Interplay between SUMOylation and NEDDylation regulates RPL11 localization and function

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ABSTRACT: The ribosomal protein L11 (RPL11) integrates different types of stress into a p53-mediated response. Here, we analyzed the impact of the ubiquitin-like protein SUMO on the RPL11-mouse double-minute 2 homolog-p53 signaling. We show that small ubiquitin-related modifier (SUMO)1 and SUMO2 covalently modify RPL11. We find that SUMO negatively modulates the conjugation of the ubiquitin-like protein neural precursor cell-expressed developmentally downregulated 8 (NEDD8) to RPL11 and promotes the translocation of the RP outside of the nucleoli. Moreover, the SUMO-conjugating enzyme, Ubc9, is required for RPL11-mediated activation of p53. SUMOylation of RPL11 is triggered by ribosomal stress, as well as by alternate reading frame protein upregulation. Collectively, our data identify SUMO protein conjugation to RPL11 as a new regulator of the p53-mediated cellular response to different types of stress and reveal a previously unknown SUMO-NEDD8 interplay.—El Motiam, A., Vidal, S., de la Cruz-Herrera, C. F., Da Silva-Álvarez, S., Baz-Martínez, M., Seoane, R., Vidal, A., Rodríguez, M. S., Xirodimas, D. P., Carvalho, A. S., Beck, H. C., Matthiesen, R., Collado, M., Rivas, C. Interplay between SUMOylation and NEDDylation regulates RPL11 localization and function. FASEB J. 33, 643–651 (2019). www.fasebj.org

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The mouse double-minute 2 homolog (Mdm2)-p53 pathway plays an essential role in coordinating cellular responses to stress. Under normal physiologic conditions, p53 is maintained at low levels by its interaction with the E3 ubiquitin ligase Mdm2 (1–5). Upon a cellular insult, Mdm2 is inhibited, leading to p53 stabilization and activation. Several mechanisms have been shown to regulate the activity and levels of Mdm2. One of the Mdm2 regulators is the ribosomal protein L11 (RPL11). In response to different types of stress, RPL11 is released from the nucleoli to the nucleoplasm, where it interacts with Mdm2, inhibits its ubiquitin ligase activity, and induces the upregulation and activation of p53. Therefore, RPL11 is generally considered as a key player in coordinating the p53 response to nucleolar stress, and it has been proposed as one of the factors involved in activating p53 under oncogenic and replicative stresses (6–11). Several proteins have been reported to influence the RPL11–Mdm2 interaction. The covalent modification of RPL11 with the ubiquitin-like protein neural precursor cell-expressed developmentally downregulated 8 (NEDD8) promotes its nucleolar localization and controls the signaling of RPL11 to p53 in response to ribosomal stress (12). In contrast, the

ABBREVIATIONS: ARF, alternative reading frame; CHX, cycloheximide; GA, ginkgolic acid; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GFP, green fluorescent protein; GFP-F, farnesylated green fluorescent protein; HA, hemagglutinin; HEK-293, human embryonic kidney 293; His6, histidine 6; MCF7, Michigan Cancer Foundation 7; Mdm2, mouse double-minute 2 homolog; NEDD8, neural precursor cell-expressed developmentally downregulated 8; RPL11, ribosomal protein L11; SENP1, sentrin-specific protease 1; siC, scramble small interfering RNA; siUbc9, Ubc9 small interfering RNA; SUMO, small ubiquitin-related modifier; WCL, whole-cell lysate

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interaction of the tumor suppressor alternate reading frame (ARF) with RPL11, which is enhanced in response to either oncogenic or nucleolar stress, positively modulates the RPL11–Mdm2 binding, leading to the inhibition of p53 ubiquitination and to p53 activation (7). How the activities of the regulatory proteins are coordinated on RPL11 to regulate p53 is currently unknown. However, it is clear that post-translational modification of the RPL11-Mdm2-p53 pathway components plays an important role in such regulation.

Small ubiquitin-related modifier (SUMO)ylation is a post-translational modification with an important role in maintaining cells and tissues homeostasis. It consists in the covalent conjugation of SUMO to target proteins through a reversible and dynamic enzymatic process. This modification may impact different properties of proteins, but it mainly regulates protein–protein interactions (13). Mammals express several SUMO family members that show homology differences. SUMO2 and SUMO3 share 97% sequence identity, differing only by 3 amino-terminal residues, and show ~50% aa identity with SUMO1. SUMO2/3 can form SUMO chains, whereas SUMO1 lacks a SUMOylation motif. Moreover, SUMO2/3 is present in greater abundance than SUMO1 and is predominantly involved in cell stress responses, including oxidative stress, osmotic stress, heat shock, or virus infection (14–20).

Analysis of the human SUMO proteome has recently revealed RPL11 as a SUMO target protein (21). Here, we evaluated whether SUMO conjugation to RPL11 can regulate the Mdm2-p53 pathway. Our results show that RPL11 can be modified by SUMO1 and SUMO2 *in vitro* and *in vivo*. SUMO negatively regulates the conjugation of NEDD8 to RPL11 and favors the release of the RP outside of the nucleoli, and its conjugating enzyme Ubc9 is required for the RPL11-mediated activation of p53. RPL11 SUMOylation is promoted by ribosomal stress or after upregulation of the tumor suppressor p14ARF.

MATERIALS AND METHODS

Cell lines and reagents

Human embryonic kidney 293 (HEK-293), human p53-null lung nonsmall cell carcinoma H1299, and the p53-wild-type Michigan Cancer Foundation 7 (MCF7) breast cancer and U2OS osteosarcoma cells were cultured in complete medium (DMEM, supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin). The cells were transiently transfected using Xtreme-Gene siRNA (Roche, Barcelona, Spain) or PEI (Polysciences, Hirschberg an der Bergstrasse, Germany) transfection reagents, as suggested by the manufacturers. Actinomycin D, cycloheximide (CHX), and ginkgolic acid (GA) were from MilliporeSigma (Madrid, Spain). NEDDylation inhibitor MLN4924 was from MilliporeSigma. Smart-pool small interfering RNAs against Ubc9 (siUbc9) and scramble small interfering RNA (siC) were purchased from Dharmacon (Madrid, Spain).

