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学 位 論 文

Structural Analysis of Nuclear Stress Bodies Formed in Response to
Thermal Stress

(熱ストレスに応答した核内ストレス体の構造解析)

2019 年 9 月

北 海 道 大 学

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List of Publications and Presentations

List of Publications:

- 1) Aly M.K., Ninomiya K., Adachi S., Natsume T., and Hirose T. Two distinct nuclear stress bodies containing different sets of RNA-binding proteins are formed with HSATIII architectural noncoding RNAs upon thermal stress exposure. *Biochemical and Biophysical Research Communications*, in press.
- 2) Aly M.K., Hirose T. GAS5 Gene. In: *eLS*. John Wiley & Sons Ltd, Chichester. doi: 10.1002/9780470015902.a0005019.
- 3) Omran M.H., Aly M.K., Nasr N., Massoud A.A., Youssef S.S., Bader El Din N.G., Dawood R.M., Atef K, Moustafa R.I., Nabil W., Tabll A.A., El Awady M.K.. A Study of CC-Chemokine Receptor 5 (CCR5) Polymorphism on the Outcome of HCV Therapy in Egyptian Patients. *Hepat Mon.* 13(12): e13721.

List of Presentations:

- 1) Mahmoud K. Aly, Kensuke Ninomiya, Tetsuro Hirose. Stress-Induced Transient m⁶A Methylation in HSATIII lncRNA-Based Nuclear Stress Bodies. The 9th IGM Exchange Meeting, 2018 December 17, Graduate School of Medicine, Hokkaido University, Japan.
- 2) Mahmoud K. Aly, Kensuke Ninomiya, Tetsuro Hirose. Stress-Induced Transient m⁶A Methylation in HSATIII lncRNA-Based Nuclear Stress Bodies. The 2nd Joint Japan-Australia/Australia-Japan Joint RNA Meeting (JAJ Joint RNA), 2018 November 5-7, Graduate School of Medicine, Hokkaido University, Japan.
- 3) Mahmoud K. Aly, Kensuke Ninomiya, Tetsuro Hirose. Unravelling The Architectural Organization of The SATIII lncRNA-Dependent Nuclear Stress Bodies. The 2nd Hokkaido University Cross-Sectional Symposium, 2017 March 14, Graduate School of Medicine, Hokkaido University, Japan.

Summary

Background and Purpose:

Nuclear stress bodies (nSBs) are primate-specific membrane-less subnuclear structures that are formed in response to thermal and chemical stresses. The assembly of nSBs is initiated alongside HSF1-dependent transcription of the primate-specific highly repetitive satellite III (HSATIII) long noncoding RNA (lncRNA) and heat shock-induced HSF1 aggregation. HSATIII lncRNAs are retained on chromosomes near their own transcription sites for several hours and recruit various RNA-binding proteins (RBPs). It is still poorly understood why nSBs are assembled in primates' stress response events. Decoding the protein composition and structural organization of nSBs were our first approach to elucidate the nSBs functions in primates' stress response events. Recently, our lab has identified 141 proteins as components of nSBs, most of which are likely RBPs involved in RNA splicing, processing, modification, and export.

Here, we elucidate the existence of two distinct nuclear stress bodies containing different sets of RNA-binding proteins that are formed with HSATIII upon thermal stress exposure, nSB-M and nSB-S. In addition, we report a previously unrecognized thermal stress-induced recruitment of the N⁶-methyladenosine (m⁶A) RNA modification machinery to nSBs. m⁶A is the most abundant RNA modification in eukaryotes and has been reported to have crucial regulatory roles in cancer proliferation, sex determination, cell differentiation, neuronal function, meiosis, circadian rhythm, and chromosomal silencing. Hence, m⁶A machinery recruitment to nSBs proposes a novel nSBs-dependent regulatory function of m⁶A in primates' stress response events.

Methods:

CRISPR/Cas9, ASO and siRNA were used for depletion of target of interest. RNA FISH and IF were used for detection of HSATIII, m⁶A RNAs, and proteins of interest. m⁶A Dot Blot was used for nSB-m⁶A RNAs enrichment detection. ChIRP was used for nSBs purification. Western blot was used for detection of proteins of interest.

Results:

We observed adjacent localization of SAFB and HNRNPM, nSBs proteins, foci formed in response to thermal stress. First we confirmed that both entities utilize HSATIII lncRNAs as

architectural scaffolds. Triple staining combined HSATIII RNA-FISH and immunofluorescence experiments have shown that both HNRNPM and SAFB foci recruit distinct RNA-binding proteins sets. We found that upon thermal stress exposure, HNRNPA1 and HNRNPH1 are strictly recruited to HNRNPM-foci, we named nSB-M, while SLTM, and NCOA5 are strictly recruited to SAFB-foci, we named nSB-S.

On the other hand, we detected a thermal stress-induced sequestration of the m⁶A writers, METTL3 and WTAP, and the m⁶A reader, YTHDC1, to nSBs. In addition, anti-m⁶A modification antibody confirmed the localization of m⁶A modified RNAs at nSBs. HSATIII knockdown was enough to abolish both stress-induced m⁶A machinery recruitment and m⁶A RNAs localization to nSBs. Moreover, we found that HSATIII transcripts are methylated and that at least HSATIII serves as target substrate for m⁶A machinery at nSBs. RNA-MS analysis has identified GGAAU, the highly repetitive sequence of HSATIII, as a novel m⁶A consensus motif. m⁶A machinery showed a unique recruitment/enrichment pattern at nSBs. During stress, the m⁶A machinery is recruited to nSBs and enriched upon stress removal 1h post-stress then releases from nSBs at 4h late recovery with exception of YTHDC1 that exhibit prolonged enrichment at nSBs. Finally, we found that TDP43 depletion dramatically inhibited nSBs-m⁶A enrichment highlighting a novel regulatory role of TDP43 in stress-induced m⁶A RNA modification.

Discussion:

We identified two distinct nSBs (nSB-S and nSB-M) containing different sets of RNA-binding proteins that are formed with HSATIII arcRNAs upon thermal stress exposure. It is possible that nSB-S and nSB-M 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. On the other hand, we report a previously unrecognized thermal stress-induced recruitment of m⁶A machinery to nSBs and m⁶A RNAs localization to most HSATIII thermal stress-induced foci. To investigate the target RNAs of the m⁶A machinery in nSBs, we re-purified HSATIII and found that HSATIII is enriched with m⁶A modification. In addition, RNA-MS has identified the third A of GGAAU in the highly repetitive HSATIII sequence as the major m⁶A sites. Moreover, RNA-MS has identified a small population of HSATIII m⁶A consensus that exhibit two adjacent m⁶A modification at AA of GGAAU. The known consensus motif of m⁶A sites is GGACU which contains one base mismatch with m⁶A

sites in HSATIII, GGAAU lacking the C residue after A. The C residue in the m⁶A consensus has been reported to be essential for m⁶A deposition. Hence, shifting m⁶A consensus motif from GGACU to GGAAU might be thermal stress-dependent. Meanwhile, we cannot rule out the possibility that m⁶A machinery components and/or mechanism of action upon stress is distinct from normal condition. To elucidate the mechanism of action of the m⁶A machinery at nSBs, we screened the dynamics of m⁶A machinery components during stress/recovery phases. Remarkably, we found that m⁶A machinery has a unique recruitment pattern to nSBs, in which m⁶A machineries are recruited during stress and deposit m⁶A modification at HSATIII with relative enrichment in the early post-stress recovery stage then released from nSBs by 4h post-stress recovery stage. Only YTHDC1, the m⁶A reader, showed prolonged enrichment that might be due to additional non-m⁶A related function in nSBs during the late recovery stage. TDP43 was reported to specifically bind to GGAAU RNA foci formed in SCA31 disease. TDP43 depletion dramatically downregulated nSBs-m⁶A. Therefore, it raises the intriguing possibility that TDP43 acts as the adaptor to link the m⁶A regulatory factors to HSATIII lncRNAs with GGAAU repeat sequences under stressed condition.

Conclusion:

- The nSBs components SAFB and HNRNPM exhibit adjacent localizations in response to thermal stress.
- Both SAFB-foci and HNRNPM-foci utilize HSATIII lncRNA as architectural scaffold.
- SAFB-foci (nSB-S) and HNRNPM-foci (nSB-M) sequester two distinct sets of RNA-binding proteins. SLTM and NCOA5 are strictly recruited to nSB-S while HNRNPA1 and HNRNPH1 are strictly recruited to nSB-M.
- m⁶A writers and readers' machineries are sequestered to nSBs upon thermal stress.
- m⁶A methylated RNAs are localized at HSATIII-based nSBs.
- HSATIII lncRNAs transcripts are the target substrates of m⁶A machineries at nSBs.
- The m⁶A consensus within HSATIII is the highly repetitive sequence GGAAU that is distinct from the global GGACU m⁶A consensus.
- With exception of YTHDC1, all m⁶A factors follow a consistent recruitment pattern at nSBs where they are gradually sequestered during thermal stress (2 hours HS) and enriched at early recovery stage (1 hour post-stress) then rapidly released from nSBs at late recovery stage (4 hours post-stress)
- TDP43 is a possible modulator of m⁶A methylation in nSBs.

List of abbreviation

ALKBH5	α -ketoglutarate-dependent dioxygenase alkB homolog 5
arcRNA	Architectural RNA
ASO	Antisense oligonucleotide
ATP	Adenosine tri-phosphate
BEAN1	Brain expressed associated with NEDD4-1 gene
BSA	Bovine serum albumin
Cas9	CRISPR associated protein 9
ChIRP	Chromatin isolation by RNA purification
CID	Collision-induced dissociation
CRISPR	Clustered regularly interspaced short palindromic repeats
DAPI	4',6-diamidino-2-phenylindole
DIG	Digoxigenin
DMEM	Dulbecco's modified Eagle's medium
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
FISH	Fluorescence in situ hybridization
FITC	Fluorescein isothiocyanate
FTO	Fat-mass and obesity-associated protein
FUS	Fused in sarcoma
GeCKO	Genome-scale CRISPR Knock-Out
HNRNP	Heterogeneous nuclear ribonucleoprotein
HS	Heat shock
HSATIII	Highly repetitive satellite III
HSF1	Heat shock transcription factor 1
IF	Immunofluorescence
IgG	Immunoglobulin
IMDM	Iscove's modified dulbecco's medium
KD	Knockdown
KO	Knockout
lncRNA	Long noncoding RNA
m ⁶ A	N ⁶ -methyleadenosine

Me	Methylated
MeRIP	Methylated RNA immunoprecipitation
METTL3	Methyltransferase Like 3
mRNA	Messenger RNA
MS	Mass spectrometry
NCOA5	Nuclear receptor coactivator 5
NEDD4	Neural precursor cell expressed developmentally down-regulated protein 4
nSB-M	HNRNPM-based nuclear stress body
nSB-S	SAFB-based nuclear stress body
nSBs	Nuclear stress bodies
PBS	Phosphate buffered salts
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PGs	Perichromatin granules
RBM14	RNA-binding protein 14
RBP	RNA-binding proteins
RNA	Ribonucleic acid
RNase	Ribonuclease
rRNAs	Ribosomal RNA
RT	Reverse transcription
SAFB	Scaffold attachment factor B
SCA31	Spinocerebellar ataxia type 31
SDS	Sodium dodecyl sulfate
sgRNA	Small guide RNA
SIM	Structured illumination microscopy
siRNA	Small interfering RNA
SLTM	SAFB like transcription modulator
SRSF1	Serine and arginine rich splicing factor 1
SRSF7	Serine and arginine rich splicing factor 7
SSC	Saline-sodium citrate
TBST	Tris buffered saline with tween 20
TDP43	TAR DNA-binding protein 43 kDa
tRNAs	Transfer RNAs
UV	Ultraviolet

WB	Western blot
WTAP	Wilms Tumor 1 Associated Protein
XICs	Extracted-ion chromatograms
YTHDC1	YTH domain-containing protein 1
ZC3H13	Zinc finger CCCH-type containing 13

Introduction

Nuclear stress bodies (nSBs)

Nuclear stress bodies are primate-specific membrane-less subnuclear structures (or nuclear bodies) that are formed in response to thermal and chemical stresses (Biamonti and Vourc'h, 2010; Mahl et al., 1989). The assembly of nSBs is initiated alongside HSF1-dependent transcription of the primate-specific highly repetitive satellite III (HSATIII) long noncoding RNA (lncRNA) (Biamonti and Vourc'h, 2010) and heat shock-induced HSF1 aggregation (Jolly et al., 1997).

Human satellite III (HSATIII) long noncoding RNA

HSATIII lncRNAs are transcribed from pericentromeric HSATIII repeated arrays on several human chromosomes (Denegri et al., 2002). The largest HSATIII chromatic domains are mapped on human chromosome 9; these domains are usually located in heterochromatic regions that are transcriptionally silent, but are rapidly euchromatinized and produce HSATIII lncRNAs following exposure to thermal stress (Jolly et al., 2004; Metz et al., 2004; Rizzi et al., 2004). HSATIII lncRNAs 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 (Denegri et al., 2001; Metz et al., 2004; Weighardt et al., 1999), as well as specific chromatin-remodeling factors (Hussong et al., 2017; Kawaguchi et al., 2015) and transcription factors (Jolly et al., 2004), which results in the assembly of nSBs.

RNA modifications

RNA sequence and structure are prone to change during its lifecycle and this change is crucial for RNA self-regulation, influencing splicing, cellular localization, translation, and RNA longevity. Structural modifications in purine and pyrimidine rings of nucleobases have the ability to change the fate of RNA transcripts (Decatur and Fournier, 2002; Motorin and Helm, 2010; Serganov and Nudler, 2013). Modifications to RNA species have been reported for over 5 decades. Modified nucleotides were first recognized in abundant cellular RNAs as early as 1960 (Cohn, 1960). In 1965, ten RNA modifications were identified in the sequencing of first biological RNA from yeast (Holley et al., 1965). mRNAs are well known

to have cap and tail modifications. 5' cap modification contributes to the stability of mRNA transcripts, while the polyadenylation tail at 3' end enhances nuclear export, translation initiation and recycling in addition to promoting mRNA stability. Shortly after identification of cap and tail modifications, internal mRNA transcripts modifications were discovered (Adams and Cory, 1975; Desrosiers et al., 1974; Dubin and Taylor, 1975; Perry et al., 1975). Currently, over 170 RNA modifications have been identified and most RNA species residues have one or more distinct chemical modifications (Boccaletto et al., 2018). These modifications serve as posttranscriptional regulatory marks in a wide range of RNA species including mRNAs, tRNAs, rRNAs, small noncoding RNAs and long noncoding RNAs (Delaunay and Frye, 2019; Liu and Pan, 2016; Nachtergaele and He, 2017; Roundtree et al., 2017a; Zhao et al., 2017a).

