the SUMO-isopeptidase inhibitor NEM, were examined for Ikaros protein expression. Three slower-migrating Ikaros protein species were detected only in the presence of NEM, indicating that a fraction of the protein is SUMOylated (Fig. 1C, +NEM/SDS, bands M1, M2, and B1; see next section). Other Ikaros-interacting proteins, i.e., mSin3A and HDAC2, were also tested and found not to be SUMOylated in thymocytes (data not shown).

Mapping of the Ikaros SUMOylation sites. We next investigated which of the Ikaros SUMOylation motifs were used in vivo. For this study, the putative lysine-SUMO acceptors were mutated to arginine singly or in combination. Vectors expressing an untagged version of Ikaros (Ik-1) or the SUMOylation motif mutant forms and GFP-SUMO1 were cotransfected in the epithelial 293T cell line. Ikaros proteins were immunoprecipitated with anti-Ikaros antibody and immunoblotted with Ikaros and GFP antibodies to better reveal the modified forms (Fig. 2A, α-Ikaros and α-GFP). In addition to the main Ikaros protein band, three slower-migrating species were observed with Ikaros antibodies (Fig. 2A, lanes 3 and 5), similar to those observed in thymocytes (Fig. 1C). Antibodies to GFP confirmed that these protein species were the result of Ikaros-GFP-SUMO1 conjugation (Fig. 2A, right half).

The first of the modified bands contains Ikaros protein that is monoSUMOylated at K58 as this disappears upon the K58 mutation (Fig. 2A, band M1, lane 6; also Fig. 1C). The second contains Ikaros that is monoSUMOylated at K240 as this is also affected by the corresponding mutation (Fig. 2A, band M2, lane 7; also Fig. 1C). Ikaros protein that is biSUMOylated at both the K58 and K240 sites runs at the third position, and this is equally affected by either of the single mutations (Fig. 2A, band B1, lane 12; also Fig. 1C). In sharp contrast to the Ikaros mutations at K58 and K240, mutations at K425 and K459 had no effect on the slower-migrating forms (Fig. 2A, lanes 8 and 9). Similar findings on SUMOylation of Ikaros and its mutant forms were obtained with the human cell line U2 OS (data not shown). Other members of the SUMO family, SUMO2 and -3, were also tested for the ability to be conjugated to Ikaros. A series of expression and immunoprecipitation studies demonstrated that all three of the SUMO proteins could be conjugated to Ikaros at positions K58 and K240 (data not shown).

The Ikaros sites of SUMOylation were confirmed in lymphoid cells, where Ikaros is normally expressed. The T-cell line NA1, derived from Ikaros-null mice, was infected with pMX-GFP retroviruses harboring either Ik-1, the Ik-1 K58 and 240R SUMOylation mutant form, or the parental vector as a control (13). pMX-GFP-Ik-1 and pMX-GFP-Ik-1 K58 and 240R, but not the parental control vector, reconstituted Ikaros protein expression, which was detected as a major band at ~65 kDa (Fig. 2B). Reconstitution with Ik-1, but not with the SUMOylation mutant form of Ikaros, also provided three Ikaros-related slower-migrating protein species (Fig. 2B). These were similar in size to those observed in primary thymocytes that express Ikaros (Fig. 1C). This study verifies that the higher-molecular-weight Ikaros species observed in thymocytes and in an Ikaros-reconstituted T-cell line represent SUMOylated forms of the Ikaros protein. Furthermore, it shows that Ikaros lysines 58 and 240 are major sites for SUMOylation in vivo in both lymphoid and nonlymphoid cells.

Ikaros SUMOylation is actively regulated by SUMO isopeptidases and E3 ligases. SUMOylation is a dynamic process that is controlled by opposing enzymatic activities. In line with previous observations, Ikaros SUMOylation was examined in the presence of differing amounts of SUMOylase and deSUMOylase.

