The SUMO Ubiquitin-Protein Isopeptide Ligase Family Member Miz1/PIASxβ/Siz2 Is a Transcriptional Cofactor for TFII-I*

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We have shown previously that a TFII-I-related protein, hMusTRD1/BEN, represses transcriptional activity of TFII-I. The repression by hMusTRD1/BEN was hypothesized to occur via a two-step competition mechanism involving a cytoplasmic shuttling factor and a nuclear cofactor required for transcriptional activation of TFII-I. Employing a two-hybrid approach with both yeast genomic and mouse cDNA libraries in parallel, we have identified the RING-like zinc finger containing Miz1/PIASxβ/Siz2, which is a ubiquitin-protein isopeptide ligase in the SUMO pathway, as the potential nuclear cofactor that interacts with both TFII-I and hMusTRD1/BEN. Our conclusion is based on the following observations. First, the interactions are biochemically confirmed in mammalian cells where Miz1/mPIASxβ interacts with both TFII-I and hMusTRD1/BEN when these proteins are ectopically co-expressed. Second, co-expression of a nuclear localization signal-deficient mutant of Miz1/mPIASxβ with wild type TFII-I fails to alter the subcellular localization of the former. Finally, ectopically expressed Miz1/mPIASxβ augments the transcriptional activity of TFII-I and relieves the repression exerted by a mutant hMusTRD1/BEN that co-localized with TFII-I in the nucleus.

TFII-I belongs to a family of proteins characterized by the presence of I-repeats first identified in the founding member TFII-I (1–8). Although TFII-I functions as a signal-induced transcriptional activator, the role of the related family member hMusTRD1/BEN is less clear. Both TFII-I and hMusTRD1/BEN are mapped to the breakpoint regions of the 7q11.23 Williams-Beuren syndrome deletion (reviewed in Ref. 2). Furthermore, genetic and biochemical analyses show that each of these proteins have multiple isoforms both in mice and in humans (7, 9–11). The transcription functions of hMusTRD1/BEN have not yet been well characterized biochemically. It was first reported (3) as a muscle specific activator of the troponin I gene. It also seems to function as an activator in yeast one-hybrid assays (6). However, clear demonstration of its activator function is not yet obtained. Results from our laboratory suggest that hMusTRD1/BEN behaves as a specific repressor of TFII-I (10). Although each protein when individually expressed exhibits predominant nuclear localization in eukaryotic cells, TFII-I is excluded from the nucleus when it is co-expressed with hMusTRD1/BEN. Nuclear exclusion of TFII-I results in the repression of the TFII-I-responsive c-fos gene. A key to this novel nuclear exclusion function appears to be the serine stretch (ss)1 in hMusTRD1/BEN because deletion of this stretch results in co-occupancy of both proteins in the nucleus (10). However, although the Δss hMusTRD1/BEN failed to prevent nuclear localization of TFII-I, the transcriptional repression of TFII-I was still observed. The latter data led us to postulate that the repression by hMusTRD1/BEN involves the following two-step mechanism: a competition for a common cytoplasmic factor required for nuclear translocation and a competition for a nuclear cofactor required for transcriptional activation.

In an attempt to identify these putative factors that are shared by both the activator (TFII-I) and the repressor (hMusTRD1/BEN), we performed a two-hybrid screen using TFII-I and hMusTRD1/BEN as baits. We screened both yeast genomic and mouse cDNA libraries with the rationale that these screens would ideally yield homologous proteins. We further set the criteria such that (a) the interacting proteins should interact with both TFII-I and hMusTRD1/BEN when these proteins are co-expressed, and (b) the nuclear cofactor should interact with both the wild type and the Δss hMusTRD1/BEN, whereas the cytoplasmic factor should not interact with the latter. By employing such a double screen, we report that we have identified protein Siz2/Miz1/PIASxβ that appears to be the nuclear cofactor that interacts with TFII-I as well as the wild type and the Δss hMusTRD1/BEN. The yeast library screen yielded NFI1 (neck filament interacting protein) also known as Siz2 (after SAP and Miz) (12–14, 19–21), whereas the mouse library screen yielded the corresponding homologue, Miz1/mPIASxβ. NFI1/Siz2 and Miz1/mPIASxβ interact more avidly with hMusTRD1/BEN than TFII-I both in yeast and in mammalian interaction assays. This RING-like zinc finger-containing factor is localized exclusively in the nucleus and failed to alter the nuclear residency of TFII-I or hMusTRD1/BEN in mammalian localization assays. Consistent with these properties, ectopic Miz1/mPIASxβ augments transcriptional activation by TFII-I and alleviates Δss hMusTRD1/BEN-mediated transcriptional repression.

EXPERIMENTAL PROCEDURES

Cell Culture

COS7 cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 50 units/ml penicillin, and 50 μg/ml streptomycin (Invitrogen).