Plasmids and antibodies

The myc-tagged RPL11 expression plasmid was previously described (11) (plasmid 20936; Addgene, Cambridge, MA, USA). Flag-RPL11 was previously described (12). Hemagglutinin (HA)-RPL11 was generated by PCR amplification of RPL11 and cloning into the pcMV5-HA vector. pcDNA3.1-SUMO1 plasmid was previously described (22). pcDNA-histidine 6 (His6)-SUMO1, pcDNA-His6-SUMO2, and pcDNA-SV5-Ubc9 plasmids were previously described (23, 24). pcDNA-His6-NEDD8 plasmid was provided by M.S.R. Anti-HA antibody was from Covance (Madrid, Spain). Anti-SUMO1 antibody was from Cell Signaling Technology (Leiden, The Netherlands). Anti-NEDD8 (Y297) was from Abcam (Cambridge, United Kingdom). Anti-p53, anti-Flag, and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were from Santa Cruz Biotechnology (Dallas, Texas, USA). Anti-green fluorescent protein (GFP) was from BioLegend (Barcelona, Spain). Antibodies against RPL11 were purchased from Thermo Fisher Scientific (Waltham, MA, USA) and Abcam. Anti-myc tag antibodies were purchased from Santa Cruz Biotechnology and Cell Signaling Technology. Antibodies against SUMO2 were from Thermo Fisher Scientific and Cell Signaling Technology.

In vitro SUMOylation assays

In vitro SUMO conjugation assays were performed on [^{35}S] methionine-labeled *in vitro*-transcribed/translated proteins, as previously described (25), using recombinant SUMO-activating enzyme E1 (Biomol, Lausen, Switzerland), Ubc9, and SUMO1 or SUMO2. The *in vitro* transcription/translation of proteins was performed by using 1 μg plasmid DNA and a rabbit reticulocyte-coupled transcription/translation system, according to the instructions provided by the manufacturer (Promega, Madrid, Spain).

In vitro deSUMOylation assay

In vitro deSUMOylation assay with recombinant glutathione *S*-transferase-sentrin-specific protease 1 (SENP1; Biomol) was performed on RPL11-SUMO1 or RPL11-SUMO2, as previously described (26).

Purification of His-tagged conjugates

The purification of His-tagged conjugates using Ni^{2+}-nitrilotriacetic acid-agarose beads was performed as previously described (27).

Immunofluorescence staining

Immunofluorescence staining and confocal analysis were performed as previously described (26). Both mouse anti-myc antibody and rabbit anti-SUMO2 antibody were used at a dilution of 1:200. Goat anti-mouse secondary antibody, Alexa Fluor 488-conjugated, and goat anti-rabbit secondary antibody, Alexa Fluor 594 conjugated were obtained from Thermo Fisher Scientific. Analysis of the samples was carried out on a Leica TCS SP5 confocal laser microscope using simultaneous scans to avoid shift between the optical channels. Images were prepared using Adobe Photoshop (Adobe Systems, San Jose, CA, USA).

Cell-cycle analysis

U2OS cells, previously transfected with siUbc9 or siC, were cotransfected with farnesylated GFP (GFP-F) and the empty vector pcDNA3 or myc-RPL11. Twenty-four hours after transfection, cells were fixed, permeabilized, and stained in a solution containing propidium iodide, RNase A, and Triton X-100. Cells were analyzed for DNA content using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) using FlowJo

software (FlowJo, Ashland, OR, USA). GFP-positive cells were gated for cell-cycle analysis.

Statistical analysis

Statistical analysis was performed using a Student's *t* test. The significance level chosen for the statistical analysis was $P < 0.05$.

RESULTS

RPL11 is modified by SUMO

To evaluate whether RPL11 can be modified by SUMO, we carried out *in vitro* SUMOylation assays using *in vitro*-translated [^{35}S]methionine-labeled RPL11 protein as a substrate. *In vitro*-translated myc-RPL11 protein was detected as a band of ~28 kDa MW, as expected. When the reaction was incubated with SUMO1, we detected at least an additional higher MW band of ~43 kDa (**Fig. 1*A***, left). When the reaction was incubated with SUMO2, we observed at least 2 additional higher MW bands: 1 of ~43 kDa and a fainter band of ~58 kDa (Fig. 1*A*, left). These results indicated that RPL11 is modified by SUMO1 and SUMO2 *in vitro*. To prove further that the bands correspond to RPL11-SUMO conjugates, we incubated RPL11-SUMO1 or RPL11-SUMO2 protein with the recombinant SUMO-specific protease SENP1. The high MW bands detected when the protein was incubated with SUMO1 or SUMO2 disappeared after incubation of the reactions with SENP1 (Fig. 1*A*, right). Similar results were observed when we carried out *in vitro* SUMOylation assays using *in vitro*-translated [^{35}S]methionine-labeled untagged RPL11 protein (Fig. 1*B*). Altogether, these results demonstrate that RPL11 is modified by SUMO1 and SUMO2 *in vitro*.

To determine SUMO conjugation of RPL11 *in vivo*, HEK-293 cells were cotransfected with myc-RPL11, together with the empty vector pcDNA3, Ubc9 and His6-SUMO1, or Ubc9 and His6-SUMO2. At 48 h after transfection, both whole-cell lysates (WCLs) and His-tagged proteins, purified under denaturing conditions using nickel beads, were analyzed by Western blot using anti-myc antibody. Western blot analysis of the purified extracts revealed bands of the expected size corresponding to RPL11-SUMO1 and RPL11-SUMO2 only in cells cotransfected with His6-SUMO1 and His6-SUMO2, respectively, indicating that transfected myc-RPL11 is modified by SUMO1 and SUMO2 in the cells (Fig. 1*C*). We also detected bands corresponding to SUMOylated RPL11 protein in the purified extracts obtained from HEK-293 cells cotransfected with Flag-RPL11 and His6-SUMO2, and from MCF7 or U2OS cells transfected with HA-RPL11 and His6-SUMO2 (Fig. 1*D*), indicating that RPL11 is SUMOylated in a tag- and cell type-independent manner.