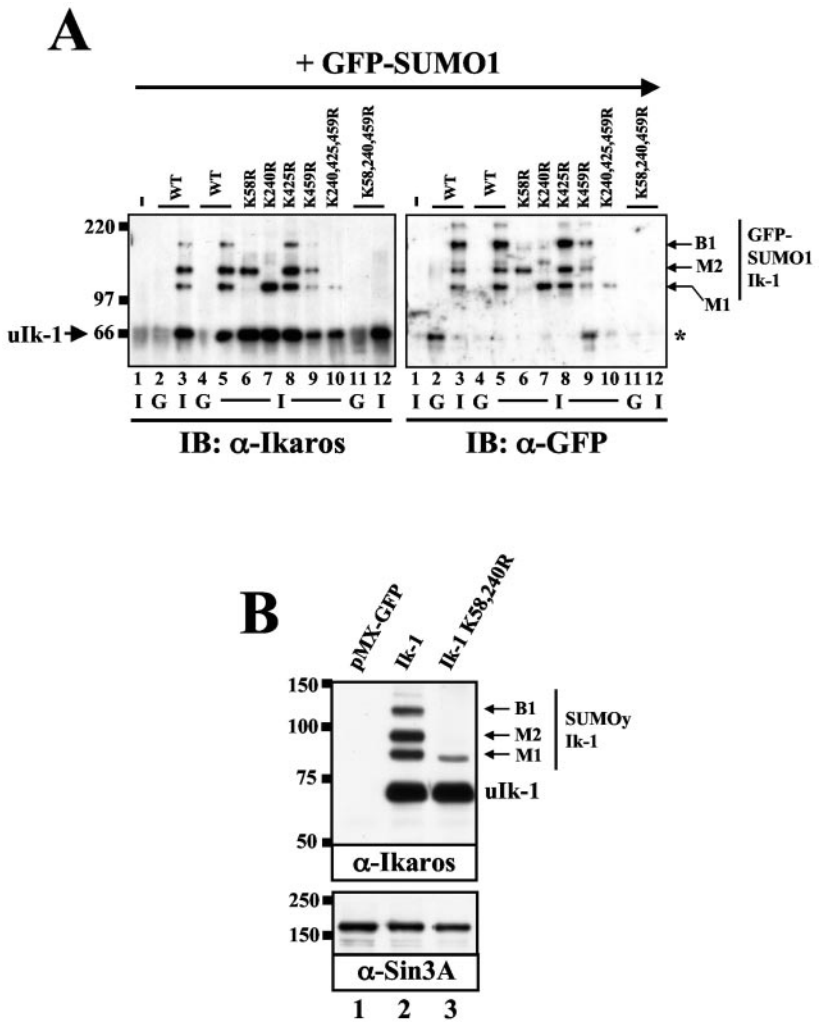

FIG. 2. Ikaros SUMOylation sites established in nonlymphoid and lymphoid cells. (A) GFP-tagged SUMO1 was coexpressed with wild-type Ik-1 (WT) or the Ikaros mutant forms Ik-1K58R, Ik-1K240R, Ik-1K425R, Ik-1K459R, Ik-1K240,425,459R, and Ik-1K58,240,459R into 293T cells. A mock transfection is also shown in lane 1 (vertical hyphen). Whole-cell extracts were immunoprecipitated with Ikaros antibody (I) or control immunoglobulin G (G). Immunoprecipitates were separated by SDS–8% PAGE and analyzed by Western blotting with Ikaros and GFP antibodies consecutively. The unmodified (uIk-1) and SUMOylated forms of Ikaros (M1 [monoSUMOylated at K58], M2 [monoSUMOylated at K240], and B1 [biSUMOylated at K58 and K240]; see Results for an explanation) are indicated (GFP-SUMO1 Ik-1). In the GFP blot, the asterisk indicates an unspecific cross-reactive band. (B) An Ikaros-null T-cell line, NA1, was infected with pMX-GFP-IRES Ik-1 (Ik-1, lane 2), the SUMO double-mutant form (K58,240R, lane 3), or the pMX-GFP-IRES control (pMX-GFP, lane 1). At 24 h after infection, cells expressing GFP were sorted and whole-cell extracts were prepared in the presence of 1% SDS and 1 mM NEM. Proteins were separated and analyzed by Western blotting with Ikaros antibodies. The unmodified (uIk-1) and SUMOylated (M1, M2, and B1) Ikaros forms are indicated (SUMOy Ik-1). A loading control immunoblot (IB) is also shown (Sin3A). The positions of approximate molecular weight markers are shown at the left of each panel.

GFP-SUMO1-Ikaros proteins were detected upon coexpression of Ik-1 and GFP-SUMO1 in 293T cells (Fig. 3A, Contr.). Ikaros SUMOylation was progressively reduced when the level of the isopeptidase Senp1 was increased (Fig. 3A, MT-Senp1). A similar effect on Ikaros deSUMOylation was also observed when the expression of another isopeptidase, Axam, was increased (data not shown).

In contrast to the effect of isopeptidases, an increase in SUMO E3 ligase (PIASxα) increased Ikaros SUMOylation (Fig. 3B; see also Fig. 5B and 7B). Immunoprecipitation studies revealed a strong interaction between Ikaros and the E3 ligase PIASxα and a weaker interaction with PIAS3 (Fig. 3C). The PIASxβ and PIAS1 family members were not detected within the Ikaros immunoprecipitates (Fig. 3C). Thus, Ikaros SUMOylation is regulated by the PIAS family of ligases, probably through Ikaros' specific interactions with two members of its family.

Ikaros SUMOylation mutant forms are more potent transcriptional repressors. We next examined whether SUMOylation of Ikaros can influence its activity as a repressor or activator of transcription. Ikaros' function as a transcriptional repressor can be revealed upon its tethering to the Gal4 DNA-binding domain (29). Gal4–Ik-1 or the Gal4–Ik-1–SUMO mutant form was coexpressed with the 5XGal4-tk-CAT reporter in U2 OS or NIH 3T3 cells (Fig. 4A and data not shown). As previously shown (29), Gal4–Ik-1 repressed transcription (Fig.

