Heat shock factor Hsf1 is involved in the regulation of a variety of cellular processes including heat shock response, development and differentiation, aging, and tumorigenesis. Hsf1 transcriptional activity is tightly controlled through phosphorylation, sumoylation, and acetylation, and through association with a number of regulatory proteins. However, regulation of Hsf1 protein stability or turnover remains unknown. We have identified a novel Hsf1-interacting protein, FILIP-1L, that was found to bind to Hsf1 through yeast two-hybrid screening. FILIP-1L encodes multiple isoforms spanning from 711 to 1135 amino acid residues. FILIP-1L contains four coiled-coil and two N-terminal leucine zipper domains. Ectopic expression of FILIP-1L reduces the expression of Hsf1 protein since FILIP-1L promotes Hsf1 ubiquitination and degradation through the ubiquitin-proteasome system (UPS), leading to a reduction in Hsf1-mediated transcription. FILIP-1L, Hsf1, and ubiquitin binding domain (UBA) of HhR23A, a receptor that transports polyubiquitinated proteins to the 19S proteasome subunit targeting them for degradation, are found in a complex. This indicates that FILIP-1L is a potential adaptor that is involved in the Hsf1 degradation pathway. Taken together, our results indicate that FILIP-1L interacts with Hsf1, controlling its stability and thus modulating the heat shock response. These data indicate a novel function for FILIP-1L and a pathway for Hsf1 degradation through the UPS.
the structure of Hsf1 and its interaction with other proteins. In addition to the above modifications, Hsf1 activity is also regulated through interactions with a number of other proteins, including Hsp70, Hsp90 (1,20,21), Hspbp1 (22), CHIP (23), RalBP1 (24), Daxx (25), MTA1 (26), 14-3-3 (27), and Cdc20 (28). These proteins either exert negative regulation on Hsf1 transcriptional activity (e.g., Hspbp1), participate in the activation of Hsf1 (e.g., CHIP, Daxx), or confer a regulatory function towards Hsf1 in a specific signaling pathway (RalBP1, MTA1). Hsf1 is expressed in a monomeric form and is in complexes with Hsp90. Upon heat shock, Hsf1 is dissociated from the Hsp90 complexes (20,24), trimerizes, and is translocated into the nucleus and binds to the promoter of Hsps and other downstream target genes, driving transcription. Hsf1 also interacts with Hsp70, and this suppresses its transcriptional activity (29). The interaction of Hsf1 with Cdc20 inhibits the interaction of Cdc20 with Cdc27, phosphorylation of Cdc27, and the ubiquitination activity of Anaphase-Promoting Complex (APC) (28). Interaction of Hsf1 with 14-3-3 at 37°C leads to cytoplasmic sequestration and repression of Hsf1 activity. Ral Binding Protein-1 (RalBP1) forms a complex with Hsf1 and Hsp90 under physiological growth conditions, and regulates the Hsf1-mediated heat shock response in the Ral-dependent signaling pathway (24). The phosphorylated Hsf1 has been shown to undergo ubiquitin-mediated degradation via the SCFβ-TrCP pathway in mitotic cells (28). However, the mechanism underlying Hsf1 protein turnover in interphase cells and during the heat shock response has not been elucidated.

In the present study, we report another novel Hsf1-associating protein named Filamin Interacting Protein 1 Like (FILIP-1L) whose biological function is presently unclear. FILIP-1L is up-regulated in response to angiogenic inhibitors (30). Overexpression of FILIP-1L in cells leads to an increase in cellular apoptosis and inhibition of cell growth and migration (31). We show that FILIP-1L forms complexes with Hsf1 and Hsp72 and ectopic expression of FILIP-1L reduces Hsf1 protein leading to inhibition of Hsf1-mediated transcription. FILIP-1L expression in cells leads to an increase in Hsf1 ubiquitination and its recruitment to the complexes with the UBA domain of hHR23A protein. We anticipate that hHR23A is a ubiquitin receptor involved in proteasomal degradation, promoting Hsf1 degradation through the UPS.

EXPERIMENTAL PROCEDURES

Cell culture
H1299 (human small cell lung carcinoma), HEK293 (human embryonic kidney epithelial), and C2C12 (murine myoblast) cell lines were maintained in Dulbecco’s Minimal Essential Medium (DMEM) supplemented to 10% Fetal Bovine Serum (FBS) and Streptomycin/Penicillin. Heat shock treatment was performed in a temperature-controlled circulating water bath.

Yeast two hybrid system
For yeast two-hybrid screening, the CytoTrap Yeast Two-Hybrid System (Stratagene, La Jolla, CA) was used as we have previously reported (24). A fragment of human Hsf1 (amino acid residues 1–378) was subcloned into the pSos vector and was used as a bait. A human heart tissue cDNA library that had been subcloned into the pMyr vector was used to find Hsf1-interacting proteins. Ten µg of the bait construct and 10 µg of the cDNA library were cotransfected into the competent yeast cdc25H strain. The transformed yeast were plated in SD (uracil/leucine-deficient) glucose plates and allowed to grow at 25°C for 48–72 hours. The yeast colonies that appeared on the plates were replica-plated on the SD/galactose plates and SD/glucose plates,
and plates were incubated at 37°C for 10 days. The positive colonies were cultured in 5 ml of SD/glucose broth and cultured at 25°C. The plasmid DNA was extracted and used to transform *Escherichia coli* DH5α and then selected on chloramphenicol-containing agar plates. The plasmids pMyr and pSos encoding Maf B transcription factor were supplied by the manufacturer and were used as a positive control (24). The isolated plasmids were verified by DNA sequencing.