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1 The abbreviations used are: ss, serine stretch; wt, wild type; GST, glutathione S-transferase; GFP, green fluorescent protein; NLS, nuclear localization signal; rhEGF, recombinant human epidermal growth factor; ADH1, alcohol dehydrogenase 1; 3-AT, 3-aminotriazole; PML, promyelocytic leukemia; X-α-gal, 5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside.
Yeast Strains and Media

The Saccharomyces cerevisiae pA69-4A strain was used in this study (Mata, trp1-901, leu1-3, 112, ural-3-52, his3-200, gal4Δ, gal80Δ, GAL2-ADE2, LYS2::GAL1-HIS3, met2::GAL7-lacZ) (15). Yeast was generally grown in rich media (YPD), and once they were transformed with 2-μm expression plasmids, they were grown in synthetic media (SD) containing the appropriate amino acids and nitrogen base supplement.

Plasmid Constructs

pGBKDU-TFII-I—A BamHI-ClaI TFII-I fragment from pEFG-TFII-I (14) was subcloned into BamHI-ClaI sites of the pGBDU-C1 vector (2 μm, ADH1 promoter driving GAL4 DNA binding domain C1, C2, C3-MCS, URA3, and Amp+) (15) to make the GAL4 DNA binding domain-TFII-I bait construct.

pGBDU-MusTRD1/BN—To make the pGBDU-MusTRD1/BEN wt, the full-length hMusTRD1/BEN was PCR-amplified from the pGEM-7Zf(-) plasmid using the following oligonucleotides: 1) EcoRI 5′-GGATCCGCTGTTCAAGGTTGC-3′ and 2) ClaI 5′-CCATGGGATCCCTTGTACAGCTCGTCCATGC-3′. The PCR product was digested with EcoRI and ClaI, gel-purified, and subcloned into the same sites of pGBDU-C1 vector (15) to get the GAL4 DNA binding domain-hMusTRD1/BEN wt bait construct.

pGBDU-MusTRD1/BN ΔN—An SmaI-BstWI fragment from pEBB-GFP-hMusTRD1/BEN ΔN (10) was subcloned into the same sites of the pGBDU-MusTRD1/BEN wt construct.

pEBG-Miz1/mPIASβ—A BamHI-NotI fragment from the pGEX-4T-1-Miz1/mPIASβ vector (kindly provided by Robert Maxson, University of Southern California School of Medicine, Los Angeles, CA), corresponding to an N-terminal truncated form of mPIASβ (amino acids 125–614 of GenBankTM accession number BC005596), was subcloned into the BamHI-NotI-digested pEBG vector. The assignment of amino acids numbers is based on the truncated form of the Miz1/mPIASβ.

pEBB-GFP-Miz1/mPIASβ—The same BamHI-NotI fragment from pGEX-4T-1-Miz1/mPIASβ was subcloned into BamHI-NotI sites of pEBB vector. The GFP was PCR-amplified from pEGFP-N1 (Clontech) using the following primers: 5′-CGGGATCCATGGTGAGCAAGGG- and 5′-CGGGATCCCTTGTACAGCTCGTCCATGC-3′. The final PCR product was digested with EcoRI and ClaI, gel-purified, and ligated into the same sites of pGBDU-GFP-Miz1/mPIASβ or pEBB-GFP-Miz1/mPIASβ.

pEBB-GFP-Miz1/mPIASβ—The same BamHI-NotI fragment from pGEX-4T-1-Miz1/mPIASβ was subcloned into BamHI-NotI sites of pEBB vector. The GFP was PCR-amplified from pEGFP-N1 (Clontech) using the following primers: 5′-CGGGATCCATGGTGAGCAAGGG- and 5′-CGGGATCCCTTGTACAGCTCGTCCATGC-3′. The final PCR product was digested with EcoRI and ClaI, gel-purified, and ligated into the same sites of pGBDU-GFP-Miz1/mPIASβ or pEBB-GFP-Miz1/mPIASβ.

pEGF-Miz1/mPIASβ—The same BamHI-NotI fragment from pGEX-4T-1-Miz1/mPIASβ was subcloned into BamHI-NotI sites of pEBB vector. The GFP was PCR-amplified from pEGFP-N1 (Clontech) using the following primers: 5′-CGGGATCCATGGTGAGCAAGGG- and 5′-CGGGATCCCTTGTACAGCTCGTCCATGC-3′. The final PCR product was digested with EcoRI and ClaI, gel-purified, and ligated into the same sites of pGBDU-GFP-Miz1/mPIASβ or pEBB-GFP-Miz1/mPIASβ.

RESULTS

Yeast Transformation and Luciferase Assays

Transfections were performed with LipofectAMINE (Invitrogen) as described (10). Before harvesting, cells were serum-starved for 14–16 h and stimulated with 25 ng/ml of recombinant human epidermal growth factor (rhEGF; Sigma) for 4 h. Total transfected DNA was kept constant by the empty vectors pEBB or pEFG. Luciferase activity was measured using the Dual Luciferase kit (Promega). Experiments were done at least three times with triplicate sets included each time.

