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Ebp1 Sumoylation, Regulated by TLS/FUS E3 Ligase, Is Required for its Anti-proliferative Activity

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Abstract

Ebp1, an ErbB3 receptor-binding protein, inhibits cell proliferation and acts as a putative tumor suppressor. Ebp1 translocates into the nucleus and functions as a transcription corepressor for E2F-1. Here, we show that Ebp1 p42 isoform can be sumoylated on both K93 and K298 residues, which mediate its nuclear translocation and is required for its anti-proliferative activity. We find that TLS/FUS, an RNA-binding nuclear protein that is involved in pre- mRNA processing and nucleocytoplasmic shuttling, has Sumo1 E3 ligase activity for Ebp1 p42. Ebp1 directly binds TLS/FUS, which is regulated by genotoxic stress-triggered phosphorylation on Ebp1. Ebp1 sumoylation facilitates its nucleolar distribution and protein stability. Overexpression of TLS enhances Ebp1 sumoylation, while depletion of TLS abolishes Ebp1 sumoylation. Moreover, Unsumoylated Ebp1 mutants fail to suppress E2F-1- regulated transcription, resulting in loss of its anti-proliferation activity. Hence, TLS-mediated sumoylation is required for Ebp1 transcription repressive activity.

Keywords

Ebp1; TLS/FUS; Sumoylation; Cell proliferation

Introduction

Ebp1, a ubiquitously expressed protein, localizes in both the nucleus and the cytoplasm and binds ErbB3 receptor in human breast cancer cells (Yoo *et al.*, 2000). Ebp1 is the human homologue of a previously identified cell cycle regulated mouse protein p38-2G4 (Radomski and Jost, 1995). ErbB3/4 ligand Heregulin (HRG) treatment of serum-starved AU565 breast cancer cells results in dissociation of Ebp1 from ErbB-3 and translocation from the cytoplasm into the nucleus (Yoo *et al.*, 2000). EBP1 overexpression inhibits proliferation of human fibroblasts, and this effect is linked to its nucleolar localization

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(Squatrito *et al.*, 2004). Northern blotting analysis reveals two transcripts with1.7 kb being the major one and 2.2 kb the minor one (Nakagawa *et al.*, 1997). These observations are consistent with the report that two Ebp1 mRNAs occur in several normal human organs (Yoo *et al.*, 2000). Recently, we report that *PA2G4* gene encodes two Ebp1 isoforms, p48 and p42, which differentially regulate PC12 cell survival and differentiation (Ahn *et al.*, 2006; Liu *et al.*, 2006). P48 is 54 amino acids longer than p42 at its N-terminus. The longer-form p48 localizes in both the cytoplasm and the nucleolus and suppresses apoptosis, whereas the shorter-form p42 predominantly resides in the cytoplasm and promotes cell differentiation (Liu *et al.*, 2006). Ebp1 is phosphorylated on serine 360 by PKC-δ, which strongly stimulates Ebp1 p48 to bind Akt and suppresses apoptosis (Ahn *et al.*, 2006). Ebp1 p42 specifically associates with NPM/B23 upon epidermal growth factor stimulation, while p48 constantly binds NPM/B23. NPM/B23 regulates cell proliferation and survival through p42 and p48, respectively (Okada *et al.*, 2007). Ebp1 also binds tumor suppressor retinoblastoma protein (Rb), leading to inhibition of the E2F1-regulated transcription (Xia *et al.*, 2001; Zhang *et al.*, 2003). Ebp1 contains an autonomous C-terminal transcriptional repression domain that binds histone deacetylase 2 (Zhang *et al.*, 2005). It strongly suppresses both androgen receptor (AR)-mediated gene transcription and tumorigenesis of prostate cancer cells and salivary adenoid carcinoma cell metastasis in mice (Yu *et al.*, 2007; Zhang *et al.*, 2005). Thus, Ebp1 p42 acts as a tumor suppressor by repressing E2F-1 and AR-mediated transcription.

TLS (translocated in liposarcoma) was initially identified as the N-terminus of TLS-CHOP, a fusion oncoprotein that is expressed as a consequence of the t(12;16) translocation, which is implicated in human myxoid and round cell liposarcomas (Crozat *et al.*, 1993; Rabbitts *et al.*, 1993). TLS or the related EWS (Ewing's sarcoma) gene is also fused to a panel of unrelated transcription factors through chromosomal translocation in some of human sarcomas and leukemias. The merged oncoproteins contain an N-terminal domain (NTD) from TLS or EWS (Sanchez-Garcia and Rabbitts, 1994). The C-terminal domain of TLS is replaced by the DNA-binding domain from the corresponding transcription factor. The NTD domain in the fusion oncoproteins plays a critical role in transformation (Kuroda *et al.*, 1997; Zinszner *et al.*, 1994). TLS possesses an SYGQ-rich region in the N-terminus, an RNA-recognition motif (RRM), a C2/C2 zinc finger motif and at least one RGG-repeat region in the C-terminus (Morohoshi *et al.*, 1998). Therefore, TLS binds RNA *in vitro* and *in vivo* (Crozat *et al.*, 1993; Prasad *et al.*, 1994; Zinszner *et al.*, 1997b); TLS is expressed at high levels in hematopoietic and non-hematopoietic tissues (Aman *et al.*, 1996), and localized primarily in the nucleus, where it may be involved in pre-mRNA processing and nucleocytoplasmic shuttling, as well as in the regulation of basal transcription (Ron, 1997; Zinszner *et al.*, 1997a).

In this report, we show that p42 but not p48 is sumoylated, which is regulated by PKC-δ-mediated phosphorylation. P42 sumoylation provokes its nucleolar translocation. TLS/Fus acts as a Sumo E3 ligase for Ebp1 p42. TLS directly binds to both Ubc9 and Ebp1 and promotes Ebp1 sumoylation. Disruption of Ebp1 sumoylation abolishes its anti-proliferative activity.

Results

Ebp1 p42 but not p48 isoform can be sumoylated

Tumor suppressors including NPM/B23 and p53 are usually regulated by sumoylation. To investigate whether both Ebp1 isoforms p42 and p48 can be sumoylated, we employed stably transfected F293 cells, which express tetracycline-inducible His-Sumo1 or His-Sumo3. F293 cells were transfected with GFP alone, GFP-p42 and p48, respectively, and the tetracycline-induced-His-Sumo modified proteins were pulled down with Nickel affinity column. Immunoblotting analysis revealed that Ebp1 p42 but not p48 was robustly sumoylated by both Sumo1 and 3 (Figure 1A, top panel). Cotransfection with Flag-Sumo1 also verified that p42 but not p48 was selectively sumoylated (Figure 1B). To examine whether Ebp1 can directly associate with SUMO1, we cotransfected GFP-sumo1 into HEK293 cells with a variety of GST-tagged Ebp1 fragments. GST-pull down assays revealed that p48 did not bind to Sumo1, and deletion of its N-terminal 22 amino acids (a.a. 23–394) elicited evident association (Figure 1C, lower left panel, lane 2 and 3), and the fragments (a.a. 292–360) and (a.a. 23–136) robustly interacted with GFP-sumo1 (lanes 1 and 4). Thus, the N-terminal 22 amino acids in p48 inhibit its association with Sumo1. Presumably, it explains why p48 cannot be sumoylated.

Genotoxic stress frequently elicits protein sumoylation (Huen and Chen, 2008; Watts, 2006). To assess whether DNA damage reagents can provoke Ebp1 sumoylation, we employed a few DNA damage agents: actinomycin D, adriamycin and VP16, which have been shown to stabilize covalent DNA intermediates of the topoisomerase I (e.g. VP16) or inhibiting RNA polymerases (e.g. actinomycin D). Compared to DMSO control, four h treatment with actinomycin D, adriamycin and VP16 markedly elevated the sumoylation of Ebp1 p42 (Figure 1D, top panel). DNA damage induces phosphorylation of p53 at Ser15 and Ser20 and leads to a reduced interaction between p53 and its negative regulator, the oncoprotein MDM2 (Meek, 1998; Shieh *et al.*, 1997). Immunoblotting analysis revealed that these three agents provoked robust p53 phosphorylation (Figure 1D, bottom panel).

