SUMO Modification of the RNA-Binding Protein La Regulates Cell Proliferation and STAT3 Protein Stability

Venkatesh Kota,^a Gunhild Sommer,^{a,e} E. Starr Hazard,^b Gary Hardiman,^{b,c} Jeffery L. Twiss,^d Tilman Heise^a

^aMedical University of South Carolina, Department of Biochemistry and Molecular Biology, Charleston, South Carolina, USA

^bCenter for Genomic Medicine Bioinformatics, Charleston, South Carolina, USA

^cMedical University of South Carolina, Departments of Medicine and Public Health Sciences, Charleston, South Carolina, USA

^dDepartment of Biological Sciences, University of South Carolina, Columbia, South Carolina, USA

^eUniversity Hospital Regensburg, Department of Pediatric Hematology, Oncology and Stem Cell Transplantation, Regensburg, Germany

ABSTRACT The cancer-associated RNA-binding protein La is posttranslationally modified by phosphorylation and sumoylation. Sumoylation of La regulates not only the trafficking of La in neuronal axons but also its association with specific mRNAs. Depletion of La in various types of cancer cell lines impairs cell proliferation; however, the molecular mechanism whereby La supports cell proliferation is not clearly understood. In this study, we address the question of whether sumoylation of La contributes to cell proliferation of HEK293 cells. We show that HEK293 cells stably expressing green fluorescent protein (GFP)-tagged wild-type La (GFP-La^{WT}) grow faster than cells expressing a sumoylation-deficient mutant La (GFP-La^{SD}), suggesting a proliferative function of La in HEK293 cells. Further, we found that STAT3 protein levels were reduced in GFP-La^{SD} cells due to an increase in STAT3 ubiquitination and that overexpression of STAT3 partially restored cell proliferation. Finally, we present RNA sequencing data from RNA immunoprecipitations (RIPs) and report that mRNAs associated with the cell cycle and ubiquitination are preferentially bound by GFP-La^{WT} and are less enriched in GFP-La^{SD} RIPs. Taken together, results of our study support a novel mechanism whereby sumoylation of La promotes cell proliferation by averting ubiquitination-mediated degradation of the STAT3 protein.

KEYWORDS La autoantigen, La protein, STAT3, proliferation, ubiquitination, SSB/La, SUMO, LARP3, RNA binding, LARP, RNA-binding proteins, ubiquitin, sumoylation

The RNA-binding protein La (La/Sjogren syndrome antigen B [SSB] or LARP3) is essential for mouse development, and cell-type-specific depletion of endogenous La impairs pre-tRNA processing and the development of specific cell types (1, 2). In addition to its well-established role in the processing of RNA polymerase III transcripts (3–6), La protein has been shown to contribute to microRNA (miRNA) processing (7–9) and to stabilize or promote translation of viral and some cellular mRNAs (10–23). In addition to this critical role in development, La is overexpressed in several different types of tumors (15, 18, 24, 25), which likely contributes to tumorigenesis by supporting cell proliferation, migration, and invasion. La regulates the translation of tumor-promoting and antiapoptotic proteins, including Mdm2, XIAP, laminin B1, cyclin D1, and Bcl2. It appears reasonable to speculate that La's aberrant regulation of those proteins contributes to its tumor-promoting activity (14, 15, 17, 18, 25).

The La protein is 47 kDa in size and binds RNA via the La motif (LAM), RNA recognition motif 1 (RRM1), and the noncanonical RNA recognition motif 2 (RRM2). RRM2 is located in the C-terminal extension that is characteristic of the mammalian La

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Address correspondence to Tilman Heise,
heise4tmf@gmail.com.

protein (5). The C-terminal region of the La protein also contains signals for nuclear localization (26), nucleolar localization (27, 28), and nuclear retention (26), as well as RNA chaperone domains (29, 30). La is primarily located in the nucleus and nucleolus. However, La can shuttle between the cytoplasm and the nucleus (31–33).

The function of the La protein is regulated by posttranslational modifications. La can be phosphorylated on serine 366 (34–36) and threonine 389 (30); phosphorylation controls La's tRNA maturation, cellular localization, and ability to facilitate mRNA translation (30, 32, 35–37). In addition, the La protein has been shown to undergo covalent modification by the small ubiquitin-like modifiers (SUMOs). Posttranslational modification of proteins by SUMO is well recognized as an important regulatory mechanism in numerous biological processes (38, 39). SUMOs are small (12-kDa) proteins expressed as four highly conserved paralogs, SUMO1, SUMO2, SUMO3, and SUMO4, in vertebrates. SUMO2 and -3 share 95% amino acid sequence homology and differ only in three N-terminal residues in their conjugated forms, whereas SUMO1 has only 50% sequence identity to the SUMO2/SUMO3 subfamily (39, 40). Several studies identified SUMO acceptor sites in La, and sumoylation regulates the retrograde transport of rodent La in neurons (41) and the RNA-binding activity of human La (42). Genome-wide studies also showed that heat shock induces SUMO2 conjugation to La (43). Proteomics studies identified the following SUMO acceptor sites in La: lysines 35, 86, and 400 in HeLa cells (44, 45) and lysines 76, 86, 105, and 116 in HEK293 cells (46).

Although the retrograde transport and RNA-binding activity of La are regulated by sumoylation, it is not clear whether sumoylation of La contributes to other cellular mechanisms. Previously, we showed that cell proliferation is La dependent in several cell lines, including HeLa, PC-3, 3T3, and UM-SCC 22B (18, 25). Here, we asked if sumoylation of La contributes to cell proliferation. By overexpression of green fluorescent protein (GFP)-tagged wild-type La (GFP-La^{WT}) or a sumoylation-deficient mutant La (GFP-La^{SD}) (42), we demonstrate that GFP-La^{SD} cell proliferation is impaired concomitant with reduced expression of signal transducer and activator of transcription 3 (STAT3). Overexpression of STAT3 partially rescued cell proliferation, suggesting that STAT3 contributes to the defect in proliferation of GFP-La^{SD} cells. Interestingly, sumoylated La counteracted, by a yet unknown mechanism, the proteasome-mediated degradation of STAT3, indicating that La's sumoylation status can determine its role in cell proliferation. High-throughput sequence analysis of RNA immunoprecipitations (RIPs) suggested that sumoylation of La facilitates the association of La with mRNAs associated with cell cycle processes and ubiquitination. This finding implies that La modulates the expression of a large variety of cellular factors.