Cell Extracts, GST Pull-down, and Immunoblotting

At 42 h post-transfection COS7 cells were harvested, washed twice in phosphate-buffered saline buffer, and lysed in lysis buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Triton-X100) containing 1X antiprotease mixture EDTA-free (Roche Molecular Biochemicals) for 30 min at 4 °C. The lysate was clarified by centrifugation for 10 min at 14,000 rpm at 4 °C. Total protein concentration was measured by the Bradford method (Bio-Rad) with a 1 μg of bovine serum albumin used as the standard. Immunoprecipitation with anti-TFII-I antibody, and 300 μg were used for GST pull down. Incubations with protein A-agarose or glutathione-agarose beads (50 μl, 1 slurry; Invitrogen and Sigma, respectively) were done for 2 h, rocking at 4 °C. At the end of the incubation, the beads were washed 2 times with 1 ml of lysis buffer without detergents. After the final wash, 4 × SDS sample buffer was added to the beads, boiled at 100 °C for 3 min, and loaded onto a 10% SDS-PAGE. Semi-dry transfer to nitrocellulose was done as described (18). The mouse monoclonal antibodies, anti-GST (GST-2, Sigma) and anti-GFP (JL-18, Clontech), were used at 1:3500 and 1:2000, respectively. Detection was done by enhanced chemiluminescence (PerkinElmer Life Sciences) using standard methods.

Immunofluorescence

COS7 cells were transfected with 200 ng of each construct, and 26 h post-transfection cells were fixed as described before (10). Cells were incubated with mouse monoclonal antibody anti-GST (GST-2, Sigma) (1:3500) followed by Alexa 594 goat anti-mouse IgG (Molecular Probes) (1:2000) and visualized on a fluorescence E400 Nikon microscope.

RESULTS

Identification of Siz2/Miz1/mPIASβ as a hMusTRD1/BEN and TFII-I Interacting Protein by Two-hybrid Screen—In an attempt to identify the putative factors that are shared by both TFII-I and hMusTRD1/BEN, we performed a two-hybrid screen using both of these proteins sequentially as baits. We screened
both yeast genomic library and a mouse cDNA library with the rationale that these screens would ideally yield homologous proteins. Indeed, by using this approach, the yeast library screen yielded NFI1/SIZ2 (12–14), whereas the mouse library screen yielded the corresponding homologue, Miz1/mPIAS\(\beta\) (19) (Fig. 1). Although in all our analyses we have used the cDNA corresponding to Miz1, sequence analysis revealed that Miz1 is actually a truncated form of mPIAS\(\beta\) missing the N-terminal 124 amino acids. The PIAS proteins belong to a large family of proteins that share two highly conserved motifs (Fig. 2) as follows: a SAP domain (a putative DNA-binding motif) presumably involved in chromosomal organization (12, 20, 21) and a RING-like zinc finger motif implicated in protein-protein interactions (22–24). Importantly, both NFI1/SIZ2 and Miz1/mPIAS\(\beta\) interacted better with \(\Delta s\) hMusTRD1/BEN. Our final selection of true positive clones was determined under extremely stringent conditions (Fig. 1). Hence, the observation that TFII-I yeast co-transformants grow much slower than wt or \(\Delta s\) hMusTRD1/BEN yeast co-transformants reflects the strength of interaction. We conclude that Siz2/Miz1/mPIAS\(\beta\) interaction with hMusTRD1/BEN in yeast is stronger than the interaction of Siz2/Miz1/mPIAS\(\beta\) with TFII-I.

**In Vivo Interactions of Miz1/mPIAS\(\beta\) with TFII-I or hMusTRD1/BEN**—After establishing interactions of Siz2/Miz1/mPIAS\(\beta\) with both TFII-I and hMusTRD1/BEN in yeast, we wanted to corroborate these interactions in mammalian cells. Furthermore, to determine which regions of Miz1/mPIAS\(\beta\) are important for potential physical and functional interactions with TFII-I and hMusTRD1/BEN, we also generated a series of C-terminal mutants (Fig. 3A). For interaction studies, we either expressed GST-tagged Miz1/mPIAS\(\beta\) (wild type or mutants) alone or with GFP-tagged TFII-I (Fig. 3B) or with GFP-tagged hMusTRD1/BEN (Fig. 4) in COS7 cells. The potential interactions were analyzed either by immunoprecipitation (for TFII-I, Fig. 3B, upper panel) or by GST pull down (for hMusTRD1/BEN, Fig. 4, upper panels). These results revealed that wild type GST-Miz1/mPIAS\(\beta\) was immunoprecipitated when co-expressed with GFP-TFII-I (Fig. 3B, compare lane 1 with lane 2). Deletion of the C-terminal 141 amino acids, containing the serine-threonine-rich regions, increased the interaction of this mutant with TFII-I (compare lanes 2 and 4). Whereas deletion of a portion of the RING/zinc finger (amino acids 231–236, AZF) or deletion of a putative nuclear localization signal (\(\Delta NLS1\)) did not alter the interaction of Miz1/mPIAS\(\beta\) with TFII-I (lanes 5 and 6), deletion of the putative second NLS (\(\Delta NLS2\)) significantly diminished the interaction with TFII-I (lane 7). The amount of immunoprecipitated TFII-I in all lanes was comparable except in lane 5 (bottom panel). However, when normalized to the precipitated protein in lane 1, it appears that the interaction of TFII-I with AZF is even better than the wild type Miz1/mPIAS\(\beta\). These results suggested that interaction of Miz1/mPIAS\(\beta\) with TFII-I is mediated largely through the N-terminal portion of Miz1/mPIAS\(\beta\) that does not include the RING/zinc finger.

