FLI-1 FUNCTIONALLY INTERACTS WITH PIASxα, A MEMBER OF THE PIAS E3-SUMO-LIGASE FAMILY

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ABSTRACT

FLI-1 is a transcription factor of the ETS family involved in several developmental processes, which becomes oncogenic when overexpressed or mutated. As the functional regulators of FLI-1 are largely unknown, we performed a yeast two hybrid screen with FLI-1 and identified the SUMO-E3-ligase PIASxα/ARIP3 as a novel in vitro and in vivo binding partner of FLI-1. This interaction involved the ETS domain of FLI-1 and requires the integrity of the SAP domain of PIASxα/ARIP3. SUMO-1 and Ubc9, the E2 component in the sumoylation pathway were also identified as interactors of FLI-1. Both PIASxα/ARIP3 and the closely related PIASxβ isoform specifically enhanced FLI-1 sumoylation on lysine residue 67, located in its amino-terminal activation domain. PIASxα/ARIP3 re-localized the normally nuclear but diffusely distributed FLI-1 into PIASxα nuclear bodies and repressed FLI-1 transcriptional activation as assessed using different EBS-dependent promoters and different cell systems. PIASxα repressive activity is independent of sumoylation and did not result from inhibition of FLI-1 DNA binding activity. Analysis of the properties of a series of ARIP3 mutants show that PIASxα/ARIP3 repressive properties required its physical interaction with FLI-1, identifying PIASxα as a novel co-repressor of FLI-1.

INTRODUCTION

FLI-1 (Friend leukemia integration 1) is an ETS (E26 Transformation Specific) transcription factor that plays an essential role in the megakaryocytic, granulocytic and erythrocytic lineages and in endothelial cell development (1-4). FLI-1 shares with other ETS family members a conserved ETS domain responsible for nuclear targeting and specific binding to DNA elements containing a consensus GGAA/T core (EBS, ETS Binding Site, for review see (5)). FLI-1 also possesses a Pointed/SAM domain embedded in its major amino-terminal activation domain and a C-terminal domain that also contributes to its transcriptional activation properties (for review, see (5)). Consistent with its biochemical and biological functions, a number of genes specific of the megakaryocytic, erythrocytic and granulocytic lineages have been found to be down-regulated in FLI-1-deficient bone marrow cells, with a subset of these genes being direct transcriptional targets of FLI-1 ((1,3,4) and references therein). Remarkably, lineage-restricted expression of many megakaryocytic-specific genes relies on the cooperation between FLI-1 and GATA-1 on composite DNA elements present in their promoter/enhancer sequences ((6) and references therein). Depending on the cellular and promoter contexts, FLI-1 can also repress EBS-driven transcription through ill-characterized mechanisms (7,8). Finally, FLI-1 can interact in trans with other transcriptional regulators including nuclear hormone receptors and erythroid-specific Kruppel-like factor.
(EKLF), thereby modifying transcriptional output in pathways involving these factors (9,10).

FLI-1 was originally identified as a common proviral integration site in erythroleukemia induced in newborn mouse by the F-MuLV component of the Friend virus complex (reviewed in (5)). Consistent with its central role in F-MuLV-induced erythroleukemia, enforced expression of FLI-1 in primary erythroblasts and erythroleukemic cell lines has been shown to strongly interfere with the normal, Epo-induced differentiation of these cells (8,11), properties that require its EBS-dependent transcriptional activation properties (12,13). Other studies show that FLI-1 might also contribute to the transformed phenotype through interference with the expression of genes important to erythroblast differentiation (8,10,14).

FLI-1 is rearranged in Ewing sarcoma, a childhood pediatric tumor of neuro-ectodermal origin as the result of a t(11;22)(q24;q12) chromosomal translocation, which fuses of the 5' end of the EWS gene to the 3' end of FLI-1 and the expression of a chimeric EWS-FLI-1 protein endowed with abnormal transcriptional regulatory properties and transforming activity (for review, see (15)).

PIAS1 and PIAS3 were identified as inhibitors of the cytokine receptor/JAK-induced activation of STAT1 and STAT3 DNA binding, respectively. In mammals, the PIAS family also includes PIASxα, PIASxβ and PIASy. PIAS proteins share three conserved domains: an amino-terminal SAP (SAF, Acinus, PIAS) domain, a central RING finger-like domain and a carboxy-terminal serine and acidic residues-rich domain. The SAP domain binds AT-rich DNA sequences known as scaffold attachment region (SAR) or nuclear matrix attachment sequences (MAR), involved in the topological organization of chromatin and in the regulation of gene expression (for review, see (16)). The RING-like domain of PIAS proteins is instrumental in their ability to function as E3-like SUMO ligase that favor the covalent modification of a variety of target proteins by the three known members of the SUMO (Small, Ubiquitin-like Modifier) family (for review, see (17)). Sumoylation is a three step process similar to protein ubiquitinylination, involving an E1 activating enzyme (AOS1/UBA2), an E2 conjugating enzyme (Ubc9) and E3 ligases, including RanBP2, Polycomb2 (Pc2) and PIAS proteins. Sumoylation regulates a number of protein-protein interactions involved in critical cellular functions including protein stability, nuclear import, transcriptional regulation, biogenesis of sub-nuclear bodies and chromosome structure.

As the functional regulators of FLI-1 are largely unknown we performed a yeast two hybrid screen to identify novel FLI-1 interactors. We identified PIASxα/ARIP3 as a new partner and co-regulator of FLI-1. ARIP3 was found to interact physically with FLI-1, to repress its transcriptional activity and to sequester it into ARIP3/PIASxα nuclear bodies. FLI-1 is sumoylated on a single lysine residue in its transcriptional activation domain and ARIP3 is shown to be an E3 SUMO ligase for FLI-1 in vivo and in vitro. Interestingly, the repressive properties of ARIP3 are not dependent upon its SUMO ligase activity and FLI-1 sumoylation, but critically depend upon its ability to physically complex FLI-1 into a transcriptionally inactive protein complex.

EXPERIMENTAL PROCEDURES

Reagents - Anti-FLI-1 (SC-356) and anti-ERK2 (SC-154) antibodies were purchased from Santa Cruz. Anti-flag (F-3164) was purchased from Sigma and anti-HA (#1667475) from Roche. The pan-ETS antibody was a kind gift from Dr. N. Bhat. The anti-ARIP3 antibody has been previously described (18).

Yeast two-hybrid screen - The DNA fragment encoding FLI-1[225-373] was subcloned in frame with the LexA DNA binding domain in the BTM116 vector to produce the bait for yeast two-hybrid studies. A human bone marrow cDNA library fused to the Gal4 activation domain (GalAD) was from Clontech. Yeast two-hybrid screening was performed as described previously (19) and a total of 5x10^6 transformants were screened. Positive clones were selected on plates lacking uracil, histidine, tryptophan, lysine and leucine, followed by β-galactosidase assays and verified by one-on-one transformation assays.