SUMOylation of endogenous RPL11 protein was evaluated in HEK-293 cells transfected with empty vector pcDNA3, Ubc9 and His6-SUMO1, or Ubc9 and His6-SUMO2. Western blot analysis of the WCLs using anti-RPL11 antibody revealed a band of ~20 kDa (Fig. 1*E*). Purification of the His-tagged proteins under denaturing conditions and Western blot analysis using anti-RPL11 antibody revealed the presence of the bands corresponding to RPL11-SUMO1 and RPL11-SUMO2 only in cells transfected with His6-SUMO1 or His6-SUMO2, respectively, demonstrating that endogenous RPL11 protein is also modified by SUMO1 and SUMO2 (Fig. 1*E*). Collectively, these results demonstrate that RPL11 is modified by SUMO1 and SUMO2 *in vitro* and in cells.

Interplay between SUMO and NEDD8 conjugation to RPL11

RPL11 can be conjugated by the ubiquitin-like protein NEDD8, with a decrease in NEDDylation only after mutation of all lysine residues in RPL11 (12). Therefore, we speculated that SUMO might compete with NEDD8 for RPL11 conjugation. To evaluate this hypothesis, we first cotransfected HEK-293 cells with myc-RPL11, together with pcDNA3, His6-NEDD8 and pcDNA3, or His6-NEDD8 and untagged SUMO1. At 48 h after transfection, both WCLs and His-tagged proteins purified under denaturing conditions were analyzed by Western blotting with anti-myc antibody. Analysis of the purified extracts revealed the appearance of bands corresponding to RPL11-NEDD8 protein exclusively in cells transfected with His6-NEDD8 (**Fig. 2*A***). Interestingly, we observed a clear decrease in the levels of NEDDylated RPL11 protein in cells cotransfected with SUMO1 (Fig. 2*A* and Supplemental Fig. 1), indicating that upregulation of SUMO1 downmodulates the NEDDylation of RPL11.

We then cotransfected HEK-293 cells with myc-RPL11, together with pcDNA3; His6-NEDD8 and pcDNA3; Ubc9, His6-SUMO2, and pcDNA3; or Ubc9, His6-SUMO2, and His6-NEDD8, and 48 h after transfection, His-tagged proteins were purified under denaturing conditions. Western blot analysis of the purified proteins using anti-myc antibody revealed the appearance of bands corresponding to RPL11-SUMO2 and RPL11-NEDD8 in cells transfected with His6-SUMO2 or His6-NEDD8, respectively (Fig. 2*B*). When we cotransfected His6-SUMO2, together with His6-NEDD8, we consistently observed reduced levels of the bands corresponding with NEDDylated RPL11 protein (a reduction of ~50% in the intensity of the 40 kDa RPL11-NEDD8 band and of ~80% of the 50 kDa RPL11-NEDD8 band relative to that detected in cells transfected only with His6-NEDD8; Fig. 2*B*), indicating that SUMO2 negatively regulates the conjugation of RPL11 to NEDD8.

We decided next to study whether treatment with SUMOylation or NEDDylation inhibitors had an effect on the conjugation of RPL11 to NEDD8 or SUMO2. We first cotransfected HEK-293 cells with myc-RPL11, together with pcDNA3, His6-NEDD8, or Ubc9 and His6-SUMO2, and 36 h after transfection, cells were treated or not with the NEDDylation inhibitor MLN4924 for 4 h. Western blot analysis of the WCLs using anti-myc antibody revealed the appearance of NEDDylated-RPL11 bands in the cells transfected with NEDD8, whose intensity decreased after treatment with MLN4924, as previously reported (28) (Fig. 2*C*). In contrast, treatment of cells with MLN4929 induced a clear upregulation in the levels of RPL11-SUMO2 protein (Fig. 2*C*). Similar results were observed after Western blot analysis of the His-tagged proteins purified from HEK-293 cells