**Plasmids**

For pSos-Hsf1, Hsf1 cDNA encoding 1-378 amino acids was subcloned into the pSos vector (24). For pcDNA3-Flag-FILIP-1L and pcDNA3-HA-FILIP-1L (both Flag and HA tags located at the N terminus of FILIP-1L cDNA), the intact FILIP-1L cDNA (amino acid residues 1-893) was amplified by PCR from plasmids pMyr-FILIP-1L and was subcloned into the pcDNA3 at the BamHI and XhoI restriction enzyme sites. For plasmid pcDNA3-Flag-FILIP-1L-N, amino acid residues 1 to 230 of FILIP-1L were released from pcDNA3-Flag-FILIP-1L using restriction enzymes BamHI and EcoRI and the fragment was subcloned into the same restriction enzyme sites of pcDNA3. For pEBG-Hsf1 mutants, pEBG-Hsf1 amino acid residues 1-80, 1-161, 1-196, 1-278, 1-378, and 379-529 were constructed by inserting the appropriate Hsf1 fragments into the BamHI and NotI restriction enzyme sites of pEBG vector. Similar strategies were used to generate pEBG-FILIP-1L (amino acid residues 1-893) and pEBG-FILIP-1L-N (amino acid residues 1-288) expression vectors. The plasmids pcDNA3-His-Hsf1 containing human Hsf1 and pcDNA3-HA-ubiquitin were gifts from Drs. R. Morimoto (Northwestern University, Evanston, IL) and T. Kamitani (Georgia Health Sciences University), respectively. All the constructs were generated by PCR and confirmed by DNA sequencing.

**Immunoblotting, immunoprecipitation, and GST pull-down assays**

For immunoblotting, cells were lysed in 50 mM Tris HCl, pH7.5, 150 mM NaCl, 1% NP-40, protease inhibitor cocktails (Roche). Equal amounts of proteins (30 µg) were subjected to SDS-PAGE gel electrophoresis and immunoblotting (24). For immunoprecipitation, 48 hours following transfection of appropriate vectors, cells were lysed in buffer. 800 µg of cell lysate was pre-cleared using 20 µl of 50% slurry of Protein A Sepharose 4B beads. 2-3 µg of primary antibody was added to the pre-cleared cell lysate and incubated overnight at 4°C. Cell lysates were then incubated with 40 µl of the protein-A beads for 2 hours. Protein A beads containing protein complexes were centrifuged, rinsed four times with lysis buffer, and boiled in SDS-sample buffer in preparation for immunoblotting. For the *in vivo* GST pull-down assays, cells were co-transfected with appropriate plasmids together with recombinant constructs of pEBG encoding full-length or truncated mutants. 48 hours after transfection, cells were lysed and 700 µg of soluble protein fractions were incubated with 30 µl of Glutathione-Sepharose 4B beads for 16 hours. The beads were rinsed four times with lysis buffer. Samples were subjected to immunoblotting analyses. For the *in vitro* GST pull-down assays, cell lysates containing overexpressed protein was incubated with bacterially purified GST or GST-conjugated purified protein at 4°C overnight. The GST and GST fusion proteins were immobilized with Glutathione Sepharose 4B beads (24). After rinsing, beads were placed in SDS-sample buffer and boiled for 10 minutes, and samples were analyzed by immunoblotting using appropriate antibody. Antibodies were from the following sources: β−Actin and Flag (Sigma); Ubiquitin and HA (Santa Cruz Biotech); Hsf1 (Cell Signaling); Hsp72 and Hsp27 (Stressgen).
For *in vivo* ubiquitination reactions, HEK293 cells were transiently transfected with empty vector or with appropriate plasmids. Cells were left untreated or treated with proteasome inhibitors and were lysed in NP-40 lysis buffer containing protease inhibitor cocktail. The proteins were pulled down and, after rinsing with high salt buffer containing Tris-HCl pH 7.4, 0.4 NaCl, and 2 mM DTT, the immunoprecipitated complexes were immunoblotted using specific antibody as indicated in figure legends.

**Taylon beads pull-down assay**

HEK293 cells were transiently cotransfected with appropriate plasmids using Trans IT-LT (Mirus). After 48 hours, cells were treated with 10 µM of MG132 for 6 hours and lysed in denaturing lysis buffer containing 6 M Guanidinium-HCl, 100 mM Na$_2$HPO$_4$, 150 mM NaCl. Cell lysates were incubated with 30-40 µl of taylon beads for 1 hour and beads were rinsed three times in 8 M urea buffer and 2 times with PBS. The proteins were eluted in SDS-sample buffer and analyzed by immunoblotting.

**Immunofluorescent analyses**

The empty vector containing the Enhanced Yellow Fluorescent Protein (EYFP) or construct expressing FILIP-1L-EYFP was transiently transfected into H1299 cells. Cells were left untreated or treated at 43°C for 1 hour and left to recover at 37°C for 30 minutes. Cells were rinsed with PBS and fixed in 4% paraformaldehyde for 15 minutes at 25°C and then permeabilized with 0.5% Triton-X-100 in PBS (PBST) for 5 minutes at 4°C. Cells were rinsed three times and blocked in 5% BSA in PBST buffer for one hour and incubated with the primary antibody for one hour at 25°C. After three rinses, cells were incubated with secondary antibody conjugated with Texas-Red for 1 hour (17). After three rinses, nuclei were stained with DAPI in PBS buffer. Cells were analyzed using Zeiss Axio-Imager Fluorescence microscope.

**Statistical analyses**

All experiments were performed at least three times. Data are presented as mean ± SD. Statistical significance between experimental groups was assessed using Student’s *t* test and *p*<0.05 was considered significant.

