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Citation	Biochemical and biophysical research communications, 516(2), 419-423 https://doi.org/10.1016/j.bbrc.2019.06.061
Issue Date	2019-08-20
Doc URL	http://hdl.handle.net/2115/79081
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Туре	article (author version)
File Information	Aly et al (BBRC) for HUSCAP .pdf



## Two distinct nuclear stress bodies containing different sets of RNA-binding proteins are formed with HSATIII architectural noncoding RNAs upon thermal stress exposure

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Abbreviations: arcRNA, architectural RNA; ASO, antisense oligonucleotide; ChIRP-MS, chromatin isolation by RNA purification-mass spectrometry; DIG, digoxigenin; HSATIII, highly repetitive satellite III; IncRNA, long noncoding RNA; nSB, nuclear stress body; PG, perichromatin granule; RBP, RNA-binding protein; SAFB, Scaffold Attachment Factor B; SLTM, SAFB-Like Transcription Modulator; SRSF, Serine and Arginine Rich Splicing Factor.

#### Abstract

Nuclear stress bodies (nSBs) are thermal stress-inducible membrane-less nuclear bodies that are formed on highly repetitive satellite III architectural noncoding RNAs (HSATIII arcRNAs). Upon thermal stress exposure, HSATIII expression is induced to sequestrate specific sets of RNA-binding proteins and form nSBs. The major population of nSBs contain SAFB as a marker, whereas the minor population are SAFB-negative. Here, we found that HNRNPM, which was previously reported to localize in nuclear foci adjacent to SAFB-positive foci upon thermal stress, localizes in a minor population of HSATIII-dependent nSBs. Hence, we used the terms nSB-S and nSB-M to distinguish the SAFB foci and HNRNPM foci, respectively. Analysis of the components of the nSBs revealed that each set contains distinct RNA-binding proteins, including SLTM and NCO5A in nSB-Ss and HNRNPA1 and HNRNPH1 in nSB-Ms. Overall, our findings indicate that two sets of nSBs containing HSATIII arcRNAs and distinct sets of RNA-binding proteins are formed upon thermal stress exposure.

**Keywords:** nuclear stress bodies, long noncoding RNA, HSATIII, RNA-binding protein, primate-specific genes, thermal stress

### Introduction

Nuclear stress bodies (nSBs) are primate-specific membrane-less subnuclear structures (or nuclear bodies) that are formed in response to thermal and chemical stresses [1, 2]. The assembly of nSBs is initiated alongside HSF1-dependent transcription of the primate-specific highly repetitive satellite III (HSATIII) long noncoding RNA (IncRNA) [1] and heat shock-induced HSF1 aggregation [3]. HSATIII IncRNAs are transcribed from pericentromeric HSATIII repeated arrays on several human chromosomes [4]. The largest HSATIII chromatic domains are mapped on human chromosome 9; these domains are usually located in heterochromatinic regions that are transcriptionally silent, but are rapidly euchromatinized and produce HSATIII IncRNAs following exposure to thermal stress [5–7]. HSATIII IncRNAs are retained on chromosomes near their own transcription sites for several hours, even after stress removal, and recruit various RNA-binding proteins (RBPs), including Scaffold Attachment Factor B (SAFB), Serine and Arginine Rich Splicing Factor 1 (SRSF1), SRSF7, and SRSF9 [6, 8, 9], as well as specific chromatin-remodeling factors [10, 11] and transcription factors [7], which results in the assembly of nSBs.

Recently, our group found that HSATIII and nSBs promote intron retention by hundreds of pre-mRNAs during recovery from thermal stress exposure. Using a chromatin isolation by RNA purification-mass spectrometry (ChIRP-MS) analysis, we identified 141 proteins as components

of nSBs, most of which are likely RBPs involved in RNA splicing, processing, modification, and export. In addition, we found that the major nSB proteins, including SAFB and SRSFs, co-localize with HSATIII in nSBs following thermal stress exposure. On the other hand, it was previously reported that the nSB component HNRNPM is localized in thermal stress-inducible nuclear foci that are adjacent to SAFB-positive foci [2, 12], suggesting the presence of distinct thermal stress-inducible nSB-like foci in human cell lines.

Here, we investigated HNRNPM-positive nSB-like foci further by examining their RNA and protein components. We found that the HNRNPM-positive foci are formed with HSATIII architectural RNAs (arcRNAs), and that two additional HNRNP proteins co-localize with HNRNPM. Our findings suggest that HSATIII arcRNAs play architectural roles in the formation of two distinct nSBs upon thermal stress exposure by sequestrating distinct sets of RBPs.