Ebp1 binds ErbB3 in breast cancer cells, and translocates into the nucleus upon heregulin stimulation (Yoo *et al.*, 2000). To examine whether endogenous Ebp1 is also sumoylated in breast cancer cells, we treated MCF7 and AU565 cells with heregulin and immunoprecipitated Ebp1 with Ebp1 antibody, which recognizes both p42 and p48. Ebp1 was sumoylated in AU565 but not MCF7 cells, which was upregulated upon heregulin treatment (Figure 1E, left top panel). As control, sumoylated Ebp1 was selectively immunoprecipitated by Ebp1 antibody but not control IgG (Figure 1E, right panels). To explore whether DNA damage agents also provoke the similar effect, we treated AU565 cells with actinomycin D for various time points. Reverse immunoprecipitation with anti-Sumo1 revealed evident endogenous Ebp1 sumoylation by actinomycin but not DMSO as compared to control IgG (Figure 1E, bottom panels). Together, these observations support that Ebp1 p42 is sumoylated upon growth factor stimulation or genotoxic stress.

TLS/FUS is a Sumo E3 ligase for Ebp1

To search for Sumo E3 ligases that regulate p42 sumoylation, we transfected mammalian expressing GST and GST-p42 constructs into His-Sumo1 and His-Sumo3 cells. The transfected proteins were pulled down with glutathione beads, and the coprecipitated proteins were eluted and resolved on SDS-PAGE, followed by silver staining. The similar protein binding profiles occurred to both His-Sumo1 and His-Sumo3 samples, and the prominent protein bands were subjected to proteomic analysis (Figure 2A, left panel). As expected, we identified nucleophasmin NPM/B23, which confirmed our previous finding that Ebp1 associates with B23 in the nucleolus (Okada *et al.*, 2007). In addition, we found numerous ribonucleoproteins, verifying previous report (Squatrito et al., 2004). Among these binding partners, we also identified a zinc-finger containing protein TLS, which is a nucleocytoplaplasmic shuttling protein that binds RNA and mediates pre-mRNA processing (Ron, 1997; Zinszner *et al.*, 1997a). Nucleolar proteins including hBRE1 and TLS were confirmed by immunoblotting to associate with Ebp1 (Figure 2A, right panel). To investigate whether TLS regulates p42 sumoylation in intact cells, we cotransfected Flag-Sumo1 and TLS and Ubc9 into HEK293 cells with GFP-p42. P42 was prominently sumoylated when either E2 (Ubc9) or E3 (TLS) enzyme was overexpressed. The maximal effect occurred, when both E2 and E3 enzymes were highly expressed (Figure 2B), suggesting that TLS can facilitate p42 sumoylation in intact cells. In vitro sumoylation assay further supported this observation. Both the N-terminus of TLS (a.a. 1–290) and full-length of TLS but not its C-terminus (a.a. 357–525) robustly stimulated Ebp1 sumoylation (Figure 2C). To ascertain that TLS is required for p42 sumoylation, we knocked down endogenous TLS with its siRNA. Knocking down of TLS significantly abolished p42 sumoylation (Figure 2D). Together, these results demonstrate that TLS acts as an E3 sumo ligase for p42 and stimulates its sumoylation.

TLS/FUS binds p42 Ebp1

To explore whether the substrate Ebp1 p42 interacts with TLS, we cotransfected GST-p42 and p48 into HEK293 cells with Flag-TLS. GST-pull down revealed that both isoforms associated with TLS (Figure 3A, left panels). By contrast, GST control failed to bind TLS (Figure 3A, right panels). To assess whether the interaction occurred between the endogenous proteins, we treated K562 cells with DNA damage agent adriamycin (3 μM) and actinomycin D (10 nM) for different time points and immunoprecipitated Ebp1. Immunoblotting revealed that TLS bound to Ebp1 in a time-dependent manner. The binding affinity correlated with Ebp1 sumoylation and S360 phosphorylation status (Figure 3B), indicating that DNA damage regulates the association between Ebp1 and TLS, and subsequently mediates Ebp1 sumoylation, for which Ebp1 S360 phosphorylation might be implicated. Moreover, Ebp1 antibody but not control IgG selectively precipitated TLS from K562 cells in the absence of stimulation, underscoring endogenous TLS and Ebp1 form a tight complex (Figure 3C). We made similar observations in AU565 cells (data not shown). To investigate the role of Ebp1 phosphorylation in its interaction with TLS, we conducted coimmunoprecipitation assay with p42 S360D, a phosphorylation mimetic mutant, and S360A, an unphosphorylable mutant. As expected, GST-p42 S360D exhibited a stronger binding affinity to TLS than wild-type p42, while p42 S360A failed to associate with TLS

(Figure 3D), supporting that p42 S360 phosphorylation is essential for the TLS/Ebp1 complex formation. Interestingly, p42 S360D revealed much stronger sumoylation activity than wild-type p42 in His-sumo1 stably transfected F293 cells. Cotransfection of TLS further enhanced the effect. In contrast, p42 S360A was barely sumoylated regardless of TLS expression (Figure 3E). Thus, these observations support that p42 phosphorylation promotes its association with TLS and augments its sumoylation.

TLS E3 ligase directly binds to Ubc9 and is sumoylated

SUMO E3 ligases, like RING domain ubiquitin E3s, do not possess intrinsic enzymatic activity, but act as adapters, which bring together the E2 (Ubc9) and the substrate (Hochstrasser, 2001; Jackson, 2001). To explore whether TLS E3 ligase indeed interacts with Ubc9, we conducted a GST-pull down assay. The N-terminus of TLS but not its C-terminus interacted with Ubc9 (Figure 4A), fitting with the in vitro sumoylation results (Figure 4A). TLS also specifically interacted with Ubc9 in the cotransfected HEK293 cells (Figure 4B). To test whether endogenous Ubc9 interacts with TLS, we treated various cells with genotoxic agents and immunoprecipitated Ubc9. Actinomycin D treatment increased the association between Ubc9 and TLS (Figure 4C). We made the similar observation with Adriamycin (data not shown). Combined with the finding that Ebp1 also coprecipitated with TLS (Figure 3), these data demonstrate that these three proteins (Ebp1/TLS/Ubc9) might form a complex upon genotoxic stress. Coexpression of SUMO-1 with Flag-TLS resulted in the presence of a slower migrating Flag-reactive band, indicating that TLS may be sumoylated (Figure 4D, left lower panel). Immunoprecipitation with anti-TLS displayed an evidently slower migrating band, suggesting the conjugation of SUMO to TLS (Figure 4D, upper left panel). Coimmunoprecipitation study demonstrated that endogenous SUMO1 also associated with TLS in intact cells (Figure 4D, right panel). Hence, TLS directly binds to the E2 conjugation enzyme Ubc9 and SUMO1, and itself is also sumoylated as well.

Ebp1 K93 and K298 residues are sumoylation residues

SUMOylation takes place on lysine residues that are usually embedded within the core consensus motif Ψ-Lys-X-Glu (where Ψ is a hydrophobic amino acid, and X is any amino acid) (Rodriguez *et al.*, 2001). In exploring the sequence of Ebp1, we noticed that amino acids 64–67, FKKE; 92–95, LKSD; 106–109, VKID and 297–300, AKHE correspond to a motif that is identified as a consensus sumoylation element present in numerous SUMO substrates. To assess the potential sumoylation sites, we cotransfected GFP-sumo1 into HEK293 cells with a variety of Myc-Ebp1 constructs with K mutated into R. Co-immunoprecipitation assay demonstrated that p42 but not p48 was potently sumoylated, and K93R or K298R mutation disrupted Ebp1 sumoylation (Figure 5A). Hence, K93 and K298 residues are major sumoylation sites on Ebp1 p42. To confirm these two residues are indeed the major sumoylation sites, we cotransfected Flag-TLS into F293 cells with various p42 constructs. As expected, wild-type p42 was readily sumoylated, and TLS overexpression markedly increased p42 sumoylation. By contrast, none of K93R, K298R and (K93,298R) mutated proteins was sumoylated even in the presence of TLS (Figure 5B). These results support that K93 and K298 are the major sumoylation sites on Ebp1, and abolishing one residue impairs the other site sumoylation.

