Human Tudor staphylococcal nuclease (Tudor-SN) protein modulates the kinetics of AGTR1-3'UTR granule formation

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\begin{abstract}
Human Tudor staphylococcal nuclease (Tudor-SN) interacts with the G3BP protein and is recruited into stress granules (SGs), the main type of discrete RNA-containing cytoplasmic foci structure that is formed during stress conditions. Here, we further demonstrate that Tudor-SN binds and co-localizes with AGTR1-3'UTR (3'-untranslated region of angiotensin II receptor, type 1 mRNA) into SG. Tudor-SN plays an important role in the assembly of AGTR1-3'UTR granules. Moreover, endogenous Tudor-SN knockdown can increase the recovery kinetics of AGTR1-3'UTR granules. Collectively, our data indicate that Tudor-SN modulates the kinetics of AGTR1-3'UTR granule formation, which provides an additional biological role of Tudor-SN in RNA metabolism during stress.
\end{abstract}

\section{1. Introduction}
Stress granules (SG) are a type of cytoplasmic RNA foci that aggregate in response to environmental stress stimuli, such as heat shock, oxidative stress, or viral infection, and play diverse roles in the regulation of mRNA translation, storage, stability, and decay [1–6]. SGs contain arrested preinitiation complexes, untranslated mRNAs, RNA-binding proteins, and other stress-related proteins, such as Ras-CAP SH3 domain-binding protein (G3BP) [7,8], TIA-1-related protein (TIAR) [7,9], and Tudor-SN [10–12]. SGs provide a temporal reservoir for mRNAs released from polysomes during stress, which also allows the resumption of translation after the stress-inducing conditions have subsided. This process is dependent on a set of RNA-binding proteins [1–7]. The study on the roles of RNA binding proteins in the stress-induced remodeling of mRNP (messenger ribonucleoprotein) complexes is an important issue.

Tudor-SN protein, also known as SND1 (staphylococcal nuclease domain containing 1) or p100, is evolutionarily conserved in humans [13–19], animals [20–22], and plants [23–25]. Human Tudor-SN is characterized by four N-terminal tandem repeats of the staphylococcal nuclease-like domain (SN) and a C-terminal TSN (Tudor-SN5) domain [13,14]. Tudor-SN plays several distinct roles by binding different protein partners or RNA substrates. For example, Tudor-SN functions as a transcriptional co-activator through an interaction between its SN domain and basal transcription machinery, such as CREB-binding protein (CBP) and RNA polymerase II [15,16], while taking part in pre-mRNA splicing through the binding of the TSN domain with symmetrical dimethylarginine-modified Sm core proteins of the spliceosome [17,18].

Our previous study indicated that Tudor-SN efficiently associated and co-localized with G3BP in SGs via the SN domain [10]. Accumulating evidence has revealed that Tudor-SN is a type of RNA-binding protein and that this function is mainly mediated through its SN domain [14,19,24–27]. It is interesting to study the role of Tudor-SN in RNA metabolism within SGs during stress. The SN domain of Tudor-SN was reported to interact with 3'UTR (3'-untranslated region) of AGTR1 (angiotensin II receptor, type 1) mRNA under normal conditions [19]. Here, we further demonstrate...
that Tudor-SN binds and co-localizes with AGTR1-3'UTR in SG under stress conditions, which leads us to study the potential post-transcriptional regulatory mechanism of Tudor-SN on AGTR1 mRNA-containing SG formation.

AGTR1 protein, a member of G protein-coupled receptor family, is involved in the physiological actions of Angiotensin II (Ang II) [28,29]. The expression of AGTR1 can be greatly affected by post-transcriptional regulation of AGTR1 mRNA following treatment of cells with estrogen, Ang II, H2O2, or insulin [30–34]. For example, insulin up-regulates AGTR1 expression by stabilizing AGTR1 mRNA in a 3'UTR dependent manner [34]. In the present study, we demonstrate that Tudor-SN is important for AGTR1-3'UTR granule aggregation, and the knockdown of endogenous Tudor-SN decreases the SG recovery kinetics.