Identical results were obtained with hMusTRD1/BEN (Fig. 4, top and bottom panels). AZF Miz1/mPIAS\(\beta\) interacted better than the wild type protein with hMusTRD1/BEN (compare lanes 3 and 8). Because hMusTRD1/BEN is expressed less than TFII-I in general, we observed fainter bands in GST pull-down assays with GST-Miz1/mPIAS\(\beta\). The GST pull-down assay was performed due to a lack of specific antibodies against either hMusTRD1/BEN or Miz1/mPIAS\(\beta\). Despite these differences in assay conditions, it is clear that both TFII-I and hMusTRD1/BEN interact with Miz1/mPIAS\(\beta\) through the N-terminal end of Miz1/mPIAS\(\beta\). It is also worth mentioning that \(\Delta s\) hMusTRD1/BEN also interacted with Miz1/mPIAS\(\beta\) in GST pull-down assays (data not shown).

**Identification of a Functional Nuclear Localization Signal (\(\Delta NLS2\)) in Miz1/mPIAS\(\beta\)**—Although members of PIAS family of proteins have been shown to exhibit nuclear residency, no definitive localization for Miz1/mPIAS\(\beta\) has been documented. To determine the functional nuclear localization signal in Miz1/mPIAS\(\beta\), either wild type or various mutants were expressed as GST fusion proteins in COS7 cells, and their localization was determined by indirect immunofluorescence (Fig. 5). Miz1/mPIAS\(\beta\) (wt) was localized exclusively to the nucleus when expressed individually. Although deletion of NLS1 (\(\Delta NLS1\)) did not have any significant effect, deletion of NLS2 (\(\Delta NLS2\)) profoundly altered the nuclear residency of Miz1/mPIAS\(\beta\), suggesting that NLS2 is the functional sequence under our conditions and in this cell type. Neither deletion of the zinc/RING finger motifs (AZF) nor deletion of the C-terminal 141 amino acids (\(\Delta C141\)) had significant effect on the nuclear localization of Miz1/mPIAS\(\beta\). Interestingly, AZF localizes in nuclear dots far more than the wild type protein. Also a slight cytoplasmic spreading out observed with overexpression of \(\Delta C141\) may be due to the fact that the deletion is too close to NLS2. As anticipated, further deletion from the C-terminal end (\(\Delta C237\) and \(\Delta C382\)) had severe effect on the
nuclear localization of Miz1/mPIASx/H9252 because both of these mutations remove the functional NLS2 (Fig. 5).

Localization of Miz1/mPIASx/H9252 NLS Mutant Is Unaltered Upon Co-expression With TFII-I or hMusTRD1/BEN—We rationalized that if Miz1/mPIASx/H9252 is indeed a nuclear cofactor for TFII-I, then it should have a nuclear residency even in the presence of TFII-I. To test this idea, either wild type or various mutants of GST-Miz1/mPIASx/H9252 were co-expressed with GFP-TFII-I or GFP-hMusTRD1/BEN in COS7 cells, and their localization was determined by indirect immunofluorescence. As expected, there was no change in the nuclear localization of either Miz1/mPIASx/H9252 or TFII-I when these proteins were co-expressed and both co-localized in the nucleus (Fig. 6). Identical results were also obtained when Miz1/mPIASx/H9252 was co-expressed with hMusTRD1/BEN (Fig. 6). However, these results do not indicate whether the interactions between Miz1/mPIASx/H9252 and TFII-I or hMusTRD1/BEN occur in the nucleus. In order to test whether the interactions are occurring in the cytoplasm or nucleus, we employed the NLS2 mutant of Miz1/mPIASx/H9252. Our rationale was that if the two proteins interact stably in the cytoplasm, wild type TFII-I or wild type hMusTRD1/BEN should be able to “carry” the NLS mutant Miz1/mPIASx/H9252 to the nucleus. However, no significant change in cytoplasmic localization of NLS2 Miz1/mPIASx/H9252 was observed when co-expressed with either TFII-I or with hMusTRD1/BEN. These data suggests that either interaction between these proteins is not strong enough or that it occurs inside the nucleus. Based on the fact that we observe a greatly diminished interaction of the NLS2 Miz1/mPIASx/H9252 with TFII-I or hMusTRD1/BEN, we prefer the latter explanation. In converse experiments both a nuclear signal deficient-TFII-I or a nuclear-deficient hMusTRD1/BEN remains in the cytoplasm when co-expressed with wild type Miz1/mPIASx/H9252, further corroborating our idea (data not shown). We have also observed that the nuclear localization of TFII-I does not change substantially when it is ectopically expressed in a yeast strain that lacks the SIZ2 gene and subsequently complemented with ectopic Miz1/mPIASx/H9252.² Because we observed an enhanced interaction between the ΔC382 and TFII-I or hMusTRD1/BEN, we also wanted to test whether such interactions might lead to alteration in nuclear localization of the latter proteins. However, upon co-expression of TFII-I or hMusTRD1/BEN with ΔC382 Miz1/mPIASx/H9252, we did not observe any significant

