Phosphorylation of Tudor-SN, a novel substrate of JNK, is involved in the efficient recruitment of Tudor-SN into stress granules

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Posttranslational modifications of certain stress granule (SG) proteins are closely related to the assembly of SGs, a type of cytoplasmic foci structure. Our previous studies revealed that the Tudor staphylococcal nuclease (Tudor-SN) protein participates in the formation of SGs. However, the functional significance of potential Tudor-SN modifications during stress has not been reported. In this study, we demonstrated that the Tudor-SN protein was phosphorylated at threonine 103 (T103) upon stimulation with arsenite. In addition, c-Jun N-terminal kinase (JNK) was found to be responsible for Tudor-SN phosphorylation at the T103 site. We further illustrated that either a T103A mutation or the suppression of phosphorylation of T103 by the JNK inhibitor SP600125 inhibited the efficient recruitment of Tudor-SN into SGs. In addition, the T103A mutation could affect the physical binding of Tudor-SN with the G3BP (Ras-GAP SH3 domain-binding protein) protein but not with the HuR (Hu antigen R) protein and AGTR1-3′UTR (3′-untranslated region of angiotensin II receptor, type 1) mRNA cargo. These data suggested that JNK-enhanced Tudor-SN phosphorylation promotes the interaction between Tudor-SN and G3BP and facilitates the efficient recruitment of Tudor-SN into SGs under conditions of sodium arsenite-induced oxidative stress. This finding provides novel insights into the physiological function of Tudor-SN modification.

Abbreviations: SG, stress granule; Tudor-SN, Tudor staphylococcal nuclease; JNK, c-Jun N-terminal kinase; PPK, Protein kinase R; PERK, PKR-like endoplasmic reticulum kinase; GCN2, general control nonderepressible 2; HRK, hemi-regulated inhibitor kinase; eIF2α, eukaryotic translation initiation factor 2 alpha; eIF4E, eukaryotic translation initiation factor 4E; mTORC1, mammalian target of rapamycin complex 1; SNDR1, staphylococcal nuclease domain containing 1; AGTR1-3′UTR, 3′-untranslated region of angiotensin II receptor, type 1; PP1, protein phosphatase 1; λ-PPase, λ-protein phosphatase; KLH, keyhole limpet hemocyanin; MAPK, mitogen activated protein kinase; CDKs, cyclin-dependent protein kinases; G3BP, Ras-GAP SH3 domain-binding protein; HuR, Hu antigen R; TTP, tristetraprolin; CDC2, cell division control 2; TCA, total cell lysates; LC-MS/MS, Liquid chromatography-tandem mass spectrometry; MALDI-TOF MS, Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; IF, Immunofluorescence; RIP, RNA-binding protein immunoprecipitation; SE, standard error; ANOVA, one-way analysis of variance.

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1. Introduction

In response to environmental stress (e.g., oxidative stress, heat shock, UV irradiation, viral infection or hyperosmotic stress), eukaryotic cells often shut down translation initiation, leading to the formation of cytoplasmic RNA foci, which are known as stress granules [1]. SGs contain non-translating mRNAs and stalled pre-initiation complexes, as well as some RNA-binding proteins, and are proposed to regulate the translation efficiency and stability of mRNA under stress conditions [1, 2]. SG assembly is considered to be a consequence of signaling cascades that are activated by environmental stress [3–5]. A crucial factor among the signaling cascades is posttranslational protein modification (particularly phosphorylation), which efficiently regulates the primary aggregation or dynamic assembly of SGs [3–5]. For example, one or more of the serine/threonine kinases, such as protein kinase R (PKR), PKR-like endoplasmic reticulum kinase (PERK), general control nonderepressible 2 (GCN2) and heme-regulated inhibitor kinase (HRI), can induce the phosphorylation of eukaryotic translation initiation factor 2 alpha.
Our previous studies revealed that Tudor-SN, a crucial component of different stress conditions [15]. Tudor-SN is also important for the aggregation type 1 breast cancer metastasis, cell cycle and stress granule assembly [10–15]. Our previous studies revealed that Tudor-SN, a crucial component of stress granules (SGs) in various cellular processes, such as gene transcription, adipogenesis, pre-mRNA splicing, oxygen deprivation, and acetylation, ubiquitination and methylation also affect SG formation through different mechanisms [8,9].

The Tudor staphylococcal nuclease (Tudor-SN) protein, also known as SND1 (staphylococcal nuclease domain containing 1) or p100, is a kind of multifunctional protein that is implicated in a variety of cellular mechanisms [8–10]. Tudor-SN is also important for the aggregation type 1 breast cancer metastasis, cell cycle and stress granule assembly [8–10].

In the present study, Tudor-SN was identified as a novel c-Jun N-terminal kinase (JNK) target. Tudor-SN can be phosphorylated by JNK at T103, which regulates the dynamic nature of G3BP-positive SGs and the stabilization of SG-associated mRNAs during cellular stress [17]. Here, we further investigate the molecular mechanism of Tudor-SN-containing SG assembly in terms of Tudor-SN protein phosphorylation.