DNA constructs - To obtain the GST-FLI-1[3-208], GST-FLI-1[3-286], GST-FLI-1[188-452], and GST-FLI-1[250-452] fusion proteins, the corresponding regions were PCR-amplified from the human FLI-1 cDNA
using pBSwFLI-1 (13) as matrix with 5'-CCG TCG ACG GGA CTA TTA AGG AGG CC TGT CG-3' as forward primer and 5'-GGG TCG ACG GTG GAG GGT GTA TTA TAG G-3' and 5'-GGG TCG ACA GGA ATT GCC ACA GCT GGA TCT GC-3' as reverse primer for FLI-1[3-208] and FLI-1[3-286], respectively; 5'-CCG GAT CTT CGC ACC TCA GTT ACC TCA GG-3' and 5'-CCG GAT CCT TGT CAC TCA GTT ACC TCA GG-3' as forward primer for FLI-1[3-208] and FLI-1[3-286], respectively; 5'-GGG TCG ACA GGA ATT GCC ACA GCT GGA TCT GC-3' as reverse primer. This resulted in bordering the PCR-amplified fragment by either a SalI restriction site for FLI-1[3-208] and FLI-1[3-286], or BamHI and SalI at their 5' and 3' extremities, respectively for the fragments encoding FLI-1[188-452] and FLI-1[250-452]. The respective SalI or BamHI-restricted PCR fragments were inserted in the SalI- or BamHI-restricted pGEX-4T. To generate pBS-HA-FLI-1, ∆EB-HA-FLI-1 (20) was partially digested with SstI+EcoRI and ligated into SstI+EcoRI-restricted pBluescript. Mutants FLI-1[K67R] and FLI-1[K217R] were generated by oligonucleotide site-directed mutagenesis using the Quikchange kit (Stratagene) following the manufacturers instructions, using pBS-HA-wtFLI-1 as matrix and the couple of mutagenic primers, 5'-GGG TCA ACG TCA GGC GGG AGT ATG ACC-3'/5'-GGT CAT ACT CCC GCC TGA GTG TGG GAG GTT GTA TTA TAG G-3' and 5'-CCT CAC GAT TGA GTG TCA GAG CTC TAG ATC TCC C-3', respectively. Mutant FLI-1(K67-217R) was generated using pBS-HA-FLI-1[K67R] as matrix and the mutagenic primers used for FLI-1[K217R]. To generate HA-tagged versions of these mutants in the corresponding ∆EB expression vector derivatives, mutagenized FLI-1 inserts were retrieved by XhoI+HindIII digestion and ligated into XhoI+HindIII-restricted ∆EB-HA (21). The expression vector pSG5-His-SUMO-1 and pSG5-His-HA-SUMO2 (22) and the pFLAG expression vectors encoding wtARIP3, ARIP3[Δ13-28], ARIP3[Δ1-102], ARIP3[Δ102-207], ARIP3[Δ198-337], ARIP3[Δ347-418], ARIP3[Δ346-475], ARIP3[Δ467-547], ARIP3[Δ467-487], ARIP3 W383A as well as in vitro translatable pTag2A-wtARIP3, pTag2A-ARIP3[Δ102-207], pTag2A-ARIP3[Δ347-418] and pTag2A-MIZ/PIASxβ have been previously described (23,24). To generate pTag2A-ARIP3[Δ13-28], pTag2A-ARIP3[Δ198-337] and pTag2A-ARIP3[Δ346-475], the Smal + BglIII cDNA insert from the respective pFLAG vectors were subcloned into the EcoRV + BamHI-restricted pTag2A (Stratagene). The ∆EB expression plasmids encoding HA-FLI-1wt, HA-FLI-1[276-373], tkD2A-Luc and (-270/-41)mFLI-1-Luc have been previously described (12,13). FLI-1[273-452] was made as follows. Using ∆EB-HA-FLI-1wt as a template, the FLI-1 DNA fragment corresponding to amino acids 273-452 was amplified using oligo's: 5'-GCC TCG AGG CCT GGA AGC GGG CAG ATC C-3' (containing a 5'-XhoI site) and 5'-CCA AGC TTC TAG TAG TAG CTG CC-3' (containing a 3'-HindIII site). The amplified DNA-fragment was digested with XhoI and HindIII and ligated in frame into XhoI/HindIII digested ∆EB-HA to generate ∆EB-HA-FLI-1[273-452], pSG513 3xHA-BirA was a kind gift from Dr. T.B. van Dijk (Erasmus medical center). pcDNA3 bio-peptide-FLI-1[M34A] was generated as follows. An oligo nucleotide encoding the Bio-peptide (biopeptide=MAS SMR QIL DS QKM EWR SNA GGS), 5'-CCG ATA TCC CAC CAT GGC CTC TTC CCT GAG ACA GAT CCT CGA CAG CCA GAA GAT GGA GTG GCG CTC CAA CGC AGG AGG CTC TAG ATC TCC C-3', was used as a PCR template using 5'-CCC GAT ATC CCA CCA TGG-3' (containing a 5' EcoRV site) and 5'-GGG AGA TCT AGA GCC TCC-3' (containing a 3' BglII site) as primers and the PCR product was ligated into pCR2.1-TOPO generating pCR2.1-TOPO-bio-peptide. pCR2.1-TOPO-bio-peptide was digested with EcoRV and BglIII and the fragment ligated into EcoRV/BglIII digested pBS-HA-FLI-1 digesting in-frame, the HA-tag with the bio-peptide-tag. Wild type FLI-1 contains an alternative translation initiation codon at position 34. To prevent alternative translation from AUG34 this codon was mutated into an alanine codon by site-directed mutagenesis kit and primers 5'-GGA CTC CCC GAG GCA GTC GCG GCC TTG GGG AGA TGG-3' and 5'-CCA TCA CCC GAA GCC CGA CGC GAC TGC TCT GCC TGG GAG AGG CTC TAG ATC TCC C-3', respectively. Mutant FLI-1(K67-217R) was released from the pBS-vector by digestion with free EcoRV and BglIII restriction enzymes. To generate pBS-HA-FLI-1 digesting in-frame, the HA-tag with the bio-peptide-tag. Wild type FLI-1 contains an alternative translation initiation codon at position 34. To prevent alternative translation from AUG34 this codon was mutation into an alanine codon by site-directed mutagenesis kit and primers 5'-GGA CTC CCC GAG GCA GTC GCG GCC TTG GGG AGA TGG-3' and 5'-CCA TCA CCC GAA GCC CGA CGC GAC TGC TCT GCC TGG GAG AGG CTC TAG ATC TCC C-3', respectively. Mutant FLI-1(K67-217R) was released from the pBS-vector by digestion with free EcoRV and BglIII restriction enzymes.
HindIII and ligated into HindIII-digested pcDNA3 to generate pcDNA3-Bio-peptide-FLI-1(M34A), referred to as biotagged-FLI-1 in the Result section. HA-ARIP3 was made as follows. Using FLAG-ARIP3 as a template, ARIP3 wt cDNA was amplified and fused N-terminally with the HA epitope through PCR with primers: 5'-GGA GAT CTG CAA CCA CTA TGG ACC CAT ACG ATG TTC CAG ATT ACG CGG CGG ATT TCG AGG AGT TGA GG-3' (containing a 5' BglII site followed by the cDNA encoding the HA-epitope fused in frame to the first 20 nucleotides of wtARIP3) and 5'-CGG AAT TCT CAC TGT TGC ACA GTC AAT CAC TGA TCA GA-3' (containing a 3' EcoRI site). The PCR product was cut with BglII and EcoRI and ligated into BamHI/EcoRI digested pcDNA3 obtaining pcDNA3-HA-ARIP3 wt.

Affinity chromatography of biotinylated proteins; immunoprecipitation and Western blot analyses - 1.0x10^6 293 cells were seeded in 60mm² petri dishes 24 hours prior to transfection and DNA transfected using the calcium phosphate co-precipitation method as described previously (25). 48 hours after transfection, cells were washed twice with ice-cold PBS and lysed in ice-cold lysis buffer (20mM Tris-HCl pH 8.0, 137mM NaCl, 10mM EDTA, 50mM NaF, 1% (vol/vol) Nonidet P-40, 10% (v/v) glycerol, 2mM Na3VO4, 1mM Pefabloc (Roche), 50mg/ml Aprotinin, 50mg/ml Leupeptin, 50mg/ml Bacitracin and 50mg/ml Iodoacetamide) on ice for 10 min., liquid nitrogen frozen and stored at -80°C until usage. Lysates were thawed on ice and cleared by centrifugation for 10 min. at 4°C for 15,000rpm. For immunoprecipitations, lysates were incubated for 1h with 1µg specific IgG as indicated in the legends, followed by 1h incubation with 15µl of 50% Protein A Agarose beads slurry (Amersham). Beads were washed three times with ice-cold lysis buffer followed by one washing step with ice-cold PBS and boiled in SDS-PAGE sample buffer for 5 min. For affinity chromatography of biotinylated FLI-1, lysates were incubated for 1h with 10µl magnetic polystyrene beads covalently coupled to streptavidin (Dynabeads; M-280; 50% slurry washed three times and reconstituted in ice-cold lysis buffer). Magnetic beads were washed three times as described above for immunoprecipitation assays except that a magnet (Promega; Z5342) was used or fast recovery of the beads. SDS-polyacrylamide gel electrophoresis and Western blots were performed as described previously (26). Membranes were stripped in 63mM Tris-HCl pH 6.1, 2% SDS and 100mM β-mercaptoethanol for 30 min. at 50°C after which they were re-used. When appropriate, fluorescence was detected using Genesnap version 6.01 (Syngene, cambridge) and quantification with Genetools software (Syngene, Cambridge). Images were processed using Adobe Photoshop 6.0 (Adobe systems incorporated).

Luciferase assays and immunofluorescence analyses - For transactivation experiments, 3x10^5 HeLa or 293 cells grown in DMEM supplemented with 10% fetal calf serum (Gibco) were plated in 6-wells plates and transfected twenty-four hours later with the indicated amount of plasmid DNA using the calcium phosphate co-precipitation of DNA as described above. The total amount of expression plasmid was kept constant by addition of the corresponding empty vector without insert and the total amount of DNA (20µg/500µl) was kept constant by addition of carrier plasmid DNA. Cell lysates were prepared 24h after transfection and assayed for luciferase activity using the luciferase assay system kit (Promega). The results shown are the mean of at least three independent transfection experiments.

For Immunofluorescence analyses, 1.5x10^5 293 cells were seeded per well in 12 wells plates containing 14mm² glass coverslips (Marienfeld, Germany). After 24h, cells were transfected with 1µg ∆EB-HA-FLI-1 and 1.5µg pFlag-ARIP3 and mutants derived thereof as indicated, using the calcium phosphate co-precipitation method. The total amount of expression plasmid was kept constant by addition of the corresponding empty vector without insert and the total amount of DNA (20µg/500µl) was kept constant by addition of carrier plasmid DNA. Two days after transfection, the cells were washed two times with PBS and were fixed with 4% paraformaldehyde in PBS (15 min.) and permeabilized by incubation in 0.3% Triton X-100 (15 min.). After washing in PBS, the cells were incubated for 1h with a 1:200 dilution of anti-FLI-1 antibody and 1:100 dilution of anti-Flag in PBS/10% fetal calf serum at 37°C. Cells were washed three times in PBS and incubated for 1h at 37°C with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulins (1:100), Texas Red-
coupled donkey anti-mouse antibody (1:100) and 50µg/ml Hoechst (all obtained from Jackson ImmunoResearch Laboratories) diluted in PBS/10% fetal calf serum. Coverslips were washed two times with PBS and mounted in Dabco. Pictures of fixed cells were collected using a 3-D deconvolution imaging system. In brief, a Leica DM RXA microscope, equipped with a piezoelectric translator (PI-FOC; PI) placed at the base of a 100x PlanApo N.A. 1.4 objective, and a 5MHz Micromax 1300Y interline CCD camera (Roper Instruments) was used. Stacks of conventional fluorescence images were collected automatically at 0.2 µm Z-intervals (Metamorph software; Universal Imaging Corp.). Wavelength selection was achieved by switching to the corresponding motorized selective Leica filter block before each stack acquisition. Automated batch deconvolution of each Z-series was computed using a measured point spread function (PSF) with a custom-made software package (J.B. Sibarita, Institut Curie).