RESULTS

Sumoylation of La is required for cell proliferation. The RNA-binding protein La can be modified by SUMO (41–43), and we recently showed that La depletion impairs cell proliferation in several cell lines (18, 25). Here, we wanted to test whether sumoylation of La impacts the proliferation of HEK293 cells.

To determine if endogenous La can be sumoylated in HEK293 cells, we treated cells and cell extracts with *N*-ethylmaleimide (NEM) to inhibit SUMO proteases (41). Immunoblot analysis of NEM-treated or untreated extracts showed high-molecular-mass La-specific bands (Fig. 1A, bands 1 to 4 [marked by arrows]), which were clearly prominent in NEM-treated extracts (Fig. 1A [short exposure] and B [long exposure]) and were designated in order of increasing apparent mass based on migration in SDS-PAGE. These and additional high-molecular-mass bands visible in Fig. 1B are indicative of posttranslational modifications of La. A similar amount of protein was loaded in Fig. 1C. To test whether some of those high-molecular-mass La-specific bands represented sumoylated La isoforms, we performed La-specific immunoprecipitations (IPs), followed by immunoblotting with La-specific and SUMO-specific antibodies. As shown, full-length La (Fig. 1D, stars), as well as the high-molecular-mass La-specific band 1, immunoprecipitated (Fig. 1D, arrows). Importantly, when using a SUMO2/SUMO3-specific antibody, we detected the high-molecular-mass La-specific band 1 (Fig. 1E),

FIG 1 Endogenous La undergoes sumoylation in HEK293 cells. (A and B) Representative immunoblots (IB) of endogenous La using La-specific antibodies (SEx, short exposure; LEx, long exposure). The cells were treated (+) or not treated (–) with NEM (10 mM). The arrows indicate high-molecular-mass La-specific bands; the stars indicate native La. (C) Another short exposure for native La; HSP70 was used as a loading control. (D and E) Cells were treated (+) or not treated (–) with NEM (10 mM) and subjected to IP with mouse La-specific antibody. The immunoprecipitate was analyzed by immunoblotting, applying a rabbit La-specific antibody (D) or with a SUMO2/SUMO3-specific antibody (E). The arrows indicate high-molecular-mass La-specific bands recognized by the SUMO2/SUMO3-specific antibody. The stars indicate the positions of native endogenous immunoprecipitated La. HSP70 was used as a loading control. (Top) Short exposures; (bottom) long exposures.

strongly suggesting that this band reflects a sumoylated La isoform. Taken together, La-specific immunoprecipitation identified a sumoylated La isoform in the HEK293 cells. As was found for other sumoylated proteins (39, 40), often only a small fraction of the La protein is sumoylated. Bands 2 to 4 (Fig. 1A) were not efficiently immunoprecipitated by the La-specific antibody (compare Fig. 1A and D), although a second, weaker high-molecular-mass La-specific band (Fig. 1D and E, band 2) was visible after longer exposures. This might be due to the remaining activity of proteases removing post-translational modifications during the immunoprecipitation reaction, as suggested by the strong decrease in the ratio between band 1 and native La in Fig. 1A compared to D (star). It also could be that the sumoylated La isoforms are not efficiently recognized by the La-specific antibody, impairing the immunoprecipitation.

Consistent with previous published work, GFP-La^{WT} is sumoylated in sensory neurons (41) and HEK293 cells (42). We reproducibly detected La bands with GFP- and La-specific antibodies that migrated at higher apparent molecular mass than native La-GFP (Fig. 2B and 3A, S-La). This pattern of the high-molecular-mass La-specific bands appears to be similar to the four extra bands detected during the analysis of endogenous La shown in Fig. 1A. In contrast, when lysines 41 and 200 were mutated to arginine (K41/200R; GFP-La^{SD}), sumoylation of GFP-La^{SD} was reduced (Fig. 2B and 3A) (42). When comparing the results of several experiments (Fig. 2B and 3A) (42), we detected some inconsistency in the intensities of high-molecular-mass bands in GFP-La^{WT} and GFP-La^{SD} extracts, probably reflecting the difficulty in stabilizing the low-abundance sumoylated La isoforms. We also observed, occasionally (Fig. 3A) but not regularly (Fig. 2B and 3B and C; see Fig. 5C and 6F), an increase in expression of native GFP-La^{SD} protein compared to GFP-La^{WT}, suggesting that sumoylation might contribute to the stability of the La protein.

To test whether the GFP-La^{WT} bands represented a sumoylated La isoform, we immunoprecipitated GFP-La^{WT} and GFP-La^{SD} using cell lysates prepared from HEK293 cells stably overexpressing GFP-La^{WT}, GFP-La^{SD}, or GFP (Fig. 2A; see Fig. 6C). As we

FIG 2 GFP-La expression and sumoylation in HEK293 cells. (A) Representative fluorescence images showing stable expression of GFP, GFP-La^{WT}, and GFP-La^{SD} in HEK293 cells. (B) Representative immunoblot of GFP-, GFP-La^{WT}-, and GFP-La^{SD}-expressing cells using anti-GFP antibody. GAPDH was used as a loading control. S-La, sumoylated La.

hadseen for the immunoprecipitation of sumoylated endogenous La (Fig. 1D), we found that the immunoprecipitation of the GFP-tagged high-molecular-mass La isoforms is very inefficient (Fig. 3A to C). In the case of GFP-La immunoprecipitation, we did not pull down the high-molecular-mass bands 1 to 4, as seen in the immunoblot

FIG 3 GFP-tagged La undergoes sumoylation in HEK293 cells. (A) Representative immunoblot of GFP-La^{WT}- and GFP-La^{SD}-expressing cells using a GFP-specific antibody; S-La, high molecular-mass bands. HSP70 was used as a loading control. #, unknown La-specific band. (B to E) GFP-La^{WT}- and GFP-La^{SD}-expressing cells were treated with NEM (10 mM), immunoprecipitated with a GFP-specific antibody, and probed with an La-specific antibody (B), a GFP-specific antibody (C), a SUMO2/SUMO3-specific antibody (D), or a SUMO1-specific antibody (E) during immunoblot analysis. The arrows indicate a band running slightly above the heavy IgG band (squares), which likely represents a GFP-La cleavage product because it is recognized by the La-specific (B), GFP-specific (C), SUMO2/SUMO3-specific (D), and SUMO1-specific (E) antibodies. Squares, heavy and light IgG bands; circles, GFP or GFP-tagged La. The arrowheads indicate additional sumoylated bands in the input lanes.