#### **Materials and Methods**

#### Cell culture

HeLa cells were maintained in DMEM (Gibco) containing 10% FBS (Gibco) and penicillin-streptomycin (Nacalai Tesque). For thermal stress induction, the cells were incubated at 42°C in an incubator with 5% CO<sub>2</sub>. HAP1 cells (Horizon Discovery) were maintained in IMDM (Gibco) supplemented with 10% FBS (Gibco) and penicillin-streptomycin (Nacalai Tesque).

## Knockdown with antisense oligonucleotide and siRNAs

For nuclear RNA knockdown,  $3 \times 10^{6}$  cells were transfected with chemically modified antisense or control oligonucleotides (1 µM) using Nucleofector (Lonza) 16–18 hours before the assay, as described previously [13]. For mRNA knockdown, siRNAs were transfected into cultured cells using Lipofectamine RNAiMAX (Invitrogen). The siRNAs used for knockdown of SAFB2 and SLTM were purchased from Thermo Fisher Scientific (HSS190510 and HSS128903, respectively).

#### Genome editing using CRISPR/Cas9

PX330 or PX330-B/B was used for genome editing of the HAP1 cells. To establish SAFB1 knockout HAP1 cells, the sgRNA (5'-CGAAATTGAAATTACCTCCG-3') was selected from

GeCKO v2 libraries [14] and cloned into the BbsI site of PX330 (Addgene). The plasmids (2 µg) and pcDNA6/TR plasmids (0.2 µg) were transfected similarly using a Nucleofector device (Lonza). To enrich the plasmid-transfected cells, the HAP1 cells were treated with 20 µg/ml blasticidin (InvivoGen) for 3 days, starting 1 day after transfection. Subsequently, the cells were diluted into 96-well plates for selection of single clones. The selected clones were lysed by proteinase K treatment (200 µg/ml proteinase K [Roche], 20 mM Tris-HCl pH 8.0, 5 mM EDTA, 400 mM NaCl, and 0.3% SDS) at 55°C for 1 hour, followed by proteinase K inactivation at 95°C for 15 min. To detect deletions or insertions, the lysates were subjected to PCR analysis using KOD FX Neo enzyme (TOYOBO) to amplify the genomic regions flanking the guide RNA target sites. To detect small deletions in the SLTM knockout cell lines, a T7 endonuclease I (NEB) cleavage assay was performed on the amplified PCR products. The indel-positive clones were further confirmed by sequencing. Finally, the absence of protein expression was confirmed by immunoblotting.

#### Fluorescence in situ hybridization (FISH) and immunofluorescence

For HSATIII RNA in situ hybridization, cells cultured on cover glasses were washed with ice-cold PBS and fixed in 4% PFA/PBS for 10 min at room temperature. The cover glasses were then washed twice with ice-cold PBS and permeabilized in cold 70% ethanol for at least 1 hour at 4°C.

The cover glasses were washed with 10% formamide/2x SSC, hybridized with a digoxigenin (DIG)-labeled HSATIII antisense oligonucleotide (ASO) in hybridization buffer (10% formamide, 2x SSC, and 10% dextran) for 16 hours at 37°C, incubated in 10% formamide/2x SSC for 30 min at 37°C, and then washed in 2× SSC for 5 min. After blocking in 3% BSA/TBST for 30 min, the cover glasses were incubated in 3% BSA/TBST containing an anti-DIG antibody (Abcam, ab420 or ab76907, 1:500 dilution) and an antibody against a protein of interest (SAFB: Abcam, ab8060; HNRNPM: LifeSpan Biosciences, LS-B2427 or Santa Cruz Biotechnology, sc-20002; NCOA5: Bethyl Laboratories, A300-790A; SLTM: Bethyl Laboratories, A302-834A; HNRNPA1: MBL, RN114PW; HNRNPH1: Bethyl Laboratories, IHC-00087). After washing in TBST, the cover glasses were incubated in 3% BSA/TBST containing Alexa 488- or Alexa 568-conjugated secondary antibodies. For immunostaining, the fixed cells were permeabilized in cold PBS containing 0.1% Triton X-100 for 15 min, and then incubated with the antibodies as described above. For the triple staining shown in Figure 1C, HSATIII was visualized with a biotin-labeled HSATIII ASO and avidin DN-FITC (Vector Laboratories). Primary antibodies against the proteins of interest were visualized with Alexa 350- or Alexa 568-conjugated secondary antibodies

### Structured illumination microscopy

Structured illumination microscopy was performed as described previously [15]. Briefly, a

DIG-labeled HSATIII ASO was used to detect nSBs. The DIG-labeled probes were detected using an anti-DIG monoclonal antibody (Abcam, 21H8, ab420, 1:100 dilution) and a Cy3-conjugated anti-mouse secondary antibody (Millipore, AP124C, 1:100 dilution). SAFB-positive and HNRNPM-positive cells were visualized using anti-SAFB and anti-HNRNPM antibodies, respectively, and a Cy2-conjugated secondary antibody (Abcam, ab6940, 1:100 dilution). Images were captured using a ELYRA PS.1 microscope (Zeiss) with a 100× objective lens, as described previously [15].