P42 predominantly occurs in the cytoplasm, while p48 resides in both the cytoplasm and the nucleolus (Liu *et al.*, 2006). Sumoylation frequently triggers protein nuclear translocation. To explore whether p42 sumoylation is required for its nucleolar translocation, we cotransfected GFP-Sumo1 into HEK293 cells with Myc-p42 wild-type and unsumoylated mutant (K93, 298R), respectively. Immunofluorescent staining revealed that Myc-p42 wild-type and unsumoylated mutant alone mainly resided in the cytoplasm, and cotransfection with Sumo1 provoked wild-type p42 evident nucleolar translocation, whereas unsumoylated mutants predominantly localized in the cytoplasm (Figure 5C and E, left panels). Cotransfection of GFP-Sumo1 elicits p42 nucleolar residency only in a portion of cotransfected HEK293 cells. Sumo fusion protein system has been employed for effective production of native proteins (Lee *et al.*, 2008). It has also been utilized to study sumoylation substrates' biological functions. For instance, Fusion of Sumo1 to the C-terminus but not N-terminus of c-Fos leads to dramatic stabilization of c-Fos and reduction of its transcriptional activity (Bossis *et al.*, 2005). We found that GFP-sumo-p42 recombinant protein exclusively localized in the nuclei of all transfected cells and displayed different subnuclear patterns. Interestingly, some of the transfected GFP-Sumo1-Ebp1 accumulated in the nucleolus, partially colocalizing with Arf (Figure 5D and F), supporting that sumoylation of Ebp1 triggers its nucleolar translocation. The data obtained with artificial fusion protein must be interpret with caution, because GFP-Sumo-p42 is localized into some nuclear aggregates that might be just artifacts, due to sumo interaction with other proteins. The nucleolar residency of Ebp1 p42 wild-type but not unsumoylated mutants was further confirmed by immunostaining with nucleolar markers: NPM/B23 and Arf, in Myc-p42 and GFP-p42 transfected HEK293 cells (Supplemental Figure 1). Unsumoylated mutants were unable to resident in the nucleolus, underscoring that sumoylation is required for p42 nucleolar localization. Moreover, VP16 also provoked wild-type GFP-p42 nucleolar translocation; by contrast, unphosphorylated mutant GFP-p42 S360A remained in the cytoplasm (Figure 5G), indicating VP16-provoked phosphorylation in p42 is required for its nucleolar translocation. Thus, sumoylation is sufficient and necessary for p42 nucleolar translocation.

Ebp1 sumoylation is required for its anti-proliferative activity

Previous studies show that sumoylation might enhance substrate stability. For instance, Sumoylation increases HIF-1α stability and its transcriptional activity (Bae *et al.*, 2004); Sumoylation of Oct4 enhances its stability, DNA binding, and transactivation (Wei *et al.*, 2007). To explore whether p42 sumoylation also affects its stability, we cotransfected Flag-Sumo1 into HEK293 cells with GST-p42 wild-type or unsumoylated mutant (K93, 298R). The transfected cells were treated with protein translation inhibitor cycloheximide for various time points. In the absence of exogenous SUMO1, wild-type p42 half-life was less than 6 h, cotransfection of SUMO1 substantially increased its stability. By contrast, (K93,298R) was almost completely degraded at 6 h regardless of SUMO1 (Figure 6A, left panels), supporting that sumoylation significantly stabilizes p42. NPM/B23 strongly binds Ebp1 (Okada *et al.*, 2007). Depletion of NPM/B23 significantly decreased its half-life even in the presence of Sumo1 (Figure 6A, right panels), supporting that NPM/B23 might stabilize Ebp1 p42. To test whether sumoylation accelerates p42's growth suppressive activity, we transfected human cancer cells with various constructs and conducted a Brdu

incorporation assay. GFP-p42 overexpression resulted in significant cell proliferation decrease, whereas cell proliferation rate remained similar for both GFP control and GFP-Sumo1. Cotransfection of both Sumo1 and p42 further suppressed cell proliferation, which was comparable to the repressive effect by the recombinant GFP-Sumo1-p42 protein (Figure 6B), supporting sumoylation of p42 stimulates its growth suppressive activity.

Ebp1 overexpression inhibits cancer cell proliferation (Yu *et al.*, 2007; Zhang *et al.*, 2005). Ebp1 has been shown to repress various cell cycle-related gene transcription including cyclin E1 and E2F1. To examine the effect of sumoylation on Ebp1's transcriptional suppressive activity, we cotransfected E2F1 luciferase plasmid into HEK293 cells with Ebp1 p42 and (K93,298R) mutant constructs. Compared with control vector, ectopic expression of p42 decreased luciferase activity by approximately 40%, fitting with previous observations that overexpression of p42 suppresses cell proliferation. Cotransfection of p42 with either TLS or SUMO1 further enhanced p42's repressive effect, and the maximal inhibitory effect occurred in TLS, SUMO1 and p42 cotransfected cells. By contrast, transfection of SUMO1 alone or cotransfection of TLS and SUMO1 slightly increased E2F-1 transcription activity (Figure 6C, left panel). Nevertheless, unsumoylated p42 (K93,298R) was unable to suppress the luciferase activity. TLS and SUMO1 lost their stimulatory effects, suggesting that Ebp1 sumoylation is required for its transcription suppressive activity (Figure 6C, right panel). To further evaluate the effect of sumoylation on p42's cell growth inhibitory activity, we conducted a colony formation assay with AU565 cells that were stably transfected with wild-type p42 and various unsumoylated mutants. Compared with control, p42 suppressed cancer cell growth. However, K298R mutant failed to inhibit colony formation. Strikingly, p42 K93R and (K93, 298R) mutants markedly enhanced cell proliferation (Figure 6D, left panel). Consequently, knocking down of endogenous TLS but not CtBP2, a nuclear transcription corepressor, selectively increased Brdu incorporation and colony formation (Figure 6D, right panel). These data indicate that abolishing p42 sumoylation somehow facilitates cell proliferation. Together, these results support the notion that sumoylation is required for the anti-proliferative activity of Ebp1 p42.

Discussion

In this report, we identify that TLS/FUS acts as a SUMO E3 ligase for Ebp1 p42. TLS promotes p42 sumoylation at both K93 and K298 residues. Moreover, p42 sumoylation provokes its nucleolar translocation and is required for its stability and repressive activity on E2F-1 transcription factor. Abolishing p42 sumoylation cripples its anti-proliferative activity.

TLS interacts directly with Ubc9, Sumo1 and its substrate Ebp1 p42, and promotes SUMO1 modification of p42, which fulfill all the criteria applicable for an Ebp1 p42 SUMO E3 ligase. Thus, TLS might form a multiple protein complex containing sumoylation machinery. Proteomic analysis identified 498 proteins in the purified nucleolar compartment, and TLS is one of them (Andersen *et al.*, 2005). TLS is localized primarily in the nucleus, where it may be involved in pre-mRNA processing and nucleocytoplasmic shuttling, as well as in the regulation of basal transcription (Ron, 1997; Zinszner *et al.*,

