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Spatial Interplay between PIASy and FIP200 in the Regulation of Signal Transduction and Transcriptional Activity[▽]

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The members of the protein inhibitor of activated STAT (PIAS) family of proteins are implicated in fundamental cellular processes, including transcriptional regulation, either through action as E3 SUMO ligases or through SUMO-independent effects. We report here the identification of FIP200 (focal adhesion kinase family-interacting protein of 200 kDa) as a new PIASy-interacting protein. We show that the interaction depends on the integrity of the RING finger of PIASy and the carboxy terminus of FIP200. Both in vitro and in vivo sumoylation assays failed to reveal any sumoylation of FIP200, suggesting that FIP200 is not a bona fide SUMO substrate. Immunofluorescence microscopy and subcellular fractionation, either upon forced PIASy expression or in the absence of PIASy, revealed that interaction with PIASy redistributes FIP200 from the cytoplasm to the nucleus, correlating with abrogation of FIP200 regulation of TSC/S6K signaling. Conversely, FIP200 enhances the transcriptional activation of the p21 promoter by PIASy whereas PIASy transcription activity is severely reduced upon FIP200 depletion by RNA interference. Chromatin immunoprecipitation analysis demonstrates that endogenous PIASy and FIP200 are corecruited to the p21 promoter. Altogether, these results provide the first evidence for the existence of a close—spatially controlled—mode of regulation of FIP200 and PIASy nucleocytoplasmic functions.

Protein inhibitors of activated STAT (PIAS) proteins were originally identified as repressors of the cytokine-induced STAT transcription factors (7). They have since been shown also to function as E3 SUMO ligases (18, 39). PIAS proteins have been found to regulate the functions of many different proteins, but a major process in which most of these proteins are involved is gene transcriptional regulation (33, 38). PIAS proteins may activate or repress transcription, with the precise mechanism of action depending on the target gene or interacting transcriptional regulator (12, 36). PIAS proteins display conservation throughout evolution, from yeast to humans. With the exception of a variable C-terminal region, PIAS proteins are highly similar, with an overall identity of about 50% at the amino acid level. At the N terminus, PIAS proteins contain a SAP domain which is thought to target proteins to AT-rich chromosomal regions known as scaffold or matrix attachment regions (30). PIAS proteins also contain a cysteine/histidine-rich domain known as the MIZ-zinc finger or SP-RING domain. This RING finger-like domain plays a key role in the E3 ligase function of these proteins, favoring the covalent modification of various target proteins by SUMO (38).

Sumoylation is a three-step process similar to ubiquitination that involves an E1-activating enzyme (Aos1/Uba2 heterodimer), an E2-conjugating enzyme (Ubc9), and E3 ligases, including the Ran-binding protein-2, Polycomb-2, and PIAS proteins (15, 17, 35). Sumoylation has been shown to regulate each of its targets in a specific way by altering its conformation, stability, or interaction and localization properties. While PIAS proteins may act as E3 SUMO ligases, modulating the function of the target protein by adding SUMO tags, a growing body of evidence indicates a SUMO-independent role for PIAS proteins in their protein partner functions (36). The human PIAS protein family has at least five members: PIAS1, PIAS3, the α and β splice variants of PIASx, and PIASy (33). PIAS1$^{-/-}$ mice show increased protection against pathogenic infection (25), and PIASxα$^{-/-}$ animals exhibit testis defects (31), whereas PIASy-deficient mice have modest defects in interferon and Wnt signaling (28, 42). We recently showed that overexpression of PIASy in human primary fibroblasts leads to activation of the Rb and p53 pathways, thus provoking a cellular senescence arrest (2). PIASy promotes p53 sumoylation and transcriptional activation of p53 downstream target genes involved in senescence such as the gene for p21. Accordingly, PIASy$^{-/-}$ murine embryo fibroblasts (MEFs) show an increased resistance to senescence which is accompanied by a lack of p21 up-regulation.

FIP200 (focal adhesion kinase family-interacting protein of 200 kDa) has been involved in fundamental cellular processes including cell size, migration, survival, and cell cycle progression through interaction with multiple signaling pathways (9). It was first identified in yeast two-hybrid screens in which this protein was found to interact with and inhibit the kinase activities of Pyk2 and FAK (1, 40). It is characterized by a large coiled-coil region containing a leucine zipper motif. More recent studies have described a role for FIP200 in cell cycle regulation mainly via p53 stabilization and cyclin D1 degrada-

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tion (26). It has been further shown that FIP200 interacts with and inhibits the tuberous sclerosis complex (TSC) function, correlating with its ability to activate S6 kinase (S6K) phosphorylation and increase cell size (6, 10). Inactivation of the gene for FIP200 in mice leads to embryonic lethality, associated with defective heart and liver development. At the cellular level, FIP200$^{-/-}$ MEFs and liver cells show increased apoptosis together with alteration of the tumor necrosis factor alpha-JNK and TSC/mTOR pathways (11). Chano et al. (3) independently identified the FIP200 protein, which they called RB1-inducible coiled-coil 1 (RB1CC1), as an inducer of RB1 expression in human leukemia cells. Moreover, the gene for FIP200 is frequently deleted in breast cancer, suggesting a potential tumor suppressor role for FIP200 (5).

To better understand the molecular mechanisms by which PIASy regulates cellular functions, we used a biochemical purification approach to identify cellular proteins that interact with PIASy. Here, we report the identification of PIASy interaction with FIP200 both in vitro and in vivo. We show that this interaction is important for the nucleocytoplasmic partitioning of FIP200. PIASy-induced accumulation of FIP200 in the nucleus impairs the cytoplasmic activity of FIP200 on TSC binding and S6K phosphorylation, thus highlighting an as-yet-unappreciated role for PIASy in the regulation of the nuclear shuttling of other proteins to modulate their activities. Conversely, nuclear PIASy and FIP200, which co-occupy the p21 promoter, combine their effects to increase the promoter's activity. Finally, we failed to detect any sumoylation of FIP200 in our experimental conditions, suggesting that the functional interaction between PIASy and FIP200 is not coupled, at least directly, to any SUMO ligase action of PIASy on FIP200.

MATERIALS AND METHODS

Plasmids, cell culture, and infection. Flag/HA-PIASy, HA-PIASy, Flag/HA-PIASxα, and HA-FIP200 were inserted into pcDNA3 (Invitrogen) by standard procedures. The N terminus of FIP200 (amino acids [aa] 1 to 633) and the C terminus of FIP200 (aa 634 to 1591) were inserted into pcDNA3 and pFlag-CMV-6A (Sigma) or His-pcDNA3, respectively. pcDNA3-HDAC1, Flag/HA-PIASy mut (PIASy-C342F), pBABE-HA-PIASy, Myc-TSC1, GST-TSC2, and HA-S6K were as previously described (2, 10, 20). Flag-FIP200 was kindly provided by T. Chano, pRC-p53 and p21-luciferase reporter by M. Oren, the PIAS1 and PIAS3 plasmids by K. Shuai, and T7-PIASy by J. Seeler.

HeLa, U2OS, HEK293, and HEK293T cells were grown in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. All cells were maintained at 37°C under an atmosphere containing 5% CO_2. FIP200$^{-/-}$ and FIP200$^{+/+}$ MEFs (11), as well as PIASy$^{-/-}$ and PIASy$^{+/+}$ MEFs (28), were isolated from embryonic day 12.5 embryos and cultured under the same conditions. Infection of MEFs by retrovirus-mediated gene transfer was performed as described previously (2).

Transfection, siRNA, and reporter gene assay. Cells were transfected with 0.3 μg p21-luciferase and 0.01 μg pCMV-β-gal reporters plus expression constructs by using Lipofectamine and Plus transfection reagents (Invitrogen) according to the manufacturers' instructions. β-Galactosidase and luciferase activities were measured with the Galacto-Star (Tropix) and Luciferase Assay Systems (Promega) kits according to the manufacturers' instructions. Luciferase activities were normalized to β-galactosidase activities. For small interfering RNA (siRNA) knockdown experiments, U2OS cells grown in six-well plates were transfected with 100 nmol duplex oligonucleotide with Oligofectamine reagent (Invitrogen) in accordance with the manufacturer's instructions. Two independent duplex FIP200 siRNA sequences, (i) 5′-GGAGAUUUGGUACUCAUCA UCA-3′ and 5′-AGCUUGAUGAUGAGUACCAAAUCUCC-3′ (10) and (ii) 5′-GGAGUGGGCUGGUGCUUUAUU-3′ and 5′-UAAAGCACCAGCCCA CUCCUU-3′, were used. A scrambled siRNA sequence (Dharmacon) was used as a negative control. On the next day, the cells were transfected with PIASy and p21 reporter constructs as described above. The cells were incubated for 3 days and then lysed for the monitoring of luciferase activity and Western blotting.