N⁶-methyleadenosine (m⁶A)

N⁶-methyleadenosine (m⁶A) is the most abundant and prevalent internal modification in mRNA and long non-coding RNA. Installation of m⁶A RNA modification is modulated by m⁶A writers, a multicomponent methyltransferase complex that install the m⁶A modification, m⁶A readers, RNA binding proteins that recognize and bind to the m⁶A modification, and m⁶A erasers, that compete to demethylase and reverse the m⁶A modification (Roundtree et al., 2017a).

m⁶A writers

m⁶A writer complex consists of the catalytic subunit Methyltransferase Like 3 (METTL3) (Bokar et al., 1997; Liu et al., 2014), the substrate-recognizing subunit METTL14 (Delaunay and Frye, 2019; Sledz and Jinek, 2016; Wang et al., 2016a; Wang et al., 2016b), Wilms Tumor 1 Associated Protein (WTAP) (Ping et al., 2014; Zhong et al., 2008), KIAA1429 (Schwartz et al., 2014), and RNA Binding Motif Protein 15 (RBM15) (Patil et al., 2016; Yang et al., 2018).

METTL3

METTL3 protein methylates the GAC and AAC sequences in the synthetic single-stranded RNA (Kan et al., 2017; Rottman et al., 1994). METTL3 also localizes at nuclear speckles enriched with mRNA splicing factors indicating a potential role of m⁶A in mRNA metabolism (Ping et al., 2014). METTL3 nuclear activity was confirmed in initial studies using nuclear lysate to perform the methyltransferase assay. However, the activity of METTL3 in the

cytoplasmic fraction was also detected, though at a minor level (Harper et al., 1990). A METTL3 methyltransferase-independent cytoplasmic activity was also reported to modulate translation (Lin et al., 2016). As the principal methyltransferase subunit, METTL3 has been showed to regulate embryonic development (Aguilo et al., 2015; Bodi et al., 2012), cell reprogramming (Aguilo et al., 2015; Chen et al., 2015), spermatogenesis (Xu et al., 2017), T cell homeostasis (Li et al., 2017b), endothelial-to-haematopoietic transition (Lv et al., 2018; Zhang et al., 2017a), and its deletion in mice develops embryonic lethal effect (Geula et al., 2015). METTL3 is a highly conserved protein, with homologs in other species including *Saccharomyces cerevisiae* (IME4), *Drosophila melanogaster* (IME4) and *Arabidopsis Thaliana* (MTA) (Bodi et al., 2012; Bujnicki et al., 2002; Clancy et al., 2002; Hongay and Orr-Weaver, 2011; Zhong et al., 2008).

METTL14

METTL14 is additional component of the m⁶A writer complex; that form a stable heterodimer with METTL3 (Liu et al., 2014; Ping et al., 2014; Wang et al., 2014b). Separately, METTL3 and METTL14 exhibit a weak methyltransferase activity. However, the METTL3- METTL14 heterodimer has a much higher methyltransferase catalytic activity (Liu et al., 2014; Wang et al., 2014b). The principal function of METTL14 in the m⁶A writer complex is to provide an RNA-binding scaffold as illustrated in the crystal structure studies of the METTL3- METTL14 methyltransferase domain (MTD) (Sledz and Jinek, 2016; Wang et al., 2016a; Wang et al., 2017). Moreover, METTL14 promotes substantial roles in different biological processes. METTL14 depletion triggers a block in embryonic stem cell self-renewal and differentiation, embryonic developmental defects, and impaired gametogenesis in different organisms (Geula et al., 2015; Lin et al., 2017; Wang et al., 2014b). Along with METTL3, METTL14 contributes in neurogenesis by moderating cell-cycle progression of cortical neural progenitor cells in an m⁶A-dependent manner (Yoon et al., 2017). Additionally, METTL14 promotes tumor-suppression in glioblastoma, and its depletion METTL14 enhances growth and self-renewal of glioblastoma stem cells (Cui et al., 2017).

WTAP

WTAP is another component of the m⁶A writer complex that stabilizes the METTL3- METTL14 heterodimer and modulates the m⁶A levels of RNA transcripts (Agarwala et al., 2012; Liu et al., 2014; Ping et al., 2014; Zhong et al., 2008). WTAP lacks the methyltransferase domains and shows no catalytic activity of m⁶A modification (Liu et al.,

2014; Ping et al., 2014). On the other hand, WTAP depletion promotes tissue-dependent defects. Zebrafish embryos lacking WTAP undergo increased apoptosis (Ping et al., 2014). In addition, WTAP is upregulated in AML where it plays an oncogenic role (Bansal et al., 2014).

KIAA1429/ VIRMA

KIAA1429 was recognized as another component of the m⁶A methyltransferase complex by both proteomic screening and cellular studies (Schwartz et al., 2014). KIAA1429 recruits the catalytic core components (METTL3/METTL14/WTAP) to guide region-selective m⁶A methylation (Yue et al., 2018). Moreover, KIAA1429 depletion induced an about 4-fold reduction in m⁶A peak scores in human A549 cells proposing an essential regulatory role of KIAA1429 in the methyltransferase complex (Schwartz et al., 2014).

RBM15/RBM15B

RBM15 and its paralogue RBM15B modulate m⁶A methylation of mRNAs and the lncRNA target XIST. Immunoprecipitation analysis showed that RBM15/RBM15B bind and recruit the METTL3/WTAP complex to specific sites. RBM15/RBM15B iCLIP-seq data and m⁶A-miCLIP-seq data found that RBM15/RBM15B binding sites are significantly enriched at the sequences adjacent to m⁶A methylation sites (Patil et al., 2016). Consistently, the RBM15 homolog, Spenito (Nito), was revealed to be a novel subunit of the methyltransferase complex required for m⁶A mRNAs methylation in *Drosophila* (Lence et al., 2016).

m⁶A erasers

m⁶A first discovery was in 1970s (Desrosiers et al., 1974). However, the intense studies on its functions have begun a few years ago after the identification of the first demethylase, fat-mass and obesity-associated protein (FTO) that provided evidence that m⁶A RNA modification is a reversible process with regulatory roles (Jia et al., 2011). Two years later, another m⁶A demethylase was discovered, α -ketoglutarate-dependent dioxygenase alkB homolog 5 (ALKBH5) (Zheng et al., 2013). Unveiling the demethylase activity of FTO and ALKBH5, the m⁶A erasers, provided the first evidence of reversible mRNA post-transcriptional modification.

FTO

FTO was first identified to be associated with increased human body mass and obesity (Dina et al., 2007; Frayling et al., 2007; Scuteri et al., 2007; Zhao et al., 2014). All adult and fetal tissues are widely expressing FTO with elevated expression in the brain. FTO was initially reported as a demethylase for N3-methylthymidine in single-stranded DNA and for N3-methyluridine in single-stranded RNA in vitro (Gerken et al., 2007; Jia et al., 2008). The first recognition that FTO demethylates m⁶A in both DNA and RNA was in 2011. FTO depletion provokes significant increase in total m⁶A levels of polyadenylated RNA (Jia et al., 2011). As FTO oxidizes m⁶A to A, N6-hydroxymethyladenosine (hm⁶A) is produced as an intermediate product, and N6-formyladenosine (f6A) as an additional oxidized product (Fu et al., 2013). In addition, FTO was recently identified to exhibit demethylase activity against m⁶A_m, a modification strictly localized at the first encoded nucleotide after the 7- methylguanosine cap structure, and result in decreasing the mRNAs stability in vivo (Mauer et al., 2017). These findings suggest FTO work on various substrates. Moreover, FTO has been reported to function as oncogene in leukemia (Li et al., 2017c; Su et al., 2018) and glioblastoma (Cui et al., 2017).

ALKBH5

Two years after identification of the demethylase activity of FTO, ALKBH5 was identified as the second m⁶A demethylase. ALKBH5 localizes to the nucleus and its depletion decreases the global polyA RNAs proposing a function in mRNA nuclear export regulation. Most tissues express ALKBH5 with abundance in the testes which impacts mouse spermatogenesis and fertility (Zheng et al., 2013). ALKBH5 modulates splicing and stability of mRNAs in spermatocytes nuclei by eliminating pre-mRNAs m⁶A and allowing the production of mRNAs containing longer 3'UTRs (Tang et al., 2018). Furthermore, ALKBH5 plays an essential role in the immune response to viral infections in macrophages. To inhibit interferon production, the nuclear protein DDX46 recruits ALKBH5 after viral infection to demethylate m⁶A at antiviral transcripts, thus sequestering them in the nucleus (Zheng et al., 2017). In addition ALKBH5 contributes to cancer progression as was reported that ALKBH5 overexpression in glioblastoma stem cells is required for tumorigenesis and proliferation and predicts poor patient survival (Zhang et al., 2017b).

Chapter#1

1.1 Title

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

1.2 Introduction

Nuclear bodies

Nuclear bodies (NBs) are non-membrane bound structures in the nucleoplasm that are microscopically visible (0.2 to 2 μm in diameter), concentrate specific nuclear factors, namely proteins and RNAs, and constantly exchange their components with the surrounding nucleoplasm. In addition, nuclear bodies are distinguished from nuclear protein aggregates that form in the nucleus during certain pathologies, for example, amyloid plaques (Courchaine et al., 2016). Nuclear bodies appear in different forms ranging from round balls to irregular shape structures (Stanek and Fox, 2017). Some of them have uniform inner structure while others show further sub-compartmentalization. Nuclear bodies' formation is generally dynamic, especially in response to a variety of stress conditions (Boulon et al., 2010). There are numerous bodies present in the nucleus of most cells, including, but not limited to, the nucleolus, Cajal body, histone locus body, PML body, nuclear speckles, paraspeckles, Sam68 body, Polycomb body and nuclear stress bodies. The nucleus may contain additional as yet uncharacterized structures containing specific sets of proteins (Fong et al., 2013). Majority of the cellular bodies have the characteristics of liquid droplets including phase-separation from the surrounding cytoplasm or nucleoplasm, fusion and becoming bigger droplets and breaking down to smaller droplets upon exposure to shearing forces (Banani et al., 2017; Brangwynne et al., 2011). This phase-separation state of most of cellular bodies is achieved by the multivalency concept of polymer chemistry through several intermolecular interactions causing self-assemble of large polymers that lead to body formation by the liquid-liquid phase separation LLPS (Molliex et al., 2015; Nott et al., 2015). Cajal body, paraspeckle, histone locus body, and nucleolus are formed upon concentrating their key components at certain

cellular loci (Berry et al., 2015; Kaiser et al., 2008; Mao et al., 2011). Proteins that are able to achieve the liquid-liquid phase separation are characterized by possessing regions with limited number of amino acid types named low-complexity domain (LCD) (Kato et al., 2012; Molliex et al., 2015). Low-complexity domains are characterized by lacking the classical three-dimensional structure of common protein domains which enhance the multivalent weak intermolecular interactions including hydrophobic, pi stacking, and electrostatic interactions (Nott et al., 2015; Petri et al., 2012; Reichheld et al., 2017). In consistence with the phase-separated liquids dynamic nature, these weak intermolecular interactions afford slight structural order to the peptide chain. In addition, the cellular bodies are usually stable for several hours and even for days. However, their protein components are often dynamic shuttling between the cellular bodies and the surrounding cellular environments (Dundr et al., 2004; Mao et al., 2011).

Architectural RNAs (arcRNAs)

A group of nuclear bodies utilizes certain RNAs as their structural scaffold that form the nuclear body's skeleton. These nuclear bodies' structural scaffolds are either long noncoding RNA or pre-mRNAs and defined as architectural RNAs (arcRNAs) (Chujo et al., 2016). The basic criteria for an RNA to be classified as architectural RNA is to localize to certain nuclear body and to construct the nuclear body structure that should be disintegrated upon depletion of that specific RNA. Examples of arcRNAs include human highly repetitive satellite III (HSATIII) lncRNA for nuclear stress bodies (Valgardsdottir et al., 2005), intergenic spacer (IGS) lncRNA for the amyloid body (Audas et al., 2012), mammalian nuclear paraspeckle assembly transcript 1 isoform 2 (NEAT1_2) lncRNA for the paraspeckle (Sasaki et al., 2009; Shevtsov and Dundr, 2011), histone pre-mRNA for the histone locus body (Shevtsov and Dundr, 2011), fission yeast meiRNA for the Mei2 dot (Yamashita et al., 1998), and *Drosophila* heat shock RNA (Hsr) omega for the omega speckle (Mallik and Lakhotia, 2009; Chujo and Hirose, 2017).

Nuclear stress bodies

Nuclear stress bodies (nSBs) are primate-specific membrane-less subnuclear structures (or nuclear bodies) that are formed in response to thermal and chemical stresses (Biamonti and Vourc'h, 2010; Mahl et al., 1989). The assembly of nSBs is initiated alongside HSF1-dependent transcription of the primate-specific highly repetitive satellite III (HSATIII) long noncoding RNA (lncRNA) (Biamonti and Vourc'h, 2010) and heat shock-induced HSF1

aggregation (Jolly et al., 1997). HSATIII lncRNAs are transcribed from pericentromeric HSATIII repeated arrays on several human chromosomes (Denegri et al., 2002). The largest HSATIII chromatic domains are mapped on human chromosome 9; these domains are usually located in heterochromatic regions that are transcriptionally silent, but are rapidly euchromatinized and produce HSATIII lncRNAs following exposure to thermal stress (Jolly et al., 2004; Metz et al., 2004; Rizzi et al., 2004). HSATIII lncRNAs 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 (Denegri et al., 2001; Metz et al., 2004; Weighardt et al., 1999), as well as specific chromatin-remodeling factors (Hussong et al., 2017; Kawaguchi et al., 2015) and transcription factors (Jolly et al., 2004), which results in the assembly of nSBs.

Recently, our group found that HSATIII and nSBs promote intron retention of 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 (Chiodi et al., 2000; Mahl et al., 1989), 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 sequestering distinct sets of RBPs.

1.3 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% CO₂. 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, 3×10^6 cells were transfected with chemically modified antisense or control oligonucleotides (1 μ M) using Nucleofector (Lonza) 16–18 hours before the assay, as described previously (Ideue et al., 2009). 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 (Sanjana et al., 2014) and cloned into the BbsI site of PX330 (Addgene). The plasmids (2 μ g) and pcDNA6/TR plasmids (0.2 μ g) were transfected similarly using a Nucleofector device (Lonza). To enrich the plasmid-transfected cells, the HAP1 cells were treated with 20 μ g/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 μ g/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 SAFB 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/2× SSC, hybridized with a digoxigenin (DIG)-labeled HSATIII antisense oligonucleotide (ASO) in hybridization buffer (10% formamide, 2× SSC, and 10% dextran) for 16 hours at 37°C, incubated in 10% formamide/2× 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 (SIM)

Structured illumination microscopy was performed as described previously (Yamazaki et al., 2018). 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 (Yamazaki et al., 2018).

1.4 Results

1.4.1 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 (HNRNPM-positive).