² M. I. Tussié-Luna and A. L. Roy, unpublished data.

FIG. 2. Schematics of PIAS family proteins. Members of this family are found in different eukaryotic species. The relative localization of the SAP domain, proline-rich domain (putative SH3 binding domain), serine/threonine/glutamine-rich domain, RING-like zinc finger, and the putative nuclear localization signals are indicated.

FIG. 3. Miz1/mPIASx/H9252 interacts physically with TFII-I in mammalian cells. A, schematics of GST-Miz1/mPIASx/H9252 constructs. B, COS7 cells were transfected with 10 µg of GFP-TFII-I vector alone (lane 1) or co-transfected with 10 µg of either GST-Miz1/mPIASx/H9252 wt (lane 2), ΔC141 (lane 3), ΔC382 (lane 4), ZF (lane 5), NLS1 (lane 6), or NLS2 (lane 7). 44 h post-transfection, whole cell lysates were prepared, and 1000 µg were immunoprecipitated (IP) with anti-TFII-I antibody. Blots were probed first with anti-GST antibody (α-GST) (upper panels), stripped, and reprobed with anti-GFP antibody (α-GFP) (lower panel). WB, Western blot.
change in nuclear localization of TFII-I or hMusTRD1/BEN. Although 77% of ectopically TFII-I remained in the nucleus when co-expressed with wild type Miz1/mPIASxβ, 70% of nuclear TFII-I was found when it was co-expressed with ΔC382 Miz1/mPIASxβ. Thus, the enhanced interaction of TFII-I or hMusTRD1/BEN with ΔC382 Miz1/mPIASxβ most likely occurred at a post-lysis stage.

Miz1/mPIASxβ Can Be a Co-activator of TFII-I Transcriptional Activity—Because TFII-I and Miz1/mPIASxβ co-localize in the nucleus, we were interested to test whether Miz1/mPIASxβ would augment or inhibit transcriptional activity of TFII-I. However, if Miz1/mPIASxβ is indeed the nuclear cofactor that we sought, then it should co-activate rather than repress. In transient co-transfection assays with c-fos as a reporter and in the presence of rhEGF, TFII-I activated the c-fos promoter in a dose-dependent fashion (Fig. 7, lanes 2 and 3) over the vector-only lanes (lane 1). Miz1/mPIASxβ alone also gave a 3-fold increase in promoter activity in the presence of rhEGF (lanes 4 and 5). Co-expression of low but constant levels of TFII-I with increasing levels of Miz1/mPIASxβ resulted in nearly an additive activation of the c-fos promoter (lanes 6 and 7). We conclude that Miz1/mPIASxβ augments transcriptional activity of TFII-I, although the co-activity is not very high. Nevertheless, the increase in transcriptional activity is not due to an increase in TFII-I expression as the levels of expression of ectopic TFII-I decreases when both proteins were co-expressed (compare lanes 2 and 3 with 6 and 7, bottom panels). The increase in transcriptional activity seen with Miz1/mPIASxβ alone most likely reflects activation of endogenous TFII-I, although this remains to be formally proven. In the same assay we also tested the co-activation potentials of two mutants of Miz1/mPIASxβ, ΔZF and ΔC382. ΔZF showed modest activation potential on its own (compare lanes 1 with 8 and 9) and retained modest co-activation potentials with TFII-I (compare lanes 2 with 10 and 11) despite the fact that the activities were lower than observed with wild type Miz1/mPIASxβ. However, ΔC382 showed neither activation (lanes 12 and 13) nor any significant co-activation (lanes 14 and 15) potentials. We conclude that the zinc finger may not be essential for co-activation function of Miz1/mPIASxβ and that nuclear residency of Miz1/mPIASxβ is required for its TFII-I-dependent transcription function.
Miz1/mPIASxβ/Siz2 Interacts with TFII-I

Miz1/mPIASxβ is indeed a nuclear cofactor for TFII-I that can be titrated out by hMusTRD1/BEN leading to transcriptional repression of TFII-I-dependent promoters. Moreover, such function of Miz1/mPIASxβ is dependent upon its nuclear localization.