**RESULTS**

**Hsf1 interacts with FILIP-1L**

Using the human Hsf1 cDNA as a bait, we screened a human heart cDNA library using the Cyto-Trap Yeast Two-Hybrid screening system (24). A novel Hsf1-interacting protein whose sequence was identical to the FILIP-1L mRNA version 2 (also known as down-regulated ovary cancer protein 1 (Doc1), gene bank designation number NP_055705) was identified (Figure 1A). FILIP-1L version 1 encodes a 1135 amino acid residue protein and has two leucine zipper domains at the N-terminal region (amino acid residues 323-344 and 458-479) and four coiled-coiled domains spanning amino acid residus 78 to 781. Amino acid residues 78 to 781 are homologous to the N terminal of the Structural Maintenance of Chromosomes (SMC), which is a chromosome segregation ATPase, and amino acid residues 875-1115 are homologous to BLLF1 (herpes virus major outer envelope glycoprotein Gp350/220) (Figure 1A). FILIP-1L also encodes a potential nuclear localization domain at amino acid residues 250-760 are homologous to the N terminal of the Structural Maintenance of Chromosomes (SMC), which is a chromosome segregation ATPase, and amino acid residues 875-1115 are homologous to BLLF1 (herpes virus major outer envelope glycoprotein Gp350/220) (Figure 1A). FILIP-1L also encodes a potential nuclear localization domain at amino acid residues 168-183. FILIP-1L has been reported to possess four versions (Figure 1A). The last seven amino acid residues of versions 1 and 4 are 1128-VEPLLLPH-1135 and 704-VEPLLLPH-711, respectively, and that of versions 2 and 3 are 1129-SNIYN-1133 and 889-SNIYN-893, respectively. The significance of these isoforms in cellular function is not known.
The interaction between Hsf1 and FILIP-1L was examined in mammalian cells using immunoprecipitation assays. H1299 cells were either mock-transfected, or transiently co-transfected with plasmids encoding His-Hsf1 together, with either plasmids encoding Flag-FILIP-1L (thereafter called FILIP-1L), or Flag-FILIP-1L-N (encoding amino acid residues 1-230) (Figure 1A-B). Cells were left untreated or were heated at 43°C for one hour to activate Hsf1. As indicated in Figure 1B, Hsf1 was co-immunoprecipitated by both Flag-FILIP-1L and Flag-FILIP-1L-N under physiological growth conditions (lanes 3 and 5). However, the interaction of Hsf1 with Flag-FILIP-1L was reduced when cells were treated at 43°C (Figure 1B, lane 4). No Hsf1 was co-immunoprecipitated in the pre-cleared beads (Figure 1B, lane 1), or in mock-transfected cells incubated with Flag antibody (Figure 1B, lane 2). Flag-FILIP-1L and Flag-FILIP-1L-N were also co-immunoprecipitated with His-Hsf1, using antibody to Hsf1 (Figure 1C, lanes 2 and 3). These results demonstrate that the leucine zipper region of the N-terminal fragment of FILIP-1L (encoded by FILIP-1N) is required for the interaction with Hsf1 (Figure 1A-C). Immunoblot analyses showing the expressions levels of Flag-FILIP-1L, Flag-FILIP-1L-N, and His-Hsf1 in the H1299 cells are presented in Figure 1D. Comparable results were also obtained when expression plasmids encoding GFP-Hsf1 and Flag-FILIP-1L were co-expressed in cells and complexes were pulled down from untreated (37°C) or heated (43°C) cells using antibody to Flag (Figure 1E). Levels of GFP-Hsf1 and Flag-FILIP-1L in cell lysates are indicated in the lower panel of Figure 1E. These results indicate that Hsf1 interacts with FILIP-1L in cultured mammalian cells.

The interaction between Hsf1 and FILIP-1L was examined in mammalian cells using immunoprecipitation assays. H1299 cells were either mock-transfected, or transiently co-transfected with plasmids encoding His-Hsf1 together, with either plasmids encoding Flag-FILIP-1L (thereafter called FILIP-1L), or Flag-FILIP-1L-N (encoding amino acid residues 1-230) (Figure 1A-B). Cells were left untreated or were heated at 43°C for one hour to activate Hsf1. As indicated in Figure 1B, Hsf1 was co-immunoprecipitated by both Flag-FILIP-1L and Flag-FILIP-1L-N under physiological growth conditions (lanes 3 and 5). However, the interaction of Hsf1 with Flag-FILIP-1L was reduced when cells were treated at 43°C (Figure 1B, lane 4). No Hsf1 was co-immunoprecipitated in the pre-cleared beads (Figure 1B, lane 1), or in mock-transfected cells incubated with Flag antibody (Figure 1B, lane 2). Flag-FILIP-1L and Flag-FILIP-1L-N were also co-immunoprecipitated with His-Hsf1, using antibody to Hsf1 (Figure 1C, lanes 2 and 3). These results demonstrate that the leucine zipper region of the N-terminal fragment of FILIP-1L (encoded by FILIP-1N) is required for the interaction with Hsf1 (Figure 1A-C). Immunoblot analyses showing the expressions levels of Flag-FILIP-1L, Flag-FILIP-1L-N, and His-Hsf1 in the H1299 cells are presented in Figure 1D. Comparable results were also obtained when expression plasmids encoding GFP-Hsf1 and Flag-FILIP-1L were co-expressed in cells and complexes were pulled down from untreated (37°C) or heated (43°C) cells using antibody to Flag (Figure 1E). Levels of GFP-Hsf1 and Flag-FILIP-1L in cell lysates are indicated in the lower panel of Figure 1E. These results indicate that Hsf1 interacts with FILIP-1L in cultured mammalian cells.

We next performed an in vivo GST pull-down assay to determine the domains of Hsf1 that associate with FILIP-1L. Thus, plasmids containing Flag-FILIP-1L were cotransfected into H1299 cells with constructs expressing GST alone, GST-Hsf1 (1-378), or GST-Hsf1 (1-278). As indicated in Figure 2A, Flag-FILIP-1L was co-immunoprecipitated together with GST-Hsf1 (1-278), and GST-Hsf1 (1-378), but not with GST alone. These results suggest that FILIP-1L-interacting region is located between amino acid residues 1 to 278 of Hsf1 protein, which contains the Hsf1 DNA binding domain, N-terminal hydrophobic domain, and a portion of the regulatory domain. To more precisely determine the domain of Hsf1 that interacts with FILIP-1L, we co-transfected H1299 cells with constructs encoding Flag-FILIP-1L together with GST alone, or GST-Hsf1 containing amino acid residues 1-80, 1-161, 1-196 or 378-529. As presented in Figure 2B, we found that Flag-FILIP-1L could be pulled down with GST-Hsf1 amino acid residues 1-196, but not with 1-80, 1-161 or 378-529, or with GST alone. The results indicate that Hsf1 amino acid residues 1-196, which contain the full-length N-terminal hydrophobic region, is the FILIP-1L-interacting domain (Figure 2B). The expression of plasmids encoding Flag-FILIP-1L, GST, and GST-Hsf1 mutant proteins in H1299 cells were detected by immunoblot analyses using antibody to Flag, GST, or β-Actin (Figure 2A and B, lower panels). Using a comparable approach, we determined that GST-FILIP-1L amino acid residues 1-288, which contain two leucine zipper domains, could pull down endogenous Hsf1 protein under normal physiological growth conditions, but not when cells were exposed to heat shock (Figure 2C). The right panel in Figure 2C shows the expression of Hsf1 in the cell lysate. Results presented in Figure 2C further support that the N-terminal leucine zipper domain of FILIP-1L is the Hsf1-interacting domain.
FILIP-1L forms a complex with Hsf1 and Hsp72