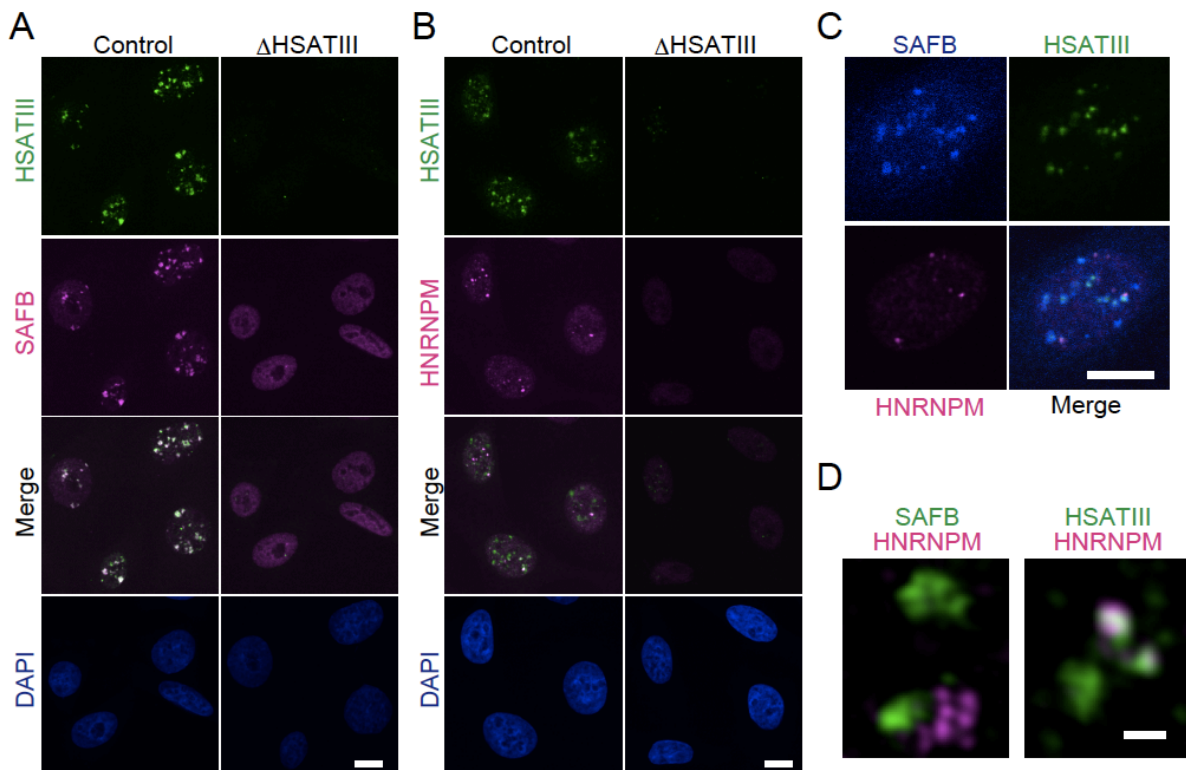


Figure 1: HNRNPM foci are distinct nSBs formed in association with HSATIII arcRNAs.

(A) Detection of the canonical nSBs via HSATIII RNA-FISH and SAFB immunofluorescence in control and Δ HSATIII 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 μ m.

(B) Detection of the HNRNPM foci via HSATIII RNA-FISH and HNRNPM immunofluorescence in control and Δ HSATIII cells. The cells were incubated as described in A. The nuclei were stained with DAPI. Scale bar: 10 μ m.

(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 μ m.

(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.

1.4.2 Identification of new components of nSB-Ss

To comprehensively characterize protein components of nSBs, Dr. Ninomiya in the laboratory recently carried out Comprehensive identification of RNA-binding proteins by mass spectrometry (ChIRP-MS) analysis. ChIRP-MS is a technique to purify RNA-protein complexes using specific biotinylated probes and streptavidin beads purification followed by mass spectrometry analysis to identify the co-precipitated RNA-binding proteins. The 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 (Norman et al., 2016), we confirmed that the anti-SLTM and anti-SAFB antibodies did not reciprocally cross-react and that they detected the specific target only (Figure 3).

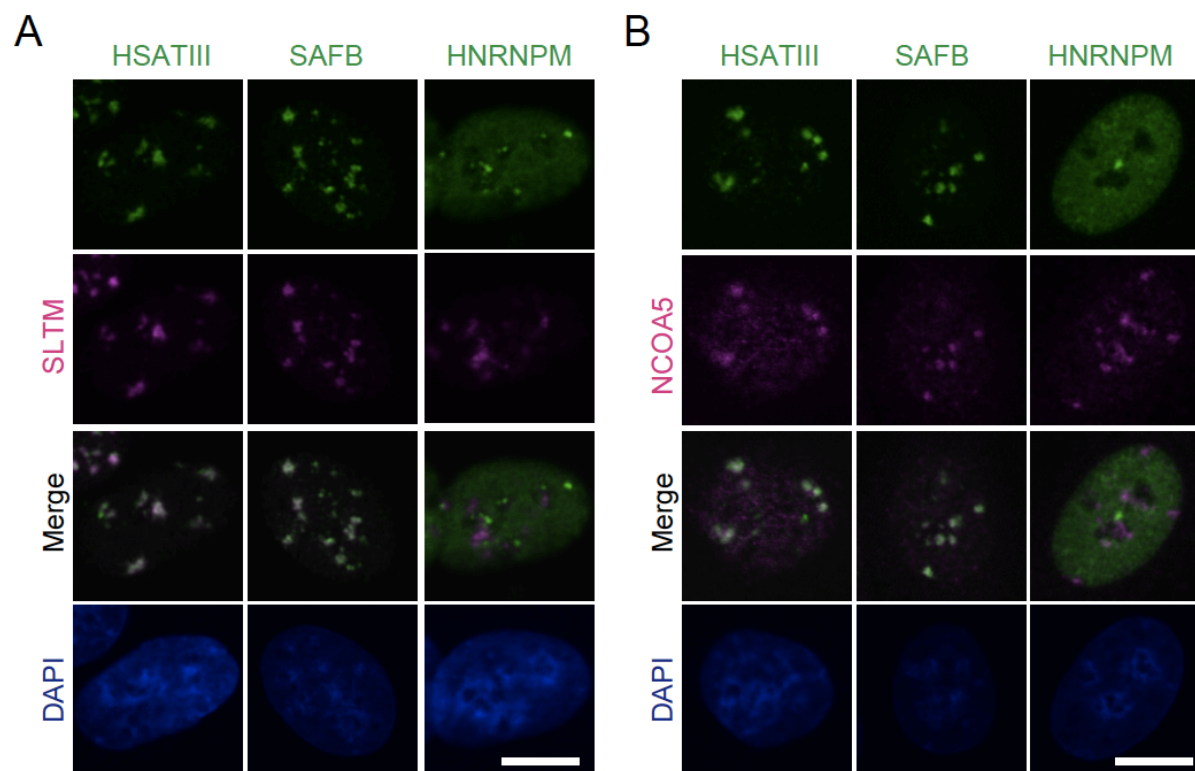


Figure 2: Identification of novel nSB-S components.

Immunofluorescent detection of SLTM (A) and NCOA5 (B) co-stained with HSAIII, SAFB, or HNRNPM. HSAIII 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 μ m.

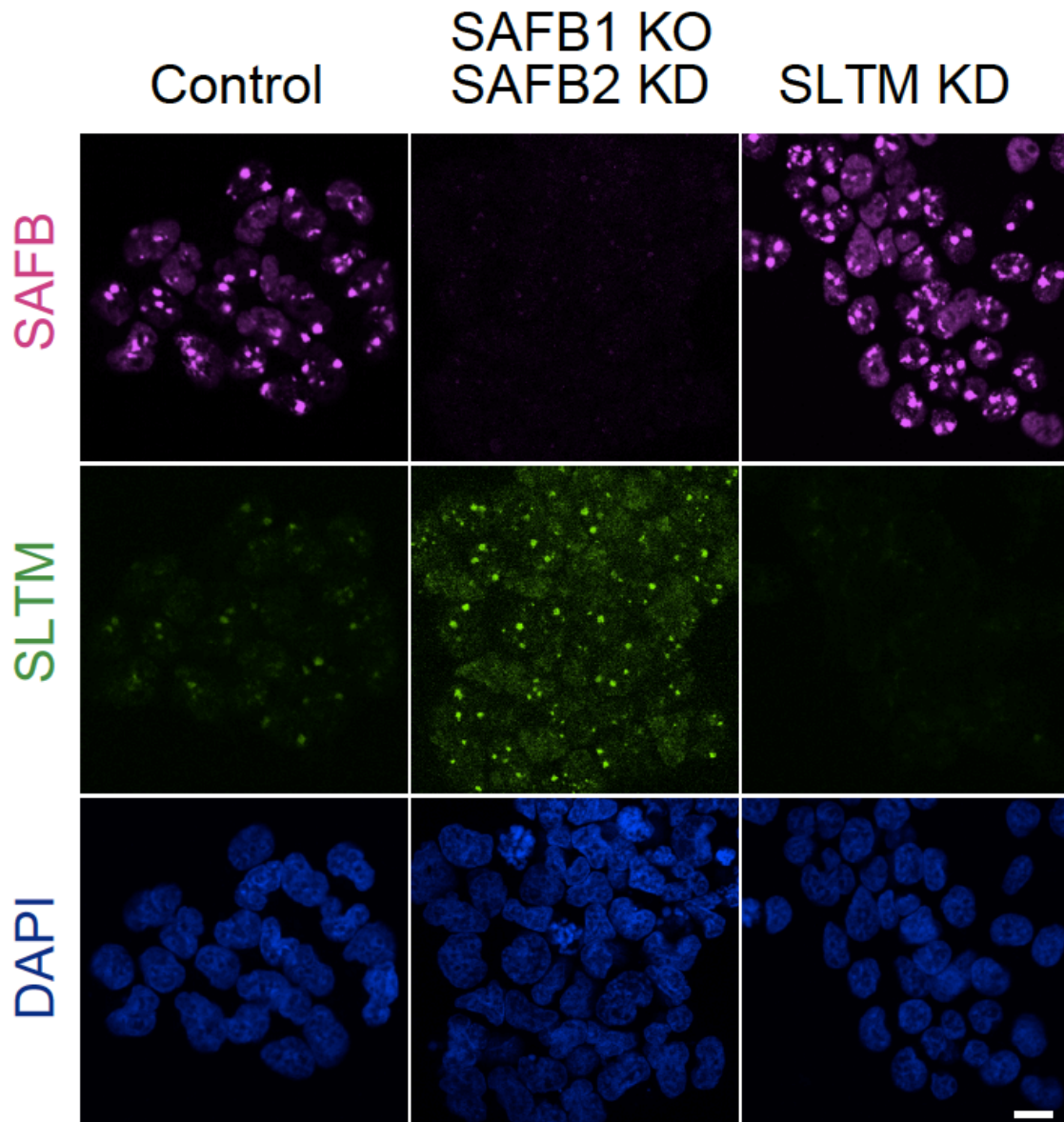


Figure 3: Specific detection of SAFB and SLTM.

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 μ m.

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 (Baltz et al., 2012; Castello et al., 2012).

1.4.3 nSB-Ms and nSB-Ss sequester distinct sets of RBPs

Among the 141 newly identified proteins, HNRNPA1 and HNRNPH1 were specifically localized in nSB-Ms but not nSB-Ss (Figure 4A and B). Like HNRNPM, both proteins are relatively abundant HNRNPs that possess canonical RNA recognition motifs as well as intrinsically disordered regions.

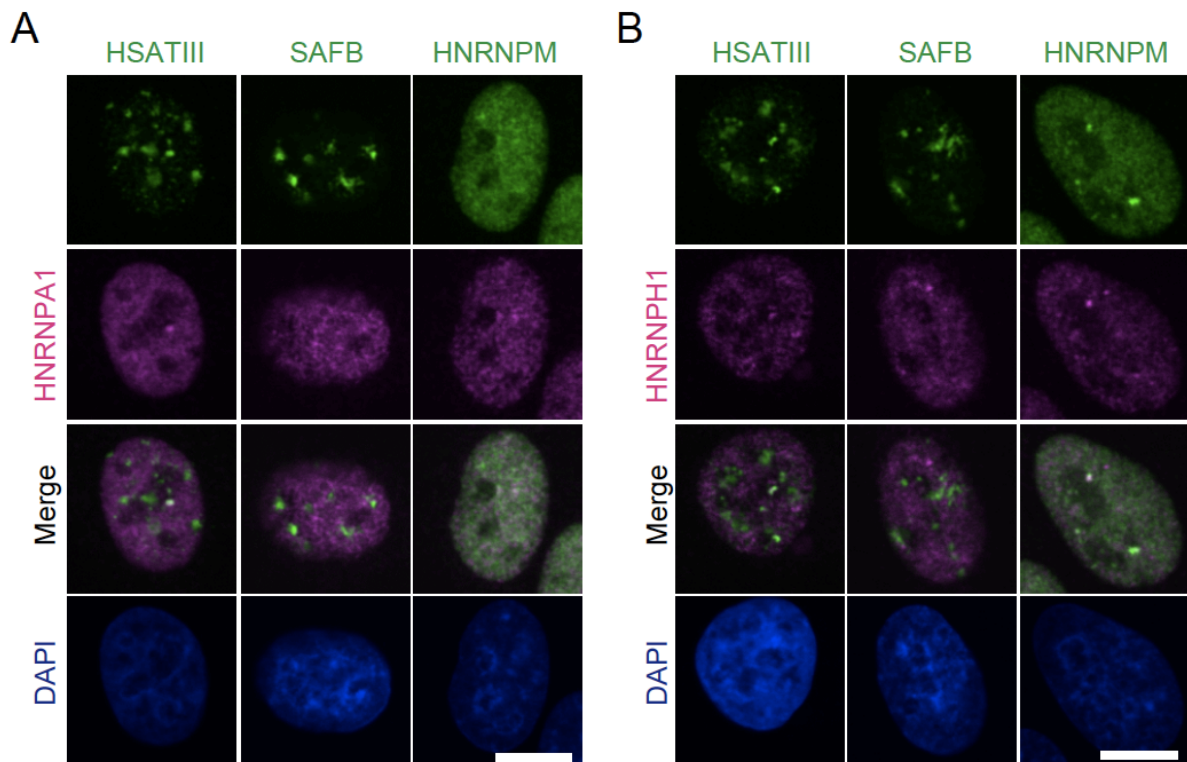


Figure 4: Identification of novel nSB-M components.

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.

1.5 Discussion

In the current study, we identified two distinct thermal stress-inducible nuclear bodies that are marked by SAFB and HNRNPM (Figure 5). 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 (HNRNPA1 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 (Chiodi et al., 2000). 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 (Chiodi et al., 2000). 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 (Denegri et al., 2002). 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.