DISCUSSION

The TFII-I family of proteins currently has two members, each with multiple isoforms (25). One distinguishing feature about these proteins is that they are genetically linked in human chromosome 7 (7q11.23) and are deleted in Williams-Beuren syndrome (25). Perhaps a more interesting feature is the fact that although TFII-I is a transcriptional activator, under the same conditions its family member hMusTRD1/BEN behaves as a repressor (10). In an attempt to elucidate the molecular mechanisms governing this family of proteins, we undertook a two-hybrid screen with the notion that interacting partners may provide useful hints to the biochemical modes of action of these proteins. We performed a parallel screen with both yeast and mammalian libraries. Although this was not the most important criterion for screening, we rationalized that if homologous proteins are pulled out from these two screens, it will provide a compelling evidence for bona fide interactions. Out of the three clones that survived this criterion, only Siz2/Miz1/mPIASxβ appeared to be a potential nuclear factor with transcriptional properties (26–30). Although we used hMusTRD1/BEN as our primary bait for screening these libraries, we also tested both TFII-I and hMusTRD1/BEN as interacting partners of the isolated putative clones. In yeast interaction studies, NFI1/Siz2 and Miz1/mPIASxβ interacted with TFII-I, hMusTRD1/BEN, as well as Δss mutant of hMusTRD1/BEN (Fig. 1). Hence, we proceeded to characterize the physical and functional interactions of Miz1/mPIASxβ with TFII-I and hMusTRD1/BEN in mammalian cells and to test whether Miz1/mPIASxβ is the nuclear cofactor of TFII-I that is competed by hMusTRD1/BEN leading to transcriptional repression.

The interactions observed in yeast were recapitulated in mammalian cells upon co-expression of Miz1/mPIASxβ with either TFII-I or hMusTRD1/BEN (Figs. 3 and 4). Although it appears that a fraction of input TFII-I or hMusTRD1/BEN interacts with Miz1/mPIASxβ under the assay conditions, whether this is also true under more physiologic conditions could not be determined because an antibody against endogenous Miz1/mPIASxβ is not available, and thus co-immunoprecipitation experiments could not be performed. Moreover, whether under normal conditions these interactions take place in the nucleus also could not be determined. Although Miz1/mPIASxβ belongs to a large family of proteins (Fig. 2), the majority of which have nuclear localization, precise subcellular localization of Miz1/mPIASxβ has not been demonstrated. Hence, to follow further characterization of Miz1/mPIASxβ and

Fig. 5. Identification of a functional nuclear localization signal NLS2 in Miz1/mPIASxβ. COS7 cells were transfected with 200 ng of expression plasmids coding for GST-Miz1/mPIASxβ wt (top row), GST-Miz1/mPIASxβ ΔNLS1 (2nd row), GST-Miz1/mPIASxβ ΔNLS2 (3rd row), GST-Miz1/mPIASxβ ΔZF (4th row), GST-Miz1/mPIASxβ ΔC141 (5th row), GST-Miz1/mPIASxβ ΔC237 (6th row), or GST-Miz1/mPIASxβ ΔC382 (last row). 30 h post-transfection, cells were fixed with 4% paraformaldehyde, and the ectopically expressed proteins were visualized by indirect immunofluorescence with monoclonal anti-GST antibody (α-GST) and Alexa 594 goat anti-mouse IgG secondary antibody. Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI). Superimposition of Alexa 594 and 4,6-diamidino-2-phenylindole images is shown at the far right panels. Images were obtained by using a Nikon E400 fluorescence microscope with a 100× objective.

Fig. 6. Wild type Miz1/mPIASxβ co-localizes with nuclear TFII-I or hMusTRD1/BEN, whereas ΔNLS2 and ΔC382 Miz1/mPIASxβ do not alter TFII-I and hMusTRD1/BEN nuclear localization. COS7 cells were transfected with 200 ng of expression plasmids coding for TFII-I-GFP (top row) or GFP-hMusTRD1/BEN (2nd row) alone or co-transfected with 200 ng of GST-Miz1/mPIASxβ wt plus 200 ng of either TFII-I-GFP or GFP-hMusTRD1/BEN (3rd and 4th rows), 200 ng of GST-Miz1/mPIASxβ ΔNLS2 plus 200 ng of either TFII-I-GFP or GFP-hMusTRD1/BEN (5th and 6th rows), or 200 ng of GST-Miz1/mPIASxβ ΔC382 plus 200 ng of either TFII-I-GFP or GFP-hMusTRD1/BEN (7th and last row). 30 h post-transfection, cells were processed as in Fig. 5.
its interactions with TFII-I family proteins, it was important for us to determine unequivocally the subcellular localization of Miz1/mPIASxβ at least when ectopically expressed. Under our assay conditions, Miz1/mPIASxβ localized exclusively to the nucleus. Interestingly, the staining revealed a speckled pattern with nuclear pod- or dot-like structures that are reminiscent of promyelocytic leukemia (PML) bodies. Our preliminary data suggest that the nuclear dots observed with Miz1/mPIASxβ indeed co-localize with PML bodies. However, the functional consequences of this co-localization are unclear at present. The localization of TFII-I and hMusTRD1/BEN in PML bodies is under study at present.