To detect S6K phosphorylation, HEK293T cells grown in 10-cm dishes were cotransfected with vectors encoding HA-S6K, T7-PIASy, and HA-FIP200. After 2 days, growing cells were directly lysed in Laemmli buffer for Western blotting.

Antibodies. We generated an anti-FIP200 polyclonal rabbit antiserum (Ab244) directed against a glutathione *S*-transferase (GST) fusion protein encompassing C-terminal aa 1350 to 1591 of human FIP200. We used the following antibodies: rat anti-PIASy monoclonal antibody recognizing mouse PIASy (28), rabbit polyclonal anti-PIASy antibody recognizing human PIASy, and rabbit polyclonal anti-SUMO antibody mainly recognizing human SUMO-1 (2), as well as antibodies against vinculin (Santa Cruz), PARP-1 (Santa Cruz), green fluorescent protein (GFP; Santa Cruz), Flag (Sigma), hemagglutinin (HA; Covance), T7 (Novagen), Myc (Santa Cruz), GST (Santa Cruz), p53 (Santa Cruz), tubulin (Oncogene), β-actin (Sigma), SUMO-2/3 (Abgent), and Thr389 phospho-S6K (Cell Technology Inc.).

ChIP. U2OS cells were cross-linked by incubation for 10 min at 20°C with formaldehyde (added directly to the culture medium at a final concentration of 1%), and chromatin immunoprecipitation (ChIP) was carried out as previously described (2). For ChIP, we used antibodies against PIASy, FIP200, p53, and GFP. DNA obtained by ChIP was amplified by PCR in a reaction mixture containing each primer pair at 10 μM. The following primers were used: for the p21 promoter, 5′-CACCACTGAGCCTTCCTCAC-3′ and 5′-CTGACTCCCAG CACACACTC-3′ (32); for the GADD45α promoter, 5′-GGATCTGTGGTAG GTGAGGGTCAGG-3′ and 5′-GGAATTAGTCACGGGAGGCAGTGCA G-3′ (34); for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene, 5′-GTATTCCCCCAGGTTTACAT-3′ and 5′-GGAGTGAGTGGAAGACAG AA-3′ (19).

Preparation of nuclear and cytoplasmic protein fractions. Phosphate-buffered saline-washed cell pellets were resuspended in 10 volumes of buffer 1 (0.5 M sucrose, 15 mM Tris [pH 7.5], 60 mM KCl, 0.25 mM EDTA, 0.125 mM EGTA, 1 Boehringer protease tablet/5 ml). The cells were then allowed to recover for 5 min at 4°C, 10% NP-40 was added dropwise, and lysis was monitored under a light microscope. The reaction was stopped by adding NP-40 when the nuclei were free of cytoplasmic contaminants. The lysed cells were centrifuged at 1,000 × *g*. The supernatant (cytoplasmic fraction) was decanted, and the pellet (corresponding to the nuclei) was resuspended in extraction buffer (20 mM Tris-Cl [pH 8.5], 10% glycerol, 2 mM dithiothreitol, 0.8 M KCl). The DNA was sheared by passage through a 25-gauge syringe and incubated for 45 min on ice. The mixture was centrifuged at 21,000 × *g*, and the nuclear fraction was dialyzed against 150 mM KCl in extraction buffer. The various cellular fractions were analyzed by immunoblotting.

GST pull-down and in vitro sumoylation assay. GST, GST-PIASy, and GST-FIP200(1350-1591) were produced in BL21DE3 *E. coli* cells and purified according to standard procedures. GST pull-down assays were carried out as previously described (2). In vitro sumoylation assays were carried out with in vitro-translated and radiolabeled HDAC1 and FIP200 and recombinant E1 (Aos1/Uba2) (370 nM), E2 (Ubc9) (630 nM), and SUMO-1 (7 μM) in 30 mM Tris–5 mM ATP–10 mM $MgCl_2$ (pH 7.5) at 30°C as previously described (20). Recombinant GST-PIASy was added at a final concentration of 500 nM.

Immunoprecipitation, immunoblotting, and immunofluorescence. Cells were lysed by adding an equal volume of 1× CHRIS buffer (50 mM Tris [pH 8.0], 0.5% Nonidet P-40, 200 mM NaCl, 0.1 mM EDTA, 1 Boehringer protease tablet/5 ml). Specific polypeptides were then recovered by immunoprecipitation from equivalent amounts of cellular proteins. Immune complexes were collected by incubation with protein A/G (Oncogene). The immunoprecipitates were washed three times in lysis buffer, and the proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein bands were then immunoblotted according to the standard procedure.

Cells were immunolabeled with a primary antibody (against FIP200 or HA) and a secondary antibody (Sigma) as previously described (2). The cells were then examined with a Leica DMIRB microscope.

PIASy complex purification and mass spectrometry. HEK293T cells were stably transfected with Flag/HA-PIASy or with an empty vector as the control. Cell lysates from 300 flasks (150-cm^2 flasks) were used for Flag immunoprecipitation, followed by Flag-peptide elution under the conditions described above. The bands visible on SDS-PAGE were digested with trypsin within the gel and extracted as described by Shevchenko et al. (37). The extracted peptides were analyzed in a liquid chromatography-coupled electrospray ionization quadrupole time-of-flight mass spectrometer (Q-ToF Ultima; Waters) under standard conditions. Proteins were identified by using the fragment spectra of sequenced peptides for searches against the NCBI nr database by using Mascot as the search engine.

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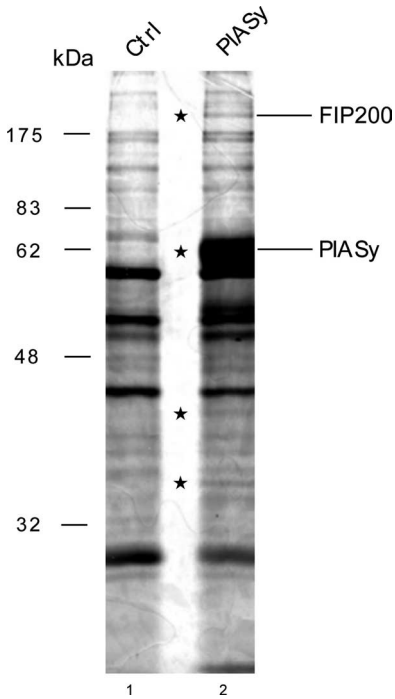

FIG. 1. Identification of FIP200 as a PIASy-associated protein by mass spectrometry. Lysates of HEK293T cells expressing Flag/HA-PIASy (PIASy) were immunoprecipitated with Flag antibody affinity resins. HEK293T cells transfected with empty Flag/HA-vector were used as a control (Ctrl). Immunoprecipitates were resolved by SDS-PAGE in a 10% polyacrylamide gel and silver staining. The four bands specifically present in the PIASy-expressing cells are marked by stars. The PIASy and FIP200 polypeptides identified by mass spectrometry analyses are indicated. Molecular masses are indicated on the left.

RESULTS

Identification of PIASy interaction with FIP200. To better characterize the cellular function of PIASy, we used a purification approach to identify proteins interacting with PIASy. We established a stable human embryonic kidney simian virus 40 large T antigen (HEK293T) cell line expressing a Flag/HA-tagged version of the E3 SUMO ligase PIASy and a HEK293T control cell line carrying the empty vector. Lysates were prepared from both stably transfected lines, incubated with anti-Flag–agarose, and subjected to Flag-peptide elution. The protein profiles of the eluted fractions were analyzed by SDS-PAGE and silver staining. Flag affinity purification gave about 30 visible protein bands in the PIASy-expressing cell line, 4 of which were not visible in the control cell line (Fig. 1). Among those, two major ones, corresponding to molecular masses of ~60 kDa and 200 kDa, were further identified by mass spectrometry as PIASy and FIP200, respectively. A yeast-two-hybrid screen performed in parallel with PIASy as bait identified FIP200 as a major PIASy-interacting partner (9 independent FIP200 clones out of 69 interacting clones) (J. Seeler and A. Dejean, unpublished results). These results indicate that PIASy associates with FIP200 in eukaryotic cells.