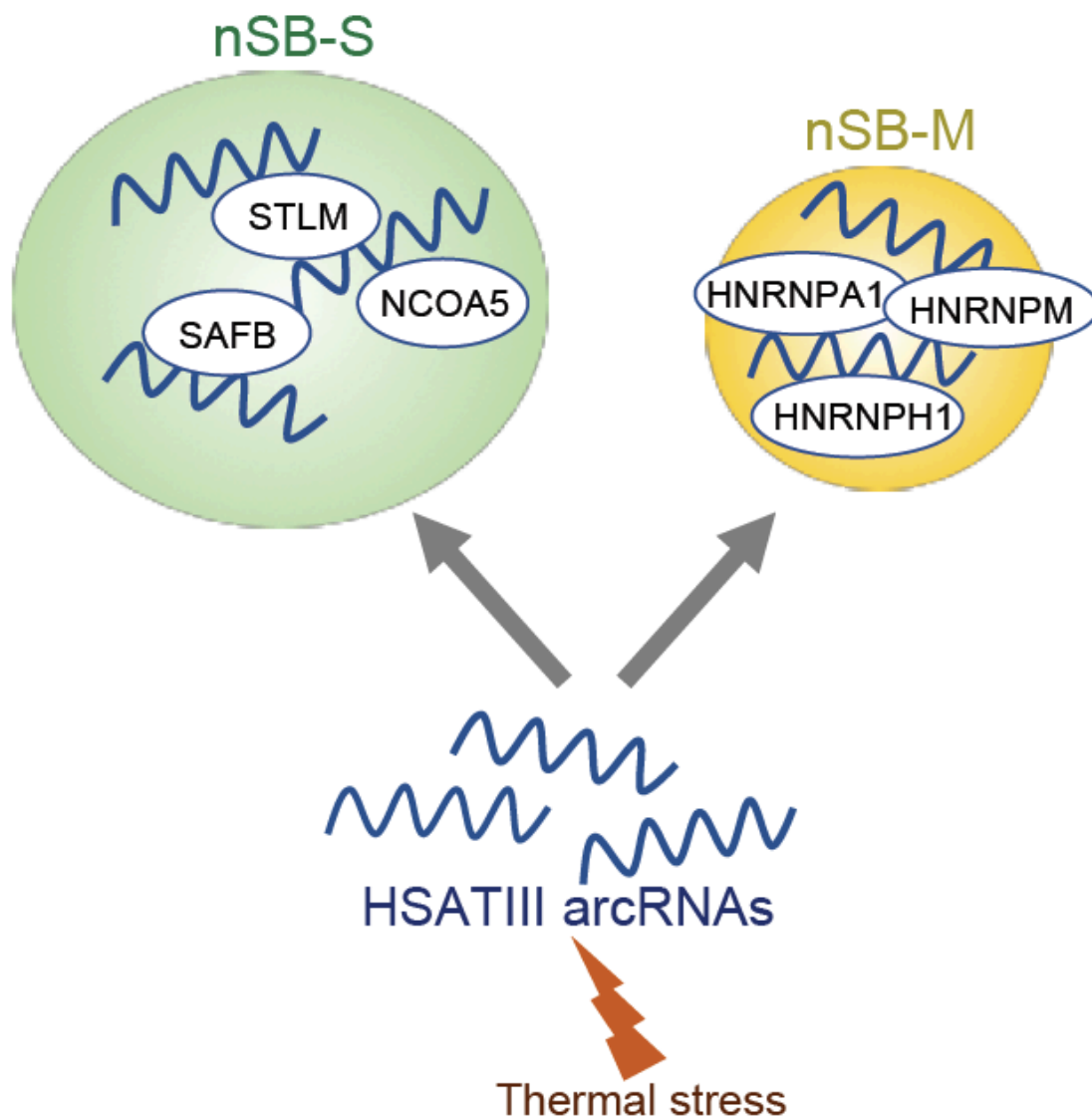


Figure 5: Model of two distinct nSBs.

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.

Chapter#2

2.1 Title

Stress-Induced Transient m⁶A Methylation in HSATIII lncRNA Based Nuclear Stress Bodies

2.2 Introduction

RNA modifications

RNA sequence and structure are prone to change during its lifecycle and this change is crucial for RNA self-regulation, influencing splicing, cellular localization, translation, and RNA longevity. Structural modifications in purine and pyrimidine rings of nucleobases have the ability to change the fate of RNA transcripts (Decatur and Fournier, 2002; Motorin and Helm, 2010; Serganov and Nudler, 2013). Modifications to RNA species have been reported for over 5 decades. Modified nucleotides were first recognized in abundant cellular RNAs as early as 1960 (Cohn, 1960). In 1965, ten RNA modifications were identified in the sequencing of first biological RNA from yeast (Holley et al., 1965). mRNAs are well known to have cap and tail modifications. 5' cap modification contributes to the stability of mRNA transcripts, while the polyadenylation tail at 3' end enhances nuclear export, translation initiation and recycling in addition to promoting mRNA stability. Shortly after identification of cap and tail modifications, internal mRNA transcripts modifications were discovered (Adams and Cory, 1975; Desrosiers et al., 1974; Dubin and Taylor, 1975; Perry et al., 1975). Currently, over 170 RNA modifications have been identified and most RNA species residues have one or more distinct chemical modifications (Boccaletto et al., 2018). These modifications serve as posttranscriptional regulatory marks in a wide range of RNA species including mRNAs, tRNAs, rRNAs, small noncoding RNAs and long noncoding RNAs (Delaunay and Frye, 2019; Liu and Pan, 2016; Nachtergaele and He, 2017; Roundtree et al., 2017a; Zhao et al., 2017a).

N⁶-methyleadenosine (m⁶A)

N⁶-methyleadenosine (m⁶A) is the most abundant and prevalent internal modification in mRNA and long non-coding RNA. Installation of m⁶A RNA modification is modulated by m⁶A writers, a multicomponent methyltransferase complex that install the m⁶A modification, m⁶A readers, RNA binding proteins that recognize and bind to the m⁶A modification, and m⁶A erasers, that compete to demethylase and reverse the m⁶A modification (Roundtree et al., 2017a).

m⁶A writers

m⁶A writer complex consists of the catalytic subunit Methyltransferase Like 3 (METTL3) (Bokar et al., 1997; Liu et al., 2014), the substrate-recognizing subunit METTL14 (Delaunay and Frye, 2019; Sledz and Jinek, 2016; Wang et al., 2016a; Wang et al., 2016b), Wilms Tumor 1 Associated Protein (WTAP) (Ping et al., 2014; Zhong et al., 2008), KIAA1429 (Schwartz et al., 2014), and RNA Binding Motif Protein 15 (RBM15) (Patil et al., 2016; Yang et al., 2018). METTL3 protein methylates the GAC and AAC sequences in the synthetic single-stranded RNA (Kan et al., 2017; Rottman et al., 1994). METTL3 also localizes at nuclear speckles enriched with mRNA splicing factors indicating a potential role of m⁶A in mRNA metabolism (Ping et al., 2014). METTL3 nuclear activity was confirmed in initial studies using nuclear lysate to perform the methyltransferase assay. However, the activity of METTL3 in the cytoplasmic fraction was also detected, though at a minor level (Harper et al., 1990). A METTL3 methyltransferase-independent cytoplasmic activity was also reported to modulate translation (Lin et al., 2016). As the principal methyltransferase subunit, METTL3 has been showed to regulate embryonic development (Aguilo et al., 2015; Bodi et al., 2012), cell reprogramming (Aguilo et al., 2015; Chen et al., 2015), spermatogenesis (Xu et al., 2017), T cell homeostasis (Li et al., 2017b), endothelial-to-haematopoietic transition (Lv et al., 2018; Zhang et al., 2017a), and its deletion in mice develops embryonic lethal effect (Geula et al., 2015). METTL3 is a highly conserved protein, with homologs in other species including *Saccharomyces cerevisiae* (IME4), *Drosophila melanogaster* (IME4) and *Arabidopsis Thaliana* (MTA) (Bodi et al., 2012; Bujnicki et al., 2002; Clancy et al., 2002; Hongay and Orr-Weaver, 2011; Zhong et al., 2008). METTL14 is additional component of the m⁶A writer complex; that form a stable heterodimer with METTL3 (Liu et al., 2014; Ping et al., 2014; Wang et al., 2014b). Separately, METTL3 and METTL14 exhibit a weak methyltransferase activity. However, the METTL3-METTL14 heterodimer has a much higher methyltransferase catalytic activity (Liu et al., 2014; Wang et al., 2014b). The principal

function of METTL14 in the m⁶A writer complex is to provide an RNA-binding scaffold as illustrated in the crystal structure studies of the METTL3-METTL14 methyltransferase domain (MTD) (Sledz and Jinek, 2016; Wang et al., 2017). Moreover, METTL14 promotes substantial roles in different biological processes. METTL14 depletion triggers a block in embryonic stem cell self-renewal and differentiation, embryonic developmental defects, and impaired gametogenesis in different organisms (Geula et al., 2015; Lin et al., 2017; Wang et al., 2014b). Along with METTL3, METTL14 contributes in neurogenesis by moderating cell-cycle progression of cortical neural progenitor cells in an m⁶A-dependent manner (Yoon et al., 2017). Additionally, METTL14 promotes tumor-suppression in glioblastoma, and its depletion METTL14 enhances growth and self-renewal of glioblastoma stem cells (Cui et al., 2017). WTAP is another component of the m⁶A writer complex that stabilizes the METTL3-METTL14 heterodimer and modulates the m⁶A levels of RNA transcripts (Agarwala et al., 2012; Liu et al., 2014; Ping et al., 2014; Zhong et al., 2008). WTAP lacks the methyltransferase domains and shows no catalytic activity of m⁶A modification (Liu et al., 2014; Ping et al., 2014). On the other hand, WTAP depletion promotes tissue-dependent defects. Zebrafish embryos lacking WTAP undergo increased apoptosis (Ping et al., 2014). In addition, WTAP is upregulated in AML where it plays an oncogenic role (Bansal et al., 2014). KIAA1429/VIRMA was recognized as another component of the m⁶A methyltransferase complex by both proteomic screening and cellular studies (Schwartz et al., 2014). KIAA1429 recruits the catalytic core components (METTL3/METTL14/WTAP) to guide region-selective m⁶A methylation (Yue et al., 2018). Moreover, KIAA1429 depletion induced an about 4-fold reduction in m⁶A peak scores in human A549 cells proposing an essential regulatory role of KIAA1429 in the methyltransferase complex (Schwartz et al., 2014). RBM15 and its paralogue RBM15B modulate m⁶A methylation of mRNAs and the lncRNA target XIST. Immunoprecipitation analysis showed that RBM15/RBM15B bind and recruit the METTL3/WTAP complex to specific sites. RBM15/RBM15B iCLIP-seq data and m⁶A-miCLIP-seq data found that RBM15/RBM15B binding sites are significantly enriched at the sequences adjacent to m⁶A methylation sites (Patil et al., 2016). Consistently, the RBM15 homolog, Spenito (Nito), was revealed to be a novel subunit of the methyltransferase complex required for m⁶A mRNAs methylation in *Drosophila* (Lence et al., 2016).

m⁶A erasers

m⁶A first discovery was in 1970s (Desrosiers et al., 1974). However, the intense studies on its functions have begun a few years ago after the identification of the first demethylase, fat-mass

and obesity-associated protein (FTO) that provided evidence that m⁶A RNA modification is a reversible process with regulatory roles (Jia et al., 2011). Two years later, another m⁶A demethylase was discovered, α -ketoglutarate-dependent dioxygenase alkB homolog 5 (ALKBH5) (Zheng et al., 2013). Unveiling the demethylase activity of FTO and ALKBH5, the m⁶A erasers, provided the first evidence of reversible mRNA post-transcriptional modification. FTO was first identified to be associated with increased human body mass and obesity (Dina et al., 2007; Frayling et al., 2007; Scuteri et al., 2007; Zhao et al., 2014). All adult and fetal tissues are widely expressing FTO with elevated expression in the brain. FTO was initially reported as a demethylase for N3-methylthymidine in single-stranded DNA and for N3-methyluridine in single-stranded RNA in vitro (Gerken et al., 2007; Jia et al., 2008). The first recognition that FTO demethylates m⁶A in both DNA and RNA was in 2011. FTO depletion provokes significant increase in total m⁶A levels of polyadenylated RNA (Jia et al., 2011). As FTO oxidizes m⁶A to A, N6-hydroxymethyladenosine (hm⁶A) is produced as an intermediate product, and N6-formyladenosine (f6A) as an additional oxidized product (Fu et al., 2013). In addition, FTO was recently identified to exhibit demethylase activity against m⁶A_m, a modification strictly localized at the first encoded nucleotide after the 7-methylguanosine cap structure, and result in decreasing the mRNAs stability in vivo (Mauer et al., 2017). These findings suggest FTO work on various substrates. Moreover, FTO has been reported to function as oncogene in leukemia (Li et al., 2017c; Su et al., 2018) and glioblastoma (Cui et al., 2017). Two years after identification of the demethylase activity of FTO, ALKBH5 was identified as the second m⁶A demethylase. ALKBH5 localizes to the nucleus and its depletion decreases the global polyA RNAs proposing a function in mRNA nuclear export regulation. Most tissues express ALKBH5 with abundance in the testes which impacts mouse spermatogenesis and fertility (Zheng et al., 2013). ALKBH5 modulates splicing and stability of mRNAs in spermatocytes nuclei by eliminating pre-mRNAs m⁶A and allowing the production of mRNAs containing longer 3'UTRs (Tang et al., 2018). Furthermore, ALKBH5 plays an essential role in the immune response to viral infections in macrophages. For inhibiting production of interferon, ALKBH5 is recruited by DDX46 after viral infection to demethylate m⁶A at antiviral transcripts that results in sequestering them in the nucleus (Zheng et al., 2017). In addition ALKBH5 contributes to cancer progression as was reported that ALKBH5 overexpression in glioblastoma stem cells is required for tumorigenesis and proliferation and predicts poor patient survival (Zhang et al., 2017b).

Biological significance of m⁶A via m⁶A readers binding

Several RNA-binding proteins have been reported to selectively bind to m⁶A-containing RNAs, named m⁶A readers. Investigations about these reader proteins have begun to reveal the ultimate role of m⁶A in different biological processes (Yang et al., 2018). m⁶A readers include YTH domain family members YTHDC1 (Patil et al., 2016; Xiao et al., 2016), YTHDF1 (Wang et al., 2015), YTHDF2 (Du et al., 2016; Wang et al., 2014a; Zhou et al., 2015), and YTHDF3 (Li et al., 2017a; Shi et al., 2017). In addition, three members of heterogeneous nuclear ribonucleoprotein (HNRNP) family HNRNPA2B1 (Alarcon et al., 2015), HNRNPC (Liu et al., 2015), and HNRNPG (Liu et al., 2017) selectively bind to m⁶A and function to regulate the processing of m⁶A-modified transcripts. The discovery and study of m⁶A readers has greatly enhanced our understanding of the regulatory role of m⁶A in various biological processes including cancer proliferation (Dai et al., 2018; Lin et al., 2016), sex determination (Lence et al., 2016), cell differentiation (Batista et al., 2014; Geula et al., 2015; Wang et al., 2014b), neuronal function (Merkurjev et al., 2018; Weng et al., 2018), meiosis (Clancy et al., 2002; Schwartz et al., 2013), circadian rhythm (Fustin et al., 2013), and chromosomal silencing (Patil et al., 2016).