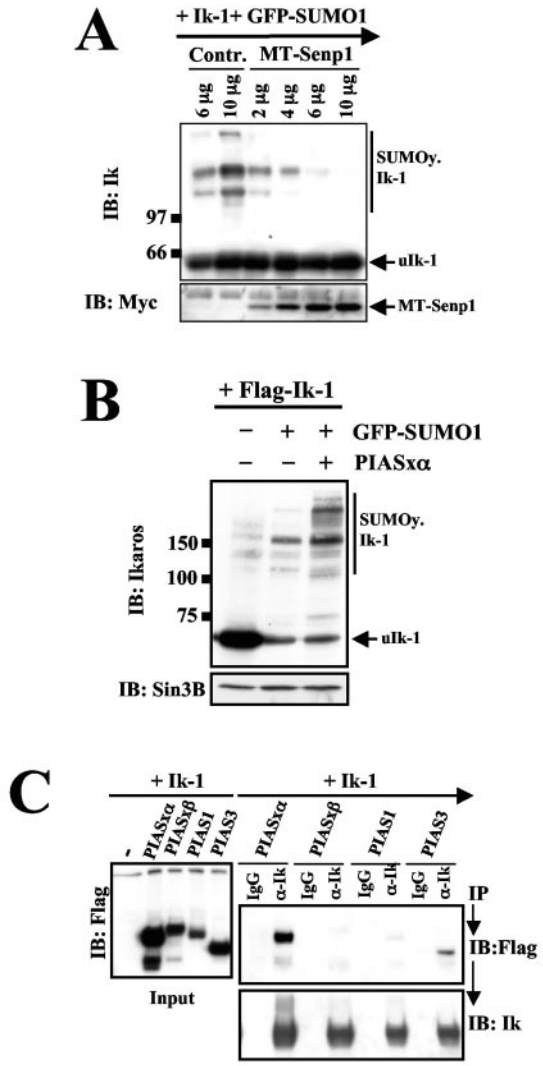

FIG. 3. SUMOylation of Ikaros is controlled by the relative activities of SUMOylases and deSUMOylases. (A) Ik-1 and GFP-SUMO1 were coexpressed in 293T cells with increasing amounts of a Myc-tagged version of the deSUMOylase Senp1 (MT-Senp1) or empty vector as a control (Contr.). Ikaros proteins were immunoprecipitated and analyzed by Western blotting. SUMOylated (SUMOy. Ik-1) and unmodified (uIk-1) Ikaros proteins are shown. The level of expression of Senp1 is shown in the lower part. (B) FlagIk-1 was coexpressed with GFP-SUMO1 with or without PIASxα in 293T cells. Whole-cell extracts were subjected to Western blot analysis with Ikaros. SUMOylated (SUMOy. Ik-1) and unmodified (uIk-1) Ikaros proteins are demarcated. A loading control for the immunoblots (IB) is shown (Sin3B). Positions of approximate molecular weight markers are also indicated. (C) Ikaros interactions with SUMO E3 ligases. Ik-1 was coexpressed with the Flag-tagged SUMO E3 ligases PIASxα, PIASxβ, PIAS1, and PIAS3 in 293T cells. Whole-cell extracts were subjected to immunoprecipitation with Ikaros antibodies (α-Ik) or control mouse immunoglobulin G (IgG) and probed with Flag (IB:Flag) and Ikaros (IB: Ik) antibodies. The expression levels of the different E3 ligases are shown at the left (Input).