In addition, the phosphorylation of T103 facilitates the efficient aggregation of Tudor-SN into SGs by promoting the binding of Tudor-SN with G3BP.

2. Materials and methods

2.1. Cell culture, plasmids and transfection

HeLa cells were grown in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum. The sodium arsenite (Sigma Aldrich, Aldrich, St Louis, MO, USA), JNK inhibitor SP600125 (S146001, Selleck Chemicals), protein phosphatase 1 (PP1, P0754, New England Biolabs) or λ-protein phosphatase (λ-Pase, P0753, New England Bio-labs) were used to treat the cells or the total cell lysates (TCLs). A HeLa stable cell line with Tudor-SN-Knockout (Tudor-SN−/−) was generated via a modified CRISPR/Cas9 double-nicking gene editing system [18,19]. Briefly, a pair of A and B sgRNAs (A: 5′-ACACGGAG GTTGATCGCCTAGCG-3′; 3′-CTCCAAAC TAGACGGCGGATCCTC-5′; B: 5′-CACCGTGCGAATACCTTC GCTGCG-3′; 3′-CGCACTTATAGCAGACGCGAAA-5′) that could specifically identify the upstream and downstream sequence within exon 2 of the Tudor-SN gene were designed and synthesized. Two recombinant eukaryotic expression plasmids (pX462-Tudor-SN-sgRNA-A, pX462-Tudor-SN-sgRNA-B) were constructed from the pX462 carrier vector (pX462-Tudor-SN-sgRNA-A, pX462-Tudor-SN-sgRNA-B). After enzyme digestion with Bsal (BD1014, Fermentas) and gene sequencing, the two recombinant plasmids were co-transfected into HeLa cells. Puromycin was then used to screen positive cells and prepare the monolayer Tudor-SN−/− HeLa cell line. The plasmids encoding RFP-tagged wild-type Tudor-SN (RFP-Tudor-SNwt) or FLAG-tagged wild type Tudor-SN (FLAG-Tudor-SNwt) were generated as described previously [15]. The FLAG-Tudor-SN (T103A) and RFP-Tudor-SN (T103A) mutant plasmids were constructed by the GENEWIZ Company (China). The GFP-G3BP plasmid was kindly provided by Dr. Jamal Tazi (Montpellier, France). Plasmids were transfected into HeLa cells using Lipofectamine 2000 transfection reagent (11668-019, Invitrogen), according to the manufacturer’s protocols.

2.2. Antibody preparation

Antibody polyclonal antibodies against pT73 or pT103 were produced by BEIJING B&M BIOTECH CO., LTD (China). Briefly, an MBL antigen retrieval system was utilized to predict the secondary structure features of the Tudor-SN region around the T73 and T103 site, including accessibility, flexibility, surface probability, antigenicity, hydrophilicity and dipole moment. Then, the unphosphorylated and phosphorylated polypeptides, including “PDADKDpTPDEPC” for T73 and “T1ENKpTPQCGR” for T103, were designed and synthesized. The C-terminal C (cytochrome) was used to efficiently bind the carrier protein, keyhole limpet hemocyanin (KLH). Antiserums were collected from New Zealand White rabbits immunized with these polypeptides four times and purified through the AKTA protein purification system (GE Healthcare Life Sciences).

2.3. LI-COR odyssey infrared imaging system

HeLa cells were untreated or treated with 0.5 mM sodium arsenite for 1 h. Total cell lysates (TCLs) of HeLa cells were collected and separated by SDS-PAGE. The PVDF membranes (Roche) were incubated with the rabbit polyclonal anti-Tudor-SN antibody (1:500 dilution, ab65078, Abcam) and mouse monoclonal anti-β-actin antibody (1:3000, A1978, Sigma Aldrich) in 10 ml Li-COR blocking buffer with gentle agitation overnight at 4 °C. The next day, the membranes were washed three times for 10 min each with 15 ml Tri-buffered saline containing 0.1% Tween (TBS-T) before addition of the secondary antibodies conjugated to a fluorescent entity, IRDye® 800CW donkey anti-Mouse IgG (H + L) (1:15000, 926-32212) and IRDye® 680 donkey anti-rabbit IgG (H + L) (1:15000, 926-32223), for 1 h at room temperature. After three washes with TBS-T, the membrane was visualized and analyzed by Odyssey IR imaging system (LI-COR Biosciences).

For the total phosphorylation of Tudor-SN, TCLs were incubated with the mouse monoclonal anti-Tudor-SN antibody bound to protein G/A agarose (20421, Pierce) overnight at 4 °C with head-over-tail rotation. The immunoprecipitated Tudor-SN proteins were separated by SDS-PAGE and blotted with the rabbit anti-Tudor-SN antibody (1:500, ab65078, Abcam) and mouse monoclonal anti-p-p38 (phospho-threonine) (1:1000, #9211, Cell Signaling Technology), mouse monoclonal anti-p-pTyr (phospho-tyrosine) (1:1000, #4970, Cell Signaling Technology), or mouse monoclonal anti-p-Ser (phospho-serine) antibody (1:1000, ab17465, Abcam). The mouse monoclonal anti-Tudor-SN antibody was used as described previously [15].