YTHDC1 is a member of the highly conserved YTH family proteins (Zhang et al., 2010b), which locates in the nucleus and forms YT bodies at transcriptionally active sites adjacent to RNA processing speckles (Zhang et al., 2010a). YTHDC1 favorably recognizes the GG(m⁶A)C sequences through its YTH domain (Xu et al., 2014). YTHDC1 modulates exon inclusion by recruiting or inhibiting different splicing factors such as Serine-arginine repeat (SR) proteins (Kasowitz et al., 2018). Additionally, YTHDC1 recognizes m⁶A on XIST and promotes XIST-mediated X chromosome silencing (Patil et al., 2016). Moreover, YTHDC1 enhance m⁶A mRNAs export through interaction with SRSF3 and nuclear RNA export factor 1 (NXF1) (Roundtree et al., 2017b) which extends the roles of YTHDC1-mediated m⁶A reading in mRNA metabolism regulation. On the other hand, YTHDC1 has been recognized as a tumor suppressor in endometrial cancer through its interaction with other splicing factors (Hirschfeld et al., 2014; Zhang et al., 2010a). YTHDC2, the largest member of the YTH family, also preferentially binds m⁶A within the consensus motif and promotes the translation efficiency while decreasing the abundance of its target mRNAs (Bailey et al., 2017; Hsu et al., 2017; Jain et al., 2018; Wojtas et al., 2017). YTHDC2 has been reported to have a role in spermatogenesis through interaction with the meiosis-specific protein MEIOC to affect the target transcripts stability during meiosis prophase I (Abby et al., 2016).(Soh et al., 2017). The lack of m⁶A binding activity of YTHDC2 was also reported in HEK cells (Patil et al.,

2016; Patil et al., 2018). The controversial findings that YTHDC2 works as a reader of m⁶A in mouse testes but not in HEK cells suggest a cell- or tissue-specific role of YTHDC2; it is also possible that YTHDC2 may indirectly modulate m⁶A transcripts through interaction with other factors. Interestingly, YTHDC2 is much larger than other YTH proteins (~160 kDa vs ~60 kDa), and holds multiple helicase domains. These unique features may provide YTHDC2 with a variety of roles, including regulatory effects on RNA binding and RNA structure, and recruitment of or binding with other protein complexes (Hsu et al., 2017; Tanabe et al., 2016). Recent studies elevated some controversies whether HNRNPA2B1 serves as m⁶A reader. Alarcon et al. reported that HNRNPA2B1 binds m⁶A and regulate alternative splicing events and microRNA processing along with METTL3 (Alarcon et al., 2015). Moreover, a structure-based study shown an “m⁶A switch” mechanism directed by HNRNPA2B1 (Wu et al., 2018). Additional two HNRNP proteins, HNRNPC1 and HNRNPG, have been reported to modulate the processing of m⁶A transcripts. They do not bind to m⁶A directly, but instead, m⁶A works as a structural switch to alter the RNA structure, which renders transcripts more accessible for binding by HNRNPC1 and HNRNPG (Liu et al., 2017). YTHDF1 was initially showed to bind m⁶A mRNA transcripts at sites near the stop codon, and its overall distribution pattern is similar to that of m⁶A sites on mRNAs. Mechanistic study has demonstrated that YTHDF1 interacts with the translation initiation machinery and enhances the translation efficiency of its target RNAs (Wang et al., 2015). YTHDF2 co-localizes with both deadenylation and decapping enzyme complexes under normal conditions and directs its targets to processing bodies (Wang et al., 2014a). By directly recruiting the CCR4-NOT deadenylase complex, YTHDF2 accelerates the degradation of m⁶A-modified transcripts (Du et al., 2016). In zebrafish, Ythdf2 deletion in embryos decelerates the decay of m⁶A-modified maternal mRNAs and impedes zygotic genome activation (Zhao et al., 2017b). YTHDF2-mediated decay of transcripts of the arterial endothelial genes notch1a and rhoca contributes to the emergence of haematopoietic stem/progenitor cells (HSPCs) during endothelial-to-haematopoietic transition (EHT) (Zhang et al., 2017a). In mice, YTHDF2 determines oocyte competence and early zygotic development by regulating maternal transcript dosage (Ivanova et al., 2017). In cells exposed to heat shock, YTHDF2 expression is upregulated with translocation of its protein to nucleus to protect m⁶A residues from demethylation by FTO in 5'UTR of stress-induced transcripts. Transcripts with enhanced 5'UTR methylation are selectively translated via a cap-independent mechanism (Zhou et al., 2015). YTHDF3, together with YTHDF1, regulates mRNA translation by interacting with a common set of ribosomal proteins (Li et al., 2017a; Shi et al., 2017). In addition, YTHDF3 could also

mediate mRNA decay by directly interacting with YTHDF2 (Shi et al., 2017). Overall, YTHDF3 serves as a hub for fine-tuning the RNA accessibility of YTHDF1 and YTHDF2. Here, we report a previously unrecognized thermal-stress induced recruitment of the m⁶A RNA modification machinery to nuclear stress bodies (nSBs). Immunofluorescence experiments along with our HSATIII ChIRP-MS data have confirmed the recruitment of the m⁶A writers METTL3 and WTAP along with the m⁶A reader, YTHDC1, to nSBs. In addition, HSATIII ChIRP-m⁶A dot blot experiments have shown that nSBs-RNAs are highly methylated. In order to identify the target nSBs-RNA transcripts for the m⁶A machinery, we followed nSBs-RNAs purification by RNA-MS analysis. Interestingly, we found that HSATIII lncRNA is highly methylated at its repetitive sequence GGAAU. To our knowledge, the reported m⁶A consensus RNA motif is restricted to RRACH (R =A or G; H = A, U, or C) (Dominissini et al., 2012; Meyer et al., 2012; Wei et al., 1976; Wei and Moss, 1977). However, the m⁶A consensus we identified is GGAAU which replaces C with A. In addition, we detected a minor population of GGAAU that exhibit adjacent double methylation at AA which is also not previously reported. Collectively, here we report a previously unrecognized stress-induced m⁶A RNA methylation localized to HSATIII-based nuclear stress bodies proposing a novel role of m⁶A methylation in stress response mechanism through the nuclear stress bodies.

2.3 Methods

2.3.1 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% CO₂. HAP1 cells (Horizon Discovery) were maintained in IMDM (Gibco) supplemented with 10% FBS (Gibco) and penicillin-streptomycin (Nacalai Tesque).

2.3.2 Knockdown with antisense oligonucleotide

For nuclear RNA knockdown, 3×10^6 cells were transfected with chemically modified antisense or control oligonucleotides (1 μ M) using Nucleofector (Lonza) 16–18 hours before the assay, as described previously (Ideue et al., 2009).

2.3.3 Genome editing using CRISPR/Cas9

PX330 or PX330-B/B was used for genome editing of the HAP1 cells. To establish the knockout HAP1 cells, the sgRNAs (5'-ACCCATACCTTCTTGGGAAG-3'), (5'-TATGTTGTCAACTATCCAAA-3'), and (5'-GCCGCAATCGCGCAGCTCAA-3') were selected for WTAP, TDP43, and RBM14, respectively, from GeCKO v2 libraries (Sanjana et al., 2014) and cloned into the BbsI site of PX330 (Addgene). The plasmids (2 μ g) and pcDNA6/TR plasmids (0.2 μ g) were transfected similarly using a Nucleofector device (Lonza). To enrich the plasmid-transfected cells, the HAP1 cells were treated with 20 μ g/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 μ g/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 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.

2.3.4 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/2× SSC, hybridized with a digoxigenin (DIG)-labeled HSATIII antisense oligonucleotide (ASO) in hybridization buffer (10% formamide, 2× SSC, and 10% dextran) for 16 hours at 37°C, incubated in 10% formamide/2× 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 or SIGMA, HPA016832; WTAP: Proteintech, 60188-1 Ig; METTL3: Abnova, H00056339-B01P; METTL14: SIGMA, HPA038002; YTHDC1: Abcam, ab122340; FTO: ab126605; m⁶A: Synaptic System, 202 003). 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 combined HSATIII RNA-FISH and m⁶A IF, cells cultured on cover glasses were fixed in 4% PFA/PBS for 30 min at room temperature followed by PBS washing overnight at 4°C. The cultured cells were then treated with proteinase K before permeabilization as follows. Cells were washed with MQW for 5 min at RT, 0.2N HCL for 20 min, MQW for 5min, prewarmed proK buffer 5 min at 37°C, proK treatment (3.5 ug/ml [Roche]) 5-7 min at 37°C, 0.2% glycine for 10 min at RT, PBS wash for 5 min at RT, 4% PFA/PBS fixation for 20 min, 3X PBS wash, permeabilized in cold PBS containing 0.1% Triton X-100 for 15 min, and then incubated with anti-m⁶A antibody (Synaptic System, 202 003) overnight at 4°C, TBST and PBS washes, 4% PFA/PBS fixation for 10 min, and then continue from hybridization step with conventional HSATIII RNA-FISH as described above.

2.3.5 m⁶A dot blot

HSATIII-ChIRPed nSBs RNAs, in vitro transcribed methylated and unmethylated RNAs were denatured at 95°C for 3 min followed by immediate 5min chill on ice. Two ul of each sample were spotted to Hybond-N+ membrane (GE Healthcare, catalog number: RPN203B), allowed to dry for 30 min. Crosslink spotted RNA to membrane in a Stratalinker 2400 UV Crosslinker twice using the Autocrosslink mode (1,200 microjoules [x100]; 25-50 sec).

Membrane was washed for 5 min with washing buffer (0.02% PBS/Tween 20) with gentle shaking, blocked with 5% skim milk/0.02% PBS/Tween 20 for 1 hour at room temperature with gentle shaking, incubated with anti-m⁶A antibody (Synaptic Systems, catalog number: 202 003, 1:1000 dilution) in 1 ml of antibody dilution buffer (5% skim milk/0.02% PBS/Tween 20) overnight at 4°C with gentle shaking, washed for 3 times 5 min each in washing buffer (0.02% PBS/Tween 20), incubated with goat anti-rabbit IgG-HRP (1:10,000) secondary antibody for 1 hour at room temperature with gentle shaking, washed 4 times 10 min each with washing buffer (0.02% PBS/Tween 20) with gentle shaking at room temperature, incubated with 2 ml of ECL Western Blotting Substrate for 5 min in darkness at room temperature, and wrapped in plastic wrap and expose with Hyperfilm ECL for a proper exposure period.

2.3.6 Chromatin isolation by RNA purification (ChIRP)

ChIRP experiments were performed in principal as described before (Chu et al., 2012) with some modifications. Cultured cells were prepared on 10-cm dishes with confluence of 80-90% before start. After thermal stress induction, cells were cross-linked by removing culture medium, adding 10 ml 1% PFA/PBS for 10 min with gentle shaking, adding 1 ml of 1.5 M glycine drop-wisely with gentle shaking for 5 min, removing crosslinking solution, washing with 10 ml of ice-cold PBS twice. Cultured cells were collected after adding 1 ml ice-cold PBS using disposable scraper and transferring the cells to new 1.5 ml tube. Cell lysates were then spun down by centrifugation at 12000 rpm for 2 min at 4°C, removing PBS then spinning again and removing residual PBS as completely as possible. Cell pellets were then measured (expected weight: 20-30 mg/dish). Cells pellets were re-suspended in 0.35 ml of lysis buffer (50 mM Tris-HCL pH 7, 10 mM EDTA, 1% SDS) containing (+1XPI, +1/500 RNase inhibitor, + 1 mM PMSF) followed by sonication (15 secs ON, 45 secs OFF) x 118 cycles followed by centrifugation (14000 rpm, 10 min, 15°C). Supernatants were transferred to new tube followed by quick freeze by liquid nitrogen then store at -80°C. Two volumes of hybridization buffer (750 mM NaCl, 1% SDS, 50 mM Tris-Cl pH 7.0, 1 mM EDTA, 15% (v/v) formamide, 1 mM PMSF, cOmpleteTM Protease Inhibitor Cocktail (Sigma), and RNase inhibitor) and the HSATIII antisense (5'-ATTCCATTCCATTCCATTCC-3') or control (5'-CTCATGGAGTTAGCCCGCTC-3') oligonucleotide were added. The mixture was incubated at 37°C for 4 hours, mixed with Dynabeads MyOne Streptavidin C1 (Thermo Fisher Scientific), and incubated for 30 min. The beads were then washed five times for 5 min at 37°C with 0.8 ml of wash buffer (2× SSC and 0.5% SDS). The bound proteins were eluted

and reverse-crosslinked by boiling in SDS sample buffer (95°C for 30 min). The bound RNAs were eluted by protease K treatment (50°C for 45 min) followed by heating (95°C for 10 min).

2.3.7 m⁶A RNA immunoprecipitation (MeRIP)

Twenty-five mL of protein G beads/sample were washed with 150 ml 1x IP buffer (10 mM Tris-HCL pH 7.5, 150 mM NaCL, 0.1% Nonidet P-40) twice and resuspended in 200 ml 1X IP buffer. 1 ml NEB anti-m⁶A antibody was added followed by 60 min rotation at 4°C then washed with 200 ml 1X IP buffer twice and resuspended in 50 ml IP buffer. 10 mg total RNAs were dissolve in 200 ml 1X IP buffer containing RNase inhibitor (1/500:0.4 ml). For 10% input, 35 ml RNase-free water were added to 17.5 ml RNA then 150 ml Tri-reagent LS (Molecular Research Center) for RNA preparation according to manufacture manual. 175 ul RNA were added to the washed m⁶A-antibody-protein G beads, rotated for 1 hour at 4°C, washed twice with 200 ml 1X IP buffer, 200 ml low salt buffer (10 mM Tris-HCL pH 7.5, 50 mM NaCl, 0.1% Nonidet P-40) twice and 200 mL high salt buffer (10 mM Tris-HCL pH 7.5 500 mM NaCl, 0.1% Nonidet P-40) twice. The final suspension was then transferred to new tube. Beads were collected using magnetic stand and supernatant was discarded. Beads pellet was dissolved with 200 ml Tri-reagent (Molecular Research Center) then proceeded with RNA preparation according to manufacturers' manual.

2.3.8 Reverse transcription

Total RNAs were purified with TRI reagent (Molecular Research Center) according to the manufacturer's instructions. To quantify HSATIII levels, the total RNAs (1 mg) were reverse-transcribed by High Capacity cDNA reverse transcription kit (Thermo Fisher Scientific) with random primers.

2.3.9 Western blot

Cells were lysed in immunoprecipitation (IP) lysis buffer (50 mM Tris-HCL pH 8.0, 150 mM NaCl, 1% Triton X-100, and complete EDTA- free protease inhibitor cocktail [Roche]) and then disrupted by five pulses of sonication for 5 sec. The cell lysates were cleared by centrifugation, and the protein concentration was determined using the Bradford method. After adding SDS sample buffer, the samples were heated at 95°C for 5 min and separated by SDS-PAGE. After SDS-PAGE, the Western blot apparatus was stacked in the order of sponge

→ filter paper → FluoroTrans W membrane → gel → filter paper → sponge. Electrical transfer was performed for 60 min at 100 V. The membrane after the transfer was immersed in blocking solution (5% skim milk/TBST) and it was then shaken for 1 hour at room temperature. After blocking, it was incubated with the first antibody in 2.5% skim milk/TBST overnight at 4°C. The membrane was washed 3 times for 10 min each time with TBST and then incubated with the secondary antibody for 90 min at room temperature. After washing three times for 10 min each time in TBST, the membrane was subjected to chemiluminescence reactions by ECL Western Blotting Detection Reagents.