DNA binding by Electrophoretic mobility shift assays and oligonucleotide pull down assay. - Electrophoretic mobility shift assays (EMSA) were essentially performed as described previously (20). For the oligonucleotide pull down assay, a double stranded DNA-oligonucleotide corresponding to a high affinity FLI-1 binding site (wt: 5'-TCG GGT CGA CAT AAC CGG AAG TGG GC-3' (+ strand)) was synthesized as a 5'-Biotinylated derivative. A version of this oligonucleotide mutated in the EBS core (5'-TCG GGT CGA CAT AAC CCC AAG TGG GC-3' (+strand)) was used a negative control. The EBS core sequence is underlined, mutations in EBS are in bold. The oligonucleotides were immobilized on streptavidin beads by overnight incubation at 4°C in binding buffer (10mM TrisHCl (pH 8.0), 10% glycerol, 6mM MgCl₂, 5mM DTT, 0.1 mM EDTA, 0.01% NP-40, 10µg/µl polydl:dC) supplemented with 1% BSA at a final ratio of 0.6µg oligonucleotide/1x10⁵ beads. After three washes, the EBS-oligonucleotides-beads were equilibrated in binding buffer. The efficiency of oligonucleotide loading to the streptavidin beads was checked on a small aliquot by detaching the EBS from the beads by incubation at 90°C in 0.1% SDS and resolving the extract in a 1.5% agarose gel in the presence of ethidium bromide. Whole cell extracts were obtained (100mm² petri-dish in 300µl immuno-precipitation lysis buffer as above) from 293 cells transfected with expression vector for HA-FLI-1 (1µg) with or without an expression vector for HA-ARIP3 (1µg) or empty vector as a control. Lysates (100µl) were diluted with 2 volumes of binding buffer, keeping polydl:dC constant at 10µg/µl and incubated with 7x10⁶ immobilized oligonucleotide-beads for 60 min. at 4°C. The DNA affinity matrix was washed three times with binding buffer and bound proteins eluted by boiling in SDS-PAGE sample buffer. Boiled extracts were subjected to SDS-PAGE and Western blotting.

In vitro and in vivo SUMO conjugation - In vitro sumoylation assays on 2µl of in vitro translated wFILI-1 protein in reticulocyte lysate (Promega), were performed as previously described (27).

To detect sumoylated FLI-1 in vivo, 0.5x10⁶ HeLa cells were seeded in 60mm² petri-dishes and transfected 24 hours later with the ΔEB-HA-FLI-1, along with pSG5-His-SUMO-1 or pSG5-His-HA-SUMO2 as indicated. After 48 hours, cells were washed twice in ice-cold PBS, lysed in 1 ml 6 M Guanidinium-HCl, 0.1 M NaH₂PO₄, 10 mM Tris-HCl, pH 8.0 and 50 µl lysate was precipitated with 1 ml 5% TCA for 2 hours at room temperature. After centrifugation (15,000 rpm, 30 min.) the TCA protein precipitates were rinsed two times with acetone, air dried and resuspended in 200 µl SDS-sample buffer. Lysates were sonificated for 20s on ice at low intensity to fragment genomic DNA and 20µl was subjected to SDS-PAGE and Western blotting. To detect ARIP3 mediated FLI-1 sumoylation in vivo, 0.5x10⁶ 293 cells were seeded in 60mm² petri-dishes and lysed directly in 200µl SDS-sample buffer. Lysates were sonificated for 20s on ice at low intensity to fragment genomic DNA and 20µl was subjected to SDS-PAGE and Western blotting.

RESULTS

Isolation of the SUMO ligase PIASxα as a binding partner of FLI-1 in a yeast two hybrid screen - In a search for potential partners of FLI-1, a yeast two-hybrid screen of a human bone marrow cDNA library was performed
using as bait a fusion between the LexA DNA binding domain and amino acid residues 225-373 of FLI-1 (LexA-FLI-1[225-373]). This region includes a domain unique to the FLI-1/ERG subfamily of ETS proteins (residues 257-276) as well as the entire ETS domain (see Fig. 1A). Three interactors involved in the post-translational modification of proteins by SUMO, including SUMO-1 itself, Ubc9 and PIASxα were identified as positive clones in this screen. These interactions are specific, since co-expression of LexA-FLI-1[225-373] with either GalAD-PIASxα, or GalAD-SUMO-1, or GalAD-Ubc9 activated the LacZ reporter present in the indicator yeast strain, whereas a control LexA-MST3 bait failed to interact with any of these GALAD fusion proteins (Fig. 1B). Of note, LexA-FLI-1[225-373] did not auto-activate the LacZ reporter (data not shown).

To analyze whether FLI-1 and PIASxα interact in vivo, 293 cells were co-transfected with expression vectors encoding a flagged version of rat PIASxα, also known as ARIP3 (18), FLI-1 and the E. coli BirA biotin ligase. The FLI-1 protein used in this study was tagged at its amino-terminal end with a 23 amino acids peptide selected as a highly specific biotinylation substrate for E. coli BirA through iterative screening of combinatorial libraries (28) and references therein). Under these conditions, biotagged-FLI-1 was efficiently biotinylated by BirA (data not shown), allowing its rapid and essentially quantitative purification from cell extracts by affinity chromatography on a streptavidin affinity matrix. As shown in Fig. 1C, co-expression of biotagged FLI-1, ARIP3 and BirA resulted in the co-purification of ARIP3 and affinity-purified FLI-1, as detected by Western blot analyses using antibodies to either Flag or ARIP3 (Fig. 1C top panel, lane 3). No such co-purification was detected when the BirA expression plasmid was omitted from the co-transfection mixture (Fig 1C, lane 5). The interaction between FLI-1 and ARIP3 was also observed using an anti-FLI-1 antibody in a conventional co-immunoprecipitation assay (Fig 1C, middle panel).

ARIP3 interacts in vivo with FLI-1 in a SAP domain-dependent fashion - To identify the domains of ARIP3 that are involved in its ability to associate with FLI-1, a series of ARIP3 deletion mutants (see Fig. 2A for a scheme) were compared to wtARIP3 for their ability to interact with FLI-1. As shown in Fig. 2B, ARIP3[Δ102-207], ARIP3[Δ198-337] and the SUMO ligase-deficient ARIP3[Δ347-418] and ARIP3[Δ346-475] co-purified with FLI-1. This shows that most of the amino-terminal domain and the zinc finger domain of ARIP3 are not essential for this interaction. In sharp contrast, disruption of ARIP3 SAP domain by deletion of amino-acids 13-28 (ARIP3 [Δ13-28]) abolished the ability of ARIP3 to interact with FLI-1 as analyzed either by affinity purification or co-immunoprecipitation (Fig. 1C and Fig. 2B).

The C-terminal part of FLI-1 (amino acid residues 225-373) is sufficient for its interaction with ARIP3 in the yeast two hybrid experiments. This region of FLI-1 includes the entire ETS-domain (amino acid residues 277-361) and an upstream region conserved in the FLI-1/ERG subfamily of ETS proteins. Besides its specific interaction with DNA, the ETS-domain mediates interactions with a number of proteins including GATA-1 (6) and EKLF (10). We next analyzed whether this region of FLI-1 is involved in the interaction with ARIP3 in vivo. As is apparent in figure 2C, wild type FLI-1 and its isolated ETS-domain (FLI-1[276-373]) were equally efficient to co-immunoprecipitate ARIP3, showing that the ETS domain is sufficient to mediate the interaction with ARIP3. This interaction was specific as the antibody directed against the ETS-domain of FLI-1 did not co-precipitate ARIP3 when wtFLI-1 or FLI-1[276-373] expression vectors were omitted from the transfection mixture (Fig 2C, lane 4). Similar to wtFLI-1, the interaction of the ETS-domain (FLI-1[276-373]) with ARIP3 depended upon the integrity of the SAP domain, as the ETS-domain failed to co-precipitate with ARIP3[Δ13-28] (Fig. 2D).

We conclude from these experiments that FLI-1 specifically interacts with ARIP3 in vivo and that this interaction involves the ETS domain of FLI-1 and requires the integrity the SAP domain of ARIP3.

The interaction between FLI-1 and ARIP3 is direct and does not require co-factors. - To analyze whether the interaction between FLI-1 and ARIP3 was direct, we next performed GST pull-down experiments using as affinity matrices a series of GST fusion proteins...
corresponding to specific functional domains of FLI-1 (see Fig. 3A for a scheme). Full-length wtARIP3 and a series of ARIP3 deletion mutants were synthesized as radio-labeled proteins by in vitro translation and their association to the different GST-FLI-1 fusion proteins and control GST was compared. As shown in figure 3B, GST-FLI-1[188-452] and GST-FLI-1[83-286], which encompasses the amino-terminal half of FLI-1 including its SAM/Pointed domain, failed to pull down ARIP3. In contrast, both GST-FLI-1[188-452] and GST-FLI-1[250-452] that span the carboxy-terminal part of FLI-1, including its ETS domain specifically bound ARIP3. In line with the results obtained in vivo, disruption of the SAP domain in ARIP3[Δ13-28] inhibited the ability of ARIP3 to interact with GST-FLI-1[250-452] (Fig. 3C). In contrast, deletion of amino-acid residues 102-207 and 198-337 in the amino-terminal half of the protein and the zinc finger domain (ARIP3[347-418]) and downstream sequences (ARIP3[346-475]) did not affect ARIP3 binding to FLI-1. PIASx is expressed in two isoforms, PIASxα and PIASxβ, which only differ at their extreme carboxy terminal ends. As expected from the results obtained with PIASxα/ARIP3, PIASxβ was co-precipitated as efficiently as ARIP3 by GST-FLI1[250-452] (Fig. 3C).

ARIP3 and PIASxβ stimulate FLI-1 sumoylation in vitro - Ubc9 and PIASxα/ARIP3 are critical components of the SUMO modification pathway of proteins, in which Ubc9 is the E2 conjugating enzyme whereas PIASxα/ARIP3 and other PIAS members are E3 ligases that facilitate SUMO conjugation to specific substrates (17). Since FLI-1 interacts physically with both Ubc9 and ARIP3, we next examined if FLI-1 can be sumoylated in vitro and whether its sumoylation can be enhanced by PIAS proteins. L-[35S]methionine/cysteine-labeled HA-tagged wtFLI-1 produced by in vitro translation was incubated in the presence of ATP, a cellular fraction containing E1 SUMO-activating activity together with recombinant Ubc9 and SUMO-1 in absence or presence of recombinant PIAS proteins. As expected, translation of the FLI-1 mRNA occurs both at the initiating methionine (M1) and at a second initiating translation AUG codon at position 34 (Fig. 4A, lane 1). In the presence of SUMO-1, two slow migrating forms were additionally observed that correspond to the expected increase in apparent molecular weight resulting from the addition of a single SUMO-1 adduct to each of the FLI-1 variants. Addition of PIAS3 or PIASy had no detectable effect on FLI-1 sumoylation levels, whereas PIAS1 clearly enhanced FLI-1 sumoylation (Fig 4A). In contrast, the addition of either PIASxα or PIASxβ strongly enhanced FLI-1 sumoylation and resulted in the appearance of FLI-1 forms that most likely correspond to the conjugation of several SUMO adducts (Fig. 4A, lanes 5 and 6). These results show that FLI-1 can be conjugated to SUMO-1 in vitro and that this modification is significantly enhanced by specific members of the PIAS family of SUMO ligases.