In mammalian cells, Hsf1 exists in a monomeric form through its interactions of the N- and C-terminal hydrophobic domains (4). Hsf1 has also been shown to form heterocomplexes with Hsp72. Hsp72 interacts with the C-terminal hydroporphic region of Hsf1 and inhibits Hsf1 transcriptional activity under physiological growth condition. Since we showed that FILIP-1L can interact with Hsf1’s N-terminal hydrophobic repeats under physiological growth conditions, we tested whether FILIP-1L/Hsf1 complexes also contain Hsp72. To this end, we performed an in vivo GST pull-down assay. The constructs expressing GST or GST-Hsf1 amino acid residues 1-529, 1-378, or 379-529 were transiently transfected alone, or co-transfected with plasmids containing Flag-FILIP-1L into HEK293 cells. As indicated in Figure 3A, GST-Hsf1 amino acid residues 1-529 could be co-immunoprecipitated with Flag-FILIP-1L and Hsp72 (Figure 3A, lanes 2 and 3). In contrast, GST-Hsf1 1-378 could only be co-immunoprecipitated with Flag-FILIP-1L but not Hsp72 (Figure 3B, compare lane 2 with lane 5). However, GST-Hsf1 amino acid residues 379-529 could pull down Hsp72, but not Flag-FILIP-1L (Figure 3B, lanes 3 and 6). No interactions between GST, Hsp72, and FILIP-1L could be detected (Figure 3A, lane 1 and Figure 3B, lanes 1 and 4). These results indicate that FILIP-1L and Hsp72 associate with different domains of Hsf1 protein. Expression levels of GST or GST-Hsf1 mutants in the cell lysates are presented in Figure 3A and B, lower panels. Expression levels of Flag-FILIP-1L and Hsp72 in the cell lysates are also presented.

These data demonstrate that full-length Hsf1 binds both Hsp72 and FILIP-1L in one complex.

To determine whether the association between Hsf1, Hsp72, and FILIP-1L is regulated by heat shock, cells were cotransfected with GST plus GST-FILIP-1L (Figure 3C, lanes 1-4), GST-Hsf1 (Figure 3C, lanes 5-8), or co-transfected with GST-Hsf1 plus Flag-FILIP-1L (Figure 3C, lanes 9-12) and left at 37°C, or were heated at 43°C for 1 hour, and then incubated at 37°C for a recovery period of 0, 1, or 3 hours. As indicated in Figure 3C, lanes 5-8, GST-Hsf1 could pull down Hsp72 under both physiological growth conditions and after cells were exposed to heat shock and left to recover from heat shock exposure. Data show that exposure of the HEK293 cells to heat shock does not significantly alter the interaction between Hsf1 and Hsp72, and that Hsp72 forms a heterodimer with Hsf1 even under physiological growth condition. Hsp72 and Hsf1 continue to interact following exposure of the cells to heat shock and during the recovery from heat shock. This is because HEK293 cells express the inducible Hsp72 constitutively. Surprisingly, GST-Hsf1, which was shown to interact with FILIP-1L under physiological growth conditions, could not pull down Flag-FILIP-1L immediately following exposure of the cells to 43°C heat shock (Figure 3C, compare lanes 9 and 10). However, Hsf1 and Flag-FILIP-1L began to interact again during 1 and 3 hours recovery following heat shock (Figure 3C, lanes 11 and 12). Cells expressing GST plus Flag-FILIP-1L did not show any interaction with Hsp72 under any condition (Figure 3C, lanes 1-4). Expression of GST and GST-Hsf1 are presented in Figure 3C, middle panel. Expression of Hsp72 and Flag-FILIP-1L in the cell lysates are presented in Figure 3C. These data further demonstrate that the interaction between Hsf1 and FILIP-1L occurs predominantly under physiological growth conditions. Under comparable conditions, neither GST protein nor GST-Hsf1 could immunoprecipitate Hsp90 (data not shown). This is consistent with previous reports that intracellular association between Hsf1 and Hsp90
requires use of a protein cross-linker (20,24).

**FILIP-1L exists as a dimer or oligomer**

To examine whether intracellular FILIP-1L exists as a monomer or oligomer, we performed immunoprecipitation and *in vivo* pull-down assays. Data show that Flag-FILIP-1L could be co-immunoprecipitated with HA-FILIP-1L (Figure 4A). Furthermore, GST-FILIP-1L or GST-FILIP-1L amino acid residues 1-288 could pull down Flag-FILIP-1L protein (Figure 4B). Expression levels of Flag-FILIP-1L and β-Actin in the cell lysate are presented as control. These results demonstrate that FILIP-1L protein is expressed in cells as either a dimer or oligomer.

**FILIP-1L expression in cells reduces Hsf1 expression**

In the human tumor cell lines, Hsf1 is normally localized in both the cytoplasm and nucleus under physiological growth conditions. Following heat shock, Hsf1 translocates into the nucleus and forms nuclear stress granules and drives transcription (17). To examine whether FILIP-1L is colocalized with endogenous Hsf1 following heat shock, cells were transiently transfected with EYFP-N1 empty vector or plasmids encoding EYFP-FILIP-1L. Cells were left untreated or were heated at 43°C for 1 hour. As presented in Figure 5 (upper panels), expression of EYFP does not affect formation of Hsf1 stress granules that forms upon exposure of the cells to heat stress. Surprisingly, we found that ectopic expression of EYFP-FILIP-1L reduced the expression of endogenous Hsf1 in cells that were untreated or cells that were heated at 43°C for 1 hour (Figure 5, lower panels, arrows). Note that Hsf1 normally form stress granules in cells exposed to heat shock (Figure 5, lower panels, arrowheads). Ectopic expression of FILIP-1L also interfered with the formation of Hsf1 stress granules following heat shock. Quantification of cells that express FILIP-1L and show reduced Hsf1 are presented in the Figure 5, right panel. As indicated in Figure 5 (lower panels), EYFP-FILIP-1L is expressed in both the cytoplasm and nucleus and often as granules; the reason for this is unclear. These structures do not colocalize with Grp75, a mitochondrial protein, Grp78, an ER resident protein, and Lamp1, a lysosomal marker (data not shown).