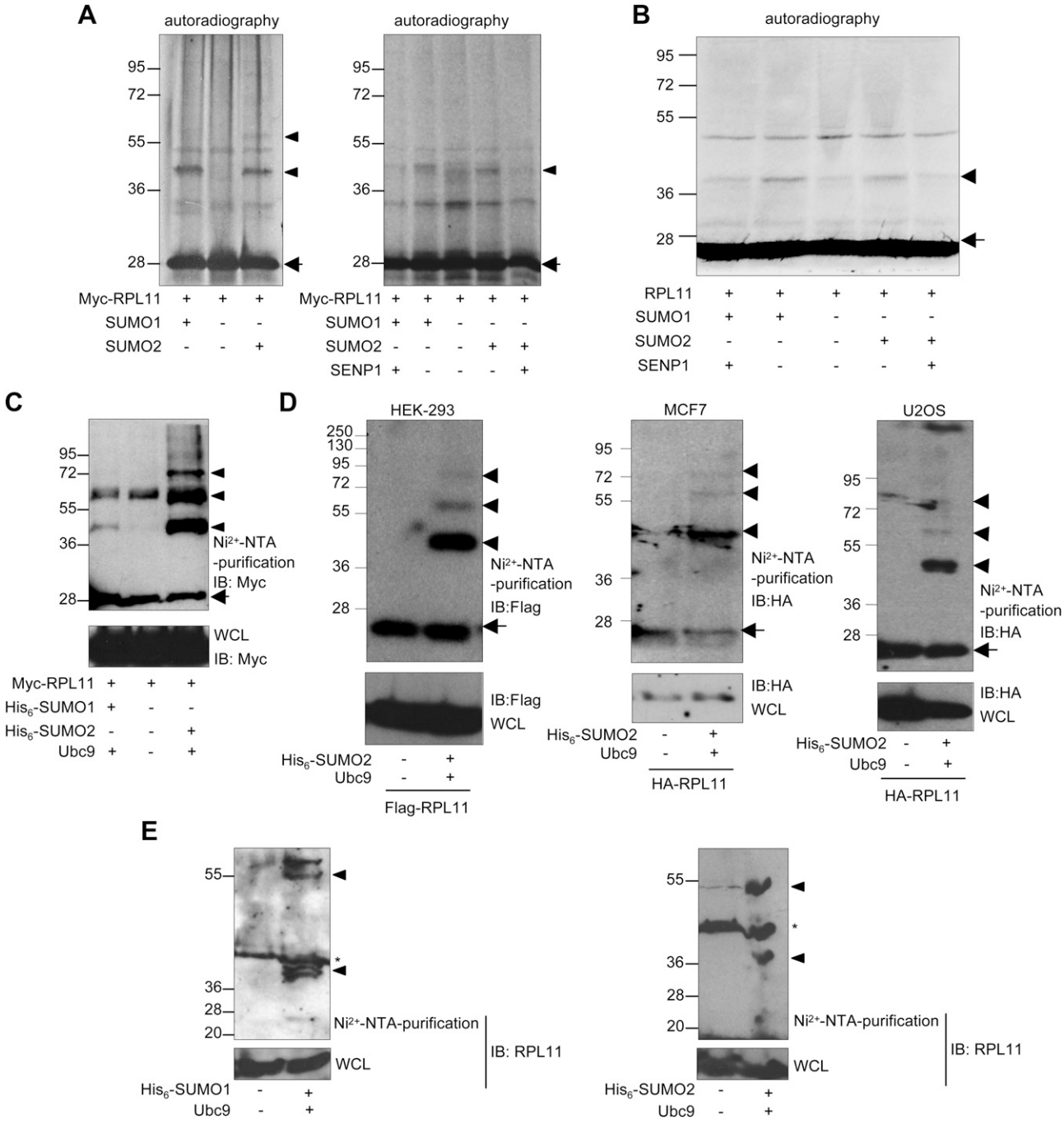

Figure 1. RPL11 is modified by SUMO1 and SUMO2 *in vitro* and *in vivo*. *A*) *In vitro*-translated [^{35}S]-labeled RPL11 was subjected to *in vitro* SUMOylation assay in the presence of SUMO1 or SUMO2 (left). SUMO1- or SUMO2-conjugated RPL11 protein was then incubated in the presence or absence of SENP1, as described in Materials and Methods (right). Proteins were resolved by SDS-PAGE and visualized by autoradiography. Arrows and arrowheads indicate the unmodified and SUMO-conjugated RPL11 protein, respectively. *B*) *In vitro*-translated, [^{35}S]-labeled, untagged RPL11 was subjected to *in vitro* SUMOylation assay in the presence of SUMO1 or SUMO2. SUMO1- or SUMO2-conjugated RPL11 protein was then incubated in the presence or absence of SENP1, as indicated. Proteins were resolved by SDS-PAGE and visualized by autoradiography. Arrow and arrowhead indicate the unmodified and SUMO-conjugated RPL11 protein, respectively. *C*) HEK-293 cells were cotransfected with myc-RPL11, together with pcDNA3, Ubc9 and His6-SUMO1, or Ubc9 and His6-SUMO2. WCLs and His-tagged purified proteins were analyzed by Western blot using anti-myc antibody. Arrow and arrowheads indicate the unmodified and SUMO-conjugated RPL11 protein, respectively. *D*) HEK-293, MCF7, or U2OS cells were cotransfected with Flag-RPL11 or HA-RPL11, together with pcDNA3 or Ubc9 and His6-SUMO2, as indicated. WCLs and His-tagged purified proteins were analyzed by Western blot using the indicated antibodies. Arrows and arrowheads indicate the unmodified and SUMO-conjugated RPL11 protein, respectively. *E*) HEK-293 cells were transfected with pcDNA3, Ubc9 and His6-SUMO1, or Ubc9 and His6-SUMO2. WCLs and His-tagged purified proteins were analyzed by Western blotting using anti-RPL11 antibody. Arrowheads indicate the SUMO-conjugated RPL11 protein. Asterisk denotes position of a nonspecific band. IB, immunoblot. NTA, nitrilotriacetic acid.