Data base scanning (PSORT) (31) revealed two consensus nuclear localization signals in Miz1/mPIASxβ, termed NLS1 and NLS2. Of these, NLS1 appears to be non-functional at least under our assay conditions because deletion of NLS1 did not alter the nuclear localization of Miz1/mPIASxβ. The NLS2 is functional because the deletion of NLS2 causes a major change in the subcellular localization of Miz1/mPIASxβ. It is also clear that co-expression of TFII-I or hMusTRD1/BEN does not increase the nuclear localization of NLS mutant Miz1/mPIASxβ. Conversely, wild type Miz1/mPIASxβ also failed to alter the localization of a nuclear localization deficient mutant of TFII-I (9) or a nuclear localization deficient mutant of hMusTRD1/BEN when both proteins were co-expressed (data not shown). Despite the fact that the wild type proteins interact, the failure of a wild type protein to alter the localization of the partner mutant could be either due to the fact that these proteins interact weakly or transiently or that they interact exclusively in the nucleus. Although these scenarios are not yet clear, the fact that protein substrates must be targeted to the nucleus in order to undergo SUMO-1 conjugation (32), a process dependent on PIAS family proteins, favors the latter idea. TFII-I, on its own, is a weak transcriptional activator. Miz1/mPIASxβ also has a weak transcriptional activity in the absence of ectopically expressed TFII-I. Together they appear to have an additive effect. It is not clear at present whether Miz1/mPIASxβ binds to c-fos promoter directly in a sequence-specific fashion or whether it is a transcriptional cofactor for TFII-I. Although Miz1/mPIASxβ can bind sequence specifically to dou-

**Fig. 7.** Miz1/mPIASxβ is a cofactor for TFII-I. A, c-fos luciferase activity of TFII-I-GFP, GST-Miz1/mPIASxβ wt, ΔZF, or ΔC382 alone or TFII-I-GFP + GST-Miz1/mPIASxβ wt, ΔZF, or ΔC382 in the presence (filled columns) or in the absence (open columns) of 25 ng/ml rhEGF (top panel). Lanes 1, empty vector; lanes 2 and 3, TFII-I-GFP, 600 and 1800 ng; lanes 4 and 5, GST-Miz1/mPIASxβ wt, 600 and 1800 ng; lanes 6 and 7, TFII-I-GFP, 600 ng + GST-Miz1/mPIASxβ wt, 600 or 1800 ng; lanes 8 and 9, GST-Miz1/mPIASxβ ΔZF, 700 and 2100 ng; lanes 10 and 11, TFII-I-GFP, 600 ng + GST-Miz1/mPIASxβ ΔZF, 700 or 2100 ng; lanes 12 and 13, GST-Miz1/mPIASxβ ΔC382, 700 and 2100 ng; lanes 14 and 15, TFII-I-GFP, 600 ng + GST-Miz1/mPIASxβ ΔC382, 700 and 2100 ng (top panel). B, cell extracts from indicated lanes in A were analyzed by Western blot probed with anti-GFP antibody (α-GFP) (top panel) and then stripped and reprobed with anti-GST antibody (α-GST) (bottom panel).