2. Materials and methods

2.1. Cell culture, plasmids and transfection

HeLa cells were cultured as described previously [16]. Tudor-SN siRNA was generated as previously reported [15,18]. The plasmid encoding GFP-epitope-tagged Tudor-SN (RFP-Tudor-SN) was generated as described previously [10]. The pGenesil-DSRed-Tudor-SN-shRNA and pGenesil-DSRed-scramble-shRNA plasmids were constructed by Wuhan Cell Marker Biotechnology (China). The GFP-MS2 system was constructed as follows: pCR4-24-MS2SL-stable, pMS2-GFP plasmid encoding GFP-MS2 were a kind gift from Dr. Robert H. Singer (Albert Einstein College of Medicine, Bronx, NY, USA) (Addgene plasmid). To generate pT7-AGTR1-3'UTR, the AGTR1-3'UTR (1430–1993 bp) fragment was amplified by polymerase chain reaction (PCR) with the primers EcoRI-forward (5'-CGAATTCCACCAAAAGGCGCTGACGATTG-3') and BamHI-reverse (5'-CGGATCCGGTGGGACAGATTGCTGTTAGTTG-3'), using AGTR1 total cDNA transcript ( OriGene) as a template. This fragment was ligated into empty pSG5 vector containing the T7 bacteriophage promoter via EcoRI and BamHI enzymes. 24×MS2 stem loop repeats were cut from pCR4-24×MS2SL-stable plasmid and subsequently ligated into pT7-AGTR1-3'UTR or pSG5 using BglII and BamHI enzymes to construct the pT7-AGTR1-3'UTR-24×MS2 or pT7-24×MS2 plasmids. All PCR products were sequenced.

siRNA and plasmids were transfected using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s protocol. GFP-MS2 system plasmids were transfected using Neofect™ DNA transfection reagent ( China).

2.2. Fast protein liquid chromatography (FPLC) assay

Different cellular fractions were prepared as described previously [35]. Total cellular lysates from HeLa cells treated with 0.5 mM arsenite sodium for 1 h were harvested with Nonident P-40 lysis buffer that was supplemented with protease inhibitor cocktail (Roche Diagnostics) and Ribolock ribonuclease inhibitor (MBI) at 4 °C. Approximately 10 mg of protein was concentrated to 500 µl using an Ultrafree centrifugal filter apparatus (10 kDa nominal molecular mass limit, Millipore) and then applied to a Superose 6 size exclusion column (850 mm × 20 mm, GE Healthcare). The column was eluted at a flow rate of 0.5 ml/min. Each 500 µl fraction was collected and divided into two portions equally. One portion was used to perform Western blotting assay with rabbit anti-Tudor-SN (Abcam), anti-Tubulin (Abcam), and anti-TIAR (Cell Signaling Technology) antibodies, and the other was used for reverse transcription-PCR to analyze the distribution of AGTR1 and GAPDH mRNA. The AGTR1 primers sequences were 5'-TCCACAAAGAACCTGGCAACCA-3' (forward); 5'-TGCGACATGCAGACACCTT-3' (reverse), which were also used in the RIP and RNA FISH assays. The primer sequences of GAPDH were reported previously [18].

2.3. RNA-binding protein immunoprecipitation (RIP) assay

RIP Assay was performed as reported previously [18]. Total cell lysates from HeLa cells were incubated with rabbit anti-Tudor-SN antibody (Santa Cruz Biotechnology) or rabbit anti-IgG (Santa Cruz Biotechnology) conjugated with Protein G Dynabeads (Invitrogen) at 4 °C overnight with head-over-tail rotation. The precipitated AGTR1 mRNAs were detected by the reverse transcription-PCR assay, and the enriched Tudor-SN was confirmed by Western blotting assay using rabbit anti-Tudor-SN antibody.