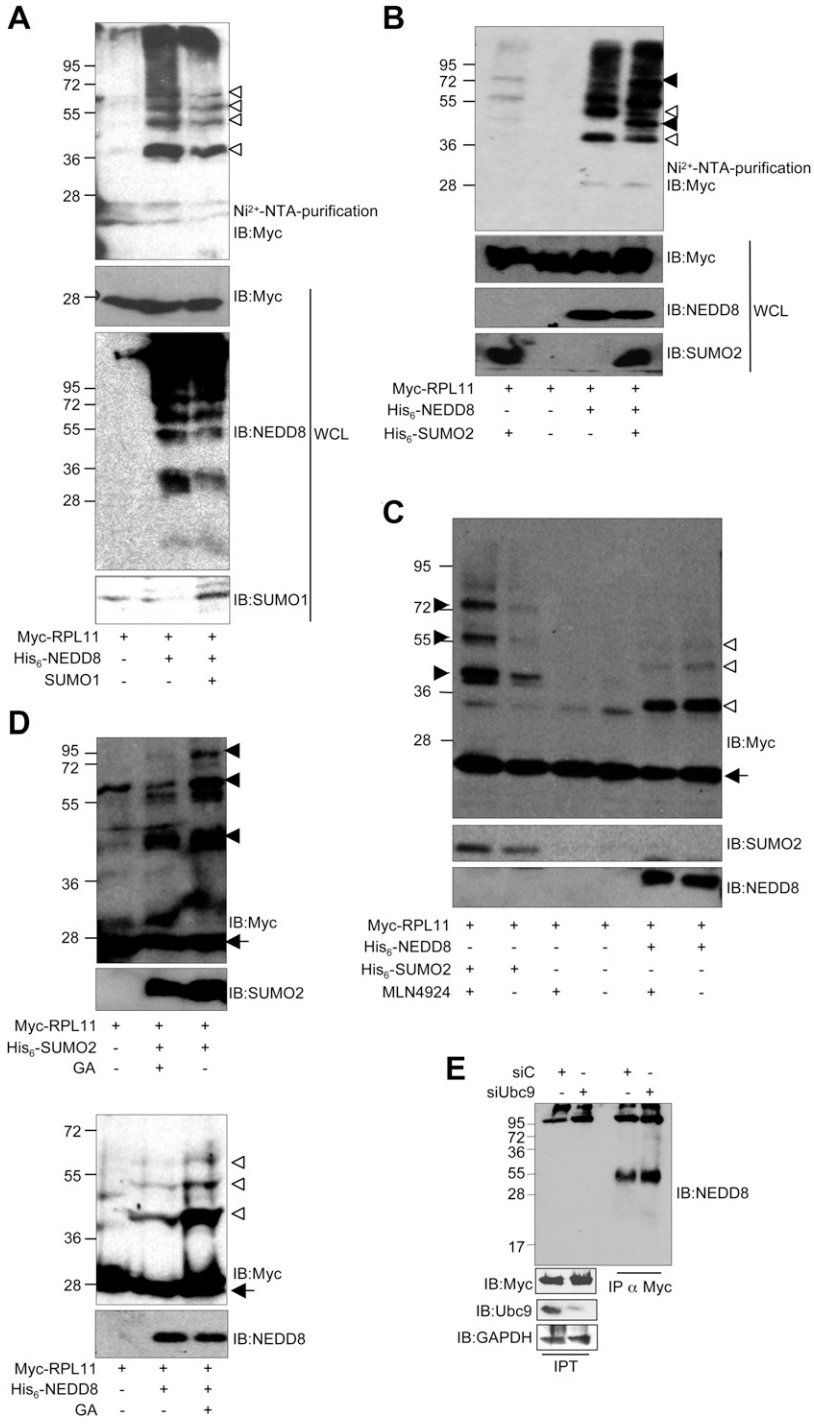

Figure 2. Interplay between SUMO and NEDD8 conjugation to RPL11. *A*) HEK-293 cells were cotransfected with myc-RPL11, together with pcDNA3, His6-NEDD8 or His6-NEDD8, Ubc9, and untagged SUMO1. At 48 h after transfection, His-tagged purified proteins were analyzed by Western blot using anti-myc antibody. Open arrowheads indicate the NEDD8-conjugated RPL11 protein. WCLs were analyzed with the indicated antibodies. *B*) HEK-293 cells were cotransfected with myc-RPL11 together with pcDNA3, His6-NEDD8, Ubc9 and His6-SUMO2 or His6-NEDD8, Ubc9, and His6-SUMO2, as indicated. At 36 h after transfection, His-tagged purified proteins were analyzed by Western blot using anti-myc antibody. Open and solid arrowheads indicate the NEDD8- and the SUMO2-conjugated RPL11 protein, respectively. WCLs were analyzed with the indicated antibodies. *C*) HEK-293 cells were cotransfected with myc-RPL11, together with pcDNA3, His6-NEDD8 or Ubc9 and His6-SUMO2, as indicated. At 36 h after transfection, cells were treated or not with the NEDDylation inhibitor MLN4924 (1 μM). At 4 h after treatment, WCLs were analyzed by Western blot using the indicated antibodies. Arrow indicates the unmodified RPL11 protein. Open and solid arrowheads indicate the NEDD8- and the SUMO2-conjugated RPL11 protein, respectively. *D*) HEK-293 cells were cotransfected with myc-RPL11, together with pcDNA3, His6-NEDD8 or Ubc9 and His6-SUMO2. At 36 h after transfection, cells were treated or not with the SUMOylation inhibitor GA (25 μM). At 4 h after treatment, WCLs were analyzed by Western blot using the indicated antibodies. Arrows indicate the unmodified RPL11 protein. Open and solid arrowheads indicate the NEDD8- and the SUMO2-conjugated RPL11 protein, respectively. *E*) HEK-293 cells were transfected with siUbc9 or siC for 48 h, and then, cells were transfected with myc-RPL11 for 24 h. Total protein extracts (IPT) and immunoprecipitated (IP) RPL11 protein, using anti-myc antibody (IP α RPL11), were analyzed by Western blot using the indicated antibodies.

cotransfected with HA-RPL11 and His6-NEDD8 or Ubc9 and His6-SUMO2 and treated or not with the NEDDylation inhibitor (Supplemental Fig. 2). Finally, HEK-293 cells were transfected, as previously described, and 36 h after transfection, cells were treated with the SUMOylation inhibitor GA. Western blot analysis of the WCLs using anti-myc antibody revealed that the RPL11-SUMO2 protein detected in cells transfected with SUMO2 decreased after treatment with GA (Fig. 2*D*, upper). In contrast, we observed a clear increase in the levels of NEDDylated RPL11 protein in the presence of the SUMOylation inhibitor (Fig. 2*D*, lower).

Finally, we examined whether downmodulation of SUMO conjugation has an impact on the endogenous NEDDylation of RPL11. HEK-293 cells were transfected with siUbc9 or siC for 48 h, and then, cells were transfected with myc-RPL11 for 24 h. RPL11 protein was then immunoprecipitated from the protein extracts using anti-myc antibody, and finally, immunoprecipitated protein was analyzed by Western blot using anti-NEDD8 antibody. As shown in Fig. 2*E*, anti-NEDD8 antibody recognized a band of the expected size corresponding to RPL11-NEDDylated protein in the immunoprecipitated extracts that increased in intensity in those cells transfected with siUbc9, suggesting that downmodulation of the SUMOylation machinery promotes the NEDDylation of RPL11. Altogether, these results demonstrate

interplay between SUMOylation and NEDDylation of RPL11.

Nucleolar stress promotes the modification of RPL11 by SUMO2

It has been reported that upon nucleolar stress, RPL11 is deNEDDylated (12). Our results indicate that there is an antagonistic relationship between SUMOylation and NEDDylation of RPL11; therefore, we decided to evaluate whether the SUMOylation of RPL11 was altered in response to nucleolar stress. U2OS cells were cotransfected with myc-RPL11 and pcDNA3 or Ubc9 and His6-SUMO2, and 36 h after transfection, cells were treated with a low dose of actinomycin D for the indicated times. As shown in **Fig. 3**, Western blot analysis of the His-tagged proteins, purified in denaturing conditions using anti-myc antibody, revealed an increase in the levels of RPL11-SUMO2 protein in response to actinomycin D, indicating that nucleolar stress promotes SUMO2 modification of RPL11 with a maximum peak after 1 h.

SUMO2 promotes the release of RPL11 from the nucleolus, and the SUMO ligase Ubc9 is required for the activation of p53 in response to RPL11 upregulation

NEDD8 promotes the nucleolar localization of RPL11 (12). Therefore, we determined the effect of SUMO on the subcellular localization of RPL11. We cotransfected MCF7 cells with myc-RPL11, together with pcDNA3 or His6-SUMO2, and 48 h after transfection, cells were analyzed by immunofluorescence using anti-myc and anti-SUMO2 antibodies. RPL11 was mainly localized in the nucleolus of those cells cotransfected with RPL11 and pcDNA3 (**Fig. 4*A***). However, RPL11 was detected in the nucleoplasm of those cells expressing high levels of SUMO2, indicating that SUMO promotes the release of RPL11 from the nucleolus to the cell nucleoplasm (Fig. 4*A*). Similar results were observed in U2OS cells (Supplemental Fig. 3).