For eIF2α phosphorylation, membranes were incubated simultaneously with rabbit monoclonal anti-phospho-eIF2α (Ser51) (1:1000, #3986, Cell Signaling Technology) and mouse monoclonal anti-eIF2α antibody (1:1000, #2103, Cell Signaling Technology). For T73 or T103 phosphorylation of Tudor-SN, membranes were incubated simultaneously with the mouse anti-Tudor-SN antibody (1:1000, sc-271590, Santa Cruz Biotechnology) and rabbit anti-pT73 or anti-pT103 antibody (1:1000).

2.4. Western blotting and antibodies

A western blotting assay was performed as previously described [14]. The following antibodies were used: rabbit polyclonal anti-pT73 or anti-pT103 (1:1000), mouse monoclonal anti-Tudor-SN (1:5), anti-p-JNK (1:1000, #9251, Cell Signaling Technology), anti-JNK (1:1000, #9252, Cell Signaling Technology), anti-p-p38 (1:1000, #4511, Cell Signaling Technology), anti-p-ERK (1:1000, #9102, Cell Signaling Technology), anti-p-ERK (1:1000, #9101, Cell Signaling Technology), anti-mTOR (1:1000, #2972, Cell Signaling Technology), anti-p-mTOR (1:1000, #2971, Cell Signaling Technology), and mouse monoclonal anti-β-actin antibody (1:3000). The grey scale value of the band was measured using Image J2X software (NIMH, Bethesda, MD, USA).
2.5. Co-immunoprecipitation (Co-IP)

A Co-IP assay was performed as previously described [15]. Briefly, TCLs of HeLa cells were incubated with specific antibodies bound to protein G/A agarose (20421, Pierce), including the mouse monoclonal anti-Tudor-SN antibody, mouse anti-IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), or mouse monoclonal anti-FLAG M2 covalently attached with agarose (A2220, Sigma Aldrich) at 4 °C for 12 h with head-over-tail rotation. Bound proteins were subjected to SDS-PAGE and detected by blotting with the corresponding antibodies. The following antibodies were used: mouse monoclonal anti-p-Thr (phosphothreonine) (Cell Signaling Technology), rabbit polyclonal anti-pT103 (1:1000), mouse monoclonal anti-FLAG (1:1000, F-1804, Sigma Aldrich), rabbit anti-p-JNK (Cell Signaling Technology), anti-JNK (Cell Signaling Technology), anti-p-38 (Cell Signaling Technology), anti-p38 (Cell Signaling Technology), mouse anti-G3BP (1:1000, ab56574, Abcam), and rabbit monoclonal anti-HuR antibody (1:1000, 07-1735, Millipore).

2.6. Dot blotting assay

The phosphorylated and unphosphorylated pT103 peptides (1 μg, 2 μg, 3 μg, 4 μg), and TCLs of HeLa cells under a normal (Control) or arsenite-treated stress (Arsenite) condition (2 μg, 4 μg, 6 μg, 8 μg) were spotted onto the PVDF membrane (Roche). After blocking with 5% BSA (Sigma Aldrich), the membrane was incubated with rabbit anti-pT103 (1:1000), mouse monoclonal anti-FLAG (1:1000, F-1804, Sigma Aldrich), rabbit anti-p-JNK (Cell Signaling Technology), anti-JNK (Cell Signaling Technology), anti-p-38 (Cell Signaling Technology), anti-p38 (Cell Signaling Technology), mouse anti-G3BP (1:1000, ab56574, Abcam), and rabbit monoclonal anti-HuR antibody (1:1000, 07-1735, Millipore).

2.7. Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

LC-MS/MS analysis for identification of Tudor-SN phosphorylation site was performed according to a previously published protocol [20]. Briefly, the protein samples were separated by SDS-PAGE, and the targeting bands were excised and digested with trypsin (Sigma Aldrich). The molecular masses of the peptide mixtures were determined by Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). The molecular masses of the tryptic peptides of the ~100 kDa protein were used to search the OWL protein sequence database for candidate proteins using the ProFound program.

2.8. Immunofluorescence (IF) assay

The IF assay was performed as previously described [17]. Briefly, HeLa cells were fixed, permeabilized, and then incubated with specific primary antibodies, including goat polyclonal anti-Tudor-SN (1:80, sc-34753, Santa Cruz Biotechnology), mouse monoclonal anti-Tudor-SN (1:2), rabbit polyclonal anti-pT103 (1:100), rabbit polyclonal anti-pT73 (1:100), and mouse monoclonal anti-G3BP (1:100, ab56574, Abcam). After washing, cells were incubated with fluorescent dye-conjugated secondary antibodies at 4 °C overnight. The following secondary antibodies were used: Alexa Fluor 488-coupled donkey anti-rabbit IgG (H + L) antibody (1:800, A21026, Molecular Probes), Donkey anti-goat IgG (H + L) (TR) antibody (1:800, ab6883, Abcam), and goat anti-mouse IgG H&L (Chromo™ 546) (1:800, ab60316, Abcam). The images were collected using an Olympus FV1000 confocal microscope (Japan) or Inverted Research Microscope Leica DMi6000 B (Germany). To determine the specificity of staining, 3 μg phosphorylated pT103 or pT73 peptides were first incubated with 10 μl the antibody to pT103 or anti-T73 antibody at 4 °C overnight, and the IF assay was then performed. The number and size of the granules were measured by the analysis particle tool in Image J 2X.