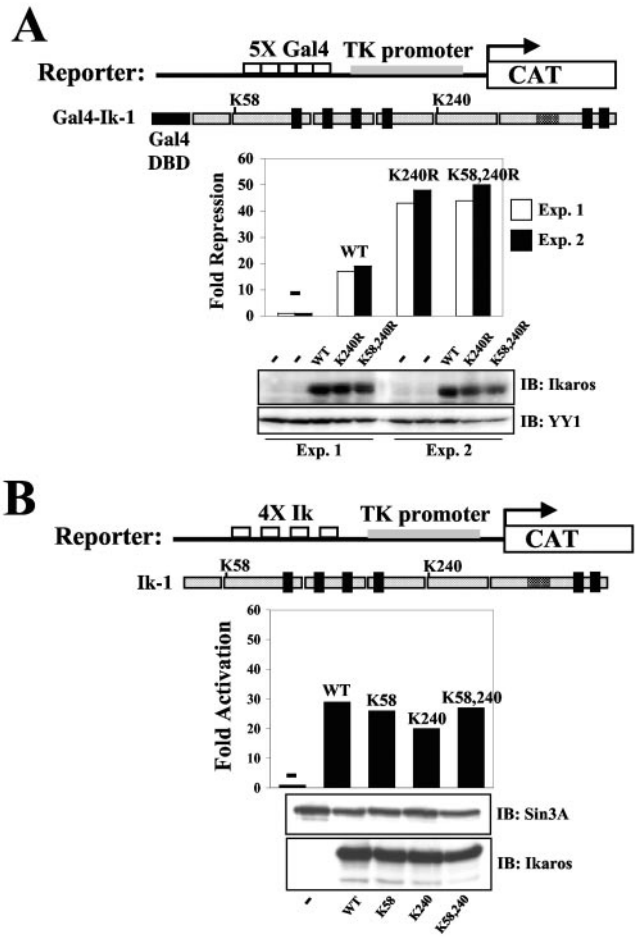

FIG. 4. SUMOylation of Ikaros specifically interferes with its ability to repress transcription. (A) Gal4 (−) or Gal4–Ik-1 fusion proteins (wild type [WT], K240R, and K58,240R) were cotransfected with the 5XGal4-tk-CAT reporter and GH control into U2 OS cells (described in Materials and Methods). Cell extracts were analyzed for CAT activity and protein expression. Reporter activity was normalized to GH levels, and fold repression relative to the Gal4 vector was calculated (29). Schematic diagrams of the Gal4-Ikaros fusion protein and the reporter used are depicted. Results of two representative experiments (Exp.), out of seven with similar findings, are displayed. Western blots (immunoblots [IB]) assessing protein expression are shown. YY1 protein expression was used as a loading control. (B) Ikaros' activity as a potentiator of gene expression is not affected by SUMOylation. Wild-type Ik-1 (WT), Ik-1 K58R (K58), Ik-1 K240R (K240), Ik-1 K58,240R (K58,240), or an expression vector control was cotransfected together with the 4XIk-tk-CAT reporter and the GH control plasmid into U2 OS cells. Reporter activity was normalized to GH levels, and fold activation relative to that obtained with the empty vector was calculated (28). One representative experiment, out of three, is shown. Protein expression is shown by immunoblots (IB), which used Sin3A as a loading control. In both panels, schematic diagrams of the Ikaros protein and the reporters are provided. In both panels, each experimental point was performed in duplicate with less than 5% variability.

4A, WT). Significantly, the Ikaros K58R (data not shown), K240R, and K58 and 240R SUMOylation mutant forms were stronger repressors of transcription than the wild-type Ikaros protein, a fraction of which is likely SUMOylated (Fig. 4A). Interestingly, a similar increase in repression was detected with either of the single-mutant forms or the double-SUMOylation mutant form of Ikaros, suggesting that co-occupancy of these SUMOylation sites is required to relieve repression. As shown by Western blot analysis, the effect of the mutations in Ikaros

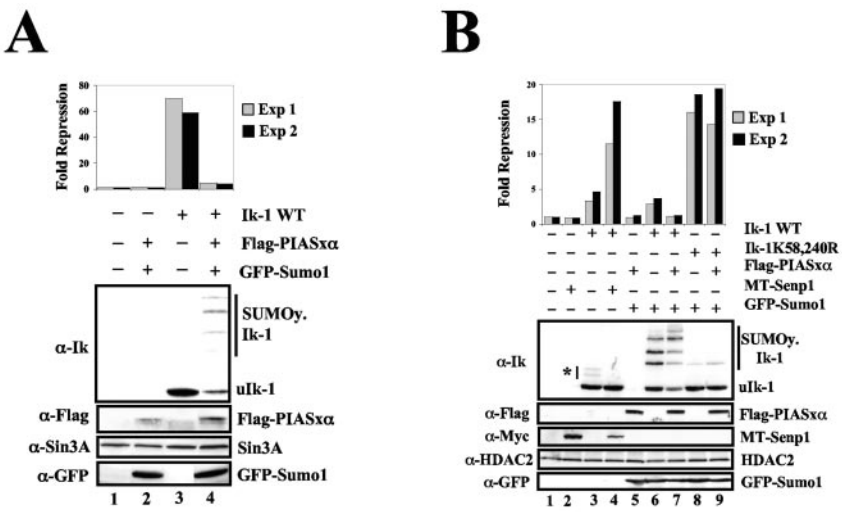

FIG. 5. Functional interactions of Ikaros with the SUMO pathway. (A) One microgram of Gal4 (lanes 1 and 2) or Gal4–Ik-1 (lanes 3 and 4) was cotransfected, together with the 5XGal4-tk-CAT reporter, Flag-PIASxα (3 μg), and GFP-SUMO1 (2 μg) where indicated, into U2 OS cells. Total extracts were prepared in the absence of NEM for CAT assay or in the presence of the deSUMOylase inhibitor for Western blotting. Western blot assays of the indicated proteins were performed to assess their expression and the levels of Ikaros SUMOylation. (B) Gal4, Gal4–Ik-1, and Gal4–Ik-1 K58,240R expression plasmids (0.5 μg of each) were cotransfected together with Gal4-tk-CAT reporter and Flag-PIASxα plus GFP-SUMO1 or MT-Senp1 (3 μg) into U2 OS cells. Samples were processed as described for panel A. Two independently performed experiments (Exp), out of four with similar findings, are displayed. Unmodified (uIk-1) and SUMOylated (SUMOy. Ik-1) Ikaros proteins are shown. The bar and asterisk indicate Ikaros modified by endogenous SUMO protein. In both panels, each experimental point was performed in duplicate with less than 5% variability. WT, wild type.

on its repression activity was not due to a change in protein expression or stability (Fig. 4A, bottom, and data not shown).

The effect of SUMOylation on Ikaros' ability to potentiate gene expression was also examined in a transient transcription assay (28). Ikaros and its SUMO mutant forms were cotransfected with a 4XIk-tk-CAT reporter (32) in U2 OS cells (Fig. 4B). Expression of either the wild-type Ikaros protein or its SUMOylation mutant forms provided a similar strong potentiation of reporter activity (Fig. 4B).

Taken together, these data support the model in which SUMOylation of Ikaros at both the K58 and K240 sites specifically negates its ability to repress gene expression.

The SUMO pathway directly regulates Ikaros' activity as a repressor. Importantly, Ikaros mutations that prevent its SUMOylation enhance its potential as a transcriptional repressor (Fig. 4A). The role of the SUMO pathway in regulating Ikaros' repressor function was examined by varying the level of enzymes that have disparate effects on SUMOylation.