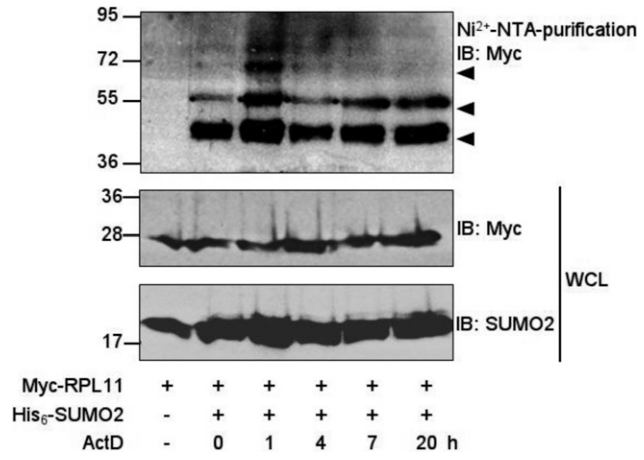

Figure 3. Ribosomal stress promotes RPL11 SUMOylation. U2OS cells were cotransfected with myc-RPL11, together with Ubc9 and His6-SUMO2. At 36 h after transfection, cells were treated with a low concentration of actinomycin D (ActD; 5 nM). At the indicated times after treatment, His-tagged, purified proteins were analyzed by Western blot using anti-myc antibody. WCLs were analyzed with the indicated antibodies. Arrowheads indicate the SUMO2-conjugated RPL11 protein.

Transfection of RPL11 led to stabilization of p53 and the induction of p53-dependent cell-cycle arrest (8, 11). Hence, we examined whether downmodulation of SUMO conjugation has an impact on the RPL11-mediated p53 stabilization or activation. U2OS cells were first transfected with siUbc9 or siC for 48 h; then cells were transfected or not with RPL11 for 24 h; and finally, they were treated with CHX for the indicated times. The levels of p53 at each time after CHX treatment were evaluated by Western blot analysis. We did not observe an alteration in the stability of p53 after Ubc9 downmodulation in cells untransfected with RPL11 (Fig. 4*B*). Transfection of RPL11 increased the stability of p53, as previously reported (Fig. 4*B*). We also observed a clear reduction in the stability of p53 in those cells with lower levels of Ubc9 (Fig. 4*B*). Altogether, these results suggest that SUMOylation contributes to p53 stabilization after RPL11 overexpression.

We then evaluated the effect of Ubc9 downmodulation on the cell-cycle arrest induced after ectopic expression of RPL11. U2OS cells, transfected with siUbc9 or siC, as described above, were cotransfected with pcDNA3 or RPL11, together with a plasmid expressing GFP-F for gating the positively transfected cells. After 24 h, the cell-cycle distribution was determined by flow cytometry analysis. We observed a significant reduction in the percentage of cells in S-phase in the cells cotransfected with siC and RPL11 relative to the percentage detected in the cells transfected with siC and pcDNA3 (Fig. 4*C*), as expected (11). However, we did not observe a significant reduction in the percentage of cells in S-phase when RPL11 was introduced in siUbc9-transfected cells (Fig. 4*C*), suggesting that SUMOylation is required for the activation of p53 in response to RPL11 overexpression.

SUMO2 modification of RPL11 is induced by ARF

RPL11 interacts with the tumor suppressor p14ARF, and it is a mediator in ARF-regulated p53 activation (7, 11). In addition, ARF has been reported to promote the SUMOylation of several proteins to which ARF binds (29, 30). Therefore, we decided to evaluate whether ARF also promotes RPL11 SUMOylation. U2OS cells (ARF null) were cotransfected with myc-RPL11, together with pcDNA3 or Ubc9 and His6-SUMO2 and in the presence or absence of GFP-ARF. At 48 h after transfection, WCLs and His-tagged proteins purified under denaturing conditions, were evaluated by Western blot using anti-myc antibody. As shown in **Fig. 5*A*** (left), expression of ARF upregulated the levels of the RPL11-SUMO2 protein. Similar results were observed when the experiment was carried out in the p53-null H1299 cell line (Fig. 5*A*, right). Moreover, as our results indicated that SUMO negatively regulates the NEDDylation of RPL11, we also decided to evaluate whether the ectopic expression of ARF has an impact on RPL11 NEDDylation. U2OS cells were cotransfected with myc-RPL11 and pcDNA3 or His6-NEDD8 and in the