**FILIP-1L represses Hsf1 transcriptional activity**

One function of Hsf1 in mammalian cells is the enhanced expression of Hsps following exposure of the cells to stress conditions (1,4,13). The data presented in Figure 5 indicated that FILIP-1L expression in cells leads to reduction in Hsf1 protein and its ability to form stress granules in the nuclei following stress, suggesting that FILIP-1L may exert a negative regulatory effect on Hsf1 transcriptional activity. To this end, we performed reporter assays using plasmids containing Hsp70 promoter fused to the luciferase (*hsp70*-luciferase) reporter gene. Cells were transiently transfected with plasmids containing *hsp70*-luciferase alone, or co-transfected with expression plasmids containing Flag-FILIP-1L. Cells were left untreated or were treated at 43 °C for one hour, and left to recover at 37 °C for 2 or 8 hours to allow luciferase expression. As the data in Figure 6A indicate, *hsp70* promoter-driven luciferase activity was significantly reduced in cells expressing FILIP-1L under both physiological growth conditions and following exposure of the cells to heat shock.

To confirm whether Hsp expression is affected in cells expressing Flag-FILIP-1L, we performed immunoblotting analyses to determine whether ectopic expression of Flag-FILIP-1L could reduce the expression level of Hsf1 target genes (Hsp72, Hsp27). HEK293 cells were
transiently transfected with plasmids encoding Flag-FILIP-1L. Cells were then heated at 43°C for one hour and allowed to recover at 37°C for 0 to 12 hours, and the expression levels of Hsp72 and Hsp27 were determined by immunoblotting. As indicated in Figure 6B, the expression of these two Hsps was significantly lower (2-fold) when cells expressed Flag-FILIP-1L. The results of immunoblotting analysis using Hsf1 antibody indicate that FILIP-1L expression in cells reduces the level of Hsf1 expression (Figure 6B). Data in Figure 6C and D also indicate that ectopic expression of Flag-FILIP-1L reduces the levels of Hsf1, αB-crystallin, Hsp27, and Hsp72 at the level of translation by 2-4-fold, while the level of Hsf1 mRNA was not affected. Taken together, the above data indicate that FILIP-1L inhibits Hsf1 transcriptional activity by altering the level of Hsf1 protein.

**FILIP-1L promotes Hsf1 polyubiquitination**

Our data indicate that ectopic expression of Flag-FILIP-1L reduces Hsf1 expression, suggesting that FILIP-1L may regulate Hsf1 protein stability. To elucidate whether FILIP-1L can mediate Hsf1 degradation through polyubiquitination and degradation through the UPS, we first determined whether Hsf1 protein could be polyubiquitinated. HEK293 cells were transiently transfected with expression vectors encoding HA-ubiquitin and His-Hsf1. Cells were then left untreated or were treated with proteasome inhibitors MG132, proteasome inhibitor 1 (PI), or lactocystein (LC). Hsf1 was immunoprecipitated using antibody to Hsf1 followed by immunoblotting using antibody to ubiquitin. As presented in Figure 7A, His-Hsf1 could be polyubiquitinated. The proteasome inhibitor LC more effectively blocked the degradation of polyubiquitinated Hsf1. The level of Hsf1 in the cell lysate is presented in Figure 7A, lower panels. To investigate whether FILIP-1L can modulate the levels of Hsf1 polyubiquitination, cells were transfected with empty vector (Figure 7B, lanes 1 and 2) or plasmids containing Flag-FILIP-1L (Figure 7B, lanes 3 and 4), and cells were left untreated (Figure 7B, lanes 1 and 3) or were treated with MG132 (Figure 7B, lanes 2 and 4). Endogenous Hsf1 was immunoprecipitated using antibody to Hsf1 followed by immunoblotting using antibody to ubiquitin. Data show that Flag-FILIP-1L can enhance Hsf1 polyubiquitination in the presence of MG132. To further confirm whether FILIP-1L can modulate the Hsf1 polyubiquitination state, we performed a talon pull-down assay. HEK293 cells were transiently transfected with expression vectors encoding His-Hsf1 alone or co-transfected with His-Hsf1 plus plasmids containing HA-ubiquitin or His-Hsf1 plus HA-ubiquitin and Flag-FILIP-1L. Cells were treated with MG132. His-Hsf1 was pulled down with talon beads followed by immunoblotting analyses using anti-HA-ubiquitin antibody. As indicated in Figure 7C, FILIP-1L could enhance His-Hsf1 polyubiquitination even under denaturation conditions (Figure 7C, lane 3). Taken together, our results demonstrate that FILIP-1L promotes Hsf1 polyubiquitination.

**FILIP-1L binds to the UBA domain of HhR23 and to Hsf1**

Ubiquitinated proteins are transported to the 19S proteasome through their association with the ubiquitin receptor proteins such as HhR23A (yeast homolog of Rad23A) (32,33). Within its protein structure, HhR23A contains two ubiquitin associating UBA domains at its C terminal domain, and the ubiquitin-like (UBL) sequences at its N-terminal domain. To elucidate whether FILIP-1L-mediated proteasome degradation of Hsf1 is regulated by adaptor protein HhR23A, we performed an in vitro GST pull-down assay. HEK293 cells were transiently transfected with GST, GST-FILIP-1L...
amino acid residues 1-893, or 1-288 and HA-HhR23A or HA-HhR23A UBA domain. GST-pull-down materials were immunoblotted using antibody to HA. Data presented in Figure 8A indicate that both fragments of FILIP-1L interact with full-length HA-HhR23A and its UBA domains. The lower panels of Figure 8A show expression of overexpressed proteins in the cell lysate. These data indicate that the FILIP-1L leucine zipper domains interact with the UBA domain of HhR23A. To examine whether Hsf1, FILIP-1L, and HhR23A are in the same complex, we performed an in vitro GST pull-down assay. HEK293 cells were transiently transfected with empty vector (Figure 8B, lanes 1 and 2), Flag-FILIP1L alone (Figure 8B, lanes 3 and 4) or His-Hsf1 alone (Figure 8B, lanes 7 and 8) or cotransfected with Flag-FILIP-1L plus His-Hsf1 (Figure 8B, lanes 5 and 6). Cells were left untreated or were treated with MG132. Cell lysates were prepared and incubated with bacterially purified protein GST-HhR23A (containing only the two UBA domains) (34). As the data in Figure 8 indicate, FILIP-1L can be pulled down using GST- HhR23A (Figure 8B, lanes 3 and 4). Further, both FILIP-1L and Hsf1 could be pulled-down using GST-HhR23A (Figure 8B, lanes 5 and 6). No Hsf1 or FILIP-1L could be pulled down using GST alone (Figure 8B, lanes 1 and 2). In addition, HhR23A alone (without FILIP-1L) could not pull down Hsf1 significantly (Figure 8B, lanes 7 and 8). In all cases, treatment of cells with MG132 enhanced the interaction of HhR23A with FILIP-1L or Hsf1. The levels of overexpressed His-Hsf1 and Flag-FILIP-1L in cell lysate are indicated in Figure 8B, lower panels.