2.9. In vitro kinase assay

HeLa cells were transfected with FLAG-Tudor-SN or FLAG-Tudor-SN*-SNT103A* plasmids. FLAG-tagged Tudor-SN fusion proteins were then immuno-purified with mouse monoclonal anti-FLAG M2 covalently attached with agarose (A2220, Sigma Aldrich) and incubated with 30 U JNK (#9219, Cell Signaling Technology) or p38 kinase (#9221, Cell Signaling Technology) in the presence of 1 mM ATP for 30 min at 30 °C. As the positive control, c-Jun (2 μg, #6093, Cell Signaling Technology) or ATF-2 (2 μg, #9224, Cell Signaling Technology) fusion proteins were incubated with 30 U JNK or p38 kinase, respectively. The reaction was quenched by the addition of 5 × SDS loading buffer. Reaction products were detected by immuno-blotting analysis using anti-p-c-Jun (1:1000, #12598, Cell Signaling Technology), rabbit anti-pT103 (1:1000), rabbit anti-p-JNK (1:1000, Cell Signaling Technology), anti-p-ATF-2 (1:1000, #9221, Cell Signaling Technology), and anti-p-p38 (1:1000, #4511, Cell Signaling Technology). The membrane was stripped and re-blotted with the mouse anti-FLAG antibody (1:1000, F-1804, Sigma Aldrich) to assess the immuno-purified FLAG fusion proteins.

2.10. RNA-binding protein immunoprecipitation (RIP) assay

HeLa cells (2.0 × 10^6) were transfected with the FLAG-Tudor-SN wild-type or FLG-FLAG-Tudor-SN*-SNT103A* plasmids. A RIP assay using mouse monoclonal anti-FLAG M2 covalently attached with agarase (Sigma Aldrich) was performed as previously reported [16]. The end-point normal PCR assay was performed to detect the presence of precipitated AGTR1-3′ UTR with the specific primers, as described previously [16].

2.11. Statistical analysis

The data were presented as the means ± standard error (SE). Student’s t-test of independent samples and a one-way analysis of variance (ANOVA) followed by a multiple mean comparisons Student-Newman-Keuls test were performed using SPSS 16.0 software. A value of P < 0.05 was considered to be statistically significant.

3. Results

3.1. Arsenite stimulation enhances the phosphorylation level of the Tudor-SN protein

Protein phosphorylation has been reported to be involved in the regulation of SG formation during stress [3–5]. Previously, we identified Tudor-SN as a component of SGs [15]. In the present study, we are interested in exploring the potential role of protein phosphorylation in the aggregation of Tudor-SN into SGs. Due to the lack of a phosphorylation antibody that could recognize the specific site of Tudor-SN, we detected the total phosphorylation level of enriched Tudor-SN on the tyrosine, threonine or serine site. The antibodies specific for phospho-tyrosine (p-Tyr), phospho-threonine (p-Thr) and phospho-serine (p-Ser) were used in the LI-COR odyssey infrared imaging system, which allows for the simultaneous visualization of the total and possible phosphorylated targeting proteins in one membrane [21]. Tudor-SN proteins were immunoprecipitated with an anti-Tudor-SN antibody from HeLa cells treated with sodium arsenite (Arsenite) or not (Control). The results in Fig. 1A–F indicated that all of the tyrosine, threonine and serine phosphorylation levels of endogenous Tudor-SN were higher with arsenite stimulation than that observed in the Control group, and the phosphorylation signal band was slightly higher than the immunoprecipitated Tudor-SN protein band. The band signal value was measured, and the phosphorylation level of threonine in the Arsenite group was ~7-fold higher than in the Control group (Fig. 1D). However, arsenite stimulation failed to affect the expression level of endogenous Tudor-SN and β-actin proteins (Fig. 1G). In addition, consistent results were obtained...
from the western blotting assays (Fig. S1). It is well known that arsenite stimulation induces SG formation dependent on the phosphorylated elf2α protein at serine 51 [3,4]. In our study, we observed that the phosphorylation level of elf2α (Serine 51) was significantly higher in the Arsenite group than in the Control group (Fig. 1H). Here, we revealed that the phosphorylation level of the Tudor-SN protein was enhanced in response to sodium arsenite-induced oxidative stress.