#### Results

#### Two distinct nSBs are formed under thermal stress conditions

Since the HSATIII arcRNA is essential for the formation of canonical nSBs, we examined whether thermal stress-inducible HNRNPM foci are also formed with HSATIII in HeLa cells. First, similar to its co-localization with SAFB in canonical nSBs, we confirmed co-localization of HNRNPM with HSATIII in nuclear foci (Figure 1A and B). ASO-mediated knockdown of HSATIII abolished both the HNRNPM-positive and SAFB-positive nSBs (Figure 1A and B), indicating that the HNRNPM foci utilize HSATIII arcRNAs as structural scaffolds. The HNRNPM-positive foci were smaller and more compact than the SAFB-positive foci (Figure 1A and B). In addition, each HeLa cell nucleus contained fewer than four HNRNPM-positive foci, but at least 15 SAFB-positive foci. Triple staining of the cells via HSATIII RNA-FISH and SAFB and HNRNPM immunofluorescence revealed a minor population of HSATIII-positive nSB foci in which HSATIII co-localized with HNRNPM but not SAFB (Figure 1C). This finding was confirmed by a super-resolution microscopic analysis of the HSATIII-positive nSB foci (Figure 1D). These observations revealed the presence of two distinct thermal stress-inducible nSBs formed on HSATIII arcRNAs, which we named nSB-S (SAFB-positive) and nSB-M (HNRNP-positive).

#### Identification of new components of nSB-Ss

Our recent ChIRP-MS analysis identified 141 proteins that co-precipitated with HSATIII arcRNAs (Ninomiya et al., submitted), which are likely nSB components. Our finding that two distinct nSBs are formed on HSATIII RNA cores raised the possibility that these 141 proteins represent components of both nSB-Ss and nSB-Ms. Consequently, we carried out immunostaining of several newly identified nSB components to determine which type of nSB they reside in. As described above, we confirmed that the known nSB component SAFB was localized in nSB-Ss but not nSB-Ms (Figure 1C and D). Among the newly identified proteins, SAFB-Like Transcription Modulator (SLTM) and Nuclear Receptor Coactivator (NCOA5) specifically localized in nSB-Ss but not nSB-Ms (Figure 2A and B). Because SLTM is structurally related to SAFB [16], we confirmed that the anti-SLTM and anti-SAFB antibodies did not reciprocally cross-react and that they detected the specific target only (Supplemental Figure S1). SLTM likely associates with HSATIII in a similar manner to SAFB. NCOA5 is a coactivator and corepressor of nuclear receptors that lacks canonical RNA-binding motifs; however, two independent studies identified NCOA5 as a protein obtained by RNA interactome capture following UV crosslinking, suggesting that it binds directly to RNAs [17, 18].

### nSB-Ms and nSB-Ss sequestrate distinct sets of RBPs

Among the 141 newly identified proteins, HNRNPA and HNRNPH1 were specifically localized in

nSB-Ms but not nSB-Ss (Figure 3A and B). Like HNRNPM, both proteins are relatively abundant HNRNPs that possess canonical RNA recognition motifs as well as intrinsically disordered regions.

## Discussion

In the current study, we identified two distinct thermal stress-inducible nuclear bodies that are marked by SAFB and HNRNPM. The HNRNPM foci were previously reported to be located adjacent to known SAFB-containing canonical nSBs. Here, we found that, like the canonical nSBs, the HNRNPM foci contain the HSATIII arcRNA as an essential structural component. Immunofluorescent detection of several proteins that were co-purified by a HSATIII ChIRP-MS analysis revealed that two additional RBPs (HNRNPA and HNRNPH1) are co-localized with HNRNPM in a minor population of HSATIII-positive nSBs. On the other hand, as a marker of the major population of nSBs, SAFB did not co-localize with HNRNPM. Similarly, the newly identified canonical nSB components (SLTM and NCOA5) did not co-localize with HNRNPM in nSBs. These findings indicate that two distinct nuclear bodies with different sets of RBPs (nSB-Ss and nSB-Ms) are formed on HSATIII arcRNAs following thermal stress exposure.