Gal4–Ik-1 and the 5XGal4-tk-CAT reporter were coexpressed with or without GFP-SUMO1 and the E3 ligase PIASxα in U2 OS cells (Fig. 5A). Cell lysates were prepared for CAT assays and for Western blot analysis. As previously shown (29), expression of Gal-4–Ikaros represses the Gal-4 reporter (Fig. 5A, lane 3). However, when Gal-4–Ikaros was coexpressed with PIASxα and GFP-SUMO1, high levels of reporter activity were observed and very little repression was obtained (Fig. 5A, lane 4). Importantly, coexpression of PIASxα resulted in a significant increase in both the level and complexity of Ikaros SUMOylation, whereas the overall Ikaros protein expressed in these two experimental points was not significantly different (Fig. 5A, Western blot assays, compare lanes 3 and 4; also data not shown). As expression of the E3 SUMOylase did not alter Gal4-dependent transcription, the observed effect is Ikaros dependent (Fig. 5A, lane 2).

Given that enzymes that promote SUMOylation attenuate Ikaros' repression activity, we set out to test whether the converse was also true; that is, whether deSUMOylases enhance Ikaros-mediated repression. To demonstrate an increase in an already strong repression potential, we adapted the experimental design to achieve lower levels of Ikaros protein. As shown in Fig. 5B, the amount of protein generated by 0.5 μg of Gal4–Ik-1 gave moderate repression (Fig. 5B, lane 3). Coexpression of the deSUMOylase Senp1 increased Ikaros-mediated repression and the level of unmodified Ikaros protein, possibly by antagonizing its SUMOylation (Fig. 5B, compare lanes 3 and 4). It is important to note that the level of repression provided by wild-type Ikaros and deSUMOylases was similar to that generated by the Ikaros SUMO mutant forms when expressed at similar levels (Fig. 5B, compare lanes 4 and 8).

As shown in Fig. 5A, coexpression of PIASxα and GFP-SUMO1 increased Ikaros SUMOylation, decreased the level of unmodified protein, and reduced its repression activity (Fig. 5B, lane 7). In contrast, coexpression of PIASxα with the Ikaros K58 and K240R SUMOylation mutant form had no such effect on its strong repression activity (Fig. 5B, lane 8).

Taken together, these studies demonstrate that SUMOylation can directly and inversely regulate transcriptional repression by Ikaros. The more SUMOylated Ikaros protein there is, the less repression it can provide.

Ikaros SUMOylation does not interfere with its nuclear localization to pericentromeric heterochromatin. To determine the mechanism by which SUMOylation regulates Ikaros-mediated repression, we examined the effects on the protein's