2.4. Immunofluorescence (IF) and live-cell imaging assays

The IF and live-cell imaging assays were performed as described previously [10]. Briefly, cells were fixed, permeabilized, and then incubated with goat anti-Tudor-SN (Santa Cruz Biotechnology) at 4 °C overnight. After washing, cells were incubated with donkey anti-goat IgG (TR) antibody (Abcam) or Alexa Fluor 488-coupled donkey anti-rabbit IgG antibody (1:800 dilution) at 4 °C overnight. Images were collected using an Olympus FV1000 confocal microscope. The OLYMPUS IX81-CSU live cell system was used for live-cell imaging.

2.5. RNA fluorescence in situ hybridization (RNA FISH) assay

HeLa cells were fixed and permeabilized as described previously [10]. After denaturation in 25% formamide at 95 °C for 3 min and placement on ice for 3 min, 6 ng/µl biotinylated AGTR1 probe was incubated with the cells in a humidified dark chamber at 40 °C overnight. The cells were then sequentially washed with 25% formamide diluted in 0.5 × SSC for 15 min, 2 × SSC for 10 min, and 4 × SSC for 10 min. The hybridization signal was detected using 2.5 µg/ml FITC-avidin (Invitrogen) in 2 × SSC containing 0.2% bovine serum albumin and 0.01% Tween 20. For biotinylated AGTR1 probe, the AGTR1-3'UTR (1430–2135 bp) fragment was amplified by PCR using AGTR1 total cDNA transcript ( OriGene) as a template. The PCR product was purified with Gel/PCR Extraction Kit (Biomiga) and biotinylated using a Biotin-Nick Translation Kit (Roche Diagnostics).

2.6. Fluorescence recovery after photobleaching (FRAP) experiment

HeLa cells were cultured in 20 mm glass-bottomed Petri dishes (NEST Biotechnology) and co-transfected with ATGR1-3'UTR-MS2-GFP system plasmids and Tudor-SN shRNA or scramble shRNA. Twenty-four hours after transfection, cells were treated with 0.5 mM sodium arsenite (Sigma) for 30 min and kept in a phenol red-free medium. FRAP experiments were carried out using an Olympus FV1000 confocal microscope. Each granule region of interest (ROI) was randomly selected in a square area, and bleaching was performed with the 405 nm wavelength operating at 50% laser power and 10 µs/pixel scan speed for 2.5 s. Fluorescence recovery was monitored by the FRAP analysis tool (Olympus) at a low laser intensity every 5 s over a 200-sec period.

2.7. Granule quantification and statistical analysis

All experiments were repeated at least three times. Approximately 100 cells per experiment were scored randomly. The number and size of granules in the cells were measured using a
blind counting method with the Image J software (National Institutes of Health). The data are presented as the means ± S.E. (Standard Error) or box-plots (grain size analysis) and compared using Independent-Sample Student’s T Tests with the SPSS 16.0 software. P values less than 0.05 were considered statistically significant.
3. Results

3.1. Tudor-SN binds and co-localizes with AGTR1-3’UTR in SG under stress conditions

Previously, it was shown that Tudor-SN binds the 3’UTR of AGTR1 mRNA under normal conditions [19]. To gain insight into the association between Tudor-SN and AGTR1-3’UTR under stress conditions, protein-RNA fractionation experiments were performed through size exclusion approach by FPLC assay. Total cell lysates from HeLa cells were fractionated by a Superose 6 gel filtration column. As shown in Fig. 1A, the elution pattern of Tudor-SN in chromatographic fractions showed a major peak at approximately 158-669 kDa and a minor peak at higher molecular masses (slightly below 2000 kDa). Moreover, the major peak largely overlapped with that of the SG protein components, including TIAR and Tudor-SN. Tubulin protein was distributed throughout the chromatographic fractions. Furthermore, both peaks of Tudor-SN co-eluted with AGTR1 mRNA, but not GAPDH mRNA. The result in Fig. S1 showed that Tudor-SN protein fails to colocalize with the PB-specific protein DCP1a, confirming that Tudor-SN is one type of SG-specific protein, in agreement with the data of Weissbach, R. [7]. These findings suggest that Tudor-SN-AGTR1 mRNA binding is associated with SG biogenesis.