3.2. Threonine 103 is the key phosphorylation site of Tudor-SN upon arsenite stimulation

To determine the specific phosphorylation sites of Tudor-SN, we immunoprecipitated the Tudor-SN protein from HeLa cells with or without arsenite treatment and analyzed the potential amino acid sites with phospho-modification using LC-MS/MS analysis. As shown in Fig. S2A–B, phosphorylation of Tudor-SN at threonine 73 (T73) and threonine 103 (T103) was observed in the Arsenite group but not the Control group. We thus targeted these two potential phosphorylation sites and generated the relative phosphorylation antibodies. First, we analyzed the secondary structure features in the Tudor-SN regions around the T73 and T103 sites via an MBL antigen retrieval system and found that a high total antigenic score was observed for T73, suggesting it is prone to exposure on the surface of the native Tudor-SN structure; in contrast, a low score value was obtained for T103, indicating it is prone to exposure on the surface of the native Tudor-SN structure. Then, we synthesized the phosphorylated peptides and immunized New Zealand White rabbits to obtain the rabbit polyclonal antibodies against p73 or pT103. LI-COR odyssey infrared imaging system and western blotting assays were then performed. As shown in Fig. 2A–D, weak band signals of T73 phosphorylation detected by the anti-pT73 antibody showed no difference in both the Control and Arsenite group; however, the anti-pT103 antibody displayed a higher band signal in the Arsenite group than in the Control group. The similar results from the western blotting assays are shown in Fig. S3A–D. IF assays were performed to detect whether the anti-pT73 or anti-pT103 antibody can recognize the Tudor-SN-containing SG structure. No SG signal was observed in the cytoplasm with the anti-pT73 antibody (Fig. 2E). In contrast, cytoplasmic foci signal was detectable with the anti-Tudor-SN (red) and anti-pT103 antibody (green) and presented as the merged yellow color in arsenite-treated cells (Fig. 2F). Therefore, the anti-pT103 antibody was used for subsequent studies.

To determine the specificity of the polyclonal phospho-specific antibodies, we conducted an immunizing peptide blocking experiment and dot blotting assay. As shown in Fig. S3E–F, the fluorescent signal was dramatically reduced for both pT73 and pT103 when the anti-pT73 or anti-pT103 antibodies were incubated with the corresponding peptides. The dot blotting assay results (Fig. 2G) showed that the anti-pT103 antibody was specific for peptides containing the phosphorylated T103 residues (lane 1) and strongly recognized the endogenous T103 in the TCLs of stressed HeLa cells (lane 4) but not the unphosphorylated peptides (lane 2). Furthermore, the data shown in Fig. 2H indicated that, compared with the Control group (lane 2–3), arsenite stress led to a greatly enhanced phosphorylation level of purified FLAG-Tudor-SNWT (lane 4) at the T103 or total threonine sites. In addition, this trend could be inhibited by treatment with protein phosphatase 1 (PP1) and λ-protein phosphatase (λ-PPase), which can remove the phosphogroup from residues (lane 5). These data provided evidence suggesting that arsenite stimulation increases the phosphorylation of the Tudor-SN protein at the T103 site (Tudor-SN\textsuperscript{T103}), which can be recognized by our anti-pT103 antibody.

3.3. JNK binds and phosphorylates Tudor-SN at the T103 site

Next, the PhosphoNET database was used to predict the potential upstream kinases responsible for the phosphorylation of Tudor-SN\textsuperscript{T103}. After categorizing the results, three main types of kinases that possessed
high kinase predictor scores were obtained: the mammalian target of rapamycin (mTOR), mitogen activated protein kinase (MAPK) and cyclin-dependent protein kinases (CDKs) (Table 1). It was reported that arsenite potently activates both the MAPK and mTOR signaling pathway [7,22]. To analyze which of the kinases of these pathways are activated and induce the phosphorylation of Tudor-SNT103 under arsenite stimulation, we performed a western blotting assay to detect the phosphorylation level of MAPK (JNK, p38, ERK) and mTOR kinases in HeLa cells stimulated with arsenite (Arsenite) or left untreated (Control). As shown in Fig. S4, compared with that in the Control group, increased phosphorylation levels of JNK, p38 kinases and mTOR but not ERK were observed in the Arsenite group. Due to the weak effect and the limitations of the experimental conditions, the role of mTOR kinases was not investigated in the present study. Thus, we carried out in vitro kinase assays to investigate whether activated JNK and p38 can directly phosphorylate the Tudor-SN protein at the T103 site. The FLAG-tagged Tudor-SNwt fusion protein was ectopically expressed and immunopurified from cells with the anti-FLAG antibody and mixed with JNK-ATP or p38-ATP complexes. The phosphorylation of Tudor-SN was detected by the anti-T103 antibody. c-Jun or ATF-2 proteins were used as positive controls. As shown in Fig. 3A, both Tudor-SNwt and TCLs of HeLa cells under normal or arsenite-treated stress condition (2 μg, 4 μg, 6 μg, 8 μg) were spotted on the film and blotted with the anti-pT103 or anti-Tudor-SN antibodies.