2.4 Results

2.4.1 Recruitment of m⁶A machinery to nSBs:

In our HSATIII-ChIRP-MS data described in Chapter 1, we detected some m⁶A writers and readers that are enriched in nSBs upon thermal stress. These factors include WTAP, RBM15, SPEN, YTHDC1 and HNRNPA2/B1. The recruitment of these m⁶A factors to nSBs propose a novel role of m⁶A RNA modification in stress response through HSATIII-based nuclear stress bodies.

2.4.1.1 Recruitment of WTAP, an essential component of m⁶A writer complex, to nSBs

First we checked the localization of WTAP to nSBs by immunofluorescence. As shown in Figure 7, in normal conditions at 37°C, WTAP diffuses in the nucleoplasm of HeLa cells. When cells are exposed to thermal stress at 42°C for 2 hours then allowed to recover at 37°C for 1 hour, we detected a clear colocalization of WTAP with SAFB, the nSBs marker. In order to confirm whether the WTAP foci depend on HSATIII, we knocked down HSATIII (Figure 6) and checked the integrity of the WTAP foci (Figure 7). The WTAP foci were abolished upon HSATIII knockdown confirming that WTAP foci correspond to the major population of HSATIII-dependent nSBs, nSB-S. We noticed the presence of a minor population of WTAP foci which are not overlapped with SAFB (Figure 7), which seem to be nSB-M or the other nSB subtype.

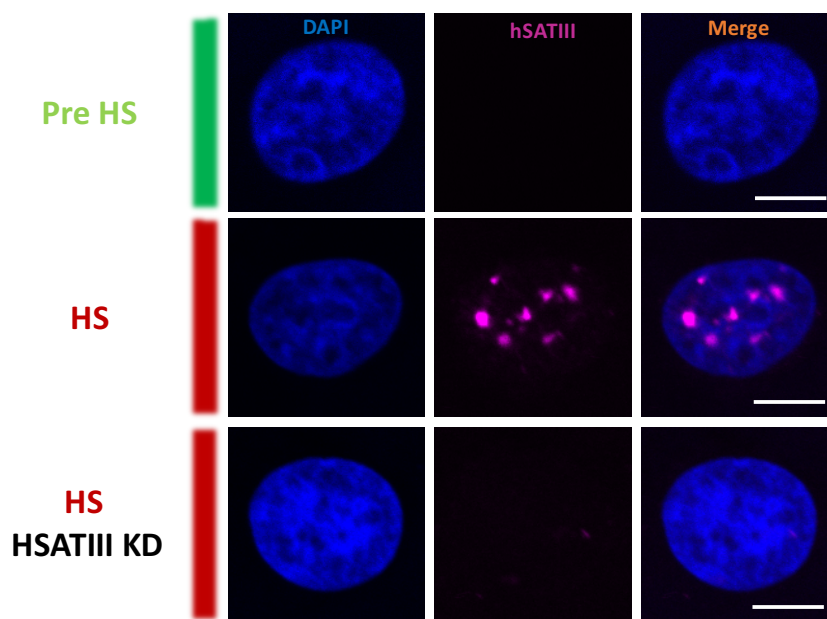


Figure 6. Confirmation of HSATIII knockdown by HSATIII RNA-FISH. HeLa cells were exposed at 42°C for 2 hours, followed by incubation at 37°C for 1 hour (HS), exposed at 42°C for 2 hours, followed by incubation at 37°C for 1 hour upon HSATIII knockdown (HS, HSATIII KD), and before thermal stress (Pre HS). The nuclei were stained with DAPI. Scale bars, 10 μ m.

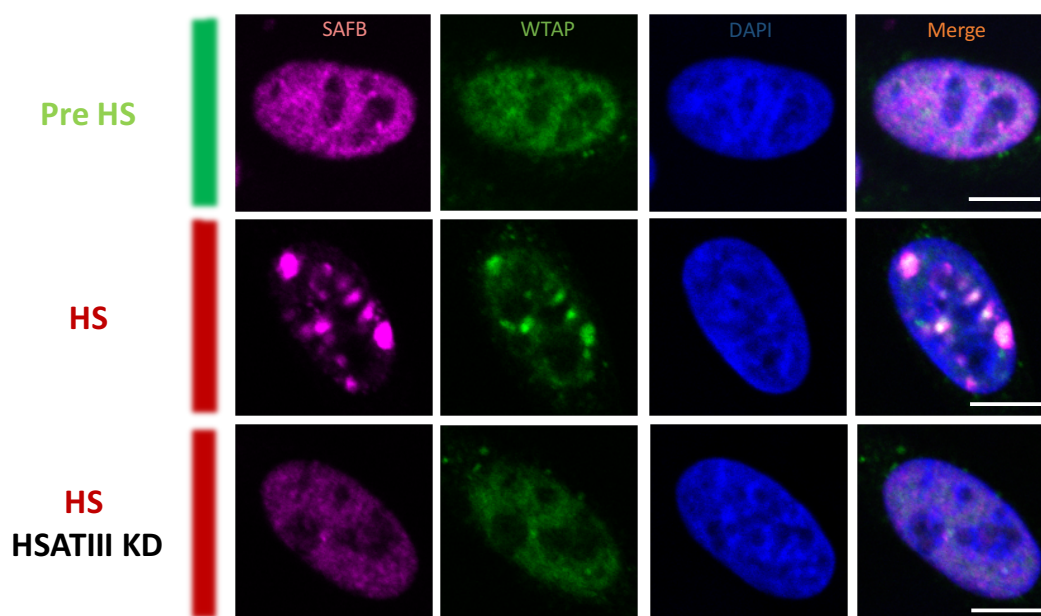


Figure 7. Detection of WTAP stress-induced recruitment to nSBs (SAFB) by immunofluorescence. HeLa cells were exposed at 42°C for 2 hours, followed by incubation at 37°C for 1 hour (HS), treated at 42°C for 2 hours, followed by incubation at 37°C for 1 hour upon HSATIII knockdown (HS, HSATIII KD), and before thermal stress (Pre HS). The nuclei were stained with DAPI. Scale bars, 10 μ m.

2.4.1.2 Recruitment of YTHDC1, m⁶A reader, to nSBs

Next we checked the localization of a well-known m⁶A reader, YTHDC1, in nSBs (Figure 8). Similar to WTAP, YTHDC1 diffuses in the nucleoplasm in normal condition at 37°C, while upon thermal stress at 42°C for 2 hours, it is recruited to the nSBs. The YTHDC1 foci were abolished upon HSATIII knockdown. In addition, the recruitment of YTHDC1 into nSBs was further investigated in our laboratory by Dr. Kensuke Ninomiya using ChIRP-WB. As shown in Figure 9, the YTHDC1 band was detectable in HSATIII-ChIRP indicating YTHDC1

enrichment in nSBs in thermal stressed cells but not in Control-ChIRP nor in Pre HS condition.

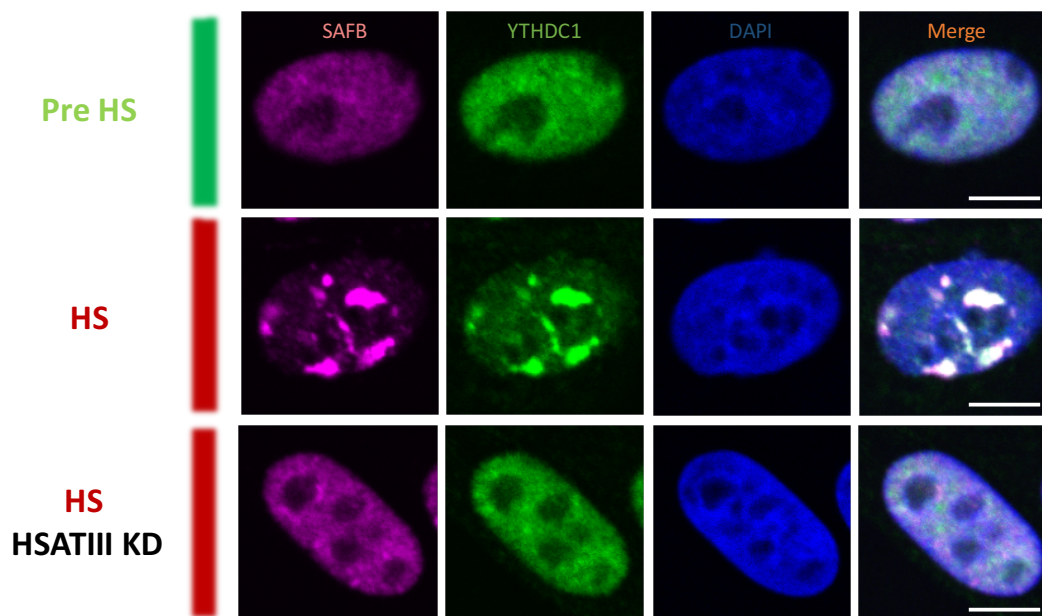


Figure 8. Detection of YTHDC1 stress-induced recruitment to nSBs (SAFB) by immunofluorescence. HeLa cells were treated at 42°C for 2 hours, followed by incubation at 37°C for 1 hour (HS), treated at 42°C for 2 hours, followed by incubation at 37°C for 1 hour upon HSATIII knockdown (HS, HSATIII KD), and before thermal stress (Pre HS). The nuclei were stained with DAPI. Scale bars, 10 μ m.

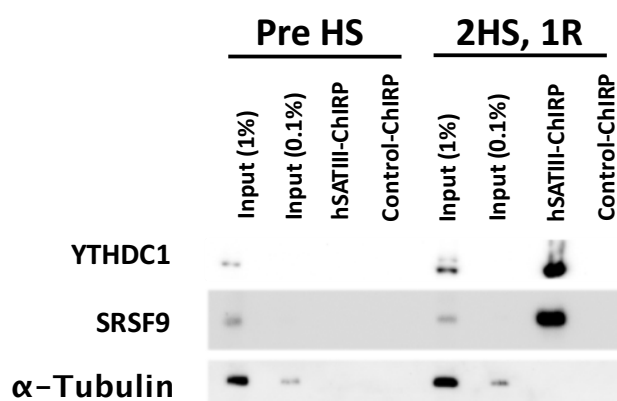


Figure 9. Western blot of YTHDC1, SRSF9, and Tubulin proteins in HSATIII-ChIRP and Control-ChIRP fractions. Samples are either derived from cells treated at 42°C for 2 hours followed by 37°C 1 hour at (2HS, 1R) or normal condition at 37°C (Pre HS).

2.4.1.3 Recruitment of METTL3, the m⁶A methylase, but not METTL14 to nSBs

METTL3, METTL14 and WTAP form the m⁶A writer complex in which METTL3 is considered as the m⁶A methylase possessing the catalytic activity for m⁶A writing, while WTAP and METTL14 contribute to stabilize the writer complex. Although WTAP was enriched in our ChIRP-MS data, neither METTL3 nor METTL14 were detectable. However, when we checked METTL3 and METTL14 recruitment to nSBs by immunofluorescence, we could detect clear localization of METTL3 but not METTL14 to nSBs (Figure 10 and 11). Similar to WTAP and YTHDC1, METTL3 foci are HSATIII-dependent as shown by its elimination upon the HSATIII knockdown. On the other hand, we also checked the recruitment of FTO, one of the m⁶A demethylases, to nSBs but we didn't detect a clear localization to nSBs (Figure 12).

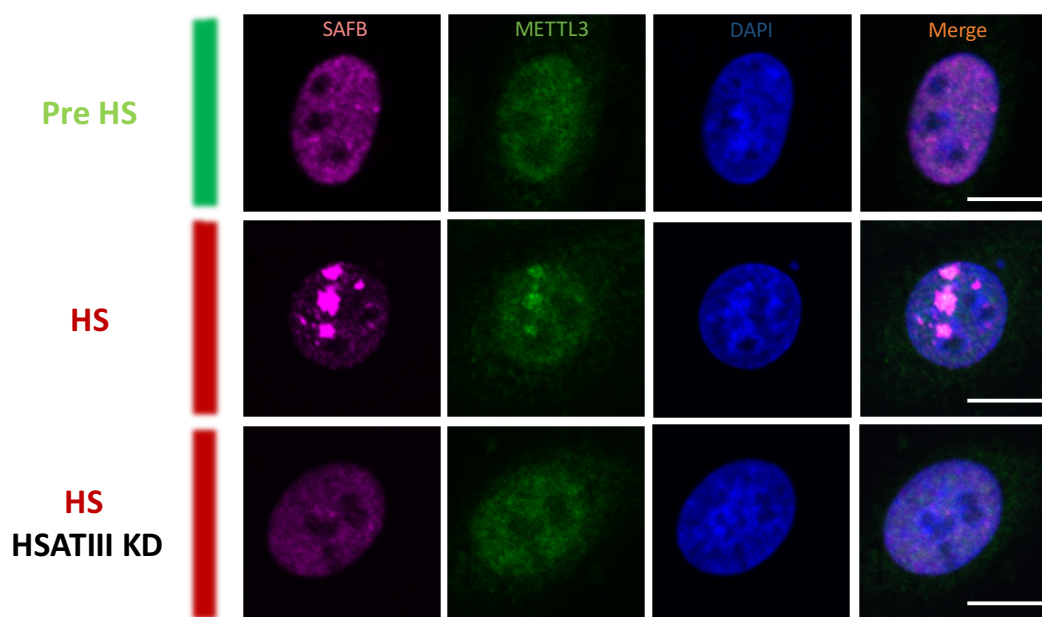


Figure 10. Detection of METTL3 stress-induced recruitment to nSBs (SAFB) by immunofluorescence. HeLa cells were treated at 42°C for 2 hours, followed by incubation at 37°C for 1 hour (HS), treated at 42°C for 2 hours, followed by incubation at 37°C for 1 hour upon HSATIII knockdown (HS, HSATIII KD), and before thermal stress (Pre HS). The nuclei were stained with DAPI. Scale bars, 10 μm.

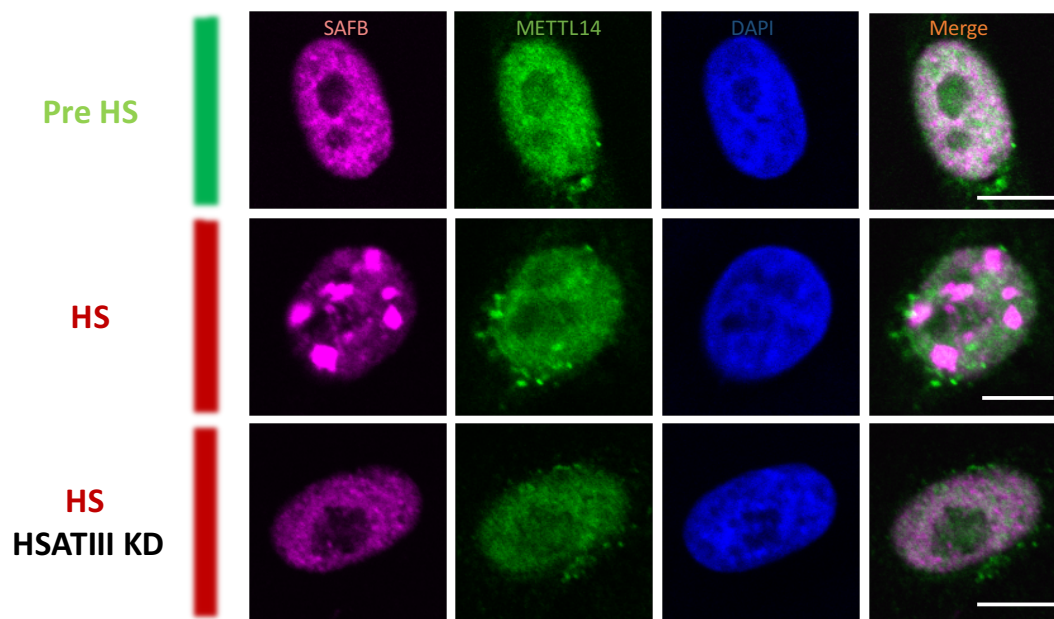


Figure 11. Immunofluorescence of METTL14 and SAFB. HeLa cells were treated at 42°C for 2 hours, followed by incubation at 37°C for 1 hour (HS), treated at 42°C for 2 hours, followed by incubation at 37°C for 1 hour upon HSATIII knockdown (HS, HSATIII KD), and before thermal stress (Pre HS). The nuclei were stained with DAPI. Scale bars, 10 μ m.