FIG 4 Sumoylation of La supports cell proliferation. (A) Representative immunoblot of La in HEK293 cells transfected with lentivirus expressing shC-, sh3-, and La shRNA5 (sh5)-specific shRNA constructs. GAPDH was used as a loading control. (B) Cell proliferation analysis of La-depleted cells as determined by the CyqQuant method. The La-depleted cells (sh5) show lower cell proliferation than control cells (shC) ($n = 3$). (C) Cell proliferation analysis of GFP-La^{WT} and GFP-La^{SD} cells by the CyqQuant method. The GFP-La^{SD} and GFP cells grew more slowly than GFP-La^{WT} cells ($n = 3$). (D) Cell cycle analysis of GFP, GFP-La^{WT}, and GFP-La^{SD} cells. The GFP and GFP-La^{SD} cells showed significant accumulation in G₀/G₁ phases, whereas GFP-La^{WT} accumulated in the G₂/M phases, ($n = 3$). The asterisks indicate significant differences: *, $P < 0.01$; **, $P < 0.05$; ***, $P < 0.001$; Student's t test. The data represent means and standard deviations (SD) of the results of independent experiments.

(Fig. 2B and 3A); however, we detected a La-specific band (Fig. 3B, arrow), which was also recognized by a GFP-specific antibody (Fig. 3C, arrow). This band was also detected with the SUMO2/SUMO3-specific (Fig. 3D, arrow) and SUMO1-specific (Fig. 3E, arrow) antibodies. Note that we have recently reported that recombinant La can be sumoylated *in vitro* by SUMO1, SUMO2, and SUMO3 (42). The intensity of the bands recognized by the SUMO2/SUMO3-specific antibody was weaker in immunoprecipitations from GFP-La^{SD} than in the GFP-La^{WT} immunoprecipitations (Fig. 3D and E). The band is very similar in size to that of endogenous sumoylated La; however, the band is also recognized by the GFP-specific antibody, strongly suggesting that it represents a sumoylated cleavage product of GFP-La. We tried several methods to stabilize modified GFP-La during cell lysis (e.g., lysis in hot SDS or 5 M urea), unfortunately without success.

Altogether, we provide evidence that endogenous, as well as GFP-tagged, La is sumoylated in HEK293 cells. The nature of the additional band detectable by immunoblotting from GFP-La^{SD} cells (Fig. 3A, hash mark) is not clear, and the band is not always clearly detectable (Fig. 2B) (42). This band appears when two lysine residues are mutated, which could lead to the use of alternative sites for sumoylation of La, and additional sumoylation sites have been reported in other cell types, such as lysines 35, 86, and 400 in HeLa cells (44, 45) and lysines 76, 86, 105, and 116 in HEK293 cells (46). Formally, we cannot rule out the possibility that the cellular changes we describe below are related to this band, which is detectable only sometimes and to different extents.

To address the question of whether sumoylation of La affects the proliferation of HEK293 cells, we first depleted La using two La-specific short hairpin RNAs (shRNAs) and a control shRNA (Fig. 4A). Consistent with other cell types, La-depleted cells grew more slowly than the control cells (Fig. 4B). Of note, the numbers of La shRNA3 (sh3)-depleted cells were too low to measure cell proliferation over time. Next, we compared the proliferation levels of GFP-expressing (GFP), GFP-La^{WT}, and GFP-La^{SD} cells. Interestingly, overexpression of GFP-La^{WT} stimulates the growth of HEK293 cells over time compared to GFP or GFP-La^{SD} cells (Fig. 4C). The slower growth of GFP or

FIG 5 Sumoylation of La increases STAT3 mRNA binding by La. (A) RT-qPCR analysis showing no significant difference in STAT3 mRNA levels in GFP-La^{WT} (Wt)- and GFP-La^{SD} (K41/200R)-expressing cells. The values are normalized against GAPDH mRNA levels ($n = 3$). (B) RT-PCR on RNA samples prepared from RIP experiments using HEK293 cells stably overexpressing GFP-La^{WT} (Wt) or GFP-La^{SD} (K41/200R). Significantly less STAT3 mRNA was associated with GFP-La^{SD} than with GFP-La^{WT}. The asterisks indicate a significant difference ($P < 0.01$), as determined by Student's t test ($n = 3$). The data represent means and SD of the results of independent experiments.

GFP-La^{SD} cells (Fig. 4C) suggests that sumoylation at lysines 41/200 is required for the proliferative function of La in HEK293 cells. Cell cycle analysis revealed a significant reduction of GFP-La^{WT} cells in the G₁ phase and a significant accumulation in the G₂/M phases of the cell cycle (Fig. 4D) compared to GFP or GFP-La^{SD} cells, which were both enriched in the G₁ phase (Fig. 4D). Previously, we have shown that La depletion did not trigger cell death or apoptosis (18, 25). Accordingly, during fluorescence-activated cell sorter (FACS) analysis, we did not observe an accumulation of GFP-La cells in the sub-G₁ fraction, which could be indicative of cells undergoing apoptosis (data not shown). Taken together, sumoylation of La at lysines 41/200 adds to the proliferative function of La in HEK293 cells.

STAT3 expression is reduced in La-depleted and GFP-La^{SD} cells but not in GFP-La^{WT} cells. Previously, it was shown that La regulates the expression of a number of proteins associated with tumorigenic processes, including cell proliferation, invasion, and chemoresistance (15–18, 25). Therefore, we considered the possibility that sumoylation of La at lysines 41/200 facilitates the expression of proteins involved in cell proliferation.

Recently, we performed a RIP of GFP-La^{WT} and GFP-La^{SD} cells, followed by sequencing, and found that specific mRNAs were preferentially enriched in GFP-La^{WT} RIPs compared to GFP-La^{SD} RIPs (42). By searching those data sets for transcription factors involved in cell proliferation and cell growth, we found that the mRNA encoding STAT3 was preferentially enriched in GFP-La^{WT} RIPs compared to GFP-La^{SD} RIPs. Because STAT3 can be aberrantly activated in cancer cells (e.g., head and neck cancers) and thereby support cell proliferation, cell cycle progression, and antiapoptotic processes (47, 48), we compared STAT3 mRNA levels in GFP-La^{WT} and GFP-La^{SD} cells (Fig. 5A) and validated the GFP-La^{WT} and GFP-La^{SD} RIP data by reverse transcription-quantitative PCR (RT-qPCR) (Fig. 5B). The analysis revealed no difference in total STAT3 mRNA levels in GFP-La^{WT} and GFP-La^{SD} cells. Interestingly, we found that STAT3 mRNA was preferentially enriched in GFP-La^{WT} compared to GFP-La^{SD} RIPs (Fig. 5B). The data support our previous study showing that sumoylation of La promotes the binding of mRNAs by La in cells (42).