1997a). TLS has been proposed to act as a proto-oncogene through chromosomal translocation. TLS's N-terminal part fuses with CHOP in myxoid liposarcoma carrying the t(12;16) translocation (Crozat *et al.*, 1993; Rabbitts *et al.*, 1993), and in different types of human myeloid leukemia in which the C-terminus of TLS is replaced by the DNA-binding domain of ERG (Panagopoulos *et al.*, 1995; Shimizu *et al.*, 1993). BCR/ABL induces increased expression of TLS by preventing its proteasome-dependent degradation (Perrotti *et al.*, 2000). TLS deficiency in mice results in defective B-lymphocyte development and activation, high levels of chromosomal instability and perinatal death (Hicks *et al.*, 2000). It has reported that TLS (−/−) mice display male sterility and enhanced radiation sensitivity (Kuroda *et al.*, 2000). All these characteristics resemble another nucleolar protein NPM/B23, which is a ubiquitously expressed nucleolar phosphoprotein that shuttles continuously between the nucleus and the cytoplasm. NPM is translocated or mutated in various lymphomas and leukemias, forming fusion proteins (NPM-ALK, NPM-RARalpha, NPM-MLF1) (Falini *et al.*, 2007). Nonetheless, NPM/B23's role in oncogenesis is controversial as NPM/B23 has been attributed with both oncogenic and tumor suppressive functions. NPM/B23 is essential for embryonic development and the maintenance of genomic stability (Grisendi *et al.*, 2005). Our previous study reveals that NPM/B23 binds Ebp1 p42 in the nucleolus, which is regulated by EGF. P42 S360D, a phosphorylation mimetic mutant strongly binds NPM/B23 (Okada *et al.*, 2007). Interestingly, p42 also strongly binds TLS, which is regulated by DNA damage stress, and Ebp1 phosphorylation increases the interaction (Figure 3). Consistently, immunofluorescent staining with p-Ebp1 S360 antibody reveals stronger colocalization by Ebp1 and TLS in the nucleus (Supplemental Figure 2). NPM/B23 is sumoylated on K263 residue, which is essential for NPM/B23 nucleolar residency (Liu *et al.*, 2007). Interestingly, TLS itself is also sumoylated (Figure 4). Conceivably, TLS sumoylation might mediate its nucleolar residency. TLS promotes p42 sumoylation and augments its anti-proliferative activity (Figure 6), indicating that TLS might possess tumor suppressive activity, although it has been proposed as a proto-oncogene due to its N-terminal fused hybrid proteins possess robust oncogenic effects. Nevertheless, whether wild-type TLS itself possesses any oncogenic activity remains unknown. However, it is worth noting that knocking down of TLS induces BrdU incorporation (Figure 6D), and this effect might not be exclusively attributed by Ebp1 p42. Nonetheless, p42 suppresses cell proliferation and TLS-promoted sumoylation further enhances its repressive effect. Moreover, knocking down TLS diminishes p42 sumoylation. Together, these data indicate that TLS siRNA-induced Brdu incorporation might be, at least in part, regulated by Ebp1 p42.

In current study, we have provided compelling evidence supports that TLS acts as a physiological E3 sumo ligase for p42. Firstly, TLS binds sumoylation machinery including both SUMO1 and Ubc9 and substrate p42. Secondly, overexpression of TLS substantially enhances p42 sumoylation in intact cells and depletion of TLS by siRNA abrogates p42 sumoylation. Thirdly, in vitro sumoylation assay reveals that TLS strongly promotes Ebp1 p42 sumoylation, for which its N-terminus, that binds Ubc9, plays an essential role. Hence, our finding establishes that TLS is a new member of growing list of SUMO E3 ligases.

SUMOylation regulates protein-protein interactions, subcellular localization, and stability (Hay, 2001; Muller *et al.*, 2001). Here we show that p42 Ebp1 sumoylation mediates its association with physiological binding partners. We found that p42 K93R and K298R single mutant decreased its binding affinity to TLS, and the double mutant K93,298R failed to bind TLS (Supplemental Figure 3). Hence, p42 sumoylation is critical for its association with TLS. Moreover, we also show that sumoylation is essential for its stability. Unsumoylated mutant displayed shorter half-life than wild-type protein (Figure 6A). Many proteins that are important for regulating gene expression including promoter-specific transcription factors, cofactors and chromatin-modifying enzymes have been found to be reversibly modified by SUMO. In most cases, SUMOylation of transcriptional regulators correlates with inhibition of transcription (Gill, 2005). Our data support that sumoylation of p42 augments its repressive effect on E2F-1 transcriptional activity, elevating its anti-proliferative activity. On the other hand, abrogating p42 sumoylation cripples its anti-proliferative activity, leading to upregulation of cancer cell growth and colony formation (Figure 6).

Ebp1 binds NPM/B23 (Okada *et al.*, 2007). Here, we show that Ebp1 p42K298R mutant fails to bind NPM/B23, whereas p42 K93R strongly binds NPM/B23 (Supplemental Figure 4A), suggesting that K298 residue plays a critical role in mediating the interaction between p42 and NPM/B23, for which p42 sumoylation might not be essential, because neither K93R nor K298R is sumoylated (Figure 5A). Ebp1 directly interacts with pRB. Overexpression of Ebp1 in MCF-7 and AU565 (Rb(+)) cells inhibits the activity of the E2F-1 (Xia *et al.*, 2001). Our previous study reveals that wild-type NPM/B23 but not unsumoylated NPM/B23 (K263R) binds Rb. Sumoylated NPM/B23 exhibits robust affinity to pRb (Liu *et al.*, 2007). We found that wild-type p42 upregulates NPM/B23 binding to pRb; in contrast, p42 K298R mutant blocks NPM/B23 binding to pRb (Supplemental Figure 4B), indicating p42/B23 interaction is indispensable for NPM/B23 interaction with pRb. It has been reported before that DNA-damaging agents, actinomycin D and ultraviolet radiation (UV), induces the dephosphorylation of pRB at Cdk phosphorylation sites and upregulates the binding of pRB to E2F-1, resulting in repression of E2F-1-mediated transcription (Inoue *et al.*, 2007). Noticeably, genotoxic stress strongly upregulates p42 sumoylation (Figure 1D). Conceivably, DNA damage activates PKC-δ, which phosphorylates p42 and provokes its sumoylation via TLS. The sumoylated p42, in turn, associates with pRB, blocking E2F-1 transcription activity. Collectively, our finding that TLS promotes Ebp1 sumoylation provides a molecular mechanism for post-translational modification mediating Ebp1 transcriptional repressive activity.

Materials and Methods

Cells and Reagents

His-Sumo stably transfected F293 cells were grown in complete medium containing 1 x Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum containing 10 μg/ml of Blasticidin and 100 μg/ml of Hygromycin in a humidified incubator at 37 °C, 5% CO2. Expression of the His-SUMO proteins was induced by adding tetracycline (Tet) to the culture medium at a final concentration of 1 μg/ml (a generous gift from Dr. Van G. Wilson, Texas A&M University). Anti-FLAG monoclonal antibody (M2),

anti-HA–HRP, anti-Myc and anti-GST-HRP monoclonal antibodies were from Sigma; Anti-GFP monoclonal antibody was from Roche. Anti-Ebp1 antibody was from Upstate Technology. Anti-Ebp1 S360 has been described previously (Ahn *et al.*, 2006). GFP-Sumo1-Ebp1 construct was prepared by three steps PCR amplification: 1) Sumo 1 was amplified using following two primers: a: HindIII Sumo1N 5′ ACTCAAGCTTC ATG TCT GAC CAG GAG GCA AA A CC 3′; b: Sumo1Ebp1R 5′ C TGT TTC TTC CAT AAT CAT AAC TGT TGA ATG ACC CCC 3′. 2) Ebp1 was amplified using following two primers: c: Sumo1Ebp1 F 5′ GGG GGT CAT TCA ACA GTT ATG ATT ATG GAA GAA ACA G 3′. d: Kpn1Ebp1 5′ ACTCGGTACC TCA GTC CCC AGC TTC ATT TTC 3′. 3) The PCR product from above two PCR reactions were mixed and amplified with primers a and d. The resulting fused PCR product was subcloned into p-EGFPC2 vector between HindIII/KpnI sites. All chemicals were from Sigma.

Preparation and Transfection of siRNA—The siRNA of TLS was purchased from Dharmacon (catelog # M180733-00); The siRNAs were transfected using Lipofectamine 2000 reagent. The sequence for shRNA of NPM/B23 is: 5′-GAT CCC G**AG GAA GAT GCA GAG TCA GAA GAT GAA GA**G AAG CTT GTC TTC ATC TTC TGA CTC TGC ATC TTC CTC TTT TTT-3′. The shRNA subcloned into pGE-1 between BamH I/Xba I, which was then cleaved by XhoI/Xba I and ligated into pAdTrack-CMV for preparing adenovirus.