Taken together, our data indicate that FILIP-1L brings Hsf1 and HhR23A together into the same complexes.

DISCUSSION

Hsf1 transcription factor activity is tightly regulated; however, whether Hsf1 is degraded during its cycles of activation and inactivation remain poorly understood. One study has found that in cells undergoing mitosis, Hsf1 can be ubiquitinated and degraded by the SCFβνTrCP complex (28). Ubiquitination and degradation of Hsf1 by SCFβνTrCP complex only occurred during mitosis when the phosphorylated Hsf1 at serine 216 was released from the Cdc20 complex. Hsf1 was then found in SCFβνTrCP complexes, ubiquitinated, and degraded (28). Another report indicates that anaphase-promoting complex/cyclosome (APC/C) ubiquitin E3 ligase mediates the ubiquitination and degradation of another Hsf family member, Hsf2 through substrate recognition by both Cdc20 and Cdh1 (5). Degradation of Hsf2 was observed during the heat shock response. Comparable interactions or ubiquitination of Hsf1 was not detected (5).

In the study reported here, we have found a protein named FILIP-1L interacts with Hsf1. The function of FILIP-1L has not clearly been characterized. FILIP-1L encodes coiled-coiled, leucine zipper, and ATPase domains and exists as multiple isoforms in many cell types (30,31). Ectopic expression of FILIP-1L results in the inhibition of cellular proliferation, migration, and apoptosis. Furthermore, endothelial cells treated with angiogenic inhibitors up-regulate FILIP-1L expression. FILIP-1L expression in tumor vasculature reduces tumor cell proliferation in vivo (31). FILIP-1L has been identified to be absent in ovarian cancer cells, but present in normal epithelial cells and was named down-regulated ovarian cancer protein 1 or DOC1 (35). We present evidence that Hsf1 interacts with FILIP-1L in the yeast two-hybrid system as well as in mammalian cells. The N-terminal fragment of FILIP-1L containing the leucine zipper domain interacts with the N-terminal hydrophobic region of Hsf1 that is required for its trimerization and transcriptional activity. Interestingly, the Hsf1 and FILIP-1L interaction, which also
contains Hsp72 in the complexes leads to polyubiquitination and degradation of Hsf1 protein. Cells co-expressing Hsf1 and FILIP-1L exhibit reduction in Hsf1 protein level, and inhibition of Hsf1 stress granule formation following exposure to heat shock. Ectopic expression of full-length FILIP-1L most efficiently degraded endogenous Hsf1 protein or Hsf1 protein containing short tags. Additionally, FILIP-1L expression in cells also inhibits Hsf1-mediated transcriptional activity. Since FILIP-1L protein appears to lack an E3 ligase domain and we were unable to show E3 ligase activity of FILIP-1L using an in vitro transcription/translation system (data not shown), we performed experiments to determine whether FILIP-1L acts as a scaffold mediating ubiquitination and degradation of Hsf1. To determine whether there could be additional proteins that may be involved in the transfer of Hsf1 to the proteasome, we used full-length hHR23A (hPlic-1, Chap1/hPlic-2) as well as the HhR23A UBA domain, which is a known ubiquitin receptor (33,34). We were able to detect Hsf1, FILIP-1L, and the hHR23A UBA domain in the same complex, suggesting that the hHR23A UBA domain could be an intermediate molecule delivering the ubiquitinated Hsf1 to the UPS. HhR23A was originally identified as a protein that has UBA and UBL domains in yeast (33). The UBL domain is recognized by S5a, which is a subunit of the proteasome.

Based on the domains present in the ubiquitin receptors, they most likely transport ubiquitinated proteins to the proteasome. HhR23A (Rad23) has been identified to bind the E6-AP ubiquitin ligase, and it has been shown to target the polyubiquitinated p53 and Png1 proteins to the UPS. The UBA domain of hHR23A binds the ubiquitinated p53, protecting the molecule from deubiquitination while the UBL domain of hHR23A transfers the ubiquitinated p53 to the UPS. Png1, which is a deglycosylating enzyme, is able to transfer ubiquitinated proteins to the UPS (36). The speculation is that the substrate specificity of Rad23A or other ubiquitin-binding proteins may be defined by other adaptor proteins. We can envision that FILIP-1L may be such an adaptor protein for the transport of ubiquitinated Hsf1 to the UPS system. As to what is the exact function of FILIP-1L, we show that hHR23A does not directly interact with Hsf1 but that Hsf1, through its interaction with the adaptor protein FILIP-1L, is transported to the UPS via the HhR23A UBA domain.

In conclusion, we have identified the adaptor protein FILIP-1L to interact with Hsf1 and facilitate its ubiquitination and degradation. FILIP-1L and Hsf1 complexes are transported to the proteasome, likely via Class 1 UBA domain containing ubiquitin receptors such as hHR23A.
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Footnotes.

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Figure legend

Figure 1. Hsf1 interacts with FILIP-L1 in mammalian cells.

(A) Schematic representation of different FILIP-1L isoforms. FILIP-1L-2 was isolated by yeast two-hybrid system to interact with Hsf1. FILIP-1L-N is not an isoform but was constructed to determine the minimal domain of the FILIP-1L interaction with Hsf1.

(B) Interaction of Flag-FILIP-1L with Hsf1. H1299 cells were mock transfected (lane 2) or co-transfected with His-Hsf1 and Flag-FILIP-1L, or Flag-FILIP-1L-N (amino acid residues 1-230) (lanes 3, 4 and 5). Cells were left at 37°C (lanes 2, 3 and 5) or were heated at 43°C for 1 hour (lane 4). Complexes of Hsf1 and FILIP-1L or FILIP-1L-N were co-immunoprecipitated using antibody to Flag and were detected using antibody to His-Hsf1. The location of IgG band is also indicated. Lane 1 represents pre-cleared protein A.