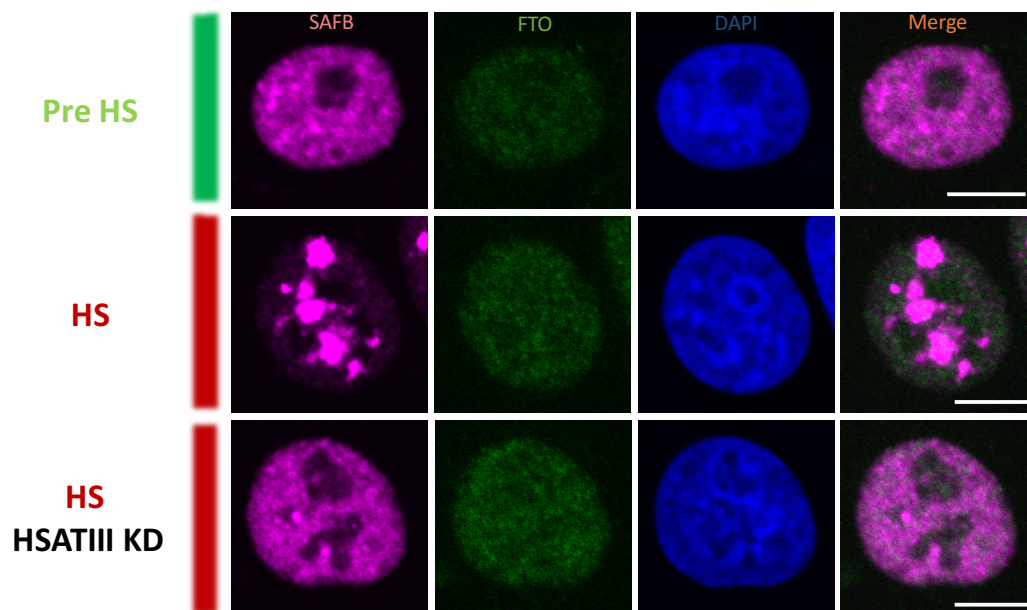


Figure 12. Immunofluorescence of FTO and SAFB. HeLa cells were treated at 42°C for 2 hours, followed by incubation at 37°C for 1 hour (HS), treated at 42°C for 2 hours, followed by incubation at 37°C for 1 hour upon HSATIII knockdown (HS, HSATIII KD), and before thermal stress (Pre HS). The nuclei were stained with DAPI. Scale bars, 10 μ m.

2.4.1.4 m⁶A RNAs localization at nuclear stress bodies

After the detection of some crucial components of m⁶A machinery at nSBs, we questioned why m⁶A machinery is recruited to nSBs and whether the machinery acts to methylate certain RNAs in nSBs. We checked the presence of the m⁶A modified RNAs in nSBs by combining FISH and IF to detect both HSATIII lncRNA as well as m⁶A modified RNAs using anti-m⁶A modification specific antibody. Since m⁶A is masked by m⁶A reader protein(s), the detection was unsuccessful at the beginning, so we attempted to use Proteinase K to partially wipe away the protein contents from the nSBs that results in exposure of any possible m⁶A modifications installed at nSB-RNAs. As shown in Figure 13, we could detect colocalization of m⁶A methylated RNAs with HSATIII lncRNA and the m⁶A RNA foci was abolished upon HSATIII knockdown.

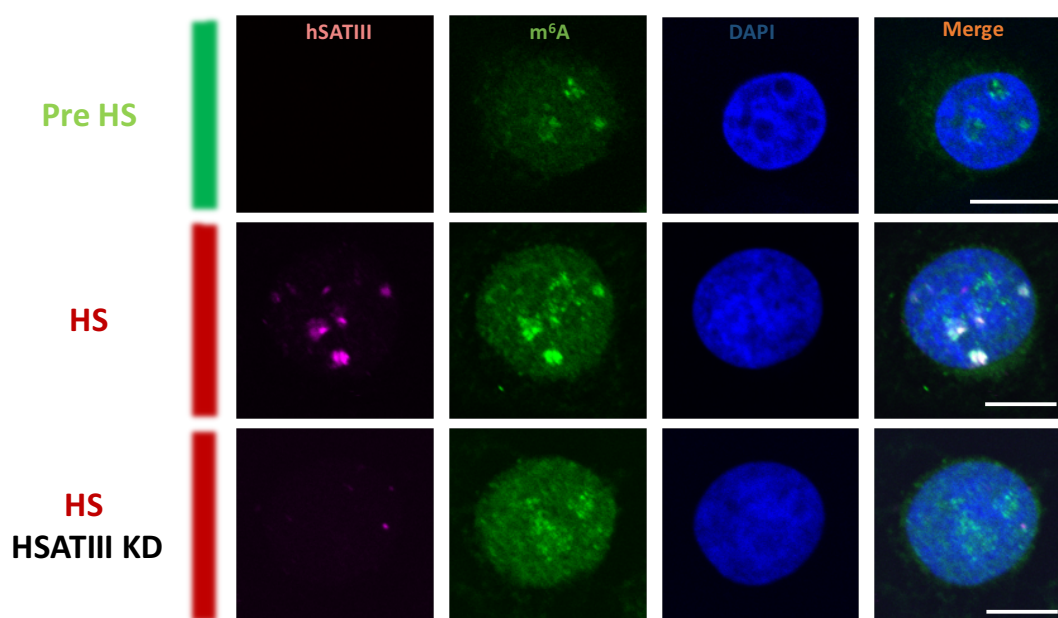


Figure 13. Detection of thermal stress-induced m⁶A methylation in nSBs via HSATIII RNA-FISH and m⁶A immunofluorescence. HeLa cells were treated at 42°C for 2 hours, followed

by incubation at 37°C for 1 hour (HS), treated at 42°C for 2 hours, followed by incubation at 37°C for 1 hour upon HSATIII knockdown (HS, HSATIII KD), and before thermal stress (Pre HS). The nuclei were stained with DAPI. Scale bars, 10 μ m.

In addition, we checked the enrichment of m⁶A RNAs in nSBs by dot blot analysis. First we purified the nSB-RNAs using HSATIII-ChIRP followed by m⁶A dot blot using anti-m⁶A specific antibody. As shown in Figure 14, we found that m⁶A methylated RNAs are enriched in nSB RNA fraction.

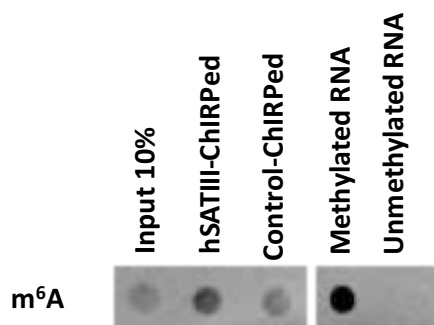


Figure 14. m⁶A dot blot of HSATIII-ChIRPed RNAs and Control-ChIRPed RNAs.

2.4.2. HSATIII lncRNA is methylated at its highly repetitive sequence GGAAU

2.4.2.1 HSATIII lncRNA methylation.

After the detection of m⁶A machinery as well as methylated RNAs in nSBs, we questioned whether HSATIII lncRNAs are target RNAs for m⁶A methylase recruited in nSBs. To assess that, Dr. Kensuke Ninomiya re-purified the HSATIII transcripts out of ChIRPed nSBs-RNAs using biotinylated antisense probes against HSATIII (nSB-HSATIII). Then we carried out m⁶A dot blot to check the m⁶A enrichment in the highly purified HSATIII lncRNAs along with serial concentrations of in vitro transcribed methylated and un-methylated RNAs. As

shown in Figure 15A, we detected m⁶A enrichment in nSB-HSATIII fraction indicating that HSATIII is highly methylated. In addition, we purified the m⁶A RNAs from total RNA by methylated RNA immunoprecipitation (MeRIP) upon thermal stress using anti-m⁶A antibody along with control anti-IgG then checked HSATIII enrichment by RT-PCR. As shown in Figure 15B, HSATIII was enriched in RIP fraction with anti-m⁶A antibody but not control anti-IgG, supporting that HSATIII is m⁶A methylated.

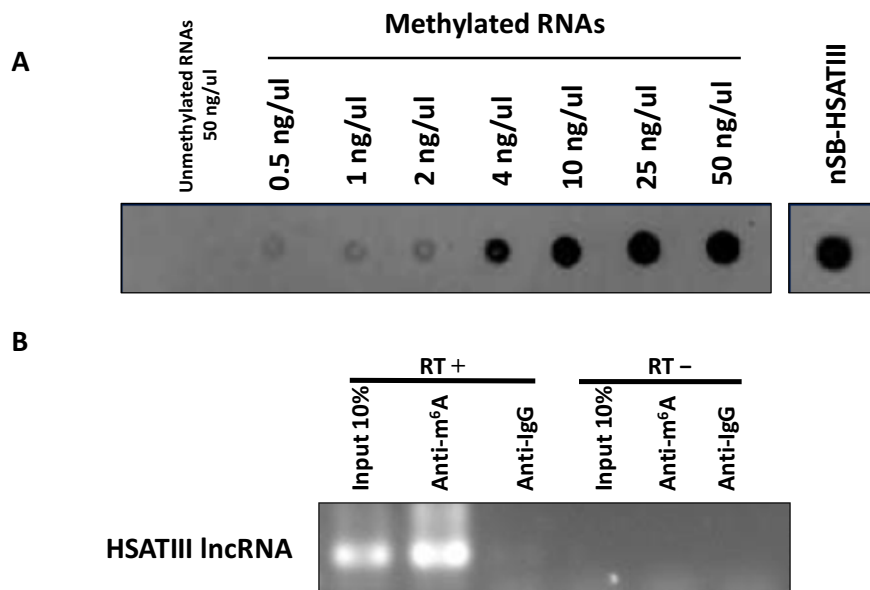


Figure 15. HSATIII lncRNA is m⁶A methylated.

(A) m⁶A dot blot using the purified HSATIII along with in vitro transcribed m⁶A RNAs. HSATIII transcripts (nSB-HSATIII) were re-purified out of ChIRPed nSBs-RNAs using biotinylated antisense probes against HSATIII. HSATIII methylation was assessed along with serial concentrations of in vitro transcribed methylated and unmethylated control RNAs.

(B) MeRIP-RT-PCR of HSATIII using anti-m⁶A antibody and anti-IgG as a control.

2.4.2.2 Identification of m⁶A methylation sites in HSATIII lncRNAs

Next we attempted to identify the m⁶A sites within the HSATIII transcripts. HSATIII lncRNAs are transcribed from the pericentromeric regions with highly repetitive sequence of GGAAU. So we questioned whether the GGAAU highly repetitive sequence of HSATIII transcripts can be a potential m⁶A sites within HSATIII. To check this possibility, we collaborated with Professor Tsutomu Suzuki at University of Tokyo to perform RNA-mass spectrometry. First we digested the purified HSATIII transcripts above with RNase T1 to

produce 5'-AAUGp-3' fragments followed by RNA-MS analysis (Figure 16). Extracted-ion chromatograms (XICs) of RNase T1-digested fragments of HSATIII show the detection of m⁶A at the HSATIII highly repetitive sequence AAUGp fragments with either single m⁶A modification (AAUGp+Me) or double m⁶A (AAUGp+2Me) (Figure 17, left). Collision-induced dissociation (CID) spectra also show the m⁶A dissociation from the HSATIII - digested fragments (Figure 17, right).

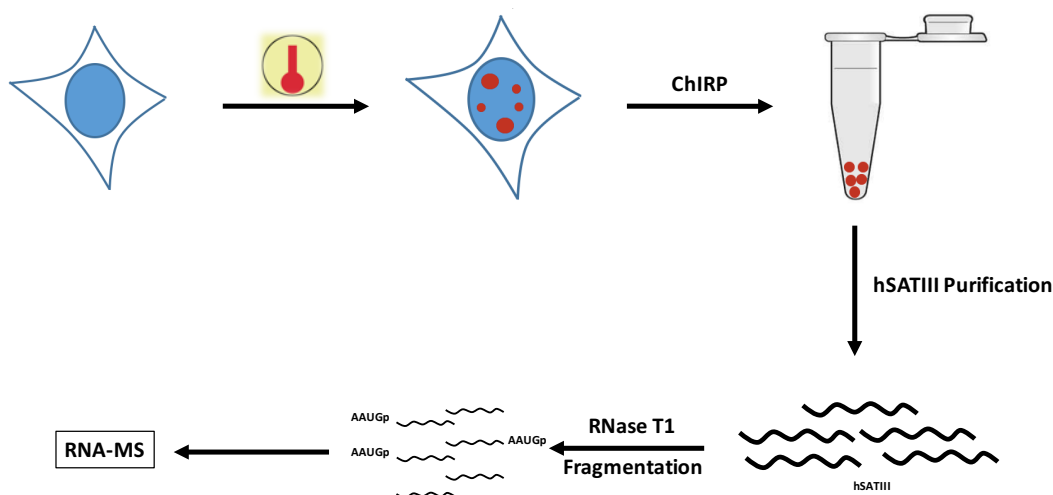


Figure 16. Schematic representation of RNA-MS analysis of HSATIII.