Using electron microscopy, Chiodi et al. found that both SAFB and HNRNPM are localized in the highly dense cluster of multiple perichromatin granules (PGs) upon thermal stress

exposure, which are located in the different district of a same cluster [12]. Intriguingly, HNRNPM tends to be located in the dense core of the PGs, whereas SAFB is seldom found within the PGs but is located in the surrounding area and on the fiber that constitutes the tail of the PGs [12]. Based on these observations, it is possible that nSB-Ss and nSB-Ms represent different stages of the synthesis and maturation of nSBs after thermal stress-induced HSATIII arcRNA synthesis. It is also possible that HNRNP proteins with high affinity to RNAs may rapidly bind to HSATIII to form the dense core of nSBs, and then SAFB and other components subsequently join the surrounding area to form the larger nSB-Ss.

In addition to the large satellite III repeat cluster in human chromosome 9q12, which is the major source of HSATIII arcRNA, chromosomes 12 and 15 possess additional satellite III repeat clusters and can direct nSB formation in human-hamster somatic cell hybrids [4]. Because of the highly repetitive nature of the chromosome regions, the exact sequences and transcribed regions of HSATIII arcRNAs have not been precisely determined; hence the HSATIII arcRNAs detected by RNA-FISH would be a heterologous mixture of closely related but partially distinct sequences. Thus, we cannot rule out the possibility that the HSATIII arcRNAs derived from specific satellite III loci preferentially associate with HNRNPM, whereas the majority of other HSATIII arcRNAs associate with SAFB. It would be interesting to investigate the determinants of the binding specificities of HSATIII sequences for nSB-S and nSB-M components. HSATIII

arcRNAs are the primate-specific lncRNAs that are induced upon thermal stress to form nSBs, suggesting the involvement of primate-specific events in the response to thermal stress. Our discovery that HSATIII forms two different nSBs raises the possibility that two distinct nuclear events are controlled by each nSB, or that the two nSBs are sequentially involved in a common event during thermal stress response and/or recovery.

## Acknowledgments

We thank K. Kondo, H. Yoshino, T. Yamazaki, and S. Nakagawa (Hokkaido University) for technical support and valuable discussions. This research was supported by a MEXT KAKENHI Grant [to TH (26113002, 16H06279, 17H03630, and 17K19335) and KN (19K06478)] and the Tokyo Biochemical Research Foundation (to TH).

## **Author Contributions**

MKA, KN, and TH conceived and designed the study. MKA, KN, SA, and TN conducted the experiments. MKA, KN, and TH wrote the manuscript.

## Additional Information

Competing Interests: The authors declare no competing interests.

### **Figure Legends**

Figure 1: HNRNPM foci are distinct nSBs formed in association with HSATIII arcRNAs.

- A. Detection of the canonical nSBs via HSATIII RNA-FISH and SAFB immunofluorescence in control and ΔHSATIII cells. The cells were treated at 42°C for 2 hours, followed by incubation at 37°C for 1 hour. The nuclei were stained with DAPI. Scale bar: 10 µm.
- B. Detection of the HNRNPM foci via HSATIII RNA-FISH and HNRNPM immunofluorescence in control and ΔHSATIII cells. The cells were incubated as described in A. The nuclei were stained with DAPI. Scale bar: 10 µm.
- C. Detection of HNRNPM foci by triple staining via HSATIII RNA-FISH and SAFB immunofluorescence. The cells were incubated as described in A. Scale bar: 10 μm.
- D. Super-resolution microscopic observation of nSBs detected via SAFB and HNRNPM immunofluorescence (left), or HSATIII RNA-FISH and HNRNPM immunofluorescence (right).
   Scale bar: 500 nm.

Figure 2: Identification of novel nSB-S components.

Immunofluorescent detection of SLTM (A) and NCOA5 (B) co-stained with HSATIII, SAFB, or HNRNPM. HSATIII was detected by RNA-FISH. The cells were treated at 42°C for 2 hours, followed by incubation at 37°C for 1 hour. The nuclei were stained with DAPI. Scale bar: 10 µm. Figure 3: Identification of novel nSB-M components.

Immunofluorescent detection of HNRNPA1 (A) and HNRNPH1 (B) co-stained with HSATIII,

SAFB, or HNRNPM. HSATIII was detected by RNA-FISH. The cells were treated at 42°C for 2

hours, followed by incubation at 37°C for 1 hour. The nuclei were stained with DAPI. Scale bar:

10 µm.

Figure 4: Model of two distinct nSBs.

Upon thermal stress exposure, HSATIII arcRNAs are transcribed from the satellite III repeats to form two distinct nSBs (nSB-S and nSB-M) containing distinct sets of RBPs.

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## **Supplementary Material**

Figure S1: Specific detection of SAFB and SLTM. Immunofluorescent detection of SAFB and

SLTM using their cognate antibodies in control HAP1 cells, and those in which SAFB1 and

SAFB2 (SAFB1 KO/SAFB2 KD) or SLTM (SLTM KD) was depleted. Scale bar: 10 µm.

## Figure 1



## Figure 2



## Figure 3