The interaction of PIASy and FIP200 was tested next by coimmunoprecipitation. Flag-FIP200 was cotransfected with either Flag/HA-PIASy or empty Flag/HA-vector in HEK293T cells. Cell lysates were immunoprecipitated with HA antibody, followed by Western blotting with Flag antibodies. As shown in Fig. 2A, Flag/HA-PIASy, but not the empty Flag/HA-vector, efficiently immunoprecipitated Flag-FIP200 (lanes 4 and 5). Interestingly, a PIASy protein (Flag/HA-PIASy mut) with a RING finger mutation that causes a defect in SUMO ligase activity due to the replacement of Cys 342 with Phe but does not affect the structure of E3 (2) failed to associate with Flag-FIP200 (lane 6). This suggests that an intact RING finger structure and, by extension, the E3 ligase activity of PIASy are required for interaction with FIP200. By contrast, deletion of the SAP domain of PIASy had no effect on the PIASy-FIP200 interaction (data not shown). Further experiments with full-length FIP200 or deletion mutant forms containing either the N-terminal (aa 1 to 633) or the C-terminal (aa 634 to 1591) domain revealed that the C-terminal domain of FIP200 mediates the association with PIASy (Fig. 2B). To evaluate the interaction at the level of endogenous proteins, we generated a polyclonal antibody against the C-terminal domain of FIP200 (Ab244). As shown in Fig. 2C, endogenous FIP200 protein immunoprecipitated a significant amount of endogenous PIASy whereas a control GFP antibody did not. Conversely, we detected no FIP200 protein with an antibody directed against the C terminus of PIASy (data not shown), probably because of the proximity between the recognition domain of the antibody and the interacting region. However, we found that ectopically expressed PIASy immunoprecipitated endogenous FIP200 (Fig. 2D, lane 5). By contrast, overexpression of other members of the PIAS family, PIAS1, PIAS3, and PIASxα, failed to reveal any association with endogenous FIP200 (Fig. 2D, lanes 2 to 4), indicating that this interaction is specific to this member of the PIAS family.

We next carried out a GST pull-down assay with recombinant GST-PIASy and in vitro translated, ^{35}S-labeled, full-length FIP200. FIP200 bound GST-PIASy with the same efficiency as HDAC1, which was used as a positive control (2, 8), but not GST alone (Fig. 2E). Consistent with the coimmunoprecipitation experiments (Fig. 2B), GST-PIASy efficiently pulled down the C-terminal domain of ^{35}S-labeled FIP200 but not its N-terminal domain (Fig. 2E). In a reciprocal experiment, ^{35}S-labeled PIASy bound a fusion protein containing the extreme C terminus of FIP200 (aa 1350 to 1591) (Fig. 2F). These data suggest a direct interaction between PIASy and FIP200.

Together, these results identify FIP200 as a PIASy-interacting protein and suggest that the RING finger of PIASy and the FIP200 1350-to-1591-aa region are required for their interaction.

PIASy regulates the subcellular localization of FIP200. To better characterize the interaction between PIASy and FIP200 at the subcellular level, we carried out immunofluorescence studies with HeLa cells transfected with expression vectors for Flag-FIP200 together with HA-PIASy, HA-PIASy mut, HA-PIASxα, or a control vector (Fig. 3A). Consistent with previous findings (24, 40), FIP200 showed a predominantly diffuse cytoplasmic localization (Fig. 3A, parts A to D) while, in contrast, PIASy presented almost exclusively nuclear staining (data not shown). Coexpression of FIP200 and PIASy induced a drastic shift of the subcellular location of FIP200 from the cytoplasm to the nucleus together with PIASy (Fig. 3A, parts E

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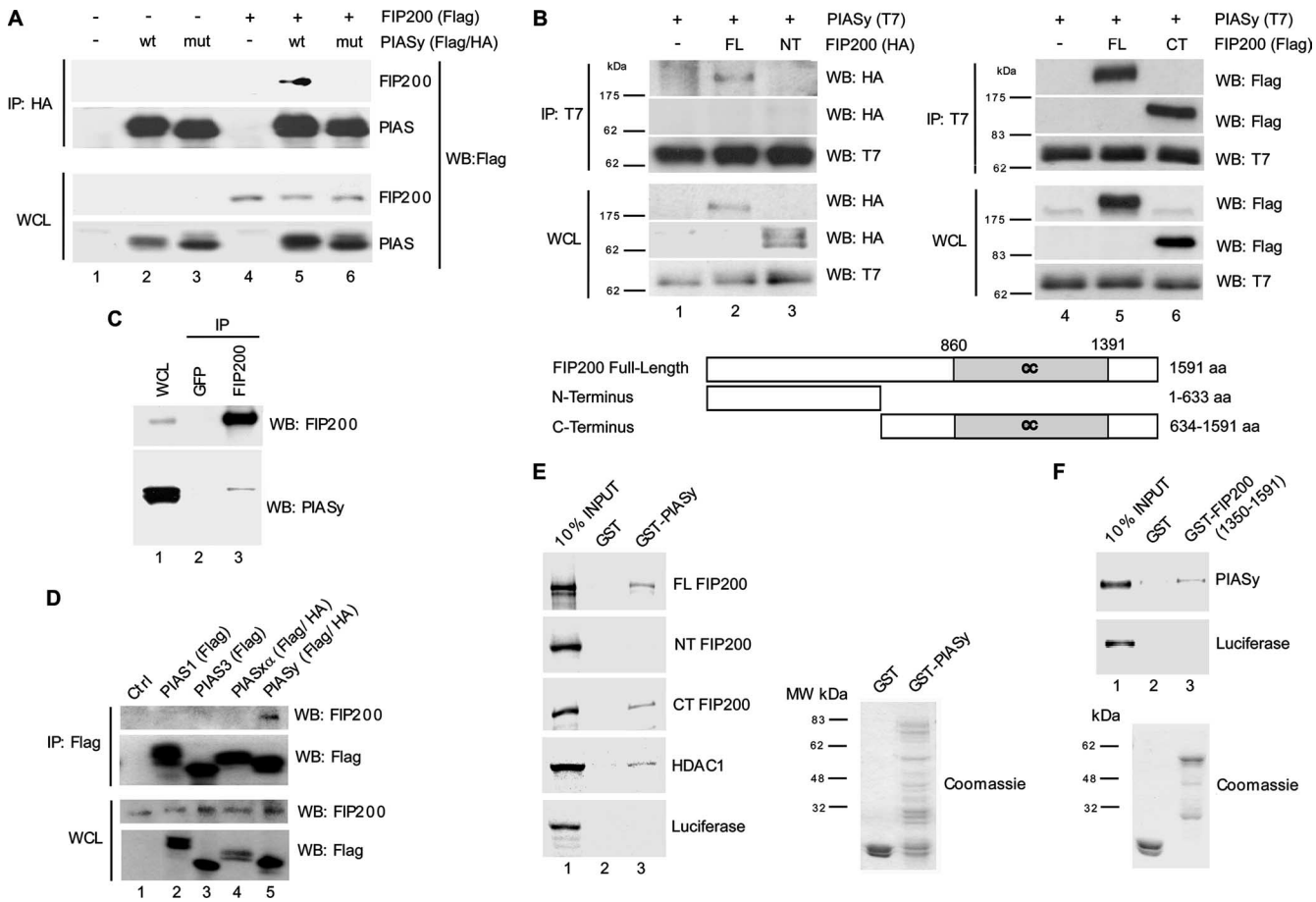