Next, we tested whether STAT3 expression is aberrantly regulated in La-depleted or GFP-La^{SD}-expressing HEK293 cells. By immunoblot analysis, we demonstrated that STAT3 protein levels are reduced in La-depleted cells (Fig. 6A), although STAT3 mRNA levels were unchanged (Fig. 6B). Similar, STAT3 protein levels were reduced in GFP-La^{SD} cells compared to GFP-La^{WT} cells (Fig. 6C and D). As described above, STAT3 mRNA

FIG 6 Sumoylation of La promotes cell proliferation via a STAT3-mediated mechanism. (A) Representative immunoblot of STAT3 in HEK293 cells transduced with shC, sh3, and sh5 lentiviral constructs. GAPDH was used as a loading control. (B) RT-qPCR analysis showing no significant difference in STAT3 mRNA levels in HEK293 cells transduced with shC, sh3, and sh5 lentiviral constructs. The values are normalized against GAPDH mRNA levels ($n = 3$). (C) Representative immunoblot showing STAT3 protein levels in GFP- La^{WT} - and GFP- La^{SD} -expressing cells. GAPDH was used as a loading control. (D) Densitometry analysis showing significantly lower STAT3 protein expression in GFP- La^{SD} -expressing cells than in GFP- La^{WT} -expressing cells. The values are normalized against GAPDH protein levels ($n = 3$). (E) Representative fluorescence images showing transient transfection of control (GFP) and RFP-STAT3 in cells transduced with lentiviral constructs expressing shC or sh5. The transfection efficiency was $\sim 30\%$. (F) Overexpression of STAT3 (RFP-STAT3) restored the numbers of La-depleted (sh5) cells ($n = 4$; $^* P = 0.0358$). (G) Representative fluorescence images showing transient transfection of control and RFP-STAT3 in GFP-, GFP- La^{WT} -, and GFP- La^{SD} -expressing cells. The transfection efficiency was $\sim 30\%$. (H) Overexpression of STAT3 (RFP-STAT3) restored GFP- La^{SD} cell numbers ($n = 3$; $^* P = 0.015$). The asterisks indicate significant differences ($P < 0.05$), as determined by Student's t test. NS, not significant. The data represent means and SD of the results of independent experiments.

levels were unchanged (Fig. 5A), suggesting that STAT3 expression is posttranscriptionally downregulated in La-depleted HEK293 cells and cells overexpressing sumoylation-deficient La. Together, these data indicate that sumoylation of La at K41/200 supports STAT3 protein expression and that low STAT3 expression might correlate with reduced proliferation of La-depleted and GFP- La^{SD} -expressing cells.

Since the STAT3 protein is linked to cell growth and cell proliferation, we aimed to restore the proliferation of La-depleted and GFP- La^{SD} cells by overexpression of red fluorescent protein (RFP)-tagged STAT3 (RFP-STAT3). As described above, we saw a significant reduction in cell proliferation with La depletion (Fig. 4B). Overexpression of STAT3 by transfection with RFP-STAT3 significantly increased the numbers of La-depleted cells (Fig. 6E and F). Similarly, transfection with the RFP-STAT3 expression plasmid significantly increased the numbers of GFP- La^{SD} cells but not of GFP or GFP- La^{WT} cells (Fig. 6G and H). The increase in cell numbers was small but significant; we attribute this to the transfection efficiency of the RFP-STAT3 expression plasmid. Nonetheless, STAT3 overexpression consistently increased the numbers of La-depleted and GFP- La^{SD} HEK293 cells, suggesting that sumoylation of La promotes the proliferation of HEK293 cells by a STAT3-mediated mechanism.

STAT3 protein stability is reduced in GFP- La^{SD} cells but not in GFP- La^{WT} cells.

To establish the mechanism by which La affects STAT3 protein levels, we first considered that La promotes STAT3 protein synthesis, because La supports the translation of a variety of cellular mRNAs, such as those of XIAP, laminin B, cyclin D1, and Bcl2 (13, 15,

FIG 7 Global translation is impaired in La-depleted HEK293 cells. Shown is autoradiography (Autorad) (A) and a corresponding Coomassie-stained gel (B) of [35 S]methionine-labeled (30 min or 60 min) total proteins of cells transduced with shC and sh5 lentiviral constructs.

17, 18, 25), and our RIP analyses showed that STAT3 mRNA associates with the La protein. First, we tested whether sumoylation of La impairs or fosters global protein synthesis. We performed 35 S metabolic-labeling experiments with La-depleted and GFP-La^{WT}- or GFP-La^{SD}-overexpressing HEK293 cells. We found that the overall signal of 35 S-labeled proteins was lower in extracts prepared from La-depleted cells than in those from control shRNA-expressing cells (Fig. 7A). Staining of the same gels with Coomassie blue demonstrated that similar amounts of protein were loaded (Fig. 7B). Next, we found that the global protein synthesis in both GFP-La^{WT} and GFP-La^{SD} cells was slightly increased compared to GFP-expressing cells (Fig. 8A, autoradiograph [Autorad], and B, Coomassie blue stain), as expected from La depletion experiments, suggesting that sumoylation of La does not facilitate global protein synthesis.

Considering that sumoylation of La might affect the synthesis of a small number of proteins in HEK293 cells, we performed polyribosomal gradient analysis of GFP-La^{WT} and GFP-La^{SD} cells. Interestingly, the 254-nm absorbance profiles of GFP-La^{SD} cell-derived gradients showed reproducibly higher 80S peaks and smaller polyribosomal areas than gradients derived from GFP-La^{WT} cells (Fig. 8C). RNA was prepared from each polysome gradient fraction, analyzed for integrity, and then reverse transcribed. The cDNA was used for quantification of STAT3 and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNAs by quantitative PCR. As shown in the representative profile, STAT3 mRNA analysis revealed a minor shift from heavy polyribosome to light polyribosome fractions in the GFP-La^{SD} cells (Fig. 8D), whereas the distribution of GAPDH mRNA was not changed (Fig. 8E). We concluded that the minor but reproducible shift of STAT3 mRNA from heavy to light polyribosome fractions might contribute to the reduction of STAT3 protein levels, but we reasoned that other mechanisms must contribute to generate the reduction in STAT3 protein levels in GFP-La^{SD} cells compared to GFP-La^{WT} cells shown in Fig. 6C and D.