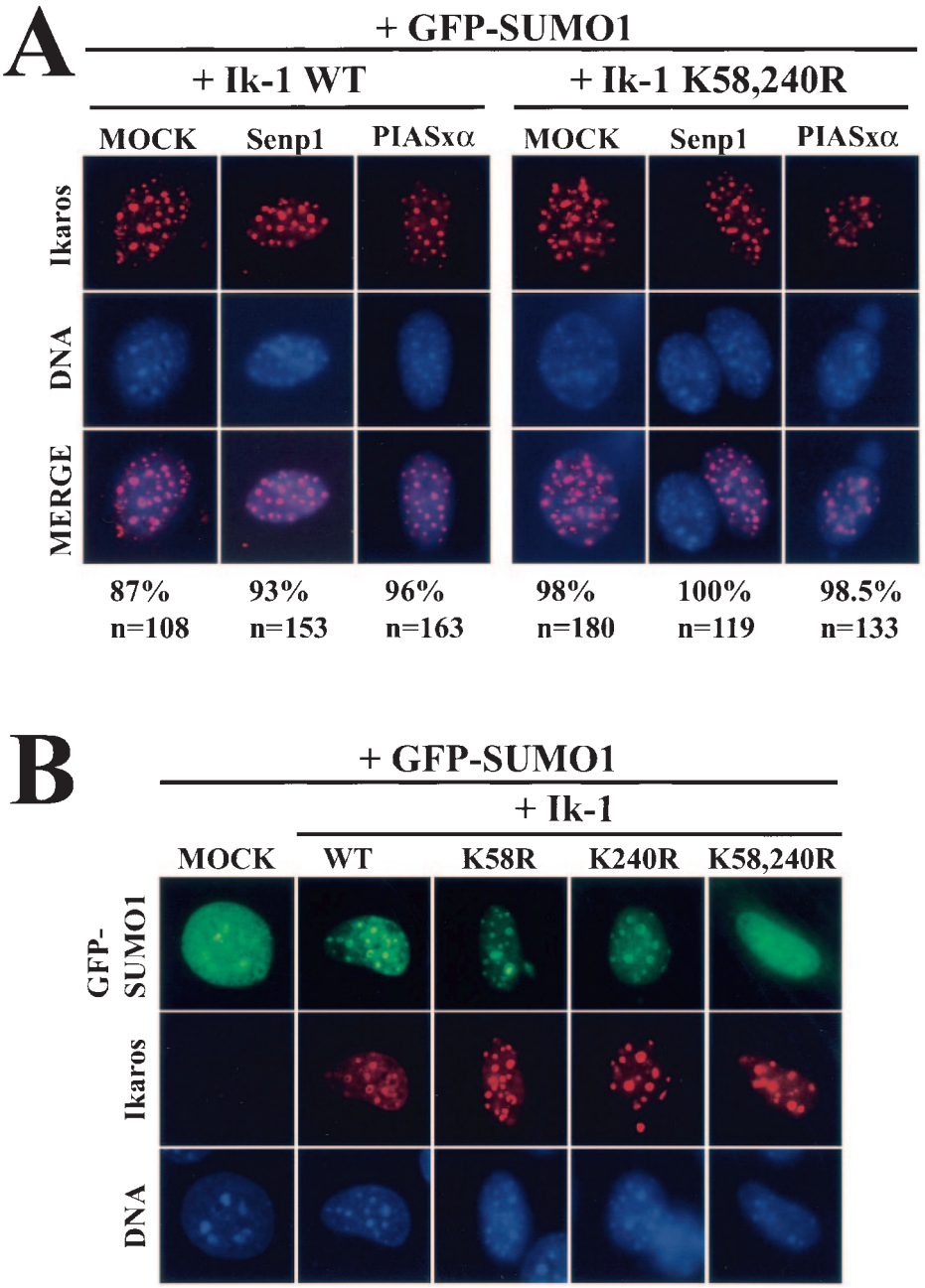

FIG. 6. SUMOylation of Ikaros does not interfere with its localization into pericentromeric heterochromatin. (A) Wild-type Ik-1 (WT) and the SUMO double-mutant form (Ik-1 K58,240R) were coexpressed together with GFP-SUMO1 and the isopeptidase Senp1 or GFP-SUMO1 and the E3 ligase PIASxα into NIH 3T3 cells as indicated. Cells were fixed and analyzed by immunofluorescence microscopy for Ikaros proteins. Condensed DNA in centromeric heterochromatin was revealed with 4′,6′-diamidino-2-phenylindole (DAPI) (DNA). The percentage of cells with Ikaros localizing in heterochromatin and the number of cells counted for each experimental condition (n) are shown. (B) Wild-type Ik-1 (WT) and the indicated SUMO mutant forms were coexpressed together with GFP-SUMO1 in NIH 3T3 cells. Cells were fixed and analyzed by fluorescence microscopy for GFP and Ikaros proteins. Condensed DNA in centromeric heterochromatin was revealed with DAPI (DNA).

nuclear localization. In proliferating cells, Ikaros undergoes a dynamic redistribution into pericentromeric heterochromatin, an event that has been correlated in the past with its function as a repressor and silencer of gene expression (1, 24).

Untagged Ikaros and its SUMOylation mutant form (K58 and K240R) were cotransfected together with GFP-SUMO1 and Senp1 or PIASxα into NIH 3T3 fibroblasts where, as in cycling lymphocytes, Ikaros localizes into pericentromeric heterochromatin (4, 28). Immunofluorescence analysis with Ikaros antibodies indicated that most of both wild-type Ikaros and its SUMOylation mutant form localized into pericentromeric heterochromatin (Fig. 6A). The localization of Ikaros (and its single- or double-SUMOylation mutant form) remained unaffected by the presence or absence of GFP-