FIG. 2. FIP200 interacts with PIASy in vivo. (A) HEK293T cells were transfected with a combination of empty Flag/HA-vector (Ctrl), wild-type (wt) Flag/HA-PIASy, C342F mutant (mut) Flag/HA-PIASy, and Flag-FIP200 as indicated. At 24 h after transfection, 100 μg of whole cell lysate (WCL) (lower part) or cell lysates immunoprecipitated with HA-conjugated agarose (upper part) were subjected to SDS-PAGE followed by Western blotting with an anti-Flag antibody. (B) HEK293T cells were transfected with T7-PIASy together with empty HA-vector (Ctrl), full-length HA-FIP200 (FL), or the HA-FIP200 N terminus (NT). Whole cell lysate was analyzed directly by Western blotting (WB) with an anti-HA or anti-T7 antibody or after immunoprecipitation (IP) with T7-conjugated agarose (left side). HEK293T cells were transfected with T7-PIASy together with empty Flag-vector (Ctrl), full-length (FL) Flag-FIP200, or the Flag-FIP200 C terminus (CT). Whole cell lysate was analyzed directly by Western blotting with anti-Flag or anti-T7 antibody or after immunoprecipitation with T7-conjugated agarose (right side). Molecular masses are indicated. In the schematic diagram of the human FIP200 protein at the bottom, full-length FIP200, the N-terminal domain of FIP200, the C-terminal domain of FIP200, and the corresponding amino acids are indicated; the FIP200 coiled-coiled domain (CC) is also indicated. (C) Lysates of HEK293T cells were subjected to immunoprecipitation with anti-FIP200 antibody and with anti-GFP antibody as a control. Whole cell lysate or precipitates were Western blotted with antibodies against FIP200 and PIASy. (D) HEK293T cells were transfected with empty Flag-vector, Flag-PIAS1, Flag-PIAS3, Flag/HA-PIASxα, and Flag/HA-PIASy. At 24 h after transfection, 100 μg of whole cell lysate (bottom) or immunoprecipitated anti-Flag antibody (top) was subjected to SDS-PAGE, followed by detection with anti-Flag antibody or anti-FIP200 antibody. (E) GST and GST-PIASy immobilized on glutathione beads were incubated with the in vitro-translated, ^{35}S-labeled full length (FL), N terminus (NT), or C terminus (CT) of FIP200; ^{35}S-labeled HDAC1 (positive control); or ^{35}S-labeled luciferase (negative control). Bound complexes were analyzed by autoradiography. Recombinant GST and GST-PIASy were detected by Coomassie staining. (F) GST and GST-FIP200 aa 1350 to 1591 immobilized on glutathione beads were incubated with in vitro-translated ^{35}S-labeled PIASy or ^{35}S-labeled luciferase. Bound complexes were analyzed by autoradiography. Recombinant GST and GST-FIP200 were detected by Coomassie staining.

to H). By contrast, overexpression of either SUMO ligase-inactive PIASy (PIASy mut) or the other PIAS family member PIASxα, both of which failed to associate with FIP200 in interaction studies (Fig. 2A and D), did not induce nuclear accumulation of FIP200.

As all attempts to detect endogenous FIP200 protein by immunofluorescence failed, we investigated the subcellular distribution of endogenous FIP200 protein by biochemical fractionation of HEK293T cell lysates, followed by Western blotting. Endogenous FIP200 was found to be present in roughly similar amounts in the nuclear and cytoplasmic fractions (Fig. 3B, lanes 3 and 4), a situation slightly different from the mostly cytoplasmic distribution of ectopically expressed FIP200 in HeLa cells as revealed by immunofluorescence (Fig. 3A). Upon overexpression of HA-PIASy, which was, as expected, found mostly in the nucleus, the levels of endogenous FIP200 in the nuclear fraction increased substantially (Fig. 3B, lane 2), whereas the amount of cytoplasmic FIP200 decreased accordingly (Fig. 3B, lane 1).

In order to confirm these results in a more physiological situation, we studied the subcellular localization of endogenous FIP200 in PIASy$^{-/-}$ and PIASy$^{+/+}$ MEFs by using

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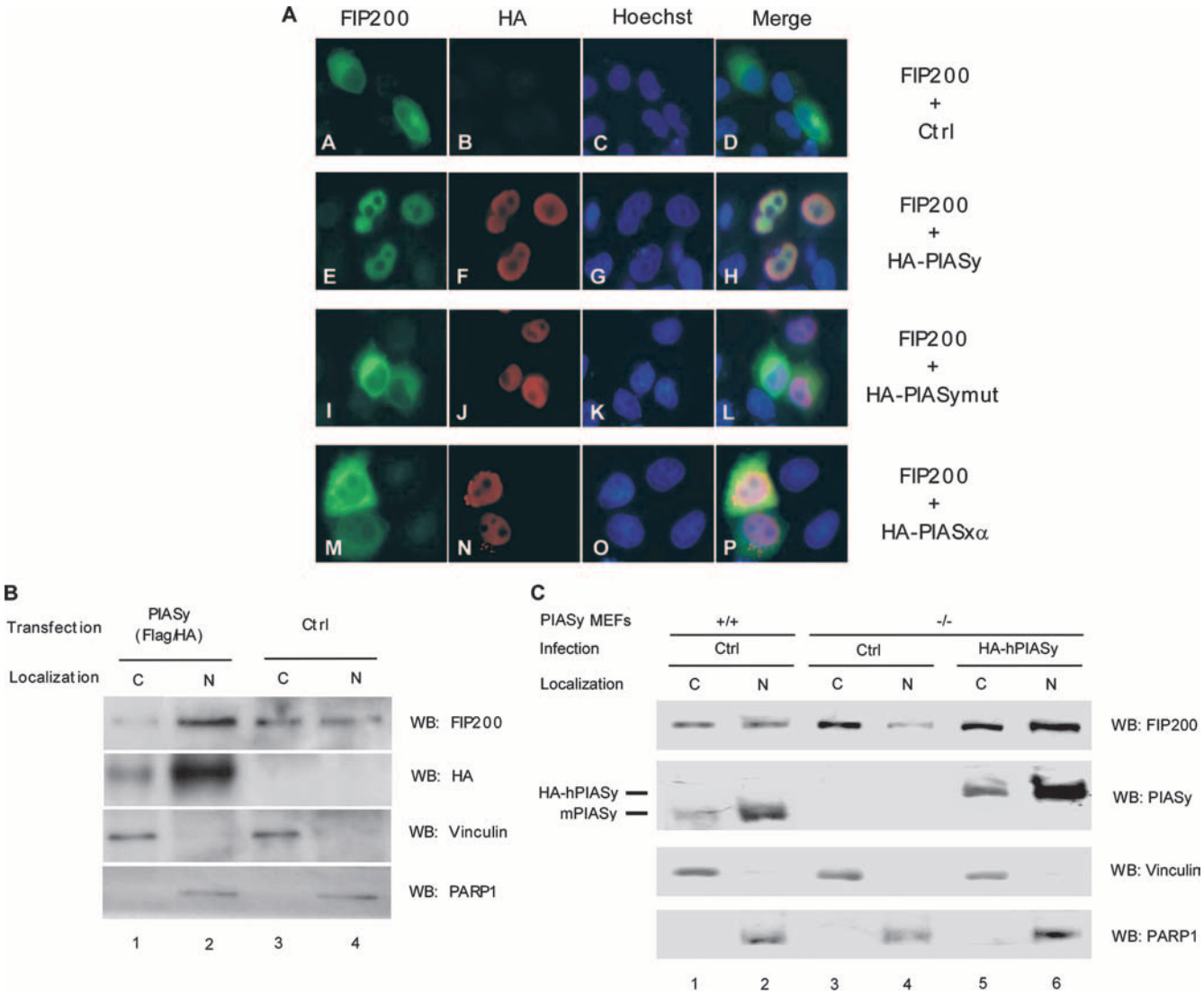