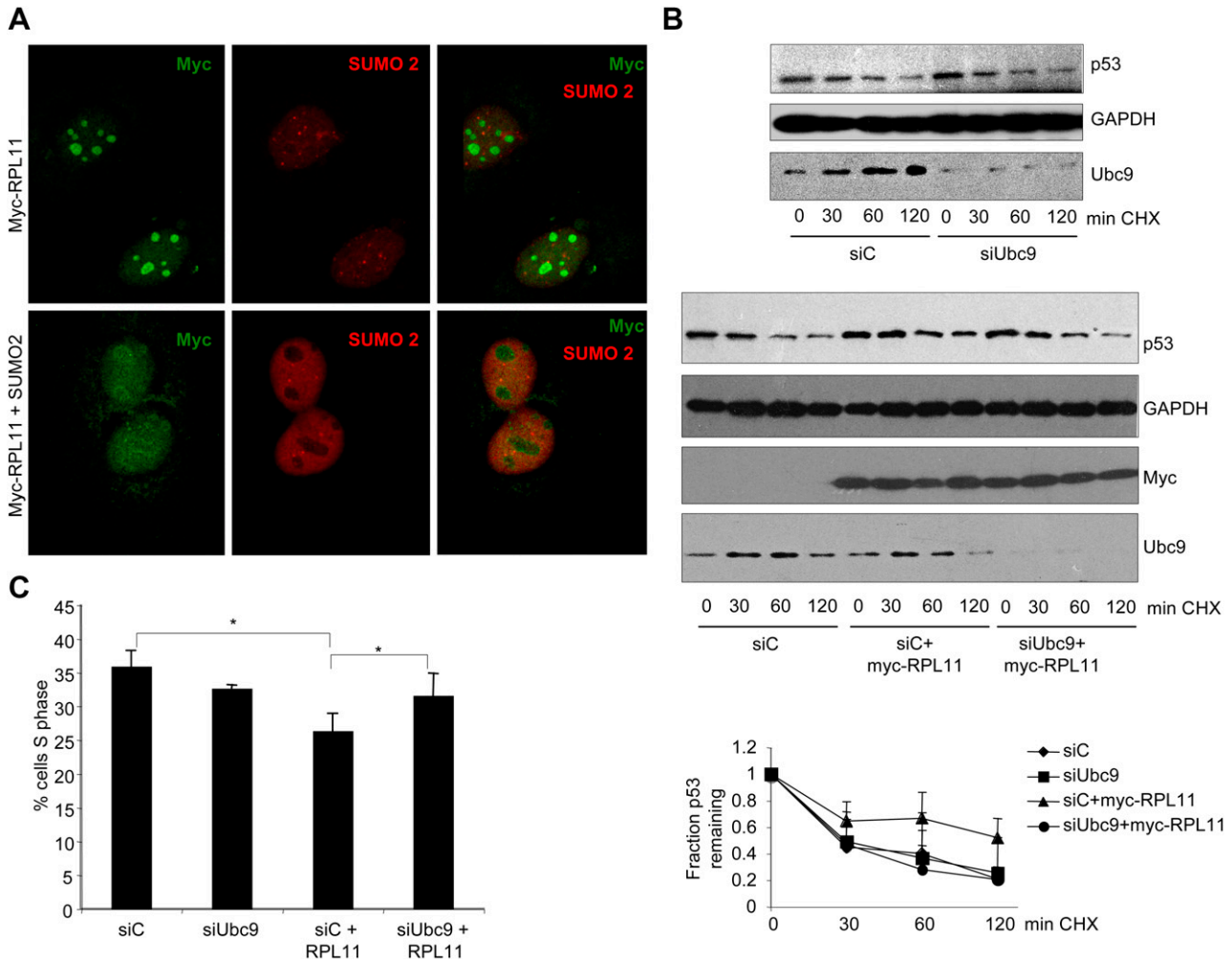

Figure 4. SUMO2 promotes the release of RPL11 from the nucleoli, and the SUMO-conjugating enzyme Ubc9 is required for the activation of p53 in response to RPL11 upregulation. *A*) MCF7 cells were cotransfected with myc-RPL11 and pcDNA3 or SUMO2, as indicated. At 48 h after transfection, cells were immunostained with anti-myc and anti-SUMO2 antibodies. Subcellular localization of the expressed proteins was analyzed under a confocal microscope. Images were processed with Adobe Photoshop. *B*) U2OS cells were transfected with siC or siUbc9 for 48 h. Then, cells were transfected with myc-RPL11 or pcDNA3; at 24 h after transfection, cells were treated with CHX (100 μg/ml). At the indicated times after CHX treatment, protein extracts were recovered and analyzed by Western blotting using the indicated antibodies. The levels of p53 remaining at each time were quantified from 3 different experiments using ImageJ, and values were normalized to time 0 samples. The average fractions of p53 remaining and their SDs are shown (lower). *C*) U2OS cells were transfected with siC or siUbc9 for 48 h. Then, cells were cotransfected with GFP-F and myc-RPL11 or pcDNA3. At 24 h after transfection, cells were harvested, fixed, permeabilized, and stained with propidium iodide. GFP-positive cells were gated for cell-cycle analysis. Mean percentage of cells in S-phase from triplicates is shown. Error bars are SD of triplicates. $*P < 0.005$ (Student's *t* test).

presence or absence of GFP-ARF. At 48 h after transfection, the WCLs, as well as the His-tagged purified proteins were analyzed by Western blotting. NEDDylation of RPL11 was dramatically downmodulated after expression of ARF (Fig. 5*B*). Interestingly, downmodulation of p53 NEDDylation by ARF (Supplemental Fig. 4) and a global downmodulation of NEDD8 conjugation were also observed (Fig. 5*B* and Supplemental Fig. 4).

DISCUSSION

Here, we identified SUMO as a regulator of the RPL11-Mdm2-p53 pathway. Our data reveal that RPL11 can be modified by SUMO1 and SUMO2 *in vitro* and *in vivo*. NEDD8 conjugation to RPL11 was only reduced after mutation of all lysine residues in RPL11 (12). Hence, we speculated that NEDD8 and SUMO might compete for conjugation to RPL11. Our results showed that NEDDylation of RPL11 decreases after SUMO overexpression and increases after treatment with the SUMOylation inhibitor GA, indicating that SUMO negatively regulates the NEDDylation of RPL11. Although SUMOylation of RPL11 was positively modulated after NEDDylation inhibitor treatment, suggesting that NEDD8 also negatively regulates the SUMOylation of RPL11, this modification was not reduced by NEDD8 overexpression. We speculate that the RPL11 stabilization or nucleolar localization promoted by NEDD8 (12) may have a positive impact on RPL11 SUMOylation. Different points of crosstalk may occur between SUMOylation and NEDDylation, including competition for common amino acid residues or regulation of