Lysine 67 is the major SUMO modification site in FLI-1 in vivo - Next we investigated whether FLI-1 was sumoylated in vivo. Expression vectors encoding an HA-tagged wtFLI-1 and either His6-tagged SUMO-1 or His-HA tagged SUMO-2 were transiently transfected into HeLa cells, cells were lysed in a GuHCl-containing buffer (see Experimental procedures) and whole cell extracts analyzed by Western blotting using an anti-FLI-1 antibody. Non-modified HA-tagged FLI-1 migrates as a major species of apparent molecular mass 50 kDa and a minor component resulting from internal initiation at methionine 34. In the presence of exogenous SUMO-1 or SUMO-2, slow migrating forms of FLI-1 were observed with an increase in apparent molecular weight corresponding to mono-sumoylation (Fig. 4B, lanes 3 and 4). Of note, a band migrating below the exogenous SUMO-1 and SUMO-2 FLI-1 adducts is present in lanes 2-4 and likely represents FLI-1 sumoylation with endogenous SUMO. Protein sumoylation often targets lysine residues in a ϕKXE consensus sequence, where K corresponds to the lysine residue to which SUMO-1 is covalently bound, ϕ to a hydrophobic amino-acid and X to any amino-acid. Perfect matches to this motif are found in FLI-1 around lysine 67(K67) and 217(K217), both localized in the amino-terminal half of the protein. To investigate whether these residues are in vivo acceptor sites for SUMO-1 or SUMO-2, they were mutated either singly or in combination into non-sumoylatable arginine to generate FLI-1[K67R], FLI-1[K217R] and FLI-1[K67R,K217R].
FLI-1 [K67,217R], respectively. Sumoylation of these mutants was compared to wtFLI-1 following transient transfection of the corresponding expression plasmids together with expression plasmids for either SUMO-1 or SUMO-2. As shown in Fig. 4C, FLI-1[K217R] was modified as efficiently as wtFLI-1. In contrast, mutation of lysine residue 67 in FLI-1[K67R] and FLI-1[K67,217R] abolished the formation of the retarded, sumoylated form of FLI-1. These results show that FLI-1 is sumoylated in vivo on a single lysine residue (K67), located in its amino-terminal domain and that both SUMO-1 and SUMO-2 can serve as substrates for this modification. Of note, the higher migrating forms of FLI-1 in vitro, presumably corresponding to multi-sumoylation of FLI-1, are not observed in vivo. Next, we studied whether ARIP3 is able to enhance sumoylation of FLI-1 in vivo. Figure 4D shows that co-expression of FLI-1 with ARIP3 enhanced sumoylation of FLI-1 in vitro, using endogenous SUMO as a substrate (Fig. 4D, compare lanes 1 and 4). Co-expression of HA-tagged SUMO-1 together with FLI-1 resulted in a second, slower migrating form of FLI-1, corresponding to the covalent attachment of HA-tagged SUMO-1, that was enhanced by co-expression with ARIP3 (Fig. 4D, compare lanes 2 and 4). These experiments indicate that FLI-1 is sumoylated at lysine residue 67 and that ARIP3 functions as a SUMO ligase for FLI-1 in vitro and in vivo.

**ARIP3 interferes with FLI-1 transcriptional activity** - PIASxα/ARIP3 was originally characterized as an androgen receptor (AR)-interacting protein that functions as a corepressor of AR and other members of the nuclear hormone receptor family (18). Later, PIASxα/ARIP3 was shown to enhance sumoylation of cellular proteins and its SUMO ligase activity can be critical for PIASxα/ARIP3 to regulate transcription (24,29). We therefore analyzed whether ARIP3 is able to modulate the transcriptional regulatory activity of FLI-1. Increasing amounts of an expression vector encoding ARIP3 were co-transfected together with fixed amounts of a FLI-1 expression plasmid and an EBS-driven luciferase reporter gene. Transactivation was monitored by luciferase activity assay. Consistent with previous results (13), FLI-1 efficiently activated luciferase expression from tkD2A-LUC, a luciferase reporter gene in which a tandem copy of ETS-responsive region-1 of the human T-cell leukemia virus type 1-long terminal repeat is inserted upstream of the -55 herpes simplex virus thymidine kinase (tk) promoter (Fig. 5A). Increasing amounts of ARIP3 inhibited FLI-1-mediated transactivation of tkD2A-Luc in a dose-dependent manner (Fig 5A). The inhibitory properties of ARIP3 on FLI-1-mediated transactivation are not restricted to model promoters since ARIP3 also efficiently and specifically inhibited the EBS-dependent (30), FLI-1-mediated transactivation of the –270/-41 mouse FLI-1 gene promoter (Fig. 5A, right panel). Importantly, ARIP3 did not affect the basal activity of either tkD2A-LUC, or –270/-41 mFLI-1-Luc, nor did it affect FLI-1 expression (Fig. 5A, bottom). Neither the sumoylation of FLI-1 on K67 nor the ligase activity of ARIP3 is essential for ARIP3 to function as a repressor of FLI-1 transactivation.

To determine whether ARIP3 repressive function depended upon FLI-1 sumoylation, we compared the ability of ARIP3 to inhibit wtFLI-1 and the sumoylation-defective FLI-1[K67R] mutant. As shown in Fig 5B, transactivation of the -270/-41 mFLI-1-Luc reporter gene by FLI-1[K67R] was similar to that induced by wtFLI-1 (Fig. 5B), indicating that the K67R mutation is without effects on FLI-1 transactivation properties. Of note, the DNA-binding ability of FLI-1[K67R] as assayed by electrophoretic mobility shift assay (EMSA) was similar to that of wtFLI-1 (data not shown). In addition, transactivation by wtFLI-1 or FLI-1[K67R] was similarly impaired in the presence of increasing amounts of ARIP3 (Fig. 5C), indicating that mutation of lysine 67 and therefore FLI-1 sumoylation is not required for ARIP3-mediated repression of FLI-1 transcriptional activity. Similar results were obtained using FLI-1[K67,217R], and using tkD2A-Luc instead of -270/41 mFLI-1-Luc as reporter gene construct (data not shown). We next examined whether the SUMO ligase activity of ARIP3 is required to inhibit wtFLI-1 transcriptional regulatory properties. To this end, we compared the inhibitory activity of wtARIP3 to either an ARIP3 mutant deleted in its RING finger region (ARIP3[A347-418]) or to a mutant in which a tryptophan residue conserved in the RING zinc finger of ARIP3 was changed into...
alanine (ARIP3[W383A]). Previous analyses have shown that both these mutations impair ARIP3 E3 ligase activity (24). As shown in Fig. 5D, both ARIP3[Δ347-418] and ARIP3[W383A] inhibited FLI-1-mediated transcriptional activation of 270/-41 mFLI-1-Luc in a manner indistinguishable from wt ARIP3. Taken together, these results show that ARIP3 inhibitory action on FLI-1-induced transactivation neither requires the SUMO-ligase activity of ARIP3 nor FLI-1 sumoylation. Notably, although PIASxβ was capable to in vitro sumoylate and interact with FLI-1, no repressive effect of PIASxβ on FLI-1 transactivation could be observed in these experimental conditions (data not shown).

ARIP3 re-localizes FLI-1 into nuclear bodies - PIAS proteins localize to specific nuclear bodies of unclear composition and function that are detected as punctate structures using confocal immunofluorescence microscopy (23,31,32). To examine whether ARIP3 could change FLI-1 subnuclear localization, indirect two-color immunofluorescence was performed in cells expressing either wtFLI-1 alone or ARIP3 alone or the combination of both. As shown in Figure 6A, single plane microscopy revealed an essentially diffuse nuclear pattern for FLI-1 when expressed alone. In contrast, ARIP3 localized into nuclear speckles in agreement with previous studies ((23), Fig. 6A). Similar results were obtained in HeLa cells (data not shown). Strikingly, co-expression of ARIP3 and FLI-1 lead to a partial re-localization of FLI-1 into ARIP3 nuclear bodies (Fig. 6A). Re-localization is independent of FLI-1 sumoylation as the non sumoylatable FLI-1[K67R] mutant also re-localized to ARIP3 nuclear bodies upon co-expression with ARIP3 (Fig. 6A). Since an intact SAP domain in ARIP3 is essential for its ability to interact physically with FLI-1, we analyzed the ability of ARIP3[Δ13-28] to re-localize FLI-1. Interestingly, ARIP3[Δ13-28] displayed a different pattern of nuclear body formation as compared to wtARIP3, forming a small number (2-15 per nucleus) of larger structures (Fig. 6B). Unlike wtARIP3, ARIP3[Δ13-28] failed to re-localize FLI-1 to nuclear bodies, indicating that integrity of the SAP domain is essential to the ability of ARIP3 to sequester FLI-1 into nuclear bodies (Fig. 6B). Deletion of amino acids 467-487, the SUMO binding region of ARIP3, which leads to the formation of fewer nuclear dots (23) and to partial cytoplasmic localization of ARIP3 also re-localized FLI-1 to nuclear dots (Fig.6B). This indicates that re-localization of FLI-1 to ARIP3 nuclear bodies is independent of SUMO binding to ARIP3. As described before for ARIP3 and other PIAS family members (23,31), E3 ligase-deficient mutants of ARIP3, namely ARIP3[Δ347-418] and ARIP3[W383A] display a diffuse nuclear localization. Co-expression of FLI-1 with both ARIP3[Δ347-418] or ARIP3[W383A] did not change the diffuse pattern normally seen with FLI-1 (Fig. 6B). Since these mutants efficiently bound FLI-1 and repressed FLI-1 transcriptional activation properties, ARIP3-mediated re-localization of FLI-1 into nuclear bodies does not seem to be sufficient to explain its ability to functionally repress FLI-1.