FIG. 3. PIASy regulates the nuclear-to-cytoplasmic ratio of FIP200. (A) HeLa cells grown on coverslips were transfected with expression vector for Flag-FIP200 together with empty HA-vector (Ctrl, A to D) or vector encoding HA-PIASy (E to H), HA-PIASy mut (I to L), or HA-PIASxα (M to P). At 24 h after transfection, cells were fixed with paraformaldehyde and stained with anti-FIP200 antibody–fluorescein-conjugated antibody (green), anti-HA antibody–Texas Red-coupled antibody (red), or Hoechst dye (blue). Merged image overlays were obtained with Meta software. Additional image processing was carried out with Canvas software. (B) HEK293T cells were transfected with empty Flag/HA-vector (Ctrl) or Flag/HA-PIASy vector. At 24 h later, cells were processed for nuclear (N)/cytoplasmic (C) fractionation as described in Materials and Methods. A 100-μg sample of each fraction was resolved by SDS-PAGE and analyzed by Western blotting (WB) with antibodies against FIP200, HA (PIASy), vinculin as the cytoplasmic marker, and poly(ADP-ribose) polymerase 1 (PARP1) as the nuclear marker. (C) PIASy$^{+/+}$ or PIASy$^{-/-}$ MEFs were retrovirally infected with empty pBABE vector (Ctrl) or pBABE-HA-hPIASy vector and selected for 2 days with puromycin (4 mg/ml). The distribution of endogenous FIP200 and endogenous or exogenous PIASy in the nuclear (N) and cytoplasmic (C) protein fractions was analyzed by Western blotting with anti-FIP200 antibody and rat anti-PIASy monoclonal antibody recognizing mouse and human PIASy (28). The quality of fractionation was verified as for panel B.

the biochemical fractionation approach. In PIASy$^{+/+}$ MEFs, endogenous PIASy was predominantly found in the nuclear fraction whereas a small amount could be detected in the cytoplasmic fraction (Fig. 3C, lanes 1 and 2). Endogenous FIP200 was found in both the nuclear and cytoplasmic fractions in roughly similar amounts (Fig. 3C, lanes 1 and 2). Interestingly, in PIASy$^{-/-}$ MEFs, the amount of nuclear FIP200 was severely reduced and FIP200 was found almost exclusively in the cytosol (Fig. 3C, lanes 3 and 4). Furthermore, reexpression of PIASy in PIASy$^{-/-}$ MEFs restored an equal distribution of FIP200 in the nuclear and cytoplasmic fractions (Fig. 3C, lanes 5 and 6), similar to what observed in PIASy$^{+/+}$ MEFs, indicating that PIASy is directly responsible for the observed change in FIP200 nuclear expression. Together, these data indicate that PIASy regulates the nuclear versus cytoplasmic pools of FIP200.

PIASy interferes with FIP200 regulation of the TSC/S6K pathway. To assess whether the shift in FIP200 subcellular localization upon the expression of PIASy may have an impact on FIP200 function, we studied the effect of PIASy on FIP200-

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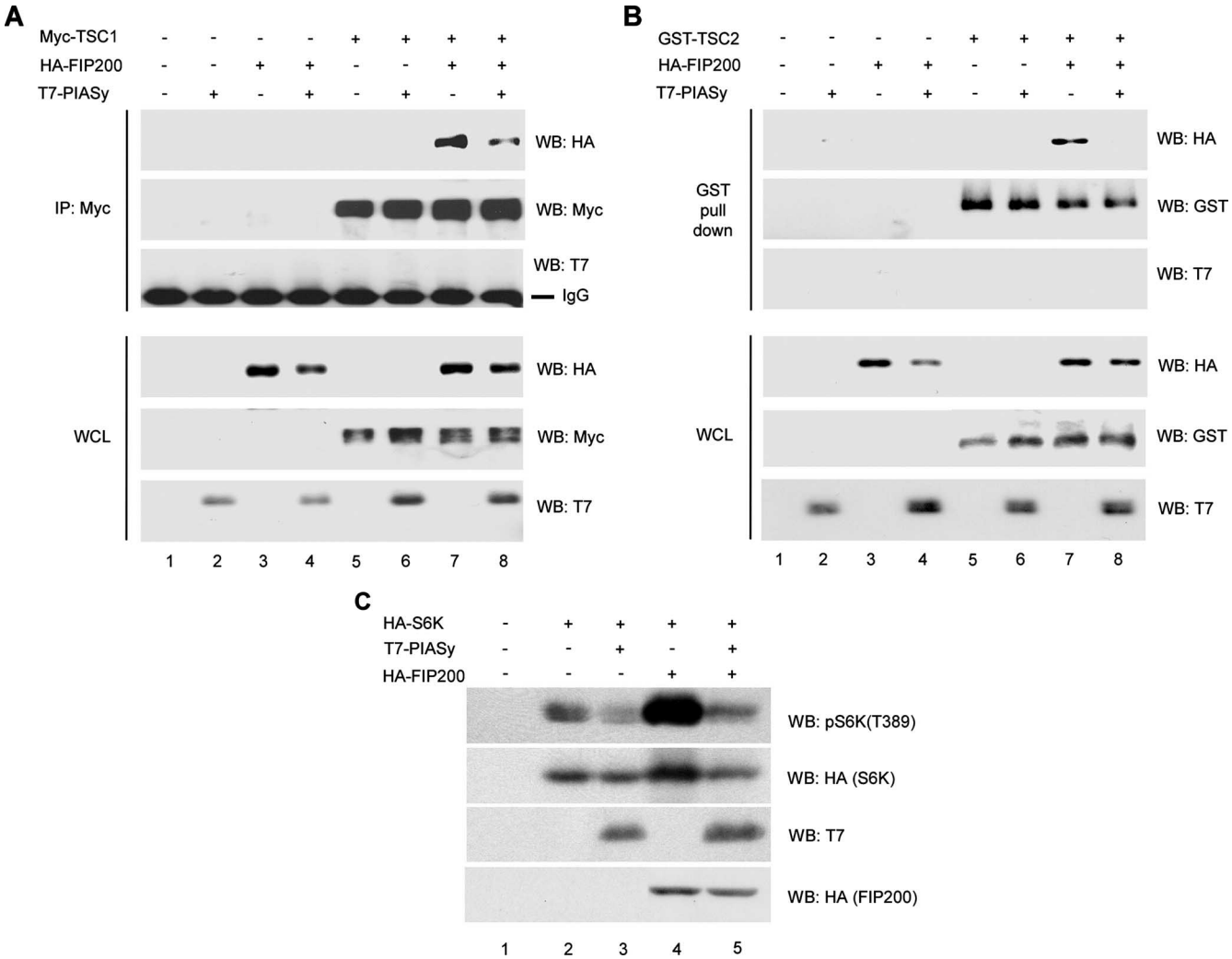

FIG. 4. PIASy inhibits FIP200-induced activation of the mTOR pathway. (A) HEK293T cells were transfected with a combination of Myc-TSC1, HA-FIP200, T7-PIASy, and empty vector as indicated. Whole cell lysates (WCL) or Myc immunoprecipitates (IP) were subjected to SDS-PAGE and analyzed by Western blotting (WB) with antibodies against HA, Myc, or T7. (B) HEK293T cells were transfected with a combination of GST-TSC2, HA-FIP200, T7-PIASy, and empty vector as indicated. Cell lysates were prepared and incubated with glutathione beads. Bound proteins were eluted, subjected to SDS-PAGE, and analyzed by Western blotting with antibodies against HA, GST, or T7. (C) PIASy abrogates FIP200-dependent S6K phosphorylation. HEK293T cells were cotransfected with vectors encoding HA-S6K, HA-FIP200, and T7-PIASy or the corresponding empty vectors. At 48 h later, cells were directly lysed in Laemmli buffer and lysates were analyzed by Western blotting. S6K phosphorylation was detected with an antibody directed against Thr389 phospho-S6K, and the expression levels of S6K, FIP200, and PIASy were verified with anti-HA or anti-T7 antibodies. IgG, immunoglobulin G.