Co-immunoprecipitation and Nickel column pull down

Ten-cm dishes of HEK293 cells were cotransfected with 5 μg of various Myc-p42 constructs and GFP-sumo1 by Nova Factor. In 24 h, the transfected cells were washed once in PBS, was lysed in 1 ml lysis buffer (50 mM Tris, pH 7.4, 40 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 1.5 mM Na_3VO_4, 50 mM NaF, 10 mM sodium pyrophosphate, 10 mM sodium β-glycerophosphate, 1 mM phenylmethylsulfonyl flouride (PMSF), 5 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A), and was centrifuged for 10 min at 14,000 × g at 4°C. After normalizing the protein concentration, 2 μl anti-myc antibody and 40 μl 50% slurry protein A/G agarose were added to the supernatant and incubated with rotation at 4°C for 2 hr. The agarose pellet was washed three times with 1000 μl lysis buffer each time. The agarose then was resuspended in 30 μl sample buffer separated by SDS-PAGE followed by immunoblotting using anti-GFP antibody. F293 cells were transfected with Myc-42 and induced with tetracycline for 24 h. His-tagged proteins were pulled down with Nickel column. After extensive washing with the above lysis buffer without EDTA, the coprecipitated proteins were analyzed by immunoblotting with anti-Myc antibody.

Recombinant protein purification and in vitro SUMOylation assay—Recombinant GST-p42 and His-SUMO1 proteins were expressed in E coli (BL21). GST-p42 proteins were purified with glutathione-sepharose beads (Amersham), and His-Sumo1 was purified with Ni2+ column and were dialysis against PBS containing 10% glycerol overnight at 4 °C. AOS1 and Ubc9 was from BIOMOL. All the purified recombinant proteins (TLS and its fragments from mammalian HEK293 cells) were dialysed against PBS containing 10% glycerol. An in vitro SUMOylation analysis of GST-p42 was carried out with the SUMOylation kit (BIOMOL, Plymouth Meeting, PA).

Immunofluorescence microscopy

Cells were fixed with either methanol at −20°C for 20 min or 10% formalin at room temperature for 20 min. The fixed cells were blocked by 10% normal goat serum in PBS for 1 h, and incubated with primary antibodies for 1 h. Cells were then incubated with secondary antibodies (Alexa Fluor 594-tagged goat anti-mouse IgG, Alexa Fluor 488-tagged goat anti-rabbit IgG or Alexa Fluor 594-tagged goat anti-mouse IgG antibodies, Molecular Probe) for 1 h, and counterstained for DNA with 4′, 6-diamidino-2-phenylindole (DAPI). After incubation with antibodies, cells were washed extensively in PBS. Cells were examined under a fluorescence microscope.

Reporter assays

The E2F-1 promoter-luciferase reporter construct contains a luciferase reporter gene. Cells (1×10^5) in 6 well plates were transfected with 0.25 μg of reporter plasmid with various Ebp1 constructs at the indicated concentrations. Renilla-TK was included as an internal control. After transfection, the cells were placed in complete medium for 48 h. Control or treated cells were harvested to analyze luciferase activity as previously described (Xia *et al.*, 2001).

Colony inhibition assays

AU565 cells (1×10^4) were seeded into individual wells of 6 well plates. Ebp1 plasmid DNA (2 μg) was stably transfected using Fugene 6 (Boerhringer Mannheim). The number of colonies surviving after 3 weeks of G418 (500 μg/ml) selection was determined microscopically.

Brdu Incorporation assay

Cells were seeded into six-well plates at 1×10^5 cells/well, cultured overnight, and transfected with various siRNA. The transfected cells were pulse labeled with BrdU (10 μm; Amersham Biosciences). The incorporation was continued for 10 min. After washing with cold PBS, the cells were fixed with 4% paraformaldehyde for 15 min. and treated with 2 M HCl at 37 °C for 30 min. The coverslips were blocked with 2% FBS/PBS, and 0.4% Triton X-100 at room temperature for 10 min. For immunostaining, mouse monoclonal antibody against BrdU (1:200) and anti-GST-FITC-conjugated antibody were used.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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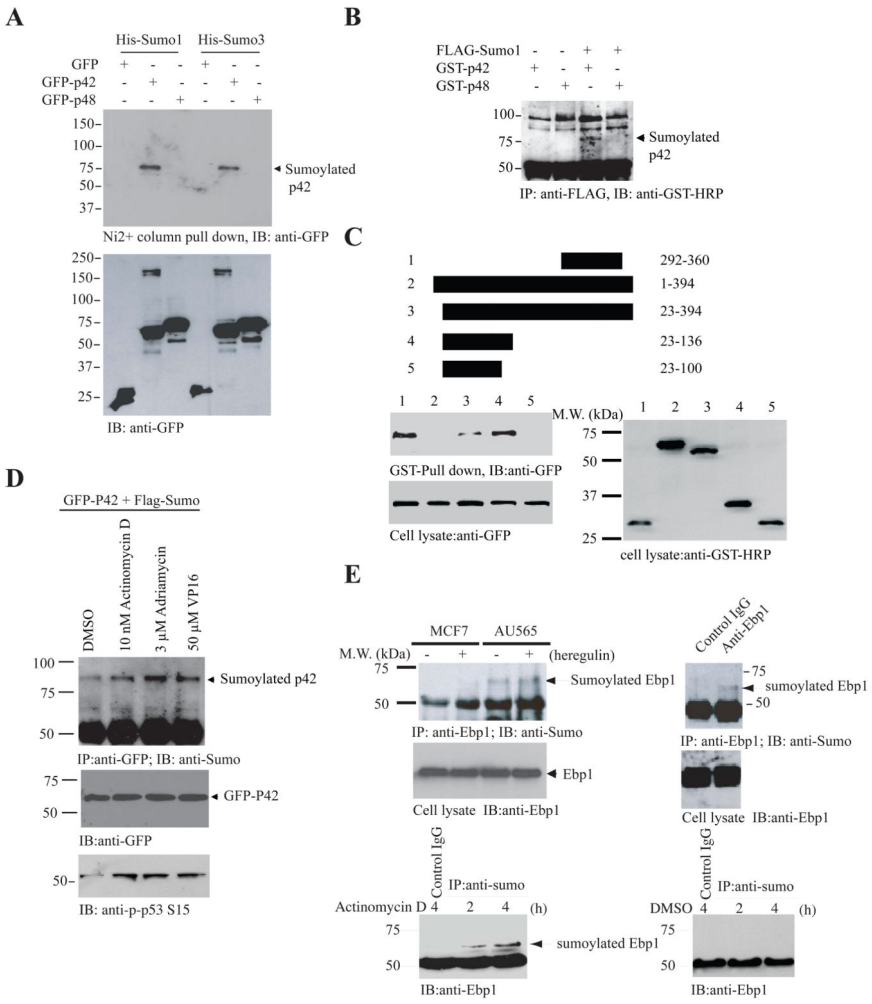

Figure 1. Ebp1 p42 but not p48 is sumoylated

(A) His-sumo1 or His-sumo3 cells were transfected with each 10 μg of GFP, GFP-p42 or GFP-p48. Total cell lysates (500μg) were pulled down with nickel resin. The beads were washed extensively and subjected to IB with anti-GFP antibody. (B) Ebp1 p42 but not p48 can be sumoylated. GST-tagged p42 and p48 were cotransfected with Flag-Sumo1 into HEK293 cells. Sumoylated proteins were immunoprecipitated with anti-Flag antibody and analyzed by immunoblotting with anti-GST-HRP (right panel). (C) Truncation of the N-terminus of p48 elicits the association between Ebp1 and Sumo. A variety of GST-Ebp1 fragments were cotransfected with GFP-Sumo1 into HEK293 cells. GST-recombinant proteins were pulled down with glutathione beads, and analyzed by immunoblotting with anti-GFP antibody. Diagram of different Ebp1 constructs (upper panel). C-terminal a.a. 292–360 and N-terminal 23–136 fragment strongly bound GFP-Sumo1 (lower left panels). Verification of transfected GST-fusion proteins (lower right panel). (D) DNA damage agents enhance p42 sumoylation. Flag-Sumo1 was cotransfected with 2 μg of GFP-p42 into HEK293 cells, followed by treatment with various DNA damage agents for 4 h. P42 was immunoprecipitated with anti-GFP, and analyzed by anti-sumo antibody (top panel). p53 phosphorylation by DNA damage agents was verified (bottom panel). (E) Heregulin triggers

Ebp1 sumoylation in AU565 breast cancer cells. MCF7 cells or AU565 cells were treated with Heregulin (10 ng/ml) for 30 min and then subjected to immunoprecipitation with anti-Ebp1 antibody and immunoblotted with anti-Sumo1 antibody (left top panel). Actinomycin D (10 nM) but not DMSO provoked Ebp1 sumoylation in AU565 cells (bottom panels).