(C) Co-immunoprecipitation of Flag-FILIP-1L or Flag-FILIP-1L-N (amino acid residues 1-230) with Hsf1 using antibody to Hsf1. Transient transfections were carried out as in (B). FILIP-1L or FILIP-1L-1N was co-immunoprecipitated using antibody to Hsf1 and immunoblotted using antibody to Flag. Lane 1 represents pre-cleared protein A; lane 2 represents cells transiently transfected with His-Hsf1 and Flag-FILIP-1L-N. Lane 3 represents cells transiently transfected with His-Hsf1 and Flag-FILIP-1L-1N. Lane 12 represents cells transiently transfected with His-Hsf1 and Flag-FILIP-1L.

(D) Immunoblot analyses showing the expression levels of ectopically expressed His-Hsf1, Flag-FILIP-1L, and Flag-FILIP-1L-N (amino acid residues 1-230) in the cell lysates used in panels B and C for co-immunoprecipitation studies.

(E) Flag-FILIP-1L interacts with GFP-Hsf1 under both physiological growth conditions and following heat shock. H1299 cells were transiently transfected with Flag-FILIP-1L alone (lane 1) or cotransfected with Flag-FILIP-1L and GFP-Hsf1 fusion protein (lanes 2 and 3).
Cells were left at 37°C (lanes 1 and 3) or were treated with heat shock at 43°C for 1 hour (lane 2). The GFP-Hsf1 and Flag-FILIP-1L complexes were co-immunoprecipitated using antibody to Flag. Immunoblotting was performed using antibody to GFP. Lower panels represent expression of GFP-Hsf1 and Flag-FILIP-1L in the cell lysate.

**Figure 2. Hsf1 N-terminal hydrophobic domain interacts with FILIP-1L leucine zipper domain.** (A and B). Top panels in (A and B) represent schematic drawings of the GST-Hsf1 mutants. DBD represents DNA binding domain, LZ, leucine zipper domain, RD, regulatory domain, TAD, transactivation domain. H1299 cells were transiently co-transfected with expression vectors containing Flag-FILIP-1L and GST alone, or with GST-Hsf1 deletion mutants. The GST and GST-Hsf1 mutants were pulled-down with Glutathione Sepharose 4B beads. Pull down materials were immunoblotted using antibody to Flag or GST. The lower panels in (A) and (B) represent immunoblots of the overexpressed Flag-FILIP-1L and β-actin in the cell lysates.

(C) GST-FILIP-1L can pull down endogenous Hsf1 under physiological growth conditions. H1299 cells were transfected with GST alone, or GST-FILIP-1L (amino acid residues 1-288). Cells were left at 37°C or were heated at 43°C for one hour. Endogenous Hsf1 was co-immunoprecipitated with GST-FILIP-1L using Glutathione Sepharose 4B beads. The immunoprecipitated materials were immunoblotted using antibody to Hsf1 and GST. The right panel represents the immunoblot analyses of cell lysate showing endogenous Hsf1 and β-actin.

**Figure 3. FILIP-1L is in Hsf1 and Hsp72 complexes.** (A) Full-length Hsf1 forms complexes with Hsp72 and Flag-FILIP-1L. HEK293 cells were transiently transfected with GST and Flag-FILIP-1L (lane 1), GST-Hsf1 alone (lane 2), or GST-Hsf1 and Flag-FILIP-1L (lane 3). The endogenous Hsp72 and Flag-FILIP-1L that were pulled down with GST-Hsf1 were immunoblotted using antibodies against Flag, Hsp72, or GST. The overexpressed Flag-FILIP-1L and endogenous Hsp72 in the cell lysates are indicated in lanes 4 and 5.

(B) Top panel represents schematic drawing of Hsf1 mutant constructs. Lower panel represents immunoblotting analyses showing Flag-FILIP-1L and Hsp72 interacting with different domains of Hsf1. HEK293 cells were transiently transfected with expression vector encoding GST (lane 1), GST-Hsf1 (amino acid residues 1-378) (lane 2), or GST-Hsf1 (amino acid residues 379-529) (lane 3), or co-transfected with Flag-FILIP-1L and GST or GST-Hsf1 mutants (lanes 4-6). The endogenous Hsp72 or overexpressed Flag-FILIP-1L and endogenous Hsp72 in the cell lysates are indicated in lanes 4 and 5.

(C) Regulation of Hsf1, Flag-FILIP-1L, and Hsp72 complexes by heat shock. HEK293 cells were transiently transfected with GST and Flag-FILIP-1L (lanes 1-4), GST-Hsf1 alone (lanes 5-8), or GST-Hsf1 and Flag-FILIP-1L (lanes 9-12). Cells were left at 37°C (lanes 1, 5 and 9) or heated at 43°C for 1 hour, and then allowed to recover at 37°C for 0 hour (lanes 2, 6 and 10), 1 hour (lanes 3, 7 and 11) or 3 hours (lanes 4, 8, and 12). The GST and GST-Hsf1 pulled down endogenous Hsp72, or ectopically expressed Flag-FILIP-1L were immunoblotted using their corresponding antibodies. Expression of GST and GST-Hsf1 are indicated in the middle panel. Lanes 13 and 14 of upper panel show expression of Hsp72 and Flag-FILIP-1L in the cell lysates. “V” indicates pcDNA3 vector alone. Levels of Hsp72, Flag-FILIP-1L, and β-actin in cell lysates are indicated in the lower panels.
Figure 4. FILIP-1L in cells exist as dimer or oligomer. (A) HEK293 cells transiently transfected with Flag-FILIP-1L alone (lane 2) or cotransfected with HA-FILIP-1L and Flag-FILIP-1L (lane 3). HA-FILIP-1L was immunoprecipitated using antibody to Flag and the immunoprecipitated materials were immunoblotted using antibodies to HA (upper panel) or Flag (lower panel). The expression of HA-, Flag- FILIP-1L and β-Actin in cell lysates is also presented. Lane 1 represents negative control where cell lysate expressing empty vector was incubated with anti-Flag antibody. (B) GST-FILIP-1L pulls down Flag-FILIP-1L. HEK293 cells were transiently transfected with Flag-FILIP-1L and GST (lane 1) or Flag-FILIP-1L with GST-FILIP-1L or GST-FILIP-1L (1-288) (lanes 2 and 3). Cell extracts were then used in pull-down experiments using GST. The pull-down materials were immunoblotted using Flag-FILIP-1L and GST. Lower panels show expression of Flag-FILIP-1L and β-actin in the cell lysates.