regulated TSC/S6K signaling. Indeed, recent studies have shown that FIP200 associates with both TSC1 and TSC2 and functions as an activator of the mammalian target of rapamycin (mTOR) pathway—possibly through disruption of TSC1-TSC2 complex formation—thus correlating with its ability to increase cell size and stimulate S6K phosphorylation (6, 10, 11). We first examined the possible effect of PIASy on FIP200 interaction with the TSC1 and TSC2 proteins. HEK293T cells were cotransfected with vectors expressing Myc-TSC1 and HA-FIP200 in the absence or presence of T7-PIASy. As expected, TSC1 efficiently immunoprecipitated FIP200. Interestingly, overexpression of PIASy significantly reduced the association between TSC1 and FIP200 (Fig. 4A). Similar experiments substituting GST-TSC2 for Myc-TSC1 showed that overexpression of PIASy almost entirely abrogated the TSC2-FIP200 interaction (Fig. 4B). These data indicate that PIASy negatively regulates TSC1/TSC2-FIP200 complex formation. No association between PIASy and TSC1 or TSC2 could be detected under these experimental conditions (Fig. 4A and B), suggesting that PIASy does not compete with FIP200 for TSC binding. To go further, we tested whether PIASy may affect FIP200-induced activation of S6K. HEK293T cells were cotransfected with HA-S6K vector or pcDNA3 empty vector along with plasmids encoding HA-FIP200 and/or T7-PIASy. Cell lysates were analyzed by Western blotting with an anti-Thr389 phosphorylated S6K antibody to determine its activation state or anti-HA and anti-T7 antibodies to verify the expression levels of the proteins. Consistent with previous findings (10), overexpression of

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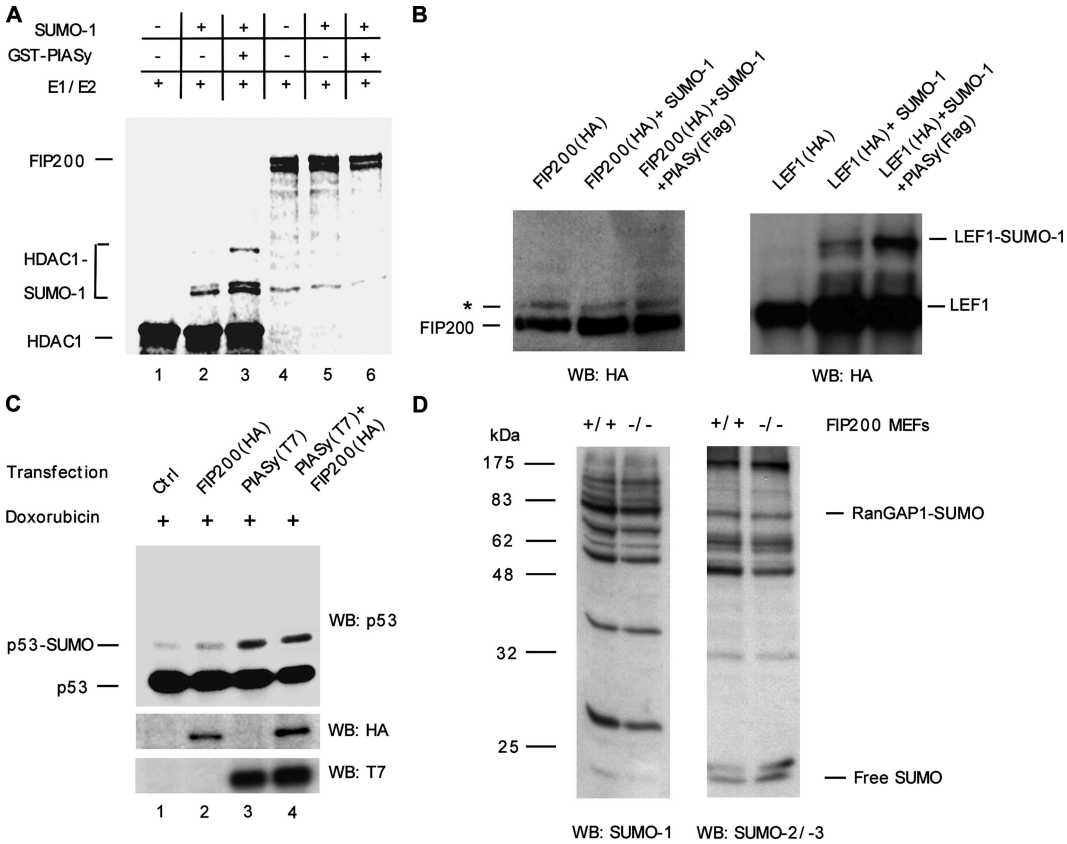

FIG. 5. FIP200 and SUMO modification. (A) In vitro-translated ^{35}S-labeled HDAC1 and ^{35}S-labeled FIP200 were incubated with the indicated recombinant proteins as described in Materials and Methods. Reaction mixtures were analyzed by SDS-PAGE and autoradiography. (B) HeLa cells were transfected with HA-FIP200 alone or together with SUMO-1 and Flag-PIASy. Whole cell lysates (WCL) were analyzed by Western blotting (WB) with anti-HA antibody as shown on the left. The same experiment but with HA-LEF1 as the substrate is shown on the right. The asterisk indicates an aspecific band. (C) FIP200 does not affect p53 sumoylation. HEK293 cells were transfected with empty HA-vector (Ctrl), HA-FIP200, T7-PIASy, and HA-FIP200 together with T7-PIASy. At 24 h later, cells were treated with 400 ng/ml doxorubicin for 5 h (+). Cell lysates were prepared in 2× Laemmli buffer with 5 mM *N*-ethylmaleimide and Western blotted with the indicated antibodies. (D) Cell lysates were prepared in CHRIS buffer with 5 mM *N*-ethylmaleimide and Western blotted with antibodies against SUMO-1 and SUMO-2/3. Sumoylated RanGAP1 and free SUMO are indicated.

FIP200 alone increased S6K phosphorylation (Fig. 4C, compare lanes 2 and 4). Cotransfection of PIASy with FIP200 returned S6K phosphorylation to its basal level (Fig. 4C, compare lanes 5 and 4). Interestingly, we observed a slight decrease in S6K phosphorylation when we overexpressed PIASy alone (Fig. 4C, compare lanes 3 and 2), an observation which most likely reflects the depletion of endogenous cytosolic FIP200 by PIASy (Fig. 3B).

Taken together, these data show that PIASy impairs the association of FIP200 with TSC1/TSC2 and abrogates FIP200-dependent S6K phosphorylation, correlating with its ability to redistribute FIP200 from the cytoplasm to the nucleus.

FIP200 and SUMO modification. Modification by SUMO has been shown to have an impact on many biological processes, including protein-protein interaction responsible for subcellular relocalization (15, 17, 35). Considering that PIASy functions as an E3 SUMO ligase, we investigated whether the interaction of FIP200 with PIASy resulted in its sumoylation, possibly accounting for its nuclear translocation. We carried out an in vitro sumoylation experiment with in vitro-translated, ^{35}S-labeled FIP200 or ^{35}S-labeled HDAC1, a bona fide SUMO substrate for PIASy used as a positive control (2, 8), to which we added or did not add recombinant PIASy. The presence of SUMO-1 in the reaction mixture did not induce the formation of higher-molecular-weight species of FIP200, a situation that remained unchanged even after the addition of PIASy (Fig. 5A, lanes 5 and 6). Under the same conditions, PIASy enhanced the formation of HDAC1–SUMO-1 conjugates, which were readily detectable in the presence of SUMO-1 (Fig. 5A, lanes 2 and 3). Studies performed in vivo similarly failed to reveal any sumoylation of FIP200 when overexpressed in HeLa cells, even upon the forced expression of SUMO-1 and/or PIASy (Fig. 5B, left side). By contrast, LEF-1, a well-known PIASy SUMO target (30), underwent efficient sumoylation in a parallel assay (Fig. 5B, right side). Thus, FIP200 does not appear to be a bona fide substrate for SUMO under either our in vitro or in vivo experimental conditions, suggesting that the effects of PIASy on FIP200 localization and function do not rely on sumoylation of FIP200 per se.