**Fig. 2.** The anti-T103 antibody recognizes the SG structure. HeLa cells were untreated (Control) or treated with 0.5 mM arsenite sodium for 1 h (Arsenite). (A-D) The LI-COR odyssey infrared imaging system was used to detect the phosphorylation level of Tudor-SN at T73 and T103, using the prepared rabbit anti-pT73 or anti-pT103 antibodies. (E-F) IF assays were also performed using the anti-pT73 or anti-pT103 antibodies. Scale bar, 10 μm. (G) The phosphorylated (phospho.), un-phosphorylated (unphospho.) pT103 peptides (1 μg, 2 μg, 3 μg, 4 μg) and TCLs of HeLa cells under normal or arsenite-treated stress condition (2 μg, 4 μg, 6 μg, 8 μg) were spotted on the film and blotted with the anti-pT103 or anti-Tudor-SN antibodies. (H) HeLa cells were transiently transfected with the FLAG-Tudor-SNwt plasmid, and the TCLs were treated with 0.5 mM arsenite sodium and PP1/λ-PPase for 1 h, either separately or together, as indicated. In addition, TCLs from transfected HeLa cells were purified with anti-FLAG beads, subjected to SDS-PAGE and then blotted with anti-p-Thr, anti-p-T103 or anti-FLAG antibodies.
that some FLAG-Tudor-SN\textsuperscript{T103} fusion proteins were phosphorylated in HeLa cells in vivo. Unlike JNK, p38 could effectively phosphorylate the positive control ATF-2 (Fig. 3B, lane 3) but not the Tudor-SN\textsuperscript{T103} (lane 1).

In addition, we performed Co-IP assays to detect the interaction between Tudor-SN and JNK or p38 under treatment with arsenite or not. Total cell lysates were immunoprecipitated with the anti-Tudor-SN antibody or anti-IgG antibody as a negative control and then detected with the indicated antibodies. The results (Fig. 3C) showed a higher binding of Tudor-SN to p-JNK and JNK (lane 1) upon arsenite treatment than in the untreated group (lane 2). In contrast, there was no detectable association between Tudor-SN and the p-p38/p38 protein under normal and stress conditions (Fig. 3D, lane 1–2). Together, these data indicated that arsenite-activated JNK is involved in the enhancement of the phosphorylation level of Tudor-SN at the T103 site.

3.4. Inactivation of JNK reduces the phosphorylation of Tudor-SN\textsuperscript{T103} and inhibits the recruitment of Tudor-SN into SGs

To further study whether JNK influences the recruitment of Tudor-SN into SGs through phosphorylating Tudor-SN at the T103 site, we performed western blotting and IF assays in HeLa cells treated with the JNK inhibitor SP600125 and arsenite, either separately or together. As shown in Fig. 4A–C, an increased phosphorylation level of JNK and Tudor-SN\textsuperscript{T103} was observed in HeLa cells treated with arsenite for 30 min (lane 3) or 60 min (lane 5) (Fig. 4B–C, *p < 0.01), suggesting that arsenite treatment led to the activation of JNK. However, the high phosphorylation levels of both JNK (lane 4) and Tudor-SN\textsuperscript{T103} (lane 6) in arsenite-treated cells could be reduced by pretreatment with SP600125 (Fig. 4B–C, **p < 0.01), which indicated that the JNK inhibitor SP600125 worked effectively, and Tudor-SN\textsuperscript{T103} is one substrate of JNK. Moreover, the results of the IF assay in Fig. 4D–F showed that the treatment of HeLa cells with arsenite for 30 min could result in the formation of SG structures (marked by anti-G3BP and anti pT103 antibodies, Fig. 4D–F, *p < 0.01), which could be impaired by pretreatment with SP600125. When arsenite treatment lasted 60 min, SP600125 pretreatment decreased the number of arsenite-induced Tudor-SN\textsuperscript{T103} granule-positive cells (Fig. 4E, **p < 0.01) but not G3BP granule-positive cells (Fig. 4F). The JNK inhibitor impaired G3BP-marked SG formation upon arsenite stimulation in the short term, but the impairment disappeared when the stimulation was prolonged. This phenomenon was similar

Table 1

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<td>191</td>
</tr>
<tr>
<td></td>
<td>CDK1</td>
<td>P06493</td>
<td>183</td>
</tr>
</tbody>
</table>

Note: The potential kinases were acquired by clicking on the orange buttons under the “Kinase Pred.” of the interesting sites for T103. After categorizing, three main types of kinases that possessed a high kinase predictor score were obtained. The table lists the predictive kinases, the UniProt ID of the kinase and the “Kinase Predictor V2 score” (the higher the KSPV2 score, the better the prospect that the kinase will phosphorylate the given site).
with the data from Chang, Y. W. [23]. It is possible that other active compensatory pathways take part in the efficient SG assembly in SP600125-pretreated cells under long durations of arsenite treatment. Even so, JNK inhibition was able to prevent Tudor-SN assembling into SGs under arsenite stimulation for both short- and long-duration exposures. JNK-mediated phosphorylation of Tudor-SN T103 promotes the SG-localization of Tudor-SN in arsenite-induced SGs.