Since STAT3 mRNA levels were not changed and STAT3 mRNA translation was only slightly changed in GFP-La^{SD} cells, we tested whether STAT3 protein stability was reduced in GFP-La^{SD} cells. We studied the STAT3 protein stability in GFP-La^{WT} and GFP-La^{SD} cells by treating the cells with the translation inhibitor cycloheximide (CHX) (20 μ M) for different time intervals and analyzed STAT3 protein levels by immunoblotting. As shown, STAT3 protein was very stable in GFP-La^{WT} cells (Fig. 9A), but in GFP-La^{SD} cells, STAT3 protein stability was strongly reduced (Fig. 9A). Quantification of protein signals revealed a STAT3 protein half-life of 6 to 7 h in GFP-La^{SD} cells, whereas the STAT3 protein was remarkably stable over the entire period of CHX treatment in GFP-La^{WT} cells (Fig. 9B).

To test whether the shortened STAT3 protein stability in GFP-La^{SD} cells was due to proteasome-mediated degradation, we cotreated the GFP-La^{SD} and GFP-La^{WT} cells with cycloheximide plus the proteasome inhibitor MG132. We found that the STAT3 protein levels were not reduced in MG132-treated GFP-La^{SD} cells (Fig. 9C). Quantification of the immunoblots did not show any significant difference in STAT3 stability between MG132-treated GFP-La^{WT} and GFP-La^{SD} cells (Fig. 9D). This finding suggests that STAT3

FIG 8 Global translation is not impaired in GFP- La^{WT} or GFP- La^{SD} cells, and sumoylation of La has a minor impact on STAT3 mRNA translation in HEK293 cells. (A and B) Autoradiography (A) and corresponding Coomassie-stained gel (B) of $[^{35}\text{S}]$ methionine-labeled total proteins of GFP- La^{WT} - and GFP- La^{SD} -expressing cells (30 min and 60 min). (C) Overlay of polyribosome fractionation profiles of two gradients loaded with extracts from GFP- La^{WT} (Wt-I/II) cells and two gradients loaded with extracts from GFP- La^{SD} (SD-I/II) cells. (D) STAT3 mRNA distribution in polyribosomal gradients from GFP- La^{WT} or GFP- La^{SD} cells as analyzed by RT-qPCR. (E) GAPDH mRNA distribution in polyribosomal gradients from GFP- La^{WT} or GFP- La^{SD} cells as analyzed by RT-qPCR. The results are representative of three independent experiments.

stability is differentially regulated in a proteasome-mediated manner in GFP- La^{WT} and GFP- La^{SD} cells.

Next, we asked whether global ubiquitination was changed in GFP- La^{WT} and GFP- La^{SD} cells. MG132-treated and untreated cell extracts were subjected to immunoblot analysis. As shown, global ubiquitination was increased in GFP- La^{SD} cells compared to GFP- La^{WT} cells (Fig. 9E), suggesting that more proteins are ubiquitinated in GFP- La^{SD} cells. We then investigated STAT3 ubiquitination in GFP- La^{WT} and GFP- La^{SD} cells. For this, we overexpressed Flag-tagged STAT3 (STAT3-Flag) and hemagglutinin (HA)-tagged ubiquitin (UB-HA) and tested for ubiquitinated isoforms by immunoblot analysis of STAT3 immunoprecipitations. There was a strong increase in STAT3 ubiquitination in GFP- La^{SD} cells compared to GFP- La^{WT} cells (Fig. 9F). This increase was not detectable in the absence of the proteasome inhibitor MG132, a situation in which

FIG 9 Sumoylation of La promotes STAT3 protein stability. (A) GFP-La^{WT}- and GFP-La^{SD}-expressing cells were treated with CHX (20 μ M) for the indicated times and analyzed for STAT3 expression by immunoblot analysis. GAPDH protein levels were analyzed as a loading control. (B) Quantification of immunoblots revealed that STAT3 stability was reduced in GFP-La^{SD} cells compared to GFP-La^{WT} ($n = 3$; *, $P = 0.022$ at 6 h and $P = 0.023$ at 12 h). The asterisks indicate significant differences ($P < 0.05$), as determined by Student's t test. (C) GFP-La^{WT}- and GFP-La^{SD}-expressing cells were treated with CHX (20 μ M) and the proteasome inhibitor MG132 (10 μ g/ml) for the indicated times and analyzed for STAT3 expression by immunoblot analysis. GAPDH protein levels were analyzed as a loading control. (D) Quantification of immunoblots revealed no significant difference in STAT3 stability in GFP-La^{SD} and GFP-La^{WT} cells ($n = 3$; $P = 0.24$ at 6 h; $P = 0.51$ at 12 h). (E) Representative immunoblot showing global ubiquitination in GFP-La^{WT} and GFP-La^{SD} cells treated or not treated with the proteasome inhibitor MG132 (10 μ g/ml). GAPDH protein levels were analyzed as a loading control. (F) Representative immunoblot showing ubiquitination of STAT3 in GFP-La^{SD} cells in the absence or presence of MG132. GFP-La^{WT}- and GFP-La^{SD}-expressing cells were cotransfected with Flag-tagged STAT3 (STAT3-Flag) and HA-tagged ubiquitin (UB-HA). After 24 h, the cells were treated or not treated with MG132 (10 μ g/ml) and subjected to immunoprecipitation applying a Flag-specific antibody. The immunoblots were analyzed with HA-specific (detection of ubiquitin) or Flag-specific (detection of STAT3) antibody. Protein levels in the extracts (Input) used for IP (bottom) were also assessed.

STAT3 stability is strongly reduced (Fig. 9A and B). Note that the very minor reduction of STAT3-Flag in the GFP-La^{SD} cells (Fig. 9F, Input) was likely due to the overexpression of STAT3. Overall, the results indicate that sumoylation of La at lysines 41/200 prevents STAT3 ubiquitination and thereby promotes STAT3 expression and cell proliferation. To our knowledge, this is the first report demonstrating that the RNA-binding protein La can regulate protein stability in a sumoylation-dependent manner.