Having established that PIASy does not act as a SUMO ligase for FIP200, we investigated whether FIP200 may rather

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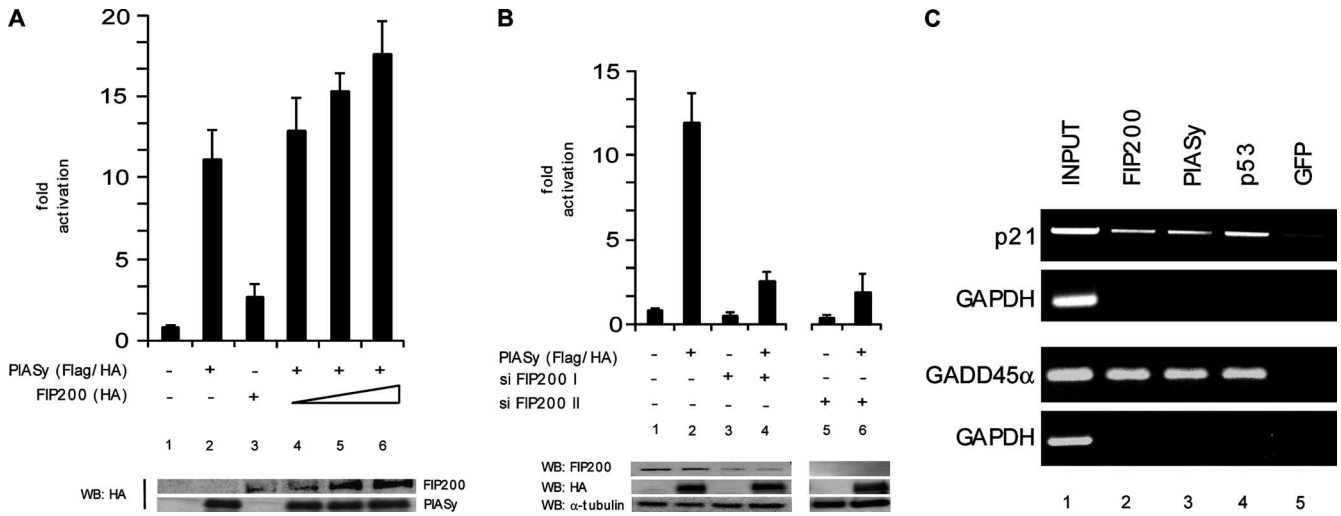

FIG. 6. Role of FIP200 in the regulation of p21 promoter activity by PIASy. (A) Flag/HA-PIASy (100 ng), HA-FIP200 (100 ng), or Flag/HA-PIASy (100 ng), together with increasing amounts of HA-FIP200 (100, 250, and 500 ng), was transfected into U2OS cells with a p21-luciferase reporter (0.3 μg) together with a cytomegalovirus–β-galactosidase vector for normalization. The expression of these constructs was checked by Western blotting (WB) with an anti-HA antibody. (B) U2OS cells were transfected with scrambled siRNA (control) or two different FIP200 siRNAs (I and II) as described in Materials and Methods. At 24 h after this initial transfection, the cells were transfected with empty Flag/HA-vector (−) or Flag/HA-PIASy (+) together with the p21 promoter reporter and the cytomegalovirus–β-galactosidase vector for normalization. The expression of these constructs was checked by Western blotting. The columns show the means of three independent experiments, and the bars show the standard errors. (C) Endogenous PIASy and FIP200 bind to the p21 and GADD45α promoters. ChIP analysis of the p21 promoter (top) and the GADD45α promoter (bottom) in U2OS cells. Cells were harvested, and ChIP was carried out with antibodies against FIP200, PIASy, p53, or GFP. The GAPDH genomic region was used as a negative control. Input, total cross-linked genomic DNA (1 ng).

act as a cofactor for the E3 activity of PIASy. Both FIP200 and PIASy associate with p53 (2, 26); in addition, PIASy functions as an E3 ligase for p53 sumoylation (2). We thus tested the possibility that FIP200 may affect PIASy-mediated sumoylation of p53. We overexpressed PIASy, FIP200, or PIASy together with FIP200 in HEK293 cells treated with doxorubicin, a DNA-damaging agent that induces sumoylation of endogenous p53 (21). As expected, ectopic expression of PIASy enhanced p53 sumoylation (Fig. 5C, lane 3). However, concomitant expression of FIP200 did not modify the profile of SUMO-modified p53 (Fig. 5C, lane 4). FIP200 alone did not affect the steady-state level of sumoylated p53 either (Fig. 5C, lane 2). Thus, FIP200 does not seem to behave as a cofactor with PIASy for p53 sumoylation. We then investigated whether the general SUMO modification pattern was affected in the absence of FIP200 by carrying out Western blot analyses of cell extracts from $FIP200^{+/+}$ and $FIP200^{-/-}$ MEFs (11) with SUMO-1 and SUMO-2/3 antibodies (Fig. 5D). The global modification profiles of SUMO-1 and SUMO-2/3 were similar in the two cell types, indicating that loss of FIP200 has no significant effect on overall sumoylation, although it does not exclude the possibility that FIP200 plays a role in the modification of specific substrates.

FIP200 is an important cofactor with PIASy in p21 promoter activation. Expression of PIASy in human fibroblasts has been shown to induce premature senescence together with activation of p53 targets such as p21 (2). Other studies have independently reported that FIP200 can induce p21 expression in a p53-dependent manner (26). We thus studied the effect of FIP200 on PIASy-mediated activation of the p21 promoter in U2OS cells. Consistent with previous findings, expression of PIASy alone led to robust activation of a p53-responsive p21-luciferase reporter, whereas under these conditions, FIP200 only slightly stimulated the promoter activity (Fig. 6A, lanes 2 and 3). Activation of the p21 promoter by PIASy and FIP200 was p53 dependent, as deletion of both p53-binding sites within the p21 promoter abolished this response (data not shown). Expression of FIP200 together with PIASy led to a modest, yet reproducible, increase in PIASy-induced p21 promoter activity in a dose-dependent manner (Fig. 6A, lanes 4 to 6), suggesting that the interaction between PIASy and FIP200 may be important for p21 activation.

To further address this point, we studied the effects of the down-regulation of endogenous FIP200 protein levels on the induction of p21 promoter activity by PIASy. Accordingly, U2OS cells were transfected with two different FIP200 siRNAs (I and II) or a scrambled siRNA as a negative control together with FLAG-HA-PIASy and the p21 reporter gene. Normal FIP200 protein levels were associated with the expected induction of p21 promoter activity by PIASy (Fig. 6B). In contrast, depletion of the FIP200 protein with either of the two FIP200-specific siRNAs almost entirely abolished PIASy-dependent transcriptional activation of the promoter (Fig. 6B). Control siRNA had no effect in a similar experiment (data not shown). These data strongly suggest that FIP200 is required for PIASy-mediated activation of the p21 promoter.

Next, we investigated whether FIP200 could cooperate with PIASy to regulate p21 expression through direct binding to the p21 promoter. To address this question, ChIP experiments were performed with U2OS cells. Both endogenous PIASy and FIP200 were found to efficiently bind the p21 promoter in the same region as p53, which was used as a positive control (Fig. 6C). ChIP assays with GFP antibody were performed as a negative control and showed only weak background binding.

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No binding to an unrelated genomic fragment of the GAPDH-encoding gene was detected either. In similar experiments, we also found occupancy of the p53-responsive GADD45α promoter by endogenous FIP200, PIASy, and p53, indicating that association of FIP200 and PIASy on p53 targets is not solely restricted to the p21 promoter (Fig. 6C).

Together, these results indicate that PIASy and FIP200 are corecruited together with p53 to the p21 promoter to regulate its activity.

DISCUSSION

PIAS proteins have been shown to have an impact on the function of many different proteins (12, 33, 36, 38). Originally identified as negative cytokine regulators (7), they have been shown also to function as E3 ligases for the SUMO modification pathway (18, 39). Evidence is accumulating that PIAS proteins act as transcriptional coregulators, and one major mechanism through which PIAS proteins operate appears to be subcellular relocalization of interacting proteins (15, 17, 35). Recently, we have shown that PIASy, one of the five PIAS family members, induces cellular senescence in part through transcriptional activation of p53 downstream targets such as p21 (2). To gain further insight into the regulatory mechanisms of PIASy, we searched for PIASy-interacting partners. We report here the identification of FIP200 as a new PIASy-interacting protein and demonstrate that this interaction is important for the subcellular localization and signal transduction activities of FIP200 such as S6K activation. Conversely, FIP200 functions as a coactivator of PIASy in p21 promoter activation. Taken together, our results reveal that PIASy and FIP200 act together to regulate signal transduction and transcriptional activity and highlight a novel mode of regulation of FIP200 function via its PIASy-dependent nucleocytoplasmic redistribution.