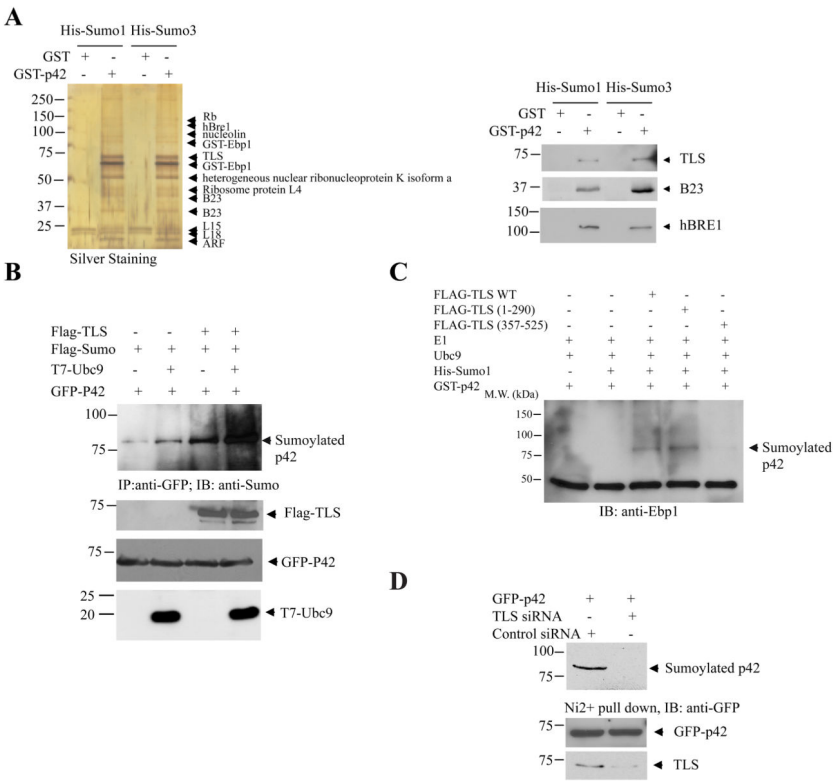

Figure 2. TLS is a Sumo E3 ligase for Ebp1 p42

(A) TLS associates the sumoylated p42. His-sumo1 or His-sumo3 cells were transfected with GST or GST-p42. Forty-eight h after transfection, cell lysates were pulled down with glutathione beads. The beads were washed extensively and eluted with 10 mM reduced glutathione. The eluted proteins were analyzed with silver staining or immunoblotting with the indicated antibodies. (B) TLS enhances p42 sumoylation. HEK293T cells were transfected with the indicated combinations. At 40 h post-transfection, cells were lysed and co-immunoprecipitated with anti-GFP antibody. The samples were subjected to IB with anti-sumo1 antibody. (C) TLS stimulates Ebp1 p42 sumoylation in vitro. A variety of purified recombinant proteins were incubated with Ebp1 p42 at 37°C for 2 h. The reaction mixture were resolved on SDS-PAGE and analyzed by immunoblotting with anti-Ebp1 antibody. (D) TLS knockdown impairs p42 sumoylation. HEK293T cells were transfected with 100μM of siRNAs directed against TLS or a scrambled siRNA as a negative control. At 24 h post-transfection, cells were seeded and transfected with GFP-p42 for additional 24 h, and then analyzed with nickel column and IB with anti-GFP antibody.

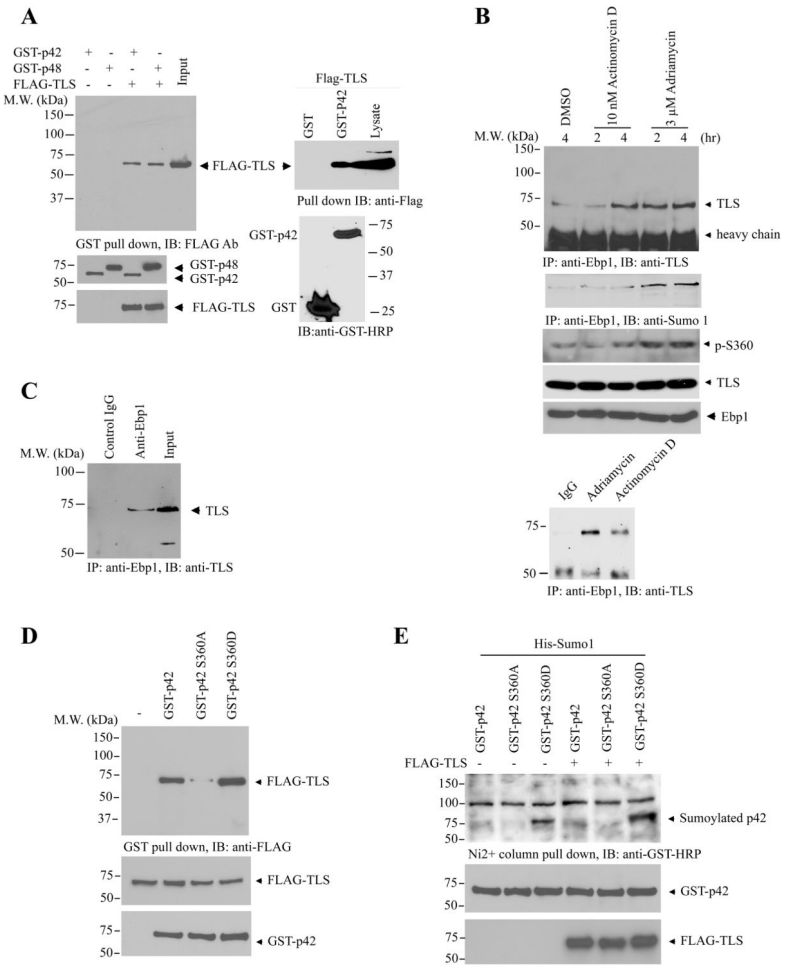

Figure 3. TLS interacts with Ebp1 and enhances its sumoylation

(A) P48 and p42 bind TLS. TLS 293T cells were transfected with FLAG-TLS and GST-p42 or GST-p48 expression vectors, and analyzed with glutathione pull down and immunoblotting as indicated. (B) Endogenous TLS interacts with Ebp1. Human leukemia K562 cells were treated with genotoxic agents at the indicated concentration for various time points. Endogenous Ebp1 was immunoprecipitated and the associated proteins were analyzed by IB with anti-TLS antibody (top panel). Ebp1 was markedly sumoylated and phosphorylated upon DNA damage drug treatment (2nd and 3rd panels). (C) Endogenous Ebp1 and TLS tightly bound to each other even in the absence of drug treatment. The cell lysates (1 mg) were subjected to immunoprecipitation with IgG or anti-Ebp1 antibodies. The immunoprecipitated samples were analyzed for anti-TLS antibody. (D) Phosphorylation of Ebp1 increases its binding affinity to TLS. Wild-type p42, S360A and S360D p42 mutants were cotransfected into HEK293 cells with Flag-TLS. Transfected GST-p42 proteins were pulled down with glutathione beads, and the coprecipitated proteins were analyzed by anti-Flag antibody (top panel). Expression of transfected constructs was verified (middle and bottom panels). (E) Phosphorylation upregulates p42 sumoylation. Wild-type p42, S360A and S360D p42 mutants were cotransfected into F293 cells with or without Flag-TLS. His-sumo modified proteins were pulled down with Ni2+ column, and the coprecipitated

proteins were analyzed by anti-GST-HRP antibody (top panel). Expression of transfected constructs was verified (middle and bottom panels).