Previous work and our findings presented here suggest that sumoylation of La affects many cellular mechanisms, including translation and ubiquitination, and there-

FIG 10 Analysis of RIP-Seq of GFP- La^{WT} and GFP- La^{SD} cells. (A) Venn diagram showing that 3,137 mRNAs were differentially bound (1.2-fold or more) in RIPs prepared from GFP- La^{WT} and GFP- La^{SD} cells; 130 mRNAs were more and 3,007 mRNAs were less enriched in GFP- La^{SD} cells. (B) GO analysis revealed terms for biological processes and biological pathways associated with the 3,007 mRNAs that were less enriched by RIPs in GFP- La^{SD} cells than in GFP- La^{WT} cells using ToppFun. The Bonferroni-corrected P values were determined by ToppFun as implemented in the ToppGene Suite.

fore has the potential to impact cellular protein levels at several points. To find support for this assumption, we sequenced the RNA prepared from independent RIP experiments performed with GFP- La^{WT} and GFP- La^{SD} HEK293 cells. As suggested by our earlier study demonstrating that sumoylation of La can increase mRNA binding (42), 3,007 mRNAs were more enriched in GFP- La^{WT} RIPs than in GFP- La^{SD} RIPs (Fig. 10A). Gene ontology (GO) analysis of the sequencing data revealed enrichment of mRNAs annotated in the cell cycle, cell cycle processes, the mitotic cell cycle (Fig. 10B), and, interestingly, ubiquitination (Fig. 10C) preferentially in GFP- La^{WT} RIPs compared to GFP- La^{SD} RIPs. This finding supports our notion that sumoylation of La regulates the binding of mRNAs and that La binds a large number of mRNAs implicated in various cellular pathways intimately linked to cell proliferation and ubiquitination. As we show here, sumoylation of La might modulate the expression of STAT3 by two different mechanisms: (i) binding of STAT3 mRNA, which might facilitate STAT3 mRNA translation, and (ii) averting STAT3 ubiquitination by an indirect mechanism.

DISCUSSION

In recent years, it has become clear that the RNA-binding protein La not only is essential for murine development but also promotes tumorigenesis. Knocking out the La protein in mice or depletion of La in various types of cancer cells has revealed that the La protein is needed for critical cellular processes, including cell proliferation, invasion, and motility. La is overexpressed in several tumor cell types, protects against cisplatin-induced apoptosis, and contributes to tumorigenesis. Nonetheless, the molecular mechanism(s) underlying these cellular outcomes is unclear.

La binds to a variety of RNAs, including RNA polymerase III transcripts, cellular mRNAs, miRNA precursors, and viral RNAs. Depending on the RNA molecules targeted, La supports processing of precursor molecules, regulates RNA translation, or regulates the stability of cellular mRNAs and viral RNAs. Therefore, it is likely that in La knockout or La-depleted cells, many RNA-mediated mechanisms and cellular functions are both directly and indirectly impaired by the decrease in La protein. This view is underscored by our RIP and sequencing (RIP-Seq) data revealing that sumoylation of La regulates its association with mRNAs that play key roles in critical cellular pathways.

In cancer cells, La has been shown to regulate the translation of mRNAs encoding tumor-promoting and antiapoptotic factors (13, 15, 17, 18, 25). Here, we show that STAT3, a transcription factor known to regulate many genes associated with cell proliferation and cell growth, is reduced in HEK293 cells upon endogenous La protein depletion or sumoylation-deficient La mutant (GFP-La^{SD}) overexpression. Although STAT3 mRNA coprecipitates with the La protein and because STAT3 mRNA levels are not affected by GFP-La^{SD} overexpression, STAT3 mRNA translation is only minimally decreased. The modest change in the STAT3-specific polysome gradient profile is unlikely to explain the reduction in STAT3 protein levels observed in GFP-La^{SD}-expressing cells.

Interestingly, the 254-nm elution profiles of GFP-La^{SD}-derived polyribosome gradients demonstrate a larger 80S peak and a smaller polyribosome area compared to a smaller 80S peak and a larger polyribosome area of translational active mRNAs in the GFP-La^{WT}-derived polyribosome gradients. A larger 80S peak may indicate that a significant number of mRNAs are halted at the translational start site and thus not elongating efficiently, as seen in GFP-La^{WT}-derived polyribosome gradients. Based on these observations, we hypothesize that differential binding of mRNAs to GFP-La^{WT} versus GFP-La^{SD} can hinder mRNAs moving into the elongation phase of translation. It has been shown that La is associated with the small ribosomal subunit (49) and that La moves into the polyribosome part of sucrose gradients (32; V. Kota, T. Heise, and G. Sommer, unpublished data), suggesting that La is associated with elongating ribosome complexes. Furthermore, La supports the formation of the 48S complex during translation initiation (12, 50) and preferentially binds RNA oligonucleotides with a start codon (17, 51). Additionally, the RNA chaperone activity of La can destabilize RNA stem-loop structures embedding the translational start site (17, 18, 30). However, the metabolic-labeling experiments did not suggest that global translation is impaired in GFP-La^{SD} cells. Hence, more mechanistic experiments are warranted to unravel the role of La sumoylation during translation initiation/elongation.

Notably, the stability of the STAT3 protein, rather than translation, is reduced in GFP-La^{SD}-expressing cells. Inhibition of proteasome activity stabilized the STAT3 protein and led to an increase in ubiquitinated STAT3 protein levels in GFP-La^{SD}-expressing cells. We conclude that sumoylation of La averts ubiquitination of STAT3. Interestingly, analysis of RIP-Seq data revealed that mRNAs associated with ubiquitination are more enriched in GFP-La^{WT} than GFP-La^{SD} RIPs. There are no previous reports showing or suggesting that La by itself can act as a ubiquitin ligase, so we presume that La indirectly facilitates STAT3 ubiquitination, which is underscored by the increase in overall ubiquitination found in extracts from GFP-La^{SD} cells compared to extracts prepared from GFP-La^{WT} cells.

Proteomic analyses indicated that STAT3 can be ubiquitinated at lysines 87, 97,

177, 180, 244, 294, and 709 (https://www.nextprot.org/entry/NX_P40763/proteomics). The ubiquitin E3 ligases TRAF6 and PDLIM2 have been shown to lead to ubiquitination and degradation of STAT3 (52, 53). Thus, it will be of high interest to study whether sumoylation of La changes the expression or activity of these or other E3 ubiquitin ligases.