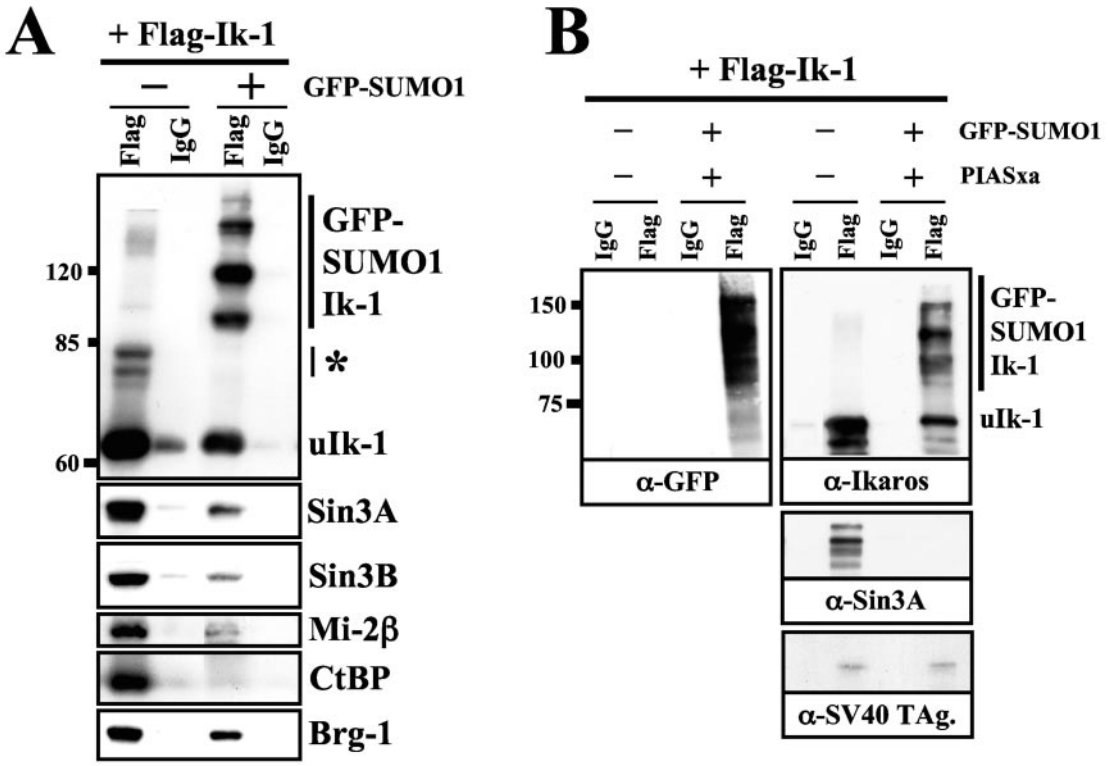

FIG. 7. SUMOylation interferes with Ikaros' ability to associate with corepressors of transcription. (A) CMV2 Flag-tagged Ikaros (Flag–Ik-1) was cotransfected together with (+) or without (−) GFP-SUMO1 into 293T cells. Ikaros proteins were immunoprecipitated (IP) with a mouse immunoglobulin G-protein G-agarose (IgG) or anti-Flag(M2)-agarose (Flag) beads. Proteins were eluted from the precipitated immunocomplexes with an excess of Flag(M2) peptide. Eluted proteins were separated by SDS–7% PAGE and analyzed by Western blotting for Brg-1, CtBP, Mi-2β, Sin3A, Sin3B, and Ikaros proteins. Unmodified (uIk-1) and GFP-SUMO1-modified (GFP-SUMO1 Ik-1) Ikaros proteins are indicated. The asterisk indicates an Ikaros species likely monomodified by endogenous SUMO. (B) Flag–Ik-1 was coexpressed with GFP-SUMO1 (+) and PIASxα (+) into 293T cells. Ikaros proteins were immunoprecipitated (IP) and analyzed as described for panel A. Western blot assays for GFP, Ikaros, Sin3A, and SV40 TAg are shown. Unmodified and SUMOylated Ikaros species are demarcated (uIk-1 and GFP-SUMO1 Ik-1, respectively). In both panels, the positions of approximate molecular weight markers are shown at the left.

SUMO1 (Fig. 6 and data not shown) or by the presence of the isopeptidase Senp1 or the E3 ligase PIASxα (Fig. 6A and data not shown).

The distribution of GFP-SUMO1 was also revealed by fluorescence. In the absence of Ikaros or in the presence of the K58 and 240R double-SUMOylation mutant form, GFP-SUMO1 was widely distributed throughout the nucleus with only a small selective presence in pericentromeric heterochromatin. However, in the presence of wild-type Ikaros or a single-SUMOylation mutant (K58 or K240) form of Ikaros, most of the GFP-SUMO1 protein was detected in pericentromeric heterochromatin together with Ikaros (Fig. 6B). This is likely the result of interaction and covalent association between Ikaros and GFP-SUMO1 that dictates the overall nuclear distribution of GFP-SUMO1.

Taken together, these results indicate that Ikaros SUMOylation does not influence its activity as a repressor by altering its nuclear localization. SUMOylated Ikaros, which has lost its function as a repressor, can still localize into pericentromeric heterochromatin.

SUMOylation of Ikaros inhibits its interactions with transcriptional corepressors. Ikaros has been proposed to regulate transcription by associating with chromatin remodeling and other transcription regulators (24, 26, 27, 29). SUMOylation of Ikaros may specifically interfere with its ability to associate with some of these factors, which are engaged in repression.

To examine this possibility, Flag-tagged Ik-1 was expressed in 293T cells without and with GFP-SUMO1, and its association with its previously reported interactors Mi-2β (NuRD complex), Sin3A, Sin3B, CtBP, and Brg-1 (Swi/Snf complex) was tested by Ikaros immunoprecipitation, followed by Western blotting (Fig. 7A). In the absence of GFP-SUMO1, a small fraction of Ikaros was SUMOylated and strong interactions with endogenously expressed Sin3A, Sin3B, CtBP, Mi-2β, and Brg-1 were detected (Fig. 7A, − GFP-SUMO1). In sharp contrast, when GFP-SUMO1 was expressed, a major fraction of Ikaros was SUMOylated and its interaction with Sin3A, Sin3B, CtBP, and Mi-2β was greatly reduced (Fig. 7A, + GFP-SUMO1). The effect was most pronounced with CtBP, which was almost absent in the Ikaros immunoprecipitate. The interactions of Ikaros with its corepressors were also evaluated upon coexpression of GFP-SUMO1 and the SUMO E3 ligase PIASxα. As shown in Fig. 7B, a dramatic increase in Ikaros SUMOylation disrupted Sin3A interactions.

In sharp contrast to Ikaros interactions with Sin3A, Sin3B, Mi-2β, and CtBP, which were greatly reduced upon its SUMOylation, Ikaros association with the Swi/Snf ATPase Brg-1 was mildly affected (Fig. 7). A previous established in-

teraction between Ikaros and the simian virus 40 (SV40) T antigen (TAg) in 293T cells was also not influenced by SUMOylation (Fig. 7B).

These data strongly support a scenario in which SUMOylation of Ikaros specifically inhibits its interactions with HDAC-dependent (Sin3 and Mi-2β), as well as HDAC-independent (CtBP), corepressors of transcription. It does not, however, significantly affect its interactions with components of the Swi/Snf complex or with the viral protein SV40 TAg.