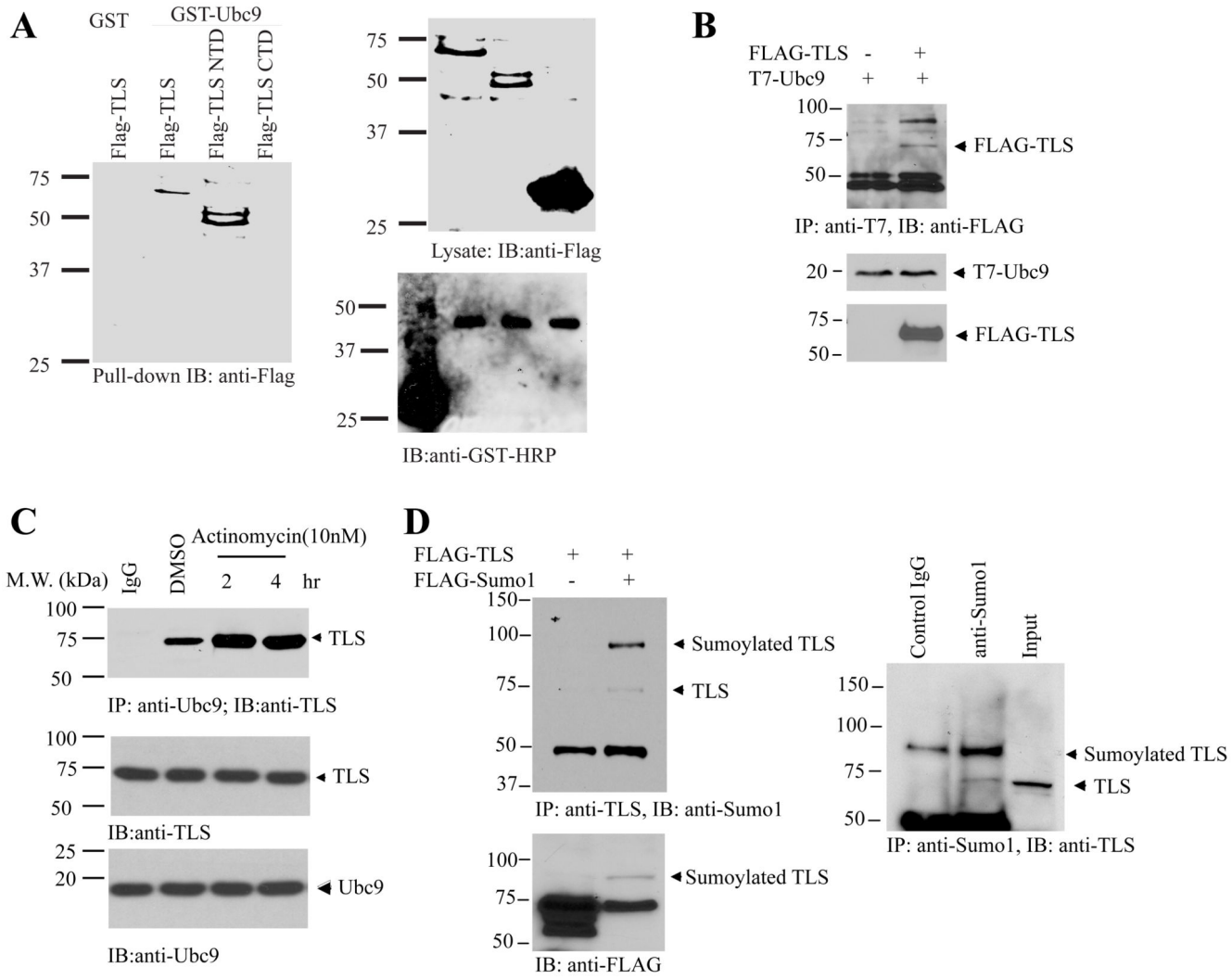

Figure 4. TLS and Ebp1 directly interact with Ubc9

(A) TLS N-terminus is required for its association with Ubc9. A variety of TLS fragments were cotransfected with GST-Ubc9 into HEK293 cells. Transfected Ubc9 was pulled down by glutathione beads and subjected to IB with anti-Flag antibody. (B) TLS interacts with Ubc9. HEK293T cells were transfected with 5 μg each of FLAG-TLS and T7-Ubc9, and total cell lysates were coimmunoprecipitated with anti-T7 antibody. The immunoprecipitated proteins were subjected to IB with anti-FLAG antibody. (C) Genotoxic stress upregulates the association between TLS and Ubc9. Human leukemic K562 cells were treated with indicated DNA damage agents for different time points. Endogenous Ubc9 was immunoprecipitated and analyzed by IB with anti-TLS antibody. (D) TLS is sumoylated. FLAG-sumo1 was coexpressed with FLAG-TLS. At 40 h post-transfection, cell lysates were immunoprecipitated with anti-TLS antibody and then, immunoblotted with anti-Sumo1 antibody (left panel). Endogenous TLS associates with sumo1. HEK293T cell lysates (1 mg) were subjected to immunoprecipitation with IgG or anti-Sumo1 antibody. The immunoprecipitated proteins were analyzed by anti-TLS antibody (right panel).

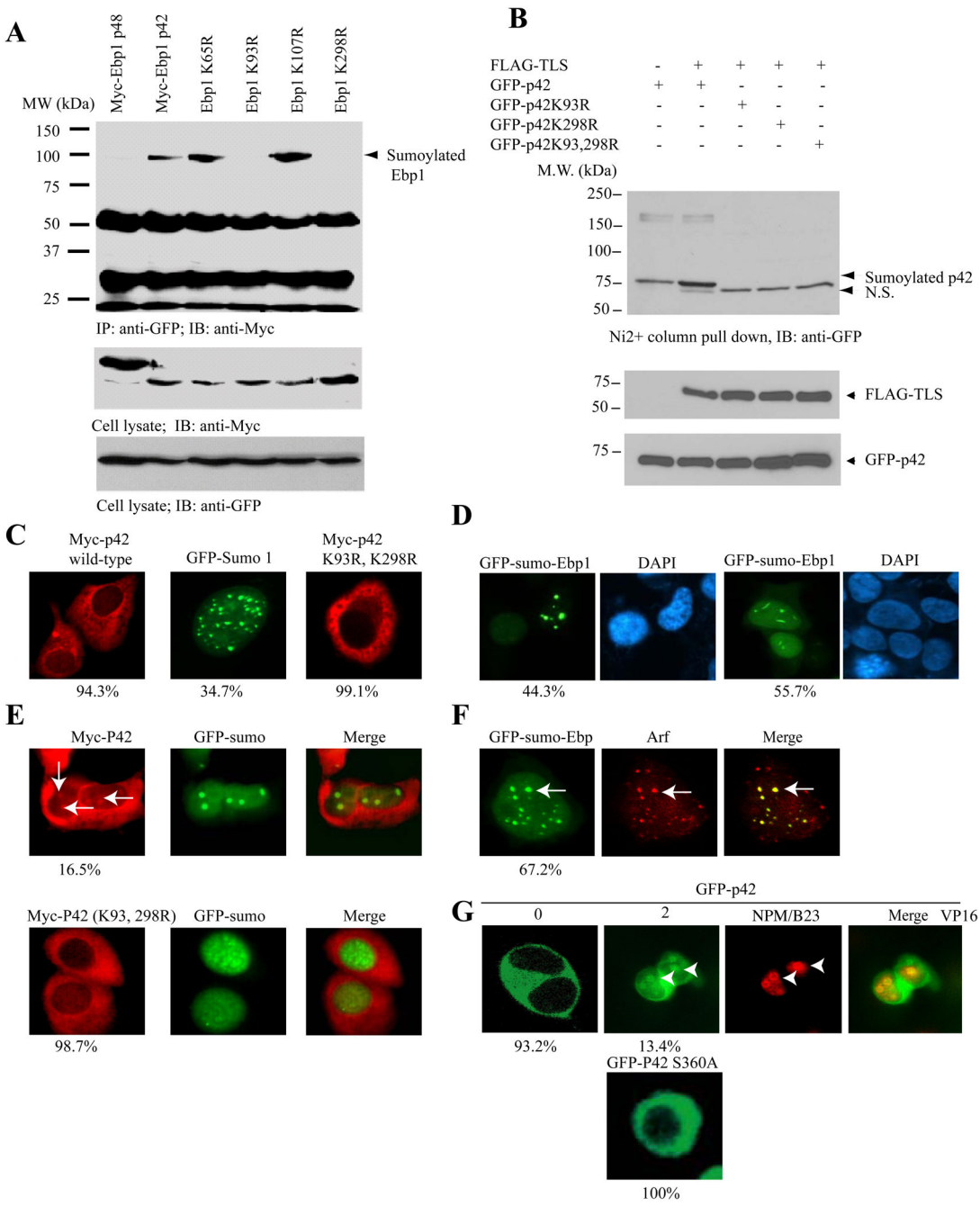

Figure 5. P42 is sumoylated at Lysine 93 and 298 residues, required for nucleolar residency