We then performed RIP assays to confirm the interaction between Tudor-SN and AGTR1-3’UTR under stress conditions. As shown in Fig. 1B, the anti-Tudor-SN antibody could efficiently precipitate with the AGTR1-3’UTR (lane 3), but not the control anti-IgG antibody (lane 2), which indicated that Tudor-SN readily interacts with the 3’UTR of AGTR1 in vivo under stress. Furthermore, we also detected the in vivo colocalization of Tudor-SN and AGTR1-3’UTR by RNA FISH with a FITC-labeled AGTR1 probe and in an IF assay using anti-Tudor-SN antibody. As shown in Fig. 1C, both endogenous Tudor-SN protein and AGTR1-3’UTR mRNA predominantly accumulated in SG under stress. The above findings indicate that Tudor-SN associates with and targets AGTR1 mRNA in the SG structure.

3.2. Tudor-SN co-localizes with AGTR1-3’UTR granules using a GFP-MS2 labeling system

To monitor the aggregation of AGTR1-3’UTR granules in living cells, we labeled the AGTR1-3’UTR using the GFP-MS2 labeling system, which is useful for visualizing the real-time motion of single mRNA molecules [36]. As shown in the schematic diagram of Fig. 2, we tethered the targeting AGTR1-3’UTR fragment with 24 copies of the bacteriophage MS2 stem loops to generate the pT7-AGTR1-3’UTR-24×MS2 plasmid, which can bind multiple...
GFP-tagged bacteriophage MS2 coat proteins (GFP-MS2) with a strong affinity, encoded by the pMS2-GFP plasmid. Hence, the localization and movement of AGTR1-3'UTR in live cells can be tracked. pT7-AGTR1-3'UTR-24×-MS2, pMS2-GFP and RFP-tagged Tudor-SN plasmids were transiently co-transfected into HeLa cells. As expected, the AGTR1-3'UTR fragment (green) co-localized with Tudor-SN (red) in arsenite-treated cells (Fig. 2A). In contrast, under normal conditions, a weak signal was detected in the cytoplasm and a strong signal was detected in the nucleus, due to the NLS (nuclear localization signal) element harbored in the pMS2-GFP plasmid. To verify the specific recognition of Tudor-SN with the AGTR1-3'UTR in vivo, HeLa cells were co-transfected with pMS2-GFP plasmid and RFP-tagged Tudor-SN plasmid, together with pT7-AGTR1-3'UTR or pT7-24×-MS2. As shown in Fig. 2B–D, neither AGTR1-3'UTR reporter nor lacking the 24×-MS2 stem loop repeats exhibits cytoplasmic foci, but the foci instead remain localized to the nucleus. Tudor-SN specifically interacts with the AGTR1-3'UTR, but not the poly(A) tail, because Tudor-SN did not colocalize with pT7-24×-MS2, which only contains the poly(A) tail (Fig. 2D). In addition, the data in Figs. S2 and S3 indicate that AGTR1-3'UTR is co-localized with another SG-specific protein, TIAR, but not the PB-specific protein DCP1a under stress conditions, confirming that these AGTR1-3'UTR granules are SG-specific, but not PB-specific. Live-cell imaging revealed the dynamic association of RFP-tagged Tudor-SN protein and GFP-tethered reporter AGTR1-3'UTR granules during cellular oxidative stress, but no interaction was observed with control plasmids (Fig. 3). These data indicate that the SG recruitment of the AGTR1 transcript is accompanied by the formation of Tudor-SN granules.