PIASy-FIP200 interaction and sumoylation. Our observation that a PIASy variant with a mutation in the RING finger structure fails to interact with FIP200 suggests that the E3 ligase activity of PIASy plays a crucial role in the PIASy-FIP200 association. Thus, a likely hypothesis was that PIASy may catalyze FIP200 sumoylation. However, the absence of detectable SUMO-modified forms of FIP200 both in vitro and in vivo, even under forced expression of PIASy, does not support this notion. Moreover, in the SUMO system, most substrates can associate with several members of the PIAS family which, in turn, stimulate their sumoylation. Further evidence for this redundancy is provided by the observation that PIASy$^{-/-}$ mice are phenotypically normal (28, 42), suggesting that other members of this protein family compensate for the lack of PIASy in mice. We show that PIASy is the only member of the PIAS family that interacts with FIP200, demonstrating the specificity of this interaction and suggesting that physiological consequences of this interaction may not be compensated for by other PIAS proteins. The present study thus reveals a physical interaction between PIASy and FIP200 that requires the RING domain of PIASy but is independent of any SUMO ligase activity of PIASy on FIP200 itself. Our in vivo and in vitro binding assays suggest that PIASy associates with FIP200 via its C-terminal 1350-to-1591-aa region. Interestingly, this C-terminal domain has been shown to mediate the interaction of FIP200 with several binding partners involved in signal transduction such as Pyk2, FAK (41), and ASK1 (11) or with *Listeria monocytogenes* ActA protein (27). Notably, the FIP200 C-terminal region was found to function like full-length FIP200 in inhibiting Pyk2 kinase activity in vitro and in blocking Pyk2-induced apoptosis (41). Moreover, this domain acts as a FIP200 dominant negative mutant on ASK1 binding to reduce JNK activation and increase tumor necrosis factor alpha-induced apoptosis in MEFs (11). In this context, it will be interesting to determine whether PIASy interaction affects the binding of FIP200 with these protein partners and their downstream functions.

PIASy has an impact on FIP200 localization and function. The fact that PIASy binds to FIP200 raised the question of the localization of this interaction. We found that either exogenous or endogenous PIASy primarily localizes in the nucleus and that exogenous FIP200 is predominantly found in the cytoplasm, whereas endogenous FIP200 is present in both the cytoplasm and the nucleus. These observations are in accordance with previous studies (4, 16, 26, 40) and consistent with a role for FIP200 in both compartments. Remarkably, expression of PIASy led to the translocation of both exogenous and endogenous FIP200 from the cytoplasm to the nucleus whereas expression of the SUMO ligase-defective mutant form had no effect. These data highlight a novel function for PIASy in the regulation of the nuclear shuttling of proteins. This finding was further supported by the observation that the level of the nuclear fraction of FIP200 was considerably lower in PIASy$^{-/-}$ MEFs than in wild-type MEFs. Other studies have implicated PIAS proteins in the relocalization of proteins in a manner that is independent from their SUMO ligase activity against such particular proteins. Notably, PIAS1 binding to the Msx1 homeoprotein is required for appropriate localization of Msx1 in proximity to target genes at the nuclear periphery, thus leading to transcriptional repression (23). In this case, neither the SUMO ligase activity of PIAS1 nor sumoylation of Msx1 is required for proper localization of Msx1. Another example is PIASy, which was shown to recruit the transcription factor LEF1 into nuclear bodies, correlating with repression of LEF1 activity (30). This situation slightly differs from the case of Msx1 in that, if sequestration of LEF1 in nuclear bodies is independent from sumoylation of LEF1 per se, it requires the integrity of the RING domain of PIASy. The requirement for this domain can be explained by the fact that, as in the case of FIP200, it mediates the interaction with LEF1 whereas mutation in the RING domain of PIAS1 does not abrogate its binding to Msx1. Though FIP200 did not appear to undergo sumoylation in our experiments, the requirement for the RING domain of PIASy for the interaction and subcellular localization of FIP200 raises the question of a possible role for the SUMO pathway in these processes.

Protein-protein interactions are critical for many signaling processes, including that of the TSC/mTOR signaling cascade, which plays an essential role in the regulation of cell growth. The tumor suppressor TSC1 and TSC2 proteins form a heterodimer which integrates signals derived from growth factors, nutrient level, energy status, and hypoxia into a common growth regulatory signal to the mTOR complex. The TSC1/TSC2 complex functions as a potent negative regulator of cell growth, mainly through inhibition of mTOR and its down-

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stream targets S6K and 4E-BP1, which play major roles in cell size and protein synthesis (14, 22, 29). FIP200 has been shown to associate with and negatively regulate TSC function, resulting in up-regulation of S6K phosphorylation and an increase in cell size (6, 10, 11). Interestingly, we show that PIASy-FIP200 physical interaction has an impact on FIP200 function. PIASy can displace FIP200 from the TSC1/TSC2 complex, possibly through depletion in the cytoplasmic fraction of FIP200, resulting in the restoration of the repressive effect of this complex on S6K phosphorylation. These data thus suggest an as-yet-unappreciated role of PIASy in S6K activation, and further studies are necessary to determine the possible contribution of PIASy to the TSC-mTOR pathway.

FIP200 has an impact on PIASy transcriptional activity. Having shown that PIASy reduces the cytoplasmic activity of FIP200, likely by inducing its nuclear transport, we sought to investigate a possible functional interaction of FIP200 with PIASy within the nucleus. PIASy has been shown to promote the sumoylation of p53 and the transcriptional activation of p53 target genes involved in senescence arrest, such as the gene for p21 (2). The finding that PIASy plays an important role in p21 promoter activation is strengthened by the observation that the p21 protein level fails to be induced by oncogenic Ras in PIASy$^{-/-}$ MEFs, correlating with a delay in the onset of Ras-induced cellular senescence in these cells (2). FIP200 has been shown to inhibit G_1-S-phase progression by interacting with and stabilizing p53, leading to an increase in the levels of the downstream target p21 (26). FIP200 was therefore a plausible candidate coregulator of PIASy for p21 promoter activity. We show here that PIASy and FIP200 functionally cooperate to activate the p21 promoter. In contrast, the E3 SUMO ligase-inactive form of PIASy had no effect on FIP200-induced p21 activation in this assay (data not shown), consistent with the requirement for a functional interaction between PIASy and FIP200 for efficient induction of p21 promoter activity. The notion that FIP200 appears to be a critical cofactor with PIASy in the transcriptional activation of p21 is underscored by our observation that acute ablation of FIP200 considerably reduced PIASy-induced p21 activation. Furthermore, the finding that PIASy and FIP200 are corecruited together with p53 to the endogenous p21 promoter is consistent with a coordinated regulation of p21 gene expression by these proteins. One possibility is that FIP200 stabilizes the recruitment of PIASy onto the p21 promoter, which would, in turn, activate p53 through sumoylation. Indeed, SUMO-modified p53 has been shown to behave as a potent activator of the p21 promoter (13). Moreover, the corecruitment of FIP200 and PIASy on the GADD45α promoter suggests that FIP200 and PIASy might be involved in the coregulation of several p53-responsive genes involved in cell cycle arrest. Finally, since FIP200 was shown to increase Rb mRNA and protein levels (3) and PIASy was shown to act as a strong corepressor of Rb on E2F target genes (2), we therefore asked whether FIP200 could exert a corepressing effect, along with PIASy, on the E2F-responsive cyclin E promoter. The repression of the cyclin E promoter mediated by PIASy was not influenced by the presence of FIP200 (data not shown), indicating that FIP200 does not act as a general cofactor with PIASy in regulating gene transcription but rather acts in a more specific way.

In summary, this study identified FIP200 as a novel interacting partner of PIASy and highlighted an unanticipated role for PIASy in the cytonuclear shuttling of FIP200. We showed that this interaction is important for both S6K-associated signal transduction activities of FIP200 and transcriptional regulation by PIASy, revealing the existence of a close interplay between PIASy and FIP200, in part mediated by spatial control. Several lines of evidence have indicated a potential role for both FIP200 and PIASy in tumor suppression (2, 5, 26). Future studies, including the use of mouse models conditionally inactivated for FIP200 and PIASy, should help in clarifying the nature of the functional interaction between FIP200 and PIASy and its potential role in tumor suppression in vivo.

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