ARIP3 does not inhibit FLI-1 DNA binding activity - The mapping studies described above indicate that the ETS domain of FLI-1 is sufficient to interact physically with ARIP3. It is possible therefore that the inhibitory properties of ARIP3 result from its ability to impair the DNA binding activity FLI-1 to EBS sequences. The specific DNA-binding activity of FLI-1 to an optimal EBS oligonucleotide was not inhibited when performed in the presence of an excess of recombinant ARIP3, as analysed by EMSA (data not shown). To further demonstrate that the binding of FLI-1 is compatible with complex formation with ARIP3 the specific DNA-binding activity of FLI-1 was assayed by oligonucleotide pull down assay, using a biotinylated double stranded EBS-oligonucleotide immobilized on streptavidin beads and cell extracts in which HA-FLI-1 was expressed in the presence or absence of HA-ARIP3 (Fig. 7A). FLI-1 was efficiently bound by the EBS-oligonucleotide affinity matrix as analyzed by western blot (Fig. 7B, lane2). This binding was specific since the DNA-binding of FLI-1 was not observed using as affinity matrix streptavidin beads loaded with a mutated EBS-oligonucleotide (Fig 7B, lanes 2, 5). Importantly, in extracts co-expressing HA-FLI-1 and HA-ARIP3, HA-ARIP3 was also specifically recovered from the wtEBS affinity matrix, showing that formation of the ARIP3-FLI-1 complex did not interfere with the DNA binding activity of FLI-1 (Fig 7B, lanes 2, 6).
The interaction between FLI-1 and ARIP3 is essential for the repressive function of ARIP3 on FLI-1 transactivation. To analyze whether ARIP3 inhibitory properties are linked to its ability to physically interact with FLI-1, we analyzed the effect of a series of ARIP3 deletion mutants that either maintain or do not maintain their ability to interact with FLI-1 (see Fig. 2, and data not shown) on FLI-1-mediated transactivation. The results of Figure 8A show that the SAP domain of ARIP3, which is required for the physical interaction between FLI-1 and ARIP3, is also critical for the transcriptional inhibitory properties of ARIP3 since both ARIP3[Δ1-102] and ARIP3[Δ13-28] failed to inhibit FLI-1 transactivation properties. In contrast, ARIP3[Δ102-207], ARIP3[Δ102-260], ARIP3[Δ198-337] and ARIP3[Δ467-547] that associated with FLI-1 in vivo and in vitro also fully inhibited FLI-1-mediated transactivation. These results, together with the data of Figure 5D show that the region of ARIP3 extending between amino-acid residues 102-418 is not critical to ARIP3 inhibitory properties and that ARIP3-mediated inhibition of FLI-1 activity is linked to its ability to physically bind FLI-1. Mutant ARIP3[Δ346-475] displayed a reduced ability to repress FLI-1. However, this protein only partially localized to the nucleus (data not shown), suggesting that the diminished inhibitory activity of this mutant is not intrinsic, but results from its incomplete accumulation to the nucleus. Of note, equal expression of FLI-1 was observed in the different conditions tested (Fig. 8B, lower panel).

DISCUSSION

In this study, several components of the posttranslational sumoylation pathway have been identified in a yeast two hybrid screen as interactors of FLI-1, including SUMO-1 itself, Ubc9, the E2 enzyme involved in the conjugation of SUMO to the ε-amino-group of specific lysine in protein substrates and PIASxα, one of the E3 ligases that facilitates SUMO conjugation to specific substrates. An interaction between FLI-1 and Ubc9 has previously been reported (33) but no link was established to neither FLI-1 sumoylation, nor regulation of FLI-1 transcriptional activity. We report here (i) that FLI-1 is sumoylated in vitro by recombinant Ubc9 and in vivo on a single lysine residue at position 67 which is part of a classical ϕKxE Ubc9 binding site, located in its transcriptional activation domain; (ii) that FLI-1 binds through its ETS domain to PIASxα and that the integrity of the SAP domain of PIASxα is required for this interaction; (iii) that the interaction of FLI-1 with PIASxα enhances FLI-1 sumoylation on K67, represses FLI-1 transcriptional activation properties and leads to the sequestering of FLI-1 into PIASxα nuclear bodies.

PIASs (Protein inhibitors of activated STATs) were first identified as repressors of the DNA binding activity of tyrosine-phosphorylated STATs, with PIAS1 and PIAS3 being specific of STAT1 and STAT3, respectively (34,35). Later, PIASx and PIASy have been shown to inhibit STAT4 and STAT1 transcriptional activity, respectively without interfering with DNA binding (36,37). Recent studies with PIASI-deficient cells have shown however that PIAS1 interfering activity is specific to a small subset of STAT1 target genes critical to the IFNβ- and IFNγ-mediated innate immune responses (38). PIAS transcriptional regulatory properties are not limited to activated STATs as they are found to regulate positively or negatively through ill-defined mechanisms the activity of other transcription factors, e.g. members of the nuclear receptor superfamilly (for review, (17)). All members of the PIAS family have been shown to be SUMO E3 ligases that can favor sumoylation of a number of DNA binding transcriptional regulators and co-regulators (for review, see (17)). Gene inactivation of SIZ1 and SIZ2, the PIAS homologues in yeast results in reduction in global protein sumoylation (39) and the Drosophila melanogaster PIAS homologue Su(var)2-10 is required for viability. In contrast, gene inactivation of PIASx, PIASI or PIASy by homologous recombination in mouse cells has no discernible effect on global protein sumoylation and is viable, indicating that PIAS proteins are likely to be redundant in mammals (38,40-42). Our in vitro and in vivo experiments show that FLI-1 conjugation to SUMO-1 is specifically enhanced by PIASxα/ARIP3 and its spliced variant PIASxβ but much less efficiently by PIAS1 and not at all by PIASy and PIAS3, indicating a clear selectivity for specific members of the PIAS
family. The reduced activity of PIAS1 does not reflect a failure to interact with FLI-1 since FLI-1 and PIAS1 were found to physically interact when co-expressed in vivo (our unpublished observations), suggesting that FLI-1 is preferentially modified by PIASx proteins.

PIASxα/ARIP3 acts as a repressor of FLI-1-dependent EBS-driven transcription. SUMO modification of DNA bound transcription factors often targets known repression domains and can result in enhanced repression (for review see (17),(43)). Although the SUMO target (K67) is located in the N-terminal activation domain of FLI-1, two lines of evidence indicate that enhanced FLI-1 sumoylation is not involved in PIASxα-mediated transcriptional repression. First, FLI-1[K67R] in which K67 is replaced by a non sumoylatable arginine, although similarly effective as wFLI-1 in transactivation, was as efficiently repressed by PIASxα/ARIP3 then wFLI-1. Second, the closely related splice variant PIASxδ despite the fact that it activated FLI-1 sumoylation as efficiently as PIASxα failed to inhibit FLI-1-mediated EBS-driven transcription. This situation is not unprecedented since repression of e.g. LEF-1 transcriptional activity by PIASy is also unlinked to its PIASy-mediated sumoylation on specific lysine residues (31). Interestingly, RING finger mutants of PIASxα/ARIP3 that are deficient for SUMO conjugation, namely ARIP3[W383A] and ARIP3[A347-418] are fully repressing FLI-1 transactivation, indicating that regulatory sumoylation of known FLI-1 co-activators such as p300 (13),(44) or co-repressors is not critically involved in the repressive properties of PIASxα on FLI-1. Other transcription factors, including GATA-2 and the androgen receptor, are also repressed by PIAS family members independent of an active RING domain (45-47), but the molecular mechanisms involved remain to be characterized.

PIASxα/ARIP3 and other PIAS proteins are localized in nuclear bodies of largely unknown composition and function but which may partially overlap with PML nuclear bodies (23,31). The association of PIAS proteins with nuclear bodies appears to require their SUMO ligase activity since ligase-deficient PIAS mutants are unable to assemble into PIAS bodies (31,45). Binding of PIASxα/ARIP3 to other sumoylated proteins and its targeting to the nuclear matrix also seems to be required for the formation of typical PIASxα bodies since deletion of the SUMO-1 binding motif of PIASxα/ARIP3 (amino-acid residues 467-487) and deletion of the MAR/SAR targeting SAP domain both impair the formation of normal PIASxα/ARIP3 nuclear bodies ((23); Fig. 6). Our data show that ARIP3 localises the normally diffusely distributed FLI-1 from the nucleoplasm into PIASxα/ARIP3 nuclear bodies, suggesting that FLI-1 repression could result from its relocalisation to PIASxα bodies. However since FLI-1 remains diffusely distributed in the nucleoplasm in cells co-expressing FLI-1 and the ligase-deficient PIASxα/ARIP3 mutants, and since these mutants repress FLI-1-mediated transcription and interact with FLI-1 as efficiently as wild type PIASxα, it appears that relocalization of FLI-1 into PIASxα nuclear bodies is not mandatory for ARIP3 to repress FLI-1 activity. However, re-localization of FLI-1 to PIASxα/ARIP3 nuclear bodies could stabilize the inhibitory complex. Re-localization of FLI-1 to nuclear bodies is also independent of FLI-1 sumoylation since the sumoylation-deficient FLI-1[K67R] mutant relocalized to PIASxα-bodies when co-expressed with PIASxα/ARIP3 in a fashion indistinguishable from wild type FLI-1. The SAP domain of PIAS proteins binds AT-rich MAR/SAR sequences in vitro (31,32) and both ARIP3/PIASxα and ligase deficient mutants of PIASxα are found to associate with detergent-insoluble nuclear fraction in a manner dependent upon their SAP domain (our unpublished observations). It is therefore possible that although recruitment of FLI-1 to mature PIASxα nuclear bodies is not instrumental in its inhibition, its targeting to the nuclear matrix could be important. Proper answer to this question will require the design of mutations in the SAP domain that dissociate the MAR/SAR targeting activity of PIASxα from its binding activity to FLI-1.