3.5. Mutation of T103 decreases Tudor-SN aggregation into SGs through suppressing the interaction between Tudor-SN and G3BP

To further investigate the molecular mechanism underlying the effect of Tudor-SN T103 phosphorylation in SG formation, we constructed mammalian expression mutant plasmids, including FLAG-Tudor-SN T103A and RFP-Tudor-SN T103A, in which the T (threonine) residue at the 103 site was mutated into an A (alanine). To rule out the interference of endogenous Tudor-SN, we also generated a Tudor-SN−/− HeLa stable cell line via a modified CRISPR/Cas9 double-nicking system. The A and B sgRNAs and complementary strands were designed and synthesized targeting regions of Tudor-SN in exon 2 (schematic diagram shown in Fig. 5A). The failure of digestion for pX462-Tudor-SN-sgRNA-A/B recombinant plasmids indicated the successful insertion of the double-stranded oligonucleotide structures (Fig. S5A, lane 5–6). Then, PCR gene sequencing (Fig. S5B), western blotting (Fig. 5B) and an IF assay (Fig. 5C) further demonstrated that the Tudor-SN gene was knocked out in the Tudor-SN−/− HeLa cells.

Then, we co-transfected the RFP-Tudor-SN wt, RFP-Tudor-SN T103A or RFP vector plasmid together with the GFP-G3BP plasmid into Tudor-SN−/− HeLa cells to detect the formation of SGs. As shown in Fig. 5D–E, the T103A mutation increased the percentage of cells without Tudor-SN granules in the normal (from 40% to 78%) and stress (from 15% to 61%) conditions, meanwhile it decreased the percentage of cells with different Tudor-SN granule numbers (0–5, 5–15, 15–30, >30). Furthermore, compared with the wild-type groups, the portion of granule with small size (0–2 μm) increased in the mutation groups (Fig. 5F, from 42% to 71% in the control condition; from 20% to 55% in the stress condition). These results suggested that mutant RFP-Tudor-SN T103A failed to efficiently form the larger SG structure in the majority of Tudor-SN−/− HeLa cells when compared with that in RFP-Tudor-SN wt cells. The presence of the exogenous G3BP-GFP fusion protein is able to form the SG structure within mammalian cells in the absence of a stress condition [3,24]. Similarly, the assembly of GFP-G3BP
granules was also observed in Tudor-SN\(^{-/-}\) HeLa cells in both the Control and Arsenite groups. It is possible that the powerful SG activation of the exogenously expressed G3BP protein and the potential compensatory mechanisms in Tudor-SN\(^{-/-}\) HeLa stable cell lines prevailed over the role of Tudor-SN in SG formation.

Next, we performed the Co-IP assay to analyze whether Tudor-SN\(^{T103A}\) phosphorylation is associated with the binding of Tudor-SN with SG components, including G3BP or HuR. FLAG-tagged Tudor-SN\(^{WT}\) or Tudor-SN\(^{T103A}\) plasmids were transfected into HeLa cells, and the FLAG vector was used as a vector control, as indicated. After the treatment with 0.5 mM sodium arsenite for 1 h (Arsenite) or not (Control), microscopy analysis was performed. Scale bar, 10 μm. (E) The number of granules per cell in RFP-Tudor-SN\(^{WT}\) or RFP-Tudor-SN\(^{T103A}\) cells were measured and divided into five categories, including 0, 0–5, 5–15, 15–30 and >30. (F) Granule size was also measured by particle analysis using Image J 2X and divided into three categories, including 0–2, 2–4 and >4. (G) Tudor-SN\(^{-/-}\) HeLa cells were transfected with FLAG-Tudor-SN\(^{WT}\), FLAG-Tudor-SN\(^{T103A}\) or FLAG vector plasmid, respectively. After treatment with 0.5 mM sodium arsenite for 1 h, a Co-IP assay was performed with monoclonal anti-FLAG agarose. The bound proteins were subjected to SDS-PAGE and blotted with the indicated antibodies. An RIP assay was also performed with a Dynabead-bound mouse anti-FLAG antibody. The precipitated AGTR1-3′ UTR was analyzed using end-point normal PCR. Five percent of the total cell lysates were included as the Input. (H) Scheme depicting the role of T103 phosphorylation in the recruitment of Tudor-SN into SGs.
2). As a control, no band signal was observed in the FLAG vector group (lane 3). Unlike G3BP, the T103A mutation did not influence the binding of Tudor-SN and HuR (lane 2). Additionally, Tudor-SN is required for the assembly of AGTR1-3′UTR granules via the binding of AGTR1-3′UTR during stress [16]. To test whether Tudor-SN T103A phosphorylation influences the interaction of Tudor-SN and AGTR1-3′UTR, we also performed an RIP assay using FLAG-tagged Tudor-SN WT and Tudor-SN T103A plasmids in the stress condition. The result (Fig. S5) showed that no difference was observed in the binding of mutant FLAG-Tudor-SN T103A and AGTR1-3′UTR compared with that of FLAG-Tudor-SN WT. These data demonstrated that phosphorylation of Tudor-SN T103 facilitates the physical interaction of Tudor-SN with G3BP but not with HuR or RNA cargo AGTR1-3′UTR during stress.