**Fig. 8.** Miz1/mPIASxβ alleviates Δss hMusTRD1/BEN mediated repression of TFII-I transcriptional activity. A, c-fos luciferase activity of TFII-I-GFP, GST-Miz1/mPIASxβ wt, ΔZF, or ΔC382; TFII-I-GFP + GST-hMusTRD1/BEN Δss and TFII-I-GFP + GST-hMusTRD1/BEN Δss + GST-Miz1/mPIASxβ wt, ΔZF, or ΔC382 in the presence (filled columns) or in the absence (open columns) of 25 ng/ml rhEGF (top panel). Lane 1, empty vector; lane 2, TFII-I-GFP, 600 ng; lane 3, GST-Miz1/mPIASxβ wt, 1500 ng; lane 4, GST-Miz1/mPIASxβ ΔZF, 2000 ng; lane 5, GST-Miz1/mPIASxβ ΔC382, 2000 ng; lanes 6–9, GST-hMusTRD1/BEN Δss, 600 ng; lane 7, + GST-Miz1/mPIASxβ wt, 1500 ng; lane 8, + GST-Miz1/mPIASxβ ΔZF, 2000 ng; lane 9, + GST-Miz1/mPIASxβ ΔC382, 2000 ng; lanes 10–16, TFII-I-GFP, 600 ng + GST-hMusTRD1/BEN Δss, 600 ng; lanes 11, 13, and 15, + GST-Miz1/mPIASxβ wt, ΔZF, or ΔC382, 650 ng, respectively; and lanes 12, 14, and 16, + GST-Miz1/mPIASxβ wt, ΔZF, or ΔC382, 2000 ng, respectively. B, cell extracts from indicated lanes in A were analyzed by Western blot probed with anti-GFP antibody (α-GFP) (top panel) and then stripped and reprobed with anti-GST antibody (α-GST) (bottom panel).
ble strand DNA in gel shift assays, the precise consensus sequence or the target genes are not well characterized yet (19). It is worthwhile to point out that various members belonging to the PIAS family (Fig. 2), viz. androgen receptor interacting protein (ARIP3) (26, 27), protein inhibitor of activated STATs (PIAS1, PIAS3, and PIASy) (28–30), and Miz1 (19), have all been shown to possess varying degrees of transcriptional activity. However, it is not clear at present whether Miz1/mPIASβ can indeed behave as a gene-specific transcriptional activator or it behaves more as a transcriptional co-regulator together with TFII-I. Although it is very likely that the full range of transcriptional activity by Miz1/mPIASβ requires its interaction with TFII-I and its N-terminal regions, the activation of c-fos by Miz1/mPIASβ in the absence of ectopic TFII-I makes it more difficult to pinpoint its mechanism of action. Accordingly, changes in transcriptional activity of the mutants of Miz1/PIASβ could be independent of TFII-I. This issue has to be addressed by determining whether Miz1/mPIASβ has a consensus binding site in the c-fos promoter and, if so, whether Miz1/mPIASβ binds to this site and activates it. Given the fact that STAT1 and STAT3 activate the c-fos promoter, we also tested the effect of PIAS1 and PIAS3 on TFII-I-dependent activation. However, in our hands, we failed to see any significant effects of either PIAS1 or PIAS3 on TFII-I-dependent activation of the c-fos promoter (data not shown). Most importantly, Miz1/mPIASβ relieved the Δss hMusTRD1/BEN-mediated transcriptional repression. Thus, under conditions in which TFII-I-dependent activation was completely abrogated by Δss hMusTRD1/BEN, co-expression of Miz1/mPIASβ rescued such repression. Miz1/mPIASβ alone, in the absence of TFII-I, also prevented Δss hMusTRD1/BEN-mediated transcriptional repression, although the relief of repression was less than achieved in the presence of TFII-I. This result suggests that Miz1/mPIASβ requires TFII-I for its full range of activity under our assay conditions, leading us to believe that Miz1/mPIASβ in this context might be acting more as a transcriptional co-regulator of TFII-I rather than as an independent transcriptional activator. Regardless, it is clear that Miz1/mPIASβ is a nuclear co-regulator or cofactor for TFII-I that is titrated away by hMusTRD1/BEN leading to transcriptional repression. The precise mechanism of this pathway remains to be elucidated.

The PIAS family of proteins is characterized by the presence of an N-terminal SAP domain believed to be involved in chromosome organization (12, 21, 22). However, because a truncated form of mPIASβ (Miz1, lacking the SAP domain) interacts with TFII-I, the SAP domain is probably dispensable for such interactions. This family is also characterized by the presence of a RING-like zinc finger motif, presumably involved in protein-protein interactions (22–24). Compelling evidence supports the involvement of a number of different and functionally distinct RING finger proteins in ubiquitin- and SUMO-dependent pathways (22, 23). Recent studies (13, 14, 33–37) have demonstrated that SIZ2, PIASx, PIASβ, PIASγ, PIAS1, and PIAS3 behave as ubiquitin-protein isopeptide-SUMO-ligases that sumoylate septsins and several transcription factors (13, 14, 33–36). In this regard, it has been shown that the enzymatic activity of PIAS family proteins is dependent on its zinc/RING finger domains (33, 34). However, our data reveal that deletion of amino acids 231–236 coding for central CTH-LQC zinc/RING finger of Miz1/mPIASβ (AZF) does not significantly affect its interaction with TFII-I or hMusTRD1/BEN and that the functional effects of these mutations are rather moderate. However, lack of enzymatic activity of this mutant needs to be formally proven.

Despite the fact that Miz1/mPIASβ is not yet well characterized, many of its family members have been shown to have exciting properties that control multiple aspects of chromosome structure and function by establishing or maintaining chromosome organization in interphase nuclei (12, 13, 21, 33, 38). Taken together these data suggest that besides its role in transcriptional activation or repression, the PIAS family of proteins will have a definite role in other chromosomal functions as well. One of the other clones obtained from the two-hybrid screens that interacted with both TFII-I and hMusTRD1/BEN was Ubc9, a ubiquitin-conjugating enzyme involved in SUMO modification. These data raise the possibility that TFII-I family of proteins may undergo SUMO modification and are also likely to be involved in SUMO-dependent pathways that control transcription. However, given that AZF interacts with TFII-I and partially functions as a co-activator, it appears that the interactions of TFII-I family proteins with Miz1/mPIASβ may be independent of enzymatic activity of Miz1/mPIASβ. It has been observed in other cases that PIAS proteins can tether other sumoylated proteins in a noncovalent fashion (37). Whether TFII-I family proteins serve a regulatory role in this latter process remains to be determined.

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The SUMO Ubiquitin-Protein Isopeptide Ligase Family Member Miz1/PIASxβ/Siz2 Is a Transcriptional Cofactor for TFII-I
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