By what mechanism is PIASxα repressing FLI-1 transcriptional activity? The isolated ETS domain of FLI-1 is sufficient to bind PIASxα/ARIP3, yet PIASxα failed to inhibit the specific binding of FLI-1 to canonical DNA response elements in vitro, suggesting that inhibition does not occur at the
level of DNA binding. Our mutant studies show that PIASxα-mediated repression correlates with physical complex formation between FLI-1 and PIASxα. A small deletion in the SAP domain of PIASxα suppressed both ARIP3/PIASxα repressive activity and complex formation with FLI-1 whereas mutations in other domains of PIASxα affected neither repression, nor binding to FLI-1. Complex formation between FLI-1 and PIASxα could result in masking of the FLI-1 activation domain thereby competing for the recruitment of co-activators. We do not favor this notion because EWS-FLI-1-mediated EBS-dependent transactivation which entirely relies on a structurally unrelated, EWS-derived activation domain (20) is also inhibited by PIASxα/ARIP3 (our unpublished observations). Previous analyses have shown that transcriptional repression of the androgen receptor by PIASy and of STAT4 by PIASxα involves the recruitment of histone deacetylase (HDAC) or HDAC-associated co-repressors (36,47). We found PIASxα-mediated repression of FLI-1 to be insensitive to trichostatin A (unpublished observation), suggesting that the members of the class I and II HDAC families are unlikely to be involved in PIASxα-mediated repression of FLI-1. PIASxα and PIASxβ are isoforms with similar primary amino-acid sequence except that the unique carboxyterminal 71 amino-acids in PIASxα are replaced by an unrelated sequence of 122 amino-acids in PIASxβ. In our experimental setting, only PIASxα acts as repressor for FLI-1 (our unpublished observations), suggesting either a direct role for the unique C-terminal domain of PIASxα in repression or an unknown role of the C-terminal domain of PIASxβ in preventing PIASx repressing activity. Experiments are in progress to compare the co-factors that associate to the repressive FLI-1/PIASxα complex in a manner dependent upon its unique C-terminal domain.

Our results did not identify any functional consequences of SUMO conjugation of FLI-1. It should be noted however that FLI-1 sumoylation occurs at low stoechiometry in vivo and could have functional consequences in a compartmentalized fashion. In accordance with the transportable repressive properties of SUMO-1 (45), the experimental covalent fusion of SUMO-1 to the amino-terminus of FLI-1 converted FLI-1 from a transcriptional activator into a repressor of EBS-mediated transcription (our unpublished observations), suggesting that stoechiometric sumoylation of FLI-1 could be repressive. Furthermore, the functional consequences of sumoylation on several DNA binding transcriptional regulators have been shown to depend upon the promoter context (18,32). In that respect, FLI-1 like other members of the ETS family functions in close synergy with other cis-bound DNA binding co-factors, often on composite DNA response elements to regulate transcriptional output. For example, in line with the requirement of FLI-1 in megakaryocytic development and differentiation (1-3), the promoters of a number of megakaryocytic lineage-specific genes contain closely juxtaposed binding sites for FLI-1 and GATA1, a configuration which is instrumental in their developmentally regulated expression (6,48). It is possible therefore that recruitment of the sumoylation machinery to specific promoters and sumoylation of co-operating cis-bound factors is important to regulate transcriptional synergy in a promoter- and cell context-specific manner.

In conclusion, we have identified PIASxα as a novel repressor of FLI-1 and FLI-1 derived oncoproteins that impairs its transcriptional activity and sequesters FLI-1 into nuclear bodies. Interestingly, repression by PIASxα is independent of its SUMO ligase activity and of FLI-1 sumoylation but depends upon the physical interaction between FLI-1 and PIASxα. The activity of FLI-1 may be repressed first through the assembly at the level of chromatin of co-repressors complexes, targeting of the FLI-1/PIASxα complex to the nuclear matrix followed by final assembly into mature PIASxα nuclear bodies.
REFERENCES

1. Hart, A., Melet, F., Grossfeld, P., Chien, K., Jones, C., Tunnacliffe, A., Favier, R., and Bernstein, A. (2000) *Immunity* 13(2), 167-177
2. Spyropoulos, D. D., Pharr, P. N., Lavenburg, K. R., Jackers, P., Papas, T. S., Ogawa, M., and Watson, D. K. (2000) *Mol Cell Biol* 20(15), 5643-5652
3. Kawada, H., Ito, T., Pharr, P. N., Spyropoulos, D. D., Watson, D. K., and Ogawa, M. (2001) *Int J Hematol* 73(4), 463-468
4. Masuya, M., Moussa, O., Abe, T., Deguchi, T., Higuchi, T., Ebihara, Y., Spyropoulos, D. D., Watson, D. K., and Ogawa, M. (2005) *Blood* 105(1), 95-102
5. Truong, A. H., and Ben-David, Y. (2000) *Oncogene* 19(55), 6482-6489
6. Eisbacher, M., Holmes, M. L., Newton, A., Hogg, P. J., Khachigian, L. M., Crossley, M., and Chong, B. H. (2003) *Mol Cell Biol* 23(10), 3427-3441
7. Czuwara-Ladykowska, J., Shirasaki, F., Jackers, P., Watson, D. K., and Trojanowska, M. (2001) *J Biol Chem* 276(24), 20839-20848
8. Tamir, A., Howard, J., Higgins, R. R., Li, Y. J., Berger, L., Zackenhaus, E., Reis, M., and Ben-David, Y. (1999) *Oncogene* 18(6), 4452-4464
9. Darby, T. G., Meissner, J. D., Ruhlmann, A., Mueller, W. H., and Scheibe, R. J. (1997) *Oncogene* 15(25), 3067-3082
10. Starck, J., Cohet, N., Gonnet, C., Sarrazin, S., Doubeikovskaia, Z., Doubeikovski, A., Verger, A., Duterque-Coquillaud, M., and Morle, F. (2003) *Mol Cell Biol* 23(4), 1390-1402
11. Pereira, R., Quang, C. T., Lesault, I., Dolznig, H., Beug, H., and Ghysdael, J. (1999) *Oncogene* 18(8), 1597-1608
12. Lesault, I., Quang, C. T., Frampton, J., and Ghysdael, J. (2002) *Embo J* 21(4), 694-703
13. Ano, S., Pereira, R., Pironin, M., Lesault, I., Milley, C., Lebigot, I., Quang, C. T., and Ghysdael, J. (2004) *J Biol Chem* 279(4), 2993-3002
14. Rabault, B., and Ghysdael, J. (1994) *J Biol Chem* 269(45), 28143-28151
15. Arvand, A., Welford, S. M., Teitell, M. A., and Denny, C. T. (2001) *Cancer Res* 61(13), 5311-5317
16. Stein, G. S., van Wijnen, A. J., Stein, J. L., Lian, J. B., Pockwinse, S. M., and McNeil, S. (1998) *J Cell Biochem Suppl* 30-31, 220-231
17. Meister, J. S., and Dejean, A. (2003) *Nat Rev Mol Cell Biol* 4(9), 690-699
18. Moilanen, A. M., Karvonen, U., Poukka, H., Yan, W., Toppari, J., Janne, O. A., and Palvimo, J. J. (1999) *J Biol Chem* 274(6), 3700-3704
19. Gyuris, J., Golemis, E., Chertkov, H., and Brent, R. (1993) *Cell* 75(4), 791-803
20. Bailly, R. A., Bosselut, R., Zachariou, F., Cormier, F., Delattre, O., Roussel, M., Thomas, G., and Ghysdael, J. (1994) *Mol Cell Biol* 14(5), 3230-3241
21. Rabault, B., and Ghysdael, J. (1994) *J Biol Chem* 269(45), 28143-28151
22. Muller, S., Berger, M., Lehembre, F., Seeler, J. S., Haupt, Y., and Dejean, A. (2000) *J Biol Chem* 275(18), 13321-13329
23. Kotaja, N., Karvonen, U., Janne, O. A., and Palvimo, J. J. (2002) *Mol Cell Biol* 22(14), 5222-5234
24. Kotaja, N., Vihinen, M., Palvimo, J. J., and Janne, O. A. (2002) *J Biol Chem* 277(20), 17781-17788
25. van den Akker, E., van Dijk, T., Parren-van Amelsvoort, M., Grossmann, K. S., Schaeper, U., Toney-Earley, K., Waltz, S. E., Lowenberg, B., and von Lindern, M. (2004) *Blood* 103(12), 4457-4465
26. van Dijk, T. B., van Den Akker, E., Amelsvoort, M. P., Mano, H., Lowenberg, B., and von Lindern, M. (2000) *Blood* 96(10), 3406-3413
27. Kirsh, O., Seeler, J. S., Pichler, A., Gast, A., Muller, S., Misra, E., Mathieu, M., Harel-Bellan, A., Kouzarides, T., Melchior, F., and Dejean, A. (2002) *Embo J* 21(11), 2682-2691
28. de Boer, E., Rodriguez, P., Bonte, E., Krijgsveeld, J., Katsantoni, E., Heck, A., Grosveld, F., and Strouboulis, J. (2003) *Proc Natl Acad Sci U S A* 100(13), 7480-7485
29. Nishida, T., and Yasuda, H. (2002) *J Biol Chem* **277**(44), 41311-41317
30. Starck, J., Mouchiroud, G., Gonnet, C., Mehlen, A., Aubert, D., Dorier, A., Godet, J., and Morle, F. (1999) *Exp Hematol* **27**(4), 630-641
31. Sachdev, S., Bruhn, L., Sieber, H., Pichler, A., Melchior, F., and Grosschedl, R. (2001) *Genes Dev* **15**(23), 3088-3103
32. Tan, J. A., Hall, S. H., Hamil, K. G., Grossman, G., Petrusz, P., and French, F. S. (2002) *J Biol Chem* **277**(19), 16993-17001
33. Hahn, S. L., Wasylyk, B., and Criqui-Filipe, P. (1997) *Oncogene* **15**(12), 1489-1495
34. Sachdev, S., Bruhn, L., Liu, B., Rao, X., Jay, P., Berta, P., and Shuai, K. (1997) *Science* **278**(5344), 1803-1805
35. Liu, B., Liao, J., Rao, X., Kushner, S. A., Chung, C. D., Chang, D. D., and Shuai, K. (1998) *Proc Natl Acad Sci U S A* **95**(18), 10626-10631
36. Arora, T., Liu, B., He, H., Kim, J., Murphy, T. L., Murphy, K. M., Modlin, R. L., and Shuai, K. (2003) *J Biol Chem* **278**(24), 21327-21330
37. Liu, B., Gross, M., ten Hoeve, J., and Shuai, K. (2001) *Proc Natl Acad Sci U S A* **98**(6), 3203-3207
38. Liu, B., Mink, S., Wong, K. A., Stein, N., Getman, C., Dempsey, P. W., Wu, H., and Shuai, K. (2004) *Nat Immunol* **5**(9), 891-898
39. Johnson, E. S., and Gupta, A. A. (2001) *Cell* **106**(6), 735-744
40. Roth, W., Sustmann, C., Kieslinger, M., Gilmozzi, A., Irmer, D., Kremmer, E., Turck, C., and Grosschedl, R. (2004) *J Immunol* **173**(10), 6189-6199
41. Wong, K. A., Kim, R., Christofk, H., Gao, J., Lawson, G., and Wu, H. (2004) *Mol Cell Biol* **24**(12), 5577-5586
42. Santti, H., Mikkonen, L., Anand, A., Hirvonen-Santti, S., Toppari, J., Panhuysen, M., Vauti, F., Perera, M., Corte, G., Wurst, W., Janne, O. A., and Palvimo, J. J. (2005) *J Mol Endocrinol* **34**(3), 645-654
43. Johnson, E. S. (2004) *Annu Rev Biochem* **73**, 355-382
44. Girdwood, D., Bumpass, D., Vaughan, O. A., Thain, A., Anderson, L. A., Snowden, A. W., Garcia-Wilson, E., Perkins, N. D., and Hay, R. T. (2003) *Mol Cell* **11**(4), 1043-1054
45. Ungureanu, D., Vanhatupa, S., Kotaja, N., Yang, J., Aittomaki, S., Janne, O. A., Palvimo, J. J., and Silvennoinen, O. (2003) *Blood* **102**(9), 3311-3313
46. Chun, T. H., Itoh, H., Subramanian, L., Iniguez-Lluhi, J. A., and Nakao, K. (2003) *Circ Res* **92**(11), 1201-1208
47. Gross, M., Yang, R., Top, I., Gasper, C., and Shuai, K. (2004) *Oncogene* **23**(17), 3059-3066
48. Lemarchandel, V., Ghysdael, J., Mignotte, V., Rahuel, C., and Romeo, P. H. (1993) *Mol Cell Biol* **13**(1), 668-676
FOOTNOTES