Figure 5. Hsf1 protein level is reduced in the presence of FILIP-1L. Upper panels: H1299 cells were transiently transfected with expression plasmids containing EYFP alone. Cells were left at 37°C or heated at 43°C for one hour. Expression of EYFP was detected using fluorescence microscopy. Level of Hsf1 was detected using antibody to Hsf1. Arrows show cells containing Hsf1 that forms stress granules following heat shock. Lower panels: H1299 cells were transiently transfected with expression plasmids containing EYFP-FILIP-1L. Cells were left at 37°C or heated at 43°C for one hour. Expression of EYFP-FILIP-1L was detected by fluorescence microscopy. Level of Hsf1 was detected using antibody to Hsf1. Arrows show cells expressing EYFP-FILIP-1L and Hsf1 in the same cell. Arrowheads show Hsf1 nuclear granules in cells that do not express EYFP-FILIP-1L. Quantification of the percent number of cells expressing EYFP-FILIP-1L with reduced Hsf1 levels compared to cells expressing EYFP is presented in the right panel. Bars are mean +/- standard deviation. Statistical significance is indicated (*p<0.05).

Figure 6. Inhibition of Hsf1 transcriptional activity by FILIP-1L. (A) HEK293 cells were transiently transfected with expression constructs containing Hsp70-luciferase plus pcDNA3-β-galactosidase (as an internal control) and Vector alone, or cotransfected with Hsp70-luciferase, pcDNA3-β-galactosidase, and Flag-FILIP-1L. Cells were left at 37°C or heated at 43°C for 1 hour and left to recover at 37°C for 0, 2 and 8 hours. Luciferase activity was determined and normalized to β-galactosidase expression levels. Bars are mean +/- standard deviation. Statistical significance is indicated for 8 hours post heat treatment (*p<0.02). (B) FILIP-1L reduces heat-induced Hsp expression. HEK293 cells were transiently transfected with empty vector (lanes 1-6) or with constructs expressing Flag-FILIP-1L (lanes 7-12). After 48 hours, cells were left at 37°C (lanes 1 and 7) or were treated at 43°C for 1 hour and then left to recover at 37°C for 0, 2 and 8 hours. The expression of Hsp72, Hsp27, Hsf1, and β-actin were determined by immunoblotting using the indicated antibodies. (C) Ectopic expression of FILIP-1L reduces level of Hsf1 and its downstream target genes. C2C12 cells were infected using empty retroviral vector (lane 1) or vector expressing Flag-FILIP-1L (lane 2). Immuno blotting experiments were performed using antibodies to detect Hsf1, αB-Crystallin (αB-Cry), Hsp27, Hsp72, or Flag. β-actin represents loading control. (D) Ectopic expression of FILIP-1L does not affect Hsf1 mRNA expression levels. C2C12 cells were infected using empty retroviral vector (lane 1) or vector expressing Flag-FILIP-1L (lane 2). After 48 hours, total RNA was isolated and Hsf1 and β-actin mRNA expression in the presence or absence of Flag-FILIP-1L were determined using semi-quantitative RT-PCR.
Figure 7. FILIP-1L mediates Hsf1 degradation through the UPS. (A) Hsf1 protein level is regulated by ubiquitination. HEK293 cells were co-transfected with plasmids containing His-Hsf1 and HA-ubiquitin (HA-Ub). Cells were left untreated or were treated with 10µM MG123 (MG), 1µg/ml of proteasome inhibitor (PI) or 5µM lactocystein (LC) for 6 hours. His-Hsf1 was immunoprecipitated and the immunoprecipitated materials were subjected to immunoblotting analyses using antibody to HA-ubiquitin. Lower panels show expression level of Hsf1 in the cell lysates.

(B) FILIP-1L enhances Hsf1 ubiquitination. HEK293 cells were transiently transfected with empty vector (lanes 1 and 2) or plasmids containing Flag-FILIP-1L (lanes 3 and 4). Cells were then left untreated (lanes 1 and 3), or were treated with 10µM of MG132 for 6 hours (lanes 2 and 4). Endogenous Hsf1 was immunoprecipitated using antibody to Hsf1 and complexes were subjected to immunoblotting analyses using antibody to ubiquitin. Lower panels show level of expression of Hsf1, Flag-FILIP-1L and β-actin in the cell lysates.

(C) Determination of FILIP-1L-mediated Hsf1 ubiquitination using nickel-pull down assay. Cells were transiently transfected with plasmids containing His-Hsf1 alone (lane 1), or cotransfected with plasmids containing HA-ubiquitin (lane 2), or HA-ubiquitin and Flag-FILIP1L (lane 3). Cells were treated with 10µM of MG132 for 6 hours and lysed in denaturing lysis buffer. The His-Hsf1 was pulled down using nickel beads, and the pull-down materials were subjected to immunoblotting using antibody to HA (upper panel) or Hsf1 (lower panel). Expression of Flag-FILIP-1L and β-actin in the cell lysate is indicated.

Figure 8. FILIP-1L and Hsf1 form complexes with HhR23A. (A) FILIP-1L interacts with UBA domain of HhR23A in an in vivo pull-down assay. HEK293 cells were transiently transfected with the indicated expression vectors (+). After 48 hours, GST pull-down assay was performed, and immunoprecipitated materials were used in immunoblotting using antibody to HA. Expression levels of HA-HhR23A and its UBA domain, GST-FILIP-1L, and GST alone are presented in the cell lysates (lower panels). Expression level of Hsp90 is presented as loading control.

(B) FILIP-1L interacts with Hsf1 and HhR23A UBA domain in an in vitro pull down assay. HEK293 cells were transiently transfected with the indicated expression vectors (+). Cells were untreated or were treated with 10µM MG132 for 6 hours. GST-HhR23A was used in pull-down assays and the level of immunoprecipitated materials was detected using antibodies to Flag and Hsf1. The level of purified GST and GST-HhR23A UBA domain are indicated. Lower panels show the expression of Flag-FILIP-1L and Hsf1 in the cell lysates. β-actin is loading control. Lanes 1-8 indicates the same groups in upper and lower panels.
Figure 2
Figure 3
Figure 4
Figure 6
Figure 7
Figure 8
Promotion of heat shock factor Hsf1 degradation via adaptor protein Filamin A-Interacting Protein 1-Like (FILIP-1L)
Yanzhong Hu and Nahid F. Mivechi

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