Although the exact mechanism by which sumoylation of La impacts proteasome-dependent degradation of STAT3 remains to be determined, we consider this finding highly significant. Indeed, our RIP-Seq data suggest that sumoylation of La plays an important role in regulating the ubiquitin/proteasome pathway. This effect is likely not limited to STAT3, and hence, the La sumoylation status likely also impacts the expression of other proteins through this mechanism. Future studies will be needed to determine the full extent of proteins regulated by this mechanism and to analyze how sumoylation of La affects the ubiquitin/proteasome pathway.

MATERIALS AND METHODS

Cell culture. HEK293 cells were purchased from the ATCC (Manassas, VA). The cells were cultured in advance in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM L-glutamine (Life Technologies) and 9% fetal bovine serum (FBS) (Atlanta Biologicals). Stable HEK293 cell lines were generated by transfecting cells with GFP, GFP-La^{WT}, and GFP-La^{SD} (La^{K41/200R}) using FuGene HD transfection reagent (Promega) and by neomycin selection. GFP-positive cells were enriched by cell sorting, and low-passage-number cells were stored in liquid nitrogen. La-specific shRNA-mediated depletion was performed with lentiviral (LV) expression vectors as described previously (17). Transduction of MISSION shRNA constructs targeting the coding sequence of La mRNA (TRCN0000062193 and TRCN0000062195; Sigma) significantly reduced La expression (>80%) compared to control shRNA (shC) (Sigma). shC was used for all shRNA LV transduction experiments according to the manufacturer's instructions. For STAT3 overexpression, the control (pEGFP-C1) or the RFP-STAT3 (Sino Biological Inc.) plasmid was transiently transfected into La-depleted or GFP-La^{SD}-expressing cells using FuGene HD transfection reagent (Promega).

RIP. RIP experiments were performed with GFP-La^{WT} and GFP-La^{SD} cells as described previously (42). The cells were washed with ice-cold PBS and lysed by incubating them for 15 min at 4°C on a tube rotator with lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% IGEPAL CA-630, 10% glycerol, 1 mM EDTA, 50 mM NaF, and 1 mM dithiothreitol [DTT]) supplemented with RNase inhibitors and protease inhibitors. The cell lysate was cleared by sonication (10 times for 3 s each time at power 3 using a Sonic Dismembrator model 100) and centrifugation at 14,000 × *g* at 4°C for 20 min. The cleared lysate was incubated with anti-GFP magnetic beads (MBL International) overnight on an orbital rotor at 4°C. The beads were washed four times with wash buffer 1 (50 mM Tris-HCl, pH 7.4, 300 mM NaCl, 0.05% IGEPAL CA-630, 20 mM EDTA, 1 mM DTT, and 1 mM MgCl₂) and three times with wash buffer 2 (50 mM Tris-HCl, pH 7.4, 300 mM NaCl, 0.05% IGEPAL CA-630, 20 mM EDTA, 1 mM DTT, 1 mM MgCl₂, and 1 M urea). For preparation of RNA, the beads were resuspended in wash buffer 2, and RNA was extracted by the phenol-chloroform-isoamyl alcohol method. Phenol-chloroform-isoamyl alcohol (25:24:1 [vol/vol/vol]; Sigma-Aldrich) was added, and the sample was vortexed and heated for 10 min at 65°C. After centrifugation for 20 min at 17,000 × *g* at room temperature, the aqueous phase was transferred to a fresh tube, and 1 μl GlycoBlue (Ambion) and 600 μl isopropanol were added. The sample was vortexed and precipitated overnight at −20°C and centrifuged for 20 min at full speed at 4°C, and the RNA pellet was washed once with 800 μl cold 70% ethanol. The RNA pellet was dissolved in RNase-DNase-free H₂O, quantitated with a NanoDrop spectrophotometer, and subjected to RIP-Seq and RT-qPCR analysis.

RIP-Seq analysis. The integrity of the input RNA and the immunoprecipitated RNA was verified on an Agilent 2200 TapeStation (Agilent Technologies, Palo Alto, CA). RNA was used to prepare RNA-Seq libraries using the TruSeq RNA Sample Prep kit (Illumina, San Diego, CA) following the protocol described by the manufacturer. Paired-end (2 times for 125 cycles each time) sequencing was performed on an Illumina HiSeq2500. Samples were demultiplexed using CASAVA (Illumina, San Diego, CA). Each sample was sequenced to a minimum depth of ~50 million reads. Data were subjected to Illumina quality control (QC) procedures (>80% of the data yielded a Phred score of 30). Fastq files were used for downstream analysis. Secondary analysis was carried out on an OnRamp Bioinformatics genomics research platform (OnRamp Bioinformatics, San Diego, CA). OnRamp's advanced genomics analysis engine utilized an automated transcriptome sequencing (RNA-seq) workflow to process the data, including (i) data validation and quality control; (ii) read alignment to the human genome using TopHat2, which revealed >93% mapping of the paired-end reads; (iii) generation of gene level count data with HTSeq; and (iv) differential expression analysis with DESeq2, which normalized sample differences and enabled the inference of differential signals with robust statistical power (Genomics Research Platform with RNA-seq workflow v1.0.1, including FastQValidator v0.1.1a, Fastqc v0.11.3, Bowtie2 v2.1.0, TopHat2 v2.0.9, HTSeq v0.6.0, and DESeq v1.8.0). The mRNA counts were normalized based on the total counts. Transcript count data from differential analysis of the samples were sorted according to their fold enrichments. Systems level analyses were performed using ingenuity pathway analysis (IPA) (Qiagen Inc.) and the transcriptome, ontology, phenotype, proteome, and pharmacome annotations-based gene list functional enrichment analysis (ToppFun) (54).

Quantitative PCR. RNA was isolated, quantified, and reverse transcribed with an RT² first-strand synthesis kit (Qiagen) for quantitative PCR. The cDNA samples were diluted with water, and qPCR was performed on the Bio-Rad iCycler-iQ or Bio-Rad CFX384 PCR system using the RT² SYBR green fluor qPCR master mix (Qiagen) and qPCR primers (Qiagen) for STAT3 (PPH00708F) and GAPDH (PPH00150F). For each primer set, a standard plot was constructed using a known concentration of template in the qPCR analysis, enabling us to calculate the mRNA copy number.