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FIGURE LEGENDS

Figure 1. Schematic diagram of the FLI-1 protein and the interaction of FLI-1 and PLASxα. A. Diagram of FLI-1 functional domains: the ETS domain is shown as a hatched box and the Pointed/SAM domain as a gray box. NTAD: N-Terminal Activation Domain; C-TAD: Carboxy-Terminal Activation Domain. The LexA fusion protein encompassing amino-acids residues 225-373 of FLI-1 that was used as a bait in the yeast two hybrid screen is depicted. LexA-MST3 (full length) was used as a negative control. B. Transformants were assayed for β-galactosidase activity and analyzed by LacZ staining. C. 293 cells were transfected with combinations of expression plasmids encoding the proteins indicated at the top. Bio-tagged FLI-1 was isolated by affinity chromatography using streptavidin beads (top panels) or by immunoprecipitation using an anti-FLI-1 antibody (middle panel) as described in Experimental procedures, and blots were processed for Western analyses using anti-FLAG, anti-ARIP3 or anti-FLI-1 to detect FLAG-ARIP3 and FLI-1, respectively. In the lower panels, whole cell lysates (WCL) used in the immunoprecipitation and affinity chromatography analyses were subjected to SDS-PAGE and processed for Western blotting using anti-ARIP3, anti-FLI-1 or anti-HA to confirm expression and input of the respective proteins.

Figure 2. The interaction between FLI-1 and ARIP3 depends on the ETS domain of FLI-1 and the integrity of the SAP domain of ARIP3. A. Schematic representation of the different N-terminal FLAG-tagged ARIP3 deletion mutants used in this study. B. 293 cells were transfected with expression vectors encoding the proteins indicated at the top of the figure. FLI-1 was isolated by affinity chromatography using streptavidin beads and isolated proteins were subjected to SDS-PAGE and the gels processed for Western blotting. Blots were stained with anti-FLAG (to detect FLAG-tagged ARIP3) and anti-FLI-1. In the lower panel the WCL (whole cell lysate) used for the affinity chromatography purification was analyzed to confirm expression of the different ARIP3 mutants (anti-FLAG) and of BirA (anti-HA). The stars point to ARIP3wt and mutants derived thereof. C. 293 cells were transfected with expression vectors encoding the indicated proteins. FLI-1 mutants were immunoprecipitated using an anti-pan-ETS antibody (recognizing the ETS-domain) and the immunoprecipitates were subjected to SDS-PAGE and Western blotting. Blots were analyzed for the presence of ARIP3 in the FLI-1 precipitates using an ARIP3 antibody (upper panel). The WCL used for the immunoprecipitation was analyzed by Western blotting to confirm the expression of ARIP3, using an anti-ARIP3 antibody. D. 293 cells were transfected with expression vectors encoding the indicated proteins. ARIP3 was immunoprecipitated using an anti-ARIP3 antibody and immunoprecipitates as well as whole cell lysates were subjected to SDS-PAGE and Western blotting. Blots were stained with the indicated antibodies.

Figure 3. ARIP3 and FLI-1 interact directly in vitro. A. Schematic representation of the different FLI-1 deletion mutants fused to GST used in the experiments. NTAD= N-terminal Transcriptional Activation Domain; CTAD= C-terminal Transcriptional Activation Domain; the ETS domain is shown as a hatched box; the Pointed/SAM domain is shown as a gray box; the GST domain is shown as a gray ellipse. B and C. GST-FLI-1 deletion mutants were isolated from transformed and IPTG-induced bacteria, purified on glutathione agarose beads and incubated with in vitro transcribed/translated [35S]-methionine and [35S]-cysteine-labeled wtARIP3 (B), or the indicated deletion mutants of ARIP3 (C). GST pulldown was as described in Experimental procedures and bound proteins were subjected to SDS-PAGE followed by fluorography of the dried gel.
Figure 4. FLI-1 is a SUMO substrate of specific PIAS-family members in vitro and is sumoylated in vivo. A. Recombinant proteins of different members of the PIAS family, HeLa cellular extract containing SUMO-E1 activating activity (SAE1/SAE2), and recombinant E2 (UBC9) and SUMO-1 were incubated with in vitro transcribed/translated \(^{35}\)S-methionine and \(^{35}\)S-cysteine-labeled wtFLI-1 for 1h. Reaction contents were subjected to SDS-PAGE and fluorography of the dried gel to visualize the sumoylation pattern of FLI-1. Non modified FLI-1 migrates as a doublet due to a natural occurring alternative translation initiation site at AUG encoding methionine 34. B-C. HeLa cells were transfected with combinations of expression vectors encoding the proteins indicated at the top of the figures, cells were lysed and whole cell proteins were insolubilized in trichloroacetic acid, resuspended in SDS-PAGE sample buffer, subjected to SDS-PAGE and processed for Western blotting using an anti-FLI-1 antibody. D. 293 cells were transfected with combinations of expression vectors encoding the proteins indicated at the top of the figures, cells were lysed as described in Experimental procedures and whole cell protein extracts were subjected to SDS-PAGE and Western blotting. The blot was successively stained with anti-FLI-1 (top panel), anti-ARIP3 (middle panel) and anti-Erk2 (lower panel; loading control). Arrows indicate the presence of FLI-1\(^{wt}\) and of sumoylated FLI-1 adducts.

Figure 5. ARIP3 represses FLI-1-induced transactivation. A. 293 cells were transfected with 600ng tkD2A-Luc (left panel) or \(-270/-41\)-mFLI-1-Luc (right panel) reporter genes together with 150ng FLI-1 and increasing amounts of ARIP3, as indicated in the figure. Cells were lysed after 24 hrs and extracts assayed for luciferase activity. Lower left panels represent the whole cell lysate subjected to SDS-PAGE and Western blotting to verify the expression of FLI-1, using the anti-FLI-1 antibody. Error bars represent the standard deviation of at least three independent experiments. B. 293 cells were transfected with the tkD2A-Luc reporter gene together with the indicated amounts of expression plasmid either wtFLI-1, or the sumoylation-defective FLI-1[K67R] mutant. C. 293 cells were transfected with the tkD2A-Luc reporter gene (600ng) together with a fixed amount (150ng) of FLI-1 expression vector either alone or together with the indicated amounts of the ARIP3 expression plasmid. Cells were harvested 24h after transfection and luciferase content was assessed as described in Experimental procedures. The effect of ARIP3 on FLI-1 transactivation is shown relative to FLI-1 or FLI-1[K67R] transactivation without ARIP3, which was put at 100 percent. Error bars represent the standard deviation of at least three independent experiments. D. 293 cells were transfected with the tkD2A-Luc reporter gene together with an expression plasmid for wtFLI-1 and increasing amounts of wtARIP3, ARIP3 [\(\Delta347-418\)] or ARIP3[W383A], as indicated. Cells were harvested 24h after transfection and luciferase activity in cellular extracts was assessed as described in Experimental procedures. Error bars represent the standard deviation of at least three independent experiments. In panels A-C, fold activation is calculated as the increase of luciferase activity induced by co-expressed FLI-1 and ARIP3 proteins compared to basal reporter gene activity

Figure 6. FLI-1 relocalises to ARIP3 nuclear bodies. A, B. 293 cells grown on coverslips were transfected with expression plasmids encoding the indicated proteins. 48 hours after transfection cells were fixed with paraformaldehyde and stained with anti-FLI-1/FITC-anti-rabbit IgG (to detect FLI-1, in green), anti-FLAG/Texas-red-anti-rabbit IgG (to detect ARIP3, in red) and Hoechst as described in Experimental procedures. Images were obtained as described in Experimental Procedures and merged image overlaps were obtained using the Metamorph software. Additional processing of images was performed using the Adobe photoshop software.