4. Discussion

SG assembly is closely associated with activated signaling cascades in response to environmental stress [3–5]. At the apex of these signaling cascades is the arrest of translation initiation, which is induced by eIF2α phosphorylation [6] or the inactivation of the translation initiation factor complex eIF4F [25]. Then, several proteins, such as G3BP, TIAR, tristetraprolin (TTP), possess the abilities of RNA-binding and self-aggregating and can assemble into the stalled pre-initiation complex to induce SG nucleation [24,26,27]. After the original aggregation, various stress-related proteins, such as HuR, will be recruited to further promote the aggregation of SGs [3]. Stress-associated protein phosphorylation modifications exhibit distinct effects on SG formation [3–5,8,9,26,27]. For example, phosphorylation of eIF2α or 4EBP regulates the initiation of SG formation, while phosphorylation of TTP reduces accumulation into SGs [26,27]. The Tudor-SN protein is a component of SGs in many species, such as Homo sapiens, silkworms and plant cells [17,28–30]. Our previous results demonstrated that human Tudor-SN can efficiently interact and co-localize with many SG nucleators [16,17]. Here, we further investigated the role of Tudor-SN phosphorylation in the recruitment of Tudor-SN into SGs.

The reported data on the phosphorylation of Tudor-SN are very limited. Leverston et al. reported that pim-1 kinase binds and phosphorylates Tudor-SN in vitro [31]. Previously, we found that Tudor-SN is phosphorylated by G1 phase Cyclin-dependent kinases (CDKs) at Ser426 and Thr429 to participate in the regulation of the G1-to-S phase transition under normal conditions [14]. Here, we provide evidence that the Tudor-SN protein can be phosphorylated at threonine 103 by JNK under arsenite-induced oxidative stress conditions. Either the mutation of threonine 103 to alanine or the inhibition of Tudor-SN T103 phosphorylation by the JNK inhibitor SP600125 can efficiently prevent Tudor-SN aggregating into SGs but cannot remarkably influence the formation of G3BP-marked SGs. This is in line with our previous report that Tudor-SN participates in SGs but is not an initiator as essential as G3BP for SG formation [15]. In addition, T103A mutation could reduce the interaction between Tudor-SN and G3BP, rather than HuR or AGTR1-3′UTR, suggesting that the recruitment of Tudor-SN into SGs depends on its efficient binding with SG nucleators G3BP, and Tudor-SN T103 phosphorylation is involved in this process. However, it is still possible that multisite phosphorylations of Tudor-SN play distinct or synergistic roles in the alteration of protein structure or kinetics during stress.

Tudor-SN exhibits high dynamic flux between the nucleus and cytoplasmic SGs [17]. In the present study, we produced the anti-pT103 antibody specific for the phosphorylated Tudor-SN T103 (p-Tudor-SN T103), which can recognize the Tudor-SN-containing SG signal within the cytoplasm in an IF assay. Surprisingly, a strong fluorescence signal of p-Tudor-SN T103 was also observed in the nucleus. The p-Tudor-SN T103 in the nucleus may be involved in the G2/M checkpoint and DNA damage process. There are three pieces of evidence in support of this speculation. 1) When cells were synchronized to different phases, the phosphorylation level of Tudor-SN T103 was higher in the G2/M phases than in the other phases (data not shown). 2) The computational prediction with KinasePhos identified Tudor-SN as a potential novel substrate of cell division control 2 (CD2) kinase, also known as cyclin-dependent kinase 1 (CDK1), which is a ubiquitously expressed serine/threonine protein kinase that controls the G2/M phase transition. An in vitro kinase assay further confirmed that the CD2/Cyclin B complex can efficiently phosphorylate Tudor-SN at T103 (data not shown). 3) Several DNA damaging stressors, such as ionizing radiation, X-ray and H2O2, could enhance the phosphorylation level of Tudor-SN T103 (data not shown). Therefore, the potential role of Tudor-SN phosphorylation in the G2/M DNA damage checkpoint merits further experiments.

SGs have been reported to be associated with resistance to cancer chemotherapy or radiotherapy [32–35]. For instance, SGs formed in the hypoxic area of the tumors may contribute to the radio-resistance of a vasculature tumor [32]. SG formation negatively regulates the stress-activated p38 and JNK MAPK (SAPK) pathways, which are relevant to the hypoxia-induced resistance to cancer chemotherapy [33]. The chemotherapeutic compound bortezomib can induce the formation of SGs, which may contribute to the resistance of bortezomib-mediated apoptosis in cancer cells [34]. Tudor-SN, a protein that is expressed highly in tumors, was also reported to drive chemoresistance of non-small cell lung carcinoma cells by regulating S100A11-dependent apoptosis [35]. Thus, it is meaningful to investigate the role of Tudor-SN-containing SG assembly in the chemo-resistance or radio-resistance of cancer cells.

5. Conclusions

In summary, this was the first study to investigate the potential role of Tudor-SN phosphorylation during stress. As shown in Fig. 5H, eukaryotic cells subjected to environmental stress activated JNK but not p38, mediating the phosphorylation of the Tudor-SN protein at T103. Enhanced Tudor-SN T103 phosphorylation can promote the binding of Tudor-SN with G3BP and efficiently drive Tudor-SN assembly into SGs in the cytoplasm. More experiments are needed to study the potential role of p-Tudor-SN T103 in the nucleus based on its nucleocytoplasmic trafficking nature.

Transparency document

The Transparency document associated with this article can be found, in online version.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2016.12.018.

References