3.3. Tudor-SN plays an important role in the assembly of AGTR1-3'UTR granules

Nascent SGs are small and then progressively fuse into larger foci [9]. Dynamic packaging of the cellular mRNA transcript by stress-related proteins is essential for the remodeling and recruitment of cytoplasmic mRNA complexes into SGs [1–6]. We previously demonstrated that the knockdown of Tudor-SN does not inhibit the formation of SG but instead retards the aggregation of small SGs into large SGs [10]. Tudor-SN is likely to be important for the assembly of AGTR1-3'UTR-containing SGs. We analyzed the number and size of AGTR1-3'UTR granules when the endogenous Tudor-SN protein was knocked down by siRNA treatment. As shown in Fig. 4A and B, the transfection of Tudor-SN siRNA significantly reduced the expression of Tudor-SN protein by approximately 70% (P < 0.05), compared with the scramble siRNA, but had no effect on the abundance of β-actin. Moreover, the knockdown of Tudor-SN significantly reduced the size of AGTR1-3'UTR granules in single cells (Fig. 4C–E, P < 0.01), while the proportion of cells containing >20 foci was also decreased (Fig. 4D, from 26% to 10%), suggesting that the knockdown of Tudor-SN hindered the emergence of AGTR1-3'UTR granules and led to a decrease in both the number and size of AGTR1-3'UTR granules. These data indicated that Tudor-SN is essential for SG recruitment of target AGTR1 mRNA.

3.4. Tudor-SN affects the recovery kinetics of SGs

SGs are proposed to be the site where the local concentration of proteins and mRNAs is increased in response to environmental stress [1,6,7]. SGs are not stable structures that often exchange dynamically with the surrounding cytosol. Different SG components show different aggregation kinetics [7,37–39]. To quantitatively analyze the role of Tudor-SN in the association/dissociation dynamic properties of AGTR1-3'UTR granules in living cells under stress, we performed the FRAP assay, which has frequently been utilized to detect the recovery kinetics of fluorescent protein labeled components [7,38]. Because the combination of the MS2-GFP labeling system and the FRAP assay can be used to analyze the kinetics of targeting RNAs [39], we photo-bleached the nascent AGTR1-3'UTR labeled with MS2-GFP to analyze its exchange kinetic in HeLa cells with Tudor-SN knockdown. HeLa cells were transfected with pGenesil-DsRed-Tudor-SN-shRNA plasmid or pGenesil-DsRed-scramble-shRNA as the negative control, and Western blot and IF assays were then performed. The transfection efficiency of shRNA was determined by the expression of DsRed. The Western blot results (Fig. 5A and B) showed that the level of endogenous Tudor-SN protein but not β-actin protein was significantly reduced (upper panel) by the Tudor-SN shRNA treatment (lower panel) compared with the scramble shRNA group. Moreover, as shown in Fig. 5C, the IF signal (red) from the Tudor-SN protein was significantly reduced with Tudor-SN shRNA in the DsRed-positive cell (green), but not with scramble shRNA. Next, an analysis of the aggregation kinetics of AGTR1-3'UTR granules was performed using the FRAP assay when Tudor-SN was knocked down. HeLa cells

![Fig. 4](image_url)
were co-transfected with the ATGR1-3'UTR-MS2-GFP system plasmid together with Tudor-SN shRNA or scramble shRNA as a control and then treated with 0.5 mM sodium arsenite. Individual regions containing GFP-tagged ATGR1-3'UTR granules in the DsRed-positive cell were photobleached with a high-power laser. Remarkably, as shown in Fig. 5C and D, when Tudor-SN was knocked down, ATGR1-3'UTR demonstrated a slower recovery rate ($t_{1/2} = 16.77 \pm 1.94$ s) and less complete recovery ($\approx 56.8\%$ compared with the control group ($t_{1/2} = 9.96 \pm 0.78$ s, $\sim 74.2\%$ recovery) (Fig. 5D, $P < 0.01$), suggesting that Tudor-SN is necessary for the efficient cytoplasmic movement of exogenous ATGR1-3'UTR under stress conditions. In addition, as shown in Fig. 5C, the knockdown of Tudor-SN fails to induce the aggregation of ATGR1-3'UTR under normal conditions, confirming that the role of Tudor-SN on ATGR1-3'UTR granule aggregation is dependent on an environmental stress. These quantitative kinetic data provide insights that Tudor-SN serves as an indispensable component to facilitate SG aggregation by interacting with SG-related mRNPs and modulating their kinetic nature under environmental stress.