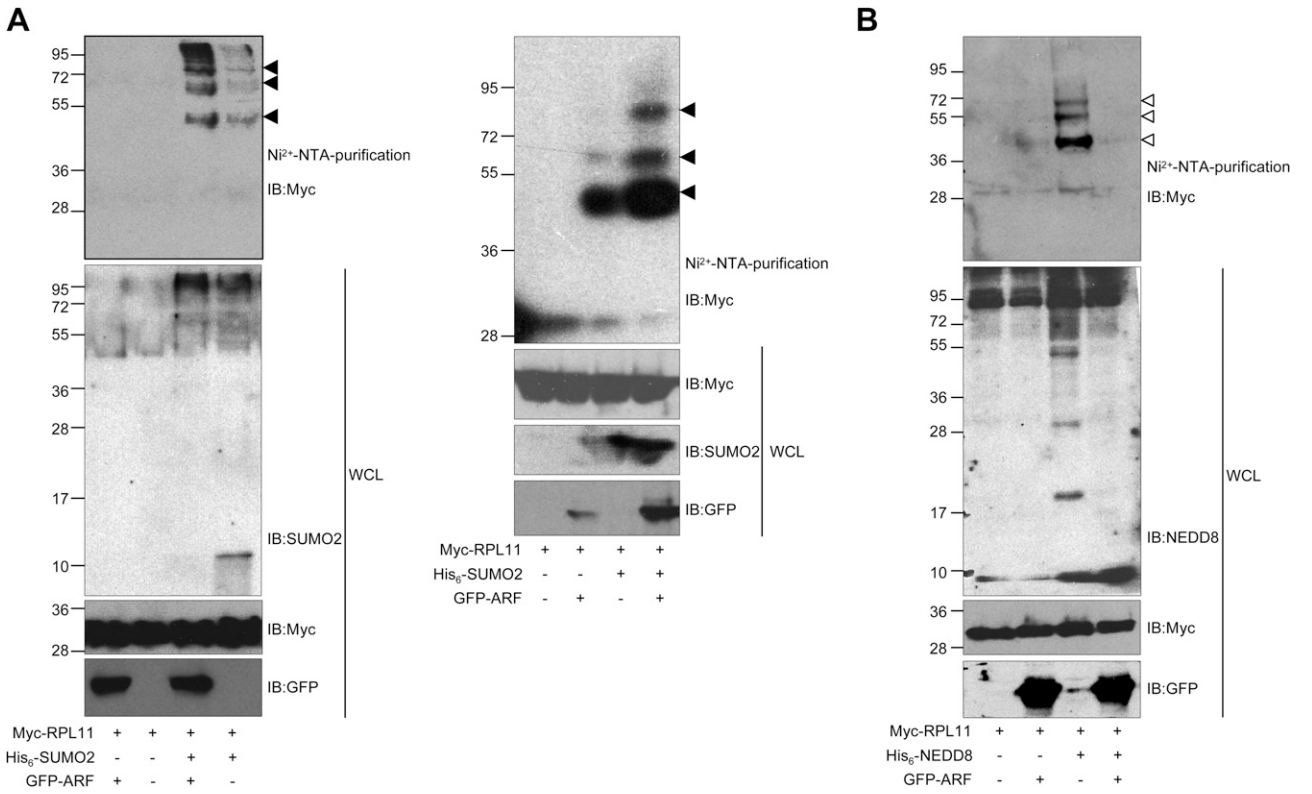

Figure 5. SUMO2 modification of RPL11 is promoted by ARF. *A*) U2OS cells (left) or H1299 cells (right) were cotransfected with myc-RPL11, together with pcDNA3 or Ubc9 and His6-SUMO2 and in the presence or absence of GFP-ARF. At 48 h after transfection, His-tagged, purified proteins were analyzed by Western blot using anti-myc antibody. Solid arrowheads indicate the SUMO2-conjugated RPL11 protein. WCLs were analyzed with the indicated antibodies. *B*) U2OS cells were cotransfected with myc-RPL11, together with pcDNA3 or His6-NEDD8 and in the presence or absence of GFP-ARF. At 48 h after transfection, His-tagged purified proteins were analyzed by Western blot using anti-myc antibody. Open arrowheads indicate the NEDD8-conjugated RPL11 protein. WCLs were analyzed with the indicated antibodies.

the NEDD8 conjugation machinery by SUMO. Accordingly, with the existence of an antagonistic relationship between NEDDylation and SUMOylation of RPL11, we observed that the SUMO2 modification of RPL11 was promoted by ribosomal stress, a stimulus that has been shown to trigger the deNEDDylation of RPL11 (12). Moreover, and in agreement with an inverse correlation between these 2 post-translational modifications, we also observed that overexpression of SUMO2 promoted the translocation of RPL11 from the nucleolus to the nucleoplasm. So far, this is the first description of interplay between SUMOylation and NEDDylation.

As a result of nucleolar stress or after ectopic expression of RPL11, the RP binds Mdm2 and promotes p53 activation (6, 8, 11, 31, 32). Our data showed that the SUMO ligase Ubc9 was required for the stabilization and activation of p53 in response to RPL11 overexpression. SUMO has been shown previously to modulate several components of the RPL11-Mdm2-p53 pathway, including p53 (33, 34) or Mdm2 (35). Therefore, we cannot exclude that the negative effect of Ubc9 downmodulation on the stability or activity of p53 that we observed is partially a result of SUMOylation inhibition of additional factors besides RPL11.

Although RPL11 is mainly known as a key protein in the control of p53 activation in response to ribosomal stress, recent reports demonstrate that RPL11 is also required for oncogenic or replicative stress-induced activation of p53 (9) and for activation of p53 by ARF (10). The molecular mechanisms underlying the RPL11-mediated p53 activation upon replicative or oncogenic stress are not known. One proposed explanation is that the increase in ARF levels, resulting from replicative or oncogenic stress, induces ribosomal stress, resulting in RPL11 suppression of Mdm2 (7, 9). We show here that upregulation of ARF triggers the SUMO2 modification of RPL11, leading us to propose that promotion of RPL11 SUMOylation by ARF may be a molecular link between the oncogenic or replicative stress and the ribosomal stress.

The ability of ARF to trigger SUMOylation of several cellular proteins and to enhance global SUMO conjugation has been previously reported (29, 30, 36, 37). The mechanism by which ARF increases SUMOylation is not clearly known. Here, we show that upregulation of ARF leads also to a decrease in RPL11 NEDDylation and importantly, in a global decline of NEDDylation. Further studies will be required to determine whether the downmodulation of global NEDD8 conjugation by ARF results from the upregulation in the SUMOylation mediated by the tumor suppressor. FJ

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AUTHOR CONTRIBUTIONS

A. El Motiam, S. Vidal, C. F. de la Cruz-Herrera, S. Da Silva-Álvarez, M. Baz-Martínez, R. Seoane, A. Vidal, A. S. Carvalho, H. C. Beck, and R. Matthiesen conducted the experiments; M. S. Rodríguez, D. P. Xirodimas, M. Collado, and C. Rivas designed the experiments; and C. Rivas wrote the paper.

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