(A) Ebp1 p42 but not p48 isoform can be sumoylated. GFP-sumo1 was cotransfected into HEK293T cells with Myc-tagged various Ebp1 constructs. Sumoylated proteins were pulled down with anti-GFP antibody and analyzed with anti-myc antibody. P42 but not p48 was sumoylated. Either K93R or K298R mutation abolished p42 sumoylation (top panel). Verification of transfected constructs (middle and bottom panels). (B) TLS enhances p42 sumoylation. HEK293T cells were transfected with indicated various expression vectors. Forty h after transfection, cells were lysed and pulled down with nickel beads. The samples

were subjected to IB with anti-GFP antibody. Wild-type but not unsumoylated mutants were sumoylated in the presence of TLS (top panel). (C-F) Sumoylation is required for p42 nucleolar translocation. HEK293 cells were transfected with the indicated expression vectors, and fixed at 40 h after transfection. Both wild-type and p42 (K93,298R) mutant resided in the cytoplasm, and GFP-Sumo1 exclusively localized in the nucleus and aggregated in the subnuclear compartments (top left panel). Cotransfection of GFP-Sumo1 elicited wild-type p42 but not p42 (K93,298R) mutant nucleolar translocation (middle and bottom left panels). GFP-Sumo-Ebp1 fusion protein distributed in the nucleus and colocalized with Arf (right panels). The percentage of cells that present the different staining patterns are labeled underneath of each picture. (G) VP16 promotes GFP-p42 wild-type but not S360A nucleolar residency.

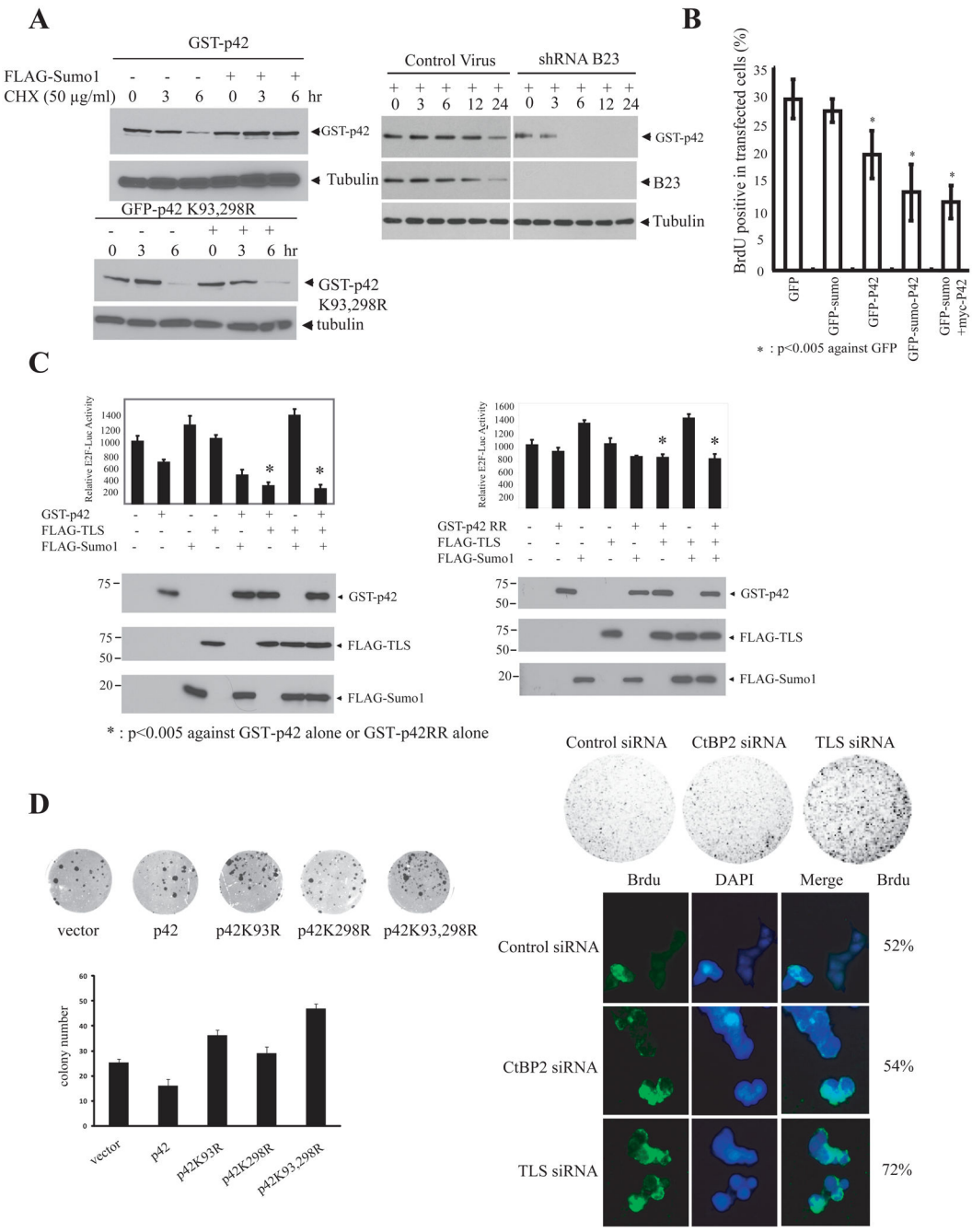

Figure 6. P42 sumoylation increases its stability and is required for its anti-proliferative activity

(A) Sumoylation is required for p42's stability. HEK293T cells were transfected with GST-p42 (upper left panel) or unsumoylated GFP-p42 (K93,298R) mutant (lower left panel) and FLAG-sumo1. Cycloheximide was added to block protein synthesis at various time points before harvesting the cells. Cell lysates were analyzed for immunoblotting with anti-GST-HRP antibody or anti-GFP antibody. Depletion of NPM/B23 decreases GST-P42 half-life in the presence of exogenous Sumo1 (right panels). (B) Sumoylation enhances p42 anti-proliferative activity. HEK293 cells were transfected with various constructs and the

transfected cells were fed with Brdu. Immunostaining showed that cotransfection of p42 and GFP-Sumo1 substantially decreased cell proliferation rate, an effect similar to GFP-Sumo1-p42 recombinant protein overexpression. (left panel) Quantitative analysis of S phase in FACS of GFP-Sumo1 and p42 cotransfected cells (right panel). (C) Sumoylation enhances p42's transcriptional suppressive activity. AU565 cells were transfected with the indicated vectors together with E2F-luciferase reporter gene. 40 h after transfection, cells were lysed and analyzed for luciferase activity. Wild-type p42 repressed E2F-1 transcription activity, and cotransfection of either TLS or Sumo1 further increased its suppressive effect. The maximal effect occurred, when both TLS and Sumo1 were cotransfected (left panel). Unsumoylated p42 lost its repressive effect on E2F-1 (right panel). Data are the mean ± SE for three independent experiments in duplicates. Significant differences were evaluated using Student's *t* test (* $p < 0.01$). (D) Colony formation assay. AU565 cells (1×10^4) were seeded into individual wells of 6 well plates. Various p42 and its sumoylation mutants were transfected into the cells. The number of colonies surviving after 3 weeks of G418 (500 mg/ml) selection was quantified (left panel). Knocking down of TLS increased colony formation and Brdu incorporation assay (right panel). Data are the representative for three independent experiments in duplicates.