### 4. Discussion

The posttranscriptional regulation of gene expression in eukaryotes involves several regulatory processes, such as mRNA nucleo-cytoplasmic export, cellular localization, translation regulation, and mRNA turnover, and often requires the interaction between RNA-binding proteins and conserved structural elements located in the 3'UTR of the mRNA molecule [40]. Several connections between Tudor-SN and 3'UTRs have been reported: (1) Tudor-SN in rice binds the 3'UTR of prolamine RNAs and is involved in RNA transport and localization in rice endosperm during rice seed development [24,25]. (2) Human Tudor-SN also interacts with the 3'UTR of the DENV (dengue virus) genome and is involved in virus replication [26]. (3) Tudor-SN binds SG-associated AGO2 protein and has been identified as a core component of RISC, which targets the 3'UTR of mRNA substrates [41]. These data favor the idea that 3'UTR binding is likely to be a type of approach for multifunctional Tudor-SN to modulate the cytoplasmic fate of specific mRNAs. Here, we present evidence that Tudor-SN binds and co-localizes with ATGR1-3'UTR granules. These data indicate that Tudor-SN is likely to act as a key positive regulator for the recruitment of specific bound mRNA cargoes into insoluble SGs under stress and to modulate the increased flow of AGTR1 mRNA that accompanies stress.

As the RNA-binding protein, Tudor-SN promotes the stabilization of certain mRNAs during stress. For example, Tudor-SN in Arabidopsis stabilizes the level of stress-responsive mRNAs encoding secreted proteins [23]. Tudor-SN enhances the expression of AGTR1 by decreasing the rate of mRNA decay in non-stressed cells, however, Tudor-SN does not mediate the posttranscriptional regulation of AGTR1 mRNA in response to atorvastatin, estrogen, insulin, or Ang II [19]. Although GAPDH was reported to increase...
AGTR1 expression via the binding of AGTR1-3'UTR in response to H$_2$O$_2$ [31], we still lack evidence that human Tudor-SN modulates the retention or stability of AGTR1-3'UTR mRNA in SG structures during stress. In addition, AGTR1-3'UTR binds several other RNA-binding proteins as well, including calreticulin [30], AUFI [32], GAPDH [31], and HuR [33,34]. Interestingly, HuR and calreticulin were confirmed to be SG components [42,43]. We also observed the binding of Tudor-SN and HuR/calreticulin (data not shown), suggesting that the post-transcriptional regulatory process of AGTR1 mRNA might be dependent on the existence of the SG-associated protein complex containing Tudor-SN, HuR, and calreticulin or binding with the 3'UTR.

PB (Processing Body), another stress-associated cytoplasmic foci structure, contains microRNA (miRNA)-mediated RNA interference (RNAi) effectors and mRNA decapping machinery, which is involved in mRNA repression and degradation [3–7]. SG and PB share some common components and exhibit intricate connections on mRNA metabolism [3–7,44]. Translationally inactive mRNA within the SG can be delivered to PB for degradation [6,7,44]. Regarding AGTR1, we fail to observe the localization of AGTR1 in the PB structure. However, we still cannot rule out the possibility of AGTR1 degradation in the PB due to the limitation of treatment time or drug concentration.

The AGTR1 protein is recognized by Ang II, a peptide hormone, and plays a pathophysiological role in atherosclerosis, hypertension, cardiac hypertrophy, kidney injury, and heart failure [28,29,45,46]. It was reported that the expression of Tudor-SN in rats is increased in response to H$_2$O$_2$-induced oxidative stress [21] or high glucose treatment [22]. The up-regulation of Tudor-SN in rats under hyperglycemic conditions leads to an increased level of AGTR1 protein expression and is involved in the modulation of glomerular injury [22]. Very recently, we demonstrated that Tudor-SN is essential for the adipogenesis [47]. It is possible that Tudor-SN functions in the pathophysiology of glucolipid metabolism-related diseases, such as atherosclerosis, by modulating the mobility or aggregation kinetics of AGTR1-containing SG under high glucose stress conditions, which merits further study.

Acknowledgments

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