Figure 7. FLI-1 binding to ETS-binding sequences is not inhibited by ARIP3. A. Expression analysis of FLI-1 and ARIP3 in whole cell extracts used in panel B. 293 cells were transfected with vectors encoding HA-FLI-1 alone or HA-FLI-1 together with HA-ARIP3, lysed after 48 hours as described in Experimental procedures and subjected to SDS-PAGE and Western blotting. Blots were stained with anti-HA to detect HA-FLI-1 and HA-ARIP3. B. 293 lysates described in A containing FLI-1 and/or ARIP3 were subjected as indicated to oligonucleotide pull-down assay using as affintiy matrix either a biotinylated-wtEBS or an mutant EBS carrying a GG to CC transition in its core. In
short, lysates were incubated with biotinylated wtEBS (wtEBS) or mutant EBS (mEBS) double stranded oligonucleotides immobilized on streptavidin beads. Bound proteins were analyzed by SDS-PAGE and Western blotting using anti-HA (top panel) and anti-ARIP3 (lower panel). C. Unbound proteins in the oligonucleotide pull-down assay of panel B were resolved by SDS-PAGE and Western blotting using anti-HA to stain for HA-FLI-1 and HA-ARIP3. Both FLI-1 and ARIP3 were present in that fraction, indicating that the experiment was performed in equilibrium conditions. A-C. Arrows indicate the different proteins and an asterisk indicates the presence of a non-specific background band.

Figure 8. ARIP3-induced repression of FLI-1 transactivation is dependent on the integrity of the SAP domain. A. 293 cells were transfected with the tkD2A-Luc reporter gene (600ng) and ΔEB-FLI-1 (150ng) in absence (top) or presence of pFLAG-ARIP3wt (250ng) or the indicated ARIP3 deletion mutants. The luciferase activity in the corresponding cell extract are shown as black bars. The white bars indicate the tkD2A-luc reporter activity when no ΔEB-FLI-1 is present. Fold activations are calculated as the increase of reporter gene activation compared to basal reporter gene activity (lane2). To account for differences in expression levels between ARIP3 and the different mutants derived thereof, the ratio between wtARIP3 expression levels and its mutants was used to normalize the transactivation values (see below). Error bars represent the standard deviation of at least three independent experiments. B. An aliquot of the pooled lysates from the transactivation experiment above were subjected to SDS-PAGE and Western blotting to assess the expression levels of wtARIP3 and ARIP3 deletion mutants (top panel; anti-FLAG) and FLI-1 (lower panel; anti-FLI-1) in the different samples. The expression levels were measured by quantification of luminescence using Genesnap/Genetools software (Syngene). The relative expression ratio of the different ARIP3 mutants as compared to wtARIP3 are indicated and were used to normalize the luciferase activity values.
Figure 1

Part A

LexA-FLI-1[225-373]  

Part B

LexA-FLI-1[225-373]  

LexA-MST3

Part C

| Condition              | α-FLI-1 | α-Flag (ARIP3) | α-ARIP3 |
|------------------------|---------|----------------|---------|
| HA-BirA + + + +        |         |                |         |
| Bio-FLI-1 + + + +      |         |                |         |
| Flag-ARIP3 wt + +      |         |                |         |
| Flag-ARIP3 Δ13-28 +    |         |                |         |

Streptavidin-beads precipitation

FLI-1 IP

WCL

1 2 3 4 5

α-HA (BirA)
Figure 2

A

| Protein | SAP domain | Acidic domain | Zn-binding region |
|---------|------------|---------------|------------------|
| ARIP3   |            |               |                  |
| ARIP3(Δ13-28) |          |               |                  |
| ARIP3(Δ102-207) |         |               |                  |
| ARIP3(Δ198-337) |        |               |                  |
| ARIP3(Δ347-418) |       |               |                  |
| ARIP3(Δ346-475) |      |               |                  |

B

Bio-FLI-1 and HA-BirA +

Streptavidin-beads precipitation

α-Flag (ARIP3)

α-FLI-1

WCL

C

ARIP3wt + + + +
FLI-1wt + + + +
FLI-1(273-452) + + + +
FLI-ETS(276-373) + + + +

ARIP3 IgG

Pan-ETS IP (FLI-1)

α-ARIP3

WCL

D

HA-FLI-1-ETS (276-373) + + + +
ARIP3wt + + + +
ARIP3Δ13-28 + + + +

α-HA ARIP3 IP

α-ARIP3

WCL

α-HA

Figure 2
Figure 3
A

In vitro sumoylation

| Protein          | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|------------------|---|---|---|---|---|---|---|
| PIAS1            | - | + | - | - | - | - | - |
| PIAS3            | - | - | + | - | - | - | - |
| ARIP3/PIASxα     | - | - | + | - | - | - | - |
| PIASxβ           | - | - | - | + | - | - | - |
| PIASxγ           | - | - | - | - | + | - | - |
| SUMO1            | + | + | + | + | + | + | + |
| E1/E2            | + | + | + | + | + | + | + |

B

| Treatment                  | 1 | 2 | 3 | 4 |
|----------------------------|---|---|---|---|
| FLI-1wt                    | - | + | + | + |
| His-SUMO1                  | - | - | + | - |
| His-HA-SUMO2               | - | - | + | - |
| His-SUMO1-FLI-1            |   |   |   |   |
| SUMO FLI-1                 |   |   |   |   |

C

| Treatment                  | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|----------------------------|---|---|---|---|---|---|---|---|---|----|----|----|
| FLI-1wt                    | + | - | - | + | - | - | + | - | - | - | - | - |
| FLI-1(K67R)                | - | + | - | - | + | - | - | - | - | - | - | - |
| FLI-1(K217R)               | - | - | + | - | - | + | - | - | - | - | - | - |
| FLI-1(K67-217R)            | - | - | + | - | - | + | - | - | - | - | - | - |
| His-SUMO1                  | - | - | - | + | + | + | - | - | - | - | - | - |
| His-HASUMO2                | - | - | - | - | + | + | + | - | - | - | - | - |
| His-SUMO1-FLI-1            | - | - | - | - | + | + | + | - | - | - | - | - |
| His-HA-SUMO2-FLI-1         | - | - | - | - | - | + | + | - | - | - | - | - |

D

| Protein          | 1 | 2 | 3 | 4 | 5 |
|------------------|---|---|---|---|---|
| ARIP3            | + | + |   |   |   |
| HA SUMO-1        | + | + |   |   |   |
| FLI-1            | + | + | + | + | + |

Figure 4
Figure 5

A

B

C

D

Fold activation (a.u.)

pFLAG-ARIP3 (ng):
- 0
- 10
- 50
- 100
- 250

Fold activation (a.u.)

pFLAG-ARIP3 (ng):
- 0
- 10
- 50
- 100
- 250

%FLI-1/FLI-1K67R transactivation

pFLAG-ARIP3 (ng):
- 0
- 10
- 50
- 100
- 250

pFLAG-ARIP3 (ng):
- 0
- 10
- 50
- 100
- 250

ARIP3wt
FLI-1wt
α-ARIP3
α-FLI-1

ARIP3wt
FLI-1wt
ARIP3wt
FLI-1 K67R

ARIP3wt
FLI-1wt
ARIP3 (Δ347-418)
ARIP3 W383A

Figure 5
Figure 6

A

|                | Hoechst | FITC | Tet RED | Merge |
|----------------|---------|------|---------|-------|
| Control        |         |      |         |       |
| HA-FLI-1wt     |         |      |         |       |
| Flag-ARIP3wt   |         |      |         |       |
| HA-FLI-1 + Flag-ARIP3 | | | | |
| HA-FLI-1 K67R  |         |      |         |       |
| HA-FLI-1 K67R + Flag-ARIP3 | | | | |

B

|                | Hoechst | FITC | Tet RED | Merge |
|----------------|---------|------|---------|-------|
| FLag-ARIP3wt   |         |      |         |       |
| HA-FLI-1wt     |         |      |         |       |
| Flag-ARIP3 (Δ13-28) | | | | |
| HA-FLI-1wt + Flag-ARIP3 (Δ13-28) | | | | |
| HA-FLI-1wt + Flag-ARIP3 (Δ467-487) | | | | |
| HA-FLI-1wt + Flag-ARIP3 (Δ347-418) | | | | |
| HA-FLI-1wt + Flag-ARIP3 W383A | | | | |
Figure 7
**Figure 8**

A. Domain structure and relative and normalized luciferase activity (a.u.) for ARIP3 variants.

B. Western blot analysis showing fluorescence ratio (A3/MUT) for ARIP3 variants.

**Legend:**
- ARIP3: Amyloidogenic region interaction protein 3
- SAP: SH3, ankyrin repeat, and positively charged domain
- Zn-domain: Zinc finger domain
- Acrid domain: Acidic domain
- ARIP3 wt: Wild-type ARIP3
- ARIP3(Δ1-102), ARIP3(Δ13-28), ARIP3(Δ102-207), ARIP3(Δ102-260), ARIP3(Δ198-337), ARIP3(Δ346-475), ARIP3(Δ467-547)

**Fluorescence ratio (A3/MUT):**
- 1.0
- 0.9
- 1.2
- 0.4
- 1.1
- 0.5
FLI-1 functionally interacts with PIASXα, a member of the PIAS E3-SUMO-ligase family

Emile van den Akker, Sabine Ano, Hsiu-Ming Shih, Ling-Chi Wang, Martine Pironin, Jorma J. Palvimo, Noora Kotaja, Olivier Kirsh, Anne Dejean and Jacques Ghysdael

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