RIP-RNA pellets from GFP-La and GFP were tested for the presence of STAT3 mRNA. Enrichment was calculated from standard curves for specific mRNAs using the RT² qPCR primer assays (Qiagen) as described previously (42). The Qiagen PH00708F qPCR primer set was used to detect STAT3 mRNA. Immunoblotting was performed using anti-GFP antibodies (Roche) to assess the pulldown efficiency of GFP-tagged La and a GFP control in the RIP assay.

Proliferation and cell cycle analyses. For proliferation analyses, cells were plated on 96-well dishes and stained with fluorescent dye (CyQ; Life Technologies) at different time intervals, and fluorescence was recorded with a microplate reader. Cell cycle analysis was performed by fixing the cells in ethanol and incubating them with 50 mg/ml propidium iodide and 50 mg/ml RNase A, followed by analysis on a FACScalibur flow cytometer (BD Biosciences).

[³⁵S]methionine labeling. Cells were plated the day before labeling and grown to 60 to 80% confluence, washed three times with methionine-free DMEM, and incubated with 250 mCi/ml [³⁵S]methionine-containing medium for 30 or 60 min. After incubation, the cells were washed twice with cold PBS and lysed with appropriate lysis buffer. The lysates were analyzed by SDS-PAGE. The gels were stained with Coomassie blue and exposed to a phosphorimager screen.

Polyribosome fractionation. The polyribosome fractionation technique has been described previously (17). For sucrose gradient centrifugation, 100 μ g/ml cycloheximide was added to the culture medium for 5 min. The cells were washed, harvested in lysis buffer (15 mM Tris-HCl, pH 7.4, 15 mM MgCl₂, 300 mM NaCl, 0.1% Triton X-100, 200 units/ml RNasin [Promega], 0.1% mercaptoethanol, 10 μ g/ml cycloheximide, including protease inhibitors), incubated for 10 min at 4°C, and centrifuged for 10 min at 4°C at 9,300 $\times$ g. The supernatant was loaded onto sucrose gradients (17.5 to 50%) and ultracentrifuged at 35,000 rpm in an SW40 rotor for 3 h. The gradients were fractionated, and RNA was extracted using the phenol-chloroform-isoamyl alcohol method, precipitated, and resuspended in water. Concentrations were measured using a NanoDrop, and the RNA quality was analyzed using a Bioanalyzer instrument. Equivalent volumes of each fraction were processed for RT, followed by qPCR as outlined above using STAT3 and GAPDH primers.

Protein stability analyses. To study the stability of the STAT3 protein, cells were incubated with 20 μ g/ml cycloheximide (Sigma, St. Louis, MO) to inhibit protein synthesis. Cells were harvested 0 to 12 h later and lysed with Laemmli buffer. The STAT3 levels in these lysates were quantified by immunoblotting (see below). To assess the effects of proteasome inhibitors on STAT3 stability, cells were incubated with 10 μ M MG132 for 0 to 12 h, followed by lysis and immunoblotting as described above.

IP. Cells were incubated in lysis buffer I (10 mM Tris-HCl, pH 7.8, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1% SDS) supplemented with protease and phosphatase inhibitors, and then samples were boiled for 10 min to completely denature the proteins and disrupt noncovalent interactions. The lysates were diluted in 900 μ l of lysis buffer II (10 mM Tris-HCl, pH 7.8, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, and a protease inhibitor mixture) and then incubated with primary antibody at 4°C for 1 h. The immunocomplexes were recovered by incubating with Dynabeads protein G (Thermo Fisher Scientific) for 1 h, and the beads were washed four times with lysis buffer II. Proteins were subjected to SDS-PAGE, followed by immunoblotting.

To detect endogenous La sumoylation, cells were incubated with or without NEM (10 mM) for 10 min, and immunoprecipitation was performed as described above with anti-La antibody (SW5) and immunoblotted with anti-SUMO2/SUMO3 antibody. To detect sumoylation of GFP-tagged La, GFP-La^{WT} and GFP-La^{SD} cells were treated with NEM (10 mM) for 10 min and immunoprecipitated with GFP-tagged magnetic beads (MBL International) as described above, followed by immunoblotting with anti-SUMO1 antibody and anti-SUMO2/SUMO3 antibody. Immunoblotting with anti-La antibody was used to confirm immunoprecipitation of GFP-tagged La.

To study STAT3 ubiquitination, GFP-La^{WT} and GFP-La^{SD} cells were transfected with STAT3-Flag (Cedarlane, Burlington, NC) and HA-ubiquitin expression plasmids (Addgene plasmid 18712 [a gift from Edward Yeh {55}]) using FuGene HD transfection reagent (Promega). Twenty-four hours thereafter, the cells were treated with 10 μ M the proteasome inhibitor MG132 for an additional 3 h or not treated, followed by cell lysis and STAT3 immunoprecipitation using anti-Flag antibodies and anti-HA-ubiquitin antibodies for immunoblotting.

Immunoblotting. Proteins were resolved by SDS-PAGE and transferred overnight onto a polyvinylidene difluoride (PVDF) membrane at 20 V using a wet-transfer system (Bio-Rad). The membranes were stained with 0.1% (wt/vol) Ponceau S to confirm equal loading and transfer of proteins. The membranes were then blocked with 5% (wt/vol) bovine serum albumin (BSA) in TBST (20 mM Tris buffer, 137 mM NaCl, and 0.1% [vol/vol] Tween 20, pH 7.6) for 1 h at room temperature, washed, and incubated with a primary antibody (1:5,000) prepared in TBST containing BSA (1% [wt/vol]) overnight at 4°C. The membranes were then washed four times with TBST for 5 min each time, followed by incubation with an appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (1:10,000) in TBST containing BSA (1% [wt/vol]) for 1 h at room temperature. Protein bands were detected using a SuperSignal West Dura extended-duration substrate (Thermo Scientific). The following antibodies were used: anti-La 3B9 (18), anti-La (Thermo Scientific), anti-GFP (Roche and Thermo Fisher), anti-GAPDH (Santa Cruz), anti-

HSP70 (Santa Cruz), anti-STAT3 (Cell Signaling), anti-HA (Enzo, Farmingdale, NY), and anti-Flag (Santa Cruz).

Statistical analysis. Two-tailed *P* values were determined by *t* test using Prism 5 (GraphPad Software).

Accession number(s). The RIP-Seq data have been submitted to the NCBI Gene Expression Omnibus under accession number [GSE102842](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE102842).

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