DISCUSSION

Here we provide the first evidence that Ikaros interacts with components of the SUMO pathway and is SUMOylated in vivo. We identify two SUMOylation sites on Ikaros and show that their simultaneous modification supports the loss of Ikaros' repression function. We demonstrate that SUMOylation of Ikaros disrupts its interactions with HDAC-dependent and HDAC-independent corepressors of transcription but does not affect its localization into pericentromeric heterochromatin.

Different lines of evidence have provided support for the idea that Ikaros directly interacts with components of the SUMO pathway. Yeast two-hybrid studies demonstrated its potential interaction with SUMO1, the component of the E1-activating enzyme heterodimer Uba2, and the E2-conjugating enzyme Ubc9. Immunofluorescence studies with mammalian cells have shown an Ikaros-dependent localization of SUMO1 into pericentromeric heterochromatin that is disrupted by mutations in the Ikaros SUMOylation motifs (data not shown). Finally, Ikaros immunoprecipitations from these cells revealed a preferential association with two of the four members of the SUMO E3 ligase family, PIASxα and PIAS3.

Ikaros is predominantly SUMOylated in vivo at lysines 58 and 240, which are located at the N-terminal half of the protein. These two Ikaros SUMOylation motifs are highly conserved across species (16), suggesting that this type of regulation of the Ikaros protein is conserved through evolution. In addition, sites corresponding to Ikaros K58 and K240 are also found in Aiolos, suggesting a similar type of control in the second Ikaros family member.

Mutations in either of the SUMOylation sites of Ikaros drastically elevated its activity as a repressor of transcription, whereas mutations in both gave no added effect. Consistent with these findings, Ikaros' repressive potential was reduced when the protein was SUMOylated at both K58 and K240 but remained unaffected when it was monoSUMOylated at either of these sites (Fig. 4). Taken together, these studies indicate that Ikaros' activity as a repressor is dependent on the complexity of SUMOylation, as both of its SUMOylation sites must be modified to relieve repression.

The level of Ikaros SUMOylation and its potential for repression can be regulated by the opposing activities of SUMOylases and deSUMOylases. Whereas SUMOylases decrease repression mediated by Ikaros, deSUMOylases like Senp1 increase its repression activity to a level that is similar to that obtained with the SUMOylation mutant forms of Ikaros. Relative expression of these enzymes, as well as accessibility of the Ikaros protein to these factors, may dictate its overall SUMOylation. For example, the Ikaros-interacting PIAS E3 ligases are distributed in promyelocytic leukemia bodies, chromatin, and nuclear matrix (19, 40), where Ikaros is likely SUMOylated. Of the deSUMOylating enzymes, Senp1 and Axam are present in the nucleoplasm whereas others are in the cytoplasm and nucleolus (34).

The mechanism by which SUMOylation controls Ikaros repression function is central to our understanding of its dual function as an activator and repressor of gene expression. Ikaros' ability to localize into pericentromeric heterochromatin has been correlated with its ability to repress and silence gene expression (2, 7). However, although SUMOylation alleviates Ikaros' repression it does not do so by affecting its pericentromeric heterochromatin localization. This argues that Ikaros' localization into this nuclear compartment is not central to the protein's ability to repress transcription. However, SUMOylation disrupts the well-established associations between Ikaros and corepressors of transcription like CtBP, Sin3, and Mi-2β of the NURD complex (24, 26, 29). The inability of Ikaros to be integrated into HDAC-dependent and HDAC-independent pathways of repression is likely to inadvertently affect its function as a repressor. SUMOylation, however, has no significant effect on Ikaros' interactions with components of the Swi/Snf complex, which can provide chromatin fluidity and, like Ikaros, can positively regulate developmentally important loci like the gene for CD8 (3, 17).

How does SUMOylation of Ikaros interfere with its interactions with HDAC-dependent and -independent corepressors? SUMOylation of Ikaros may simply block access to their binding sites. For example, the Ikaros K58 SUMOylation site lies next to the CtBP interaction motif (amino acids 34 to 38) and may be responsible for the more severe disruption of Ikaros-CtBP interactions relative to the other corepressors. The proteins Mi-2β and Sin3 share binding domains located at the N- and C-terminal regions of the Ikaros proteins, and accessibility to these common interaction domains is likely to be similarly affected by SUMOylation. SUMOylation may affect Ikaros-corepressor associations by inducing conformational changes that alter their interaction interface.

Studies with primary thymocytes and cycling T cells show that a small but significant fraction of the total Ikaros protein is SUMOylated. Nonetheless, mutation of the SUMOylation sites of Ikaros has a strong effect on its activity as a repressor. Given the dynamic and transient nature of SUMOylation, it may be required to initiate the disassembly of Ikaros-repressor complexes but not to maintain them in a separate state. DeSUMOylated Ikaros may then be preferentially retained in a different type of protein complex, i.e., Swi/Snf, that is not greatly affected by SUMOylation. SUMO modifications regulating the disassembly of septin ring structures during mitosis have been reported in yeast (20).

In conclusion, SUMO conjugation may play a pivotal role in dictating Ikaros' ability to interact with distinct transcriptional cofactors. This type of posttranslational modification may underlie the protein's ability to be integrated into a pool of potentiators versus repressors of gene expression. In the future, it will be important to determine how signaling pathways, for example, those that control Ikaros phosphorylation, impact SUMOylation. It is likely that coordinating these distinct posttranslational modifications is critical for effecting key events in gene expression during lymphocyte development.

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