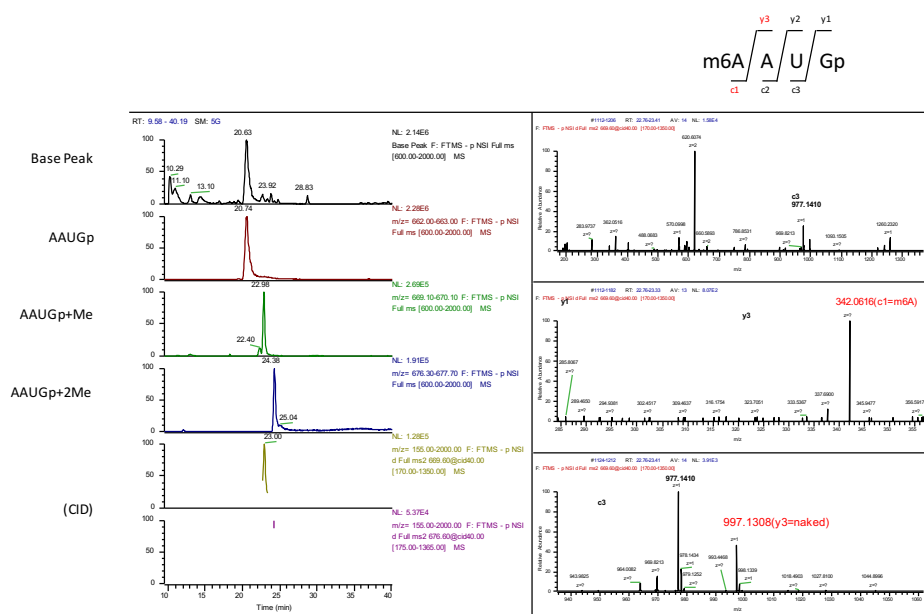


Figure 17. Extracted-ion Chromatograms (XICs) and Collision-induced dissociation (CID) spectra of RNase T1-digested fragments of HSATIII.

Collectively, these data show that the thermal stress-induced recruitment of m⁶A machinery to nSBs is likely for the methylation of adenosine residues of GGAAU the highly repetitive sequence of HSATIII lncRNA.

2.4.3 m⁶A machinery recruitment pattern to nSBs

2.4.3.1 Dynamics of protein composition in nSBs

Next we asked how the m⁶A writer and reader machineries work within nSBs. For that, we attempted to know more about the nature of nSBs themselves whether they are relatively static bodies with same protein content at different stress and recovery time points (Figure 18A) or they are dynamic with changeable protein contents at different time points (Figure 18B). So, we purified nSBs at different stress and recovery time points using HSATIII-ChIRP followed by mass-spectrometry to monitor the nSBs composition change. HSATIII-ChIRP was performed by Dr. Kensuke Ninomiya and mass-spectrometry was performed in collaboration with Dr. Shungo Adachi in National Institute of Advanced Industrial Science and Technology, Tokyo. Surprisingly, we found that nSBs are highly dynamic with changeable protein composition at different stress and recovery time points. As shown in Figure 19, we found that group of nSBs proteins are enriched only upon stress and they are released from nSBs once stress is recovered. Other nSBs proteins are recruited to nSBs only during post-stress recovery, while a third group are enriched in both upon stress and post-stress recovery. These data widen our understanding about nSB dynamics and highlight potential nSBs roles in stress response mechanism.

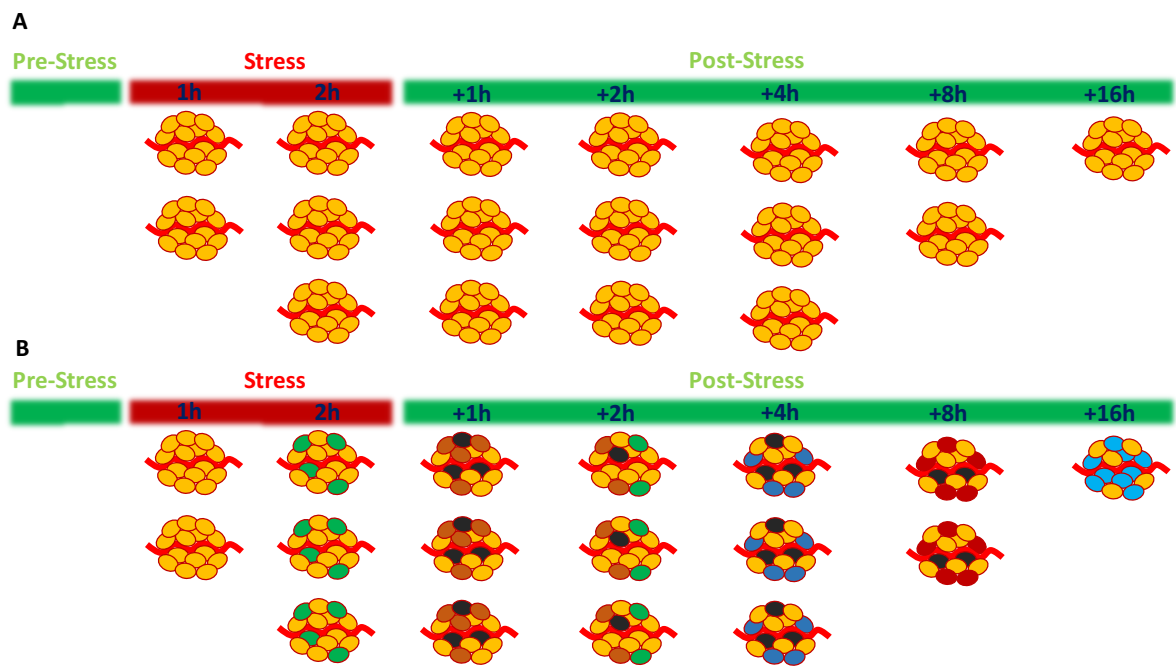


Figure 18. Schematic representation of possible nSBs dynamics state.

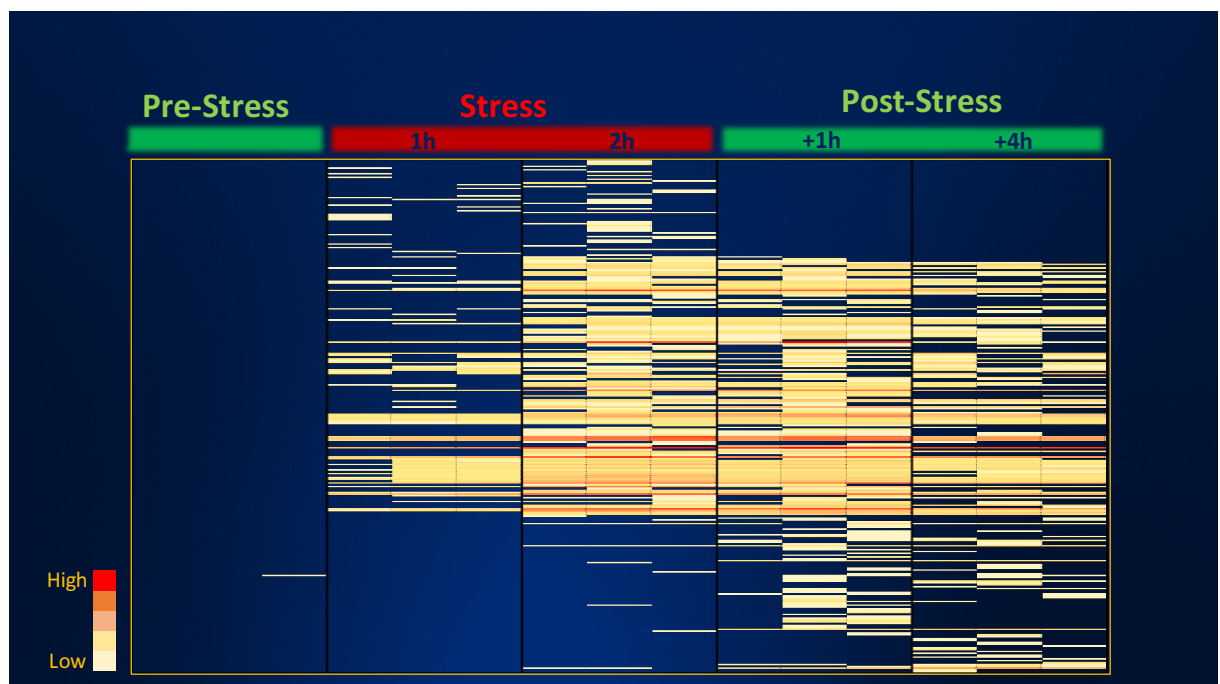


Figure 19. nSBs changeable protein composition at different stress and recovery time points.

2.4.3.2 m⁶A machineries are mainly recruited to nSBs in the early stress recovery stage

Here we monitored how the m⁶A machinery work within nSBs through different stress and recovery time points. Figure 20 shows the peptides enrichment of different m⁶A machinery components. m⁶A writers (WTAP, RBM15 and SPEN) seem to be gradually recruited to nSBs during thermal stress (2 hours HS) and enriched at early recovery stage (1hour post-stress) then are rapidly released from nSBs at late recovery stage (4 hours post-stress)

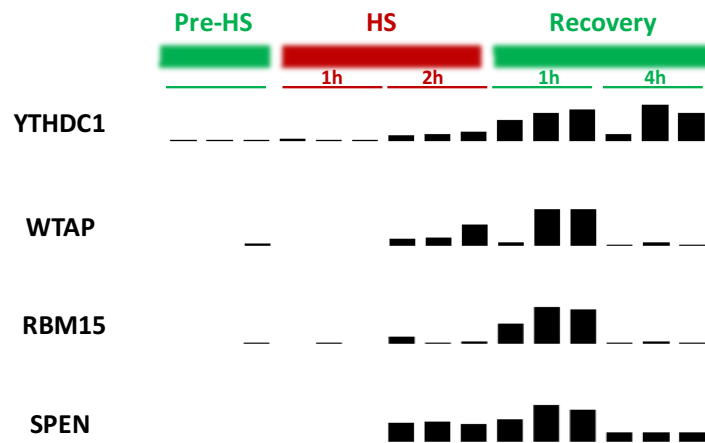


Figure 20. Mass-spectrometry peptides enrichment of different m⁶A factors.

On the other hand, we found that the m⁶A reader, YTHDC1, has a distinct enrichment pattern compared to the m⁶A writers in which it is recruited at stress phase and enriched in the early post stress recovery stage and stay enriched at nSBs until the late recovery stage. We checked the m⁶A machinery recruitment and enrichment at nSBs using immunofluorescence. As shown in Figures 21 and 22, the m⁶A writers WTAP and METTL3 are recruited to nSBs during stress and enriched in the early post-stress recovery stage (1 hour and 2 hours post-stress) then are released from nSBs by the late post-stress stage (4 h). YTHDC1 showed a continuous enrichment in nSBs until the late recovery stage (8 h) (Figure 23). The prolonged enrichment of YTHDC1 may be caused by two possibilities, either to maintain the m⁶A modification until late recovery stage or YTHDC1 might have additional non-m⁶A related function. Moreover, using combined HSATIII RNA-FISH and m⁶A immunofluorescence, we checked the m⁶A RNA dynamics at stress/recovery stages. We detected two distinct nuclear aggregations of m⁶A RNAs either HSATIII-based or HSATIII-free, likely in the nucleolus

(Figure 24). In consistence with m⁶A machineries enrichment pattern, we found that the HSATIII-based m⁶A RNA are enriched upon thermal stress and early post stress while 4 hours and 8 hours post stress, the m⁶A RNAs became more enriched at the HSATIII-free aggregation, likely in the nucleolus (Figure 24).

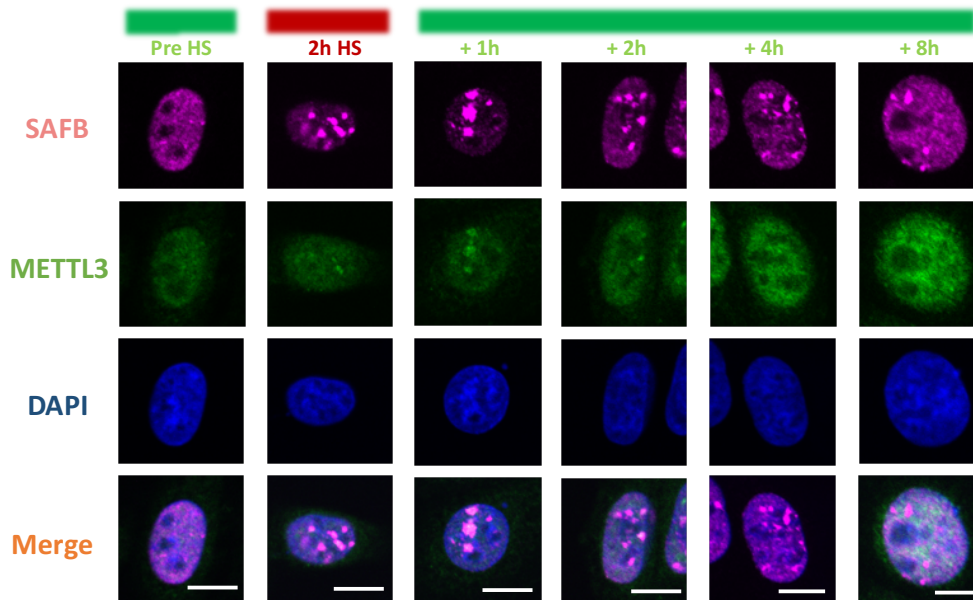


Figure 21. Immunofluorescence of SAFB and METTL3. HeLa cells were treated at 42°C for 2 hours (2 hours HS) then allowed to recover at 37°C for 1, 2, 4, or 8 hours, and before thermal stress (Pre HS). The nuclei were stained with DAPI. Scale bars, 10 μ m.

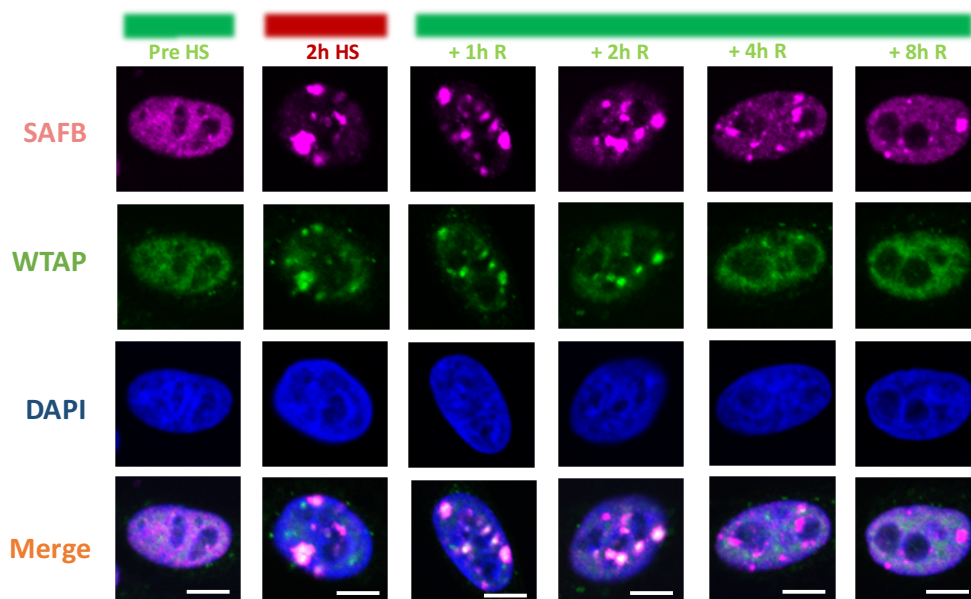


Figure 22. Immunofluorescence of SAFB and WTAP. HeLa cells were treated at 42°C for 2 hours (2 hours HS) then allowed to recover at 37°C for 1h, 2h, 4h, or 8h., and before thermal stress (Pre HS). The nuclei were stained with DAPI. Scale bars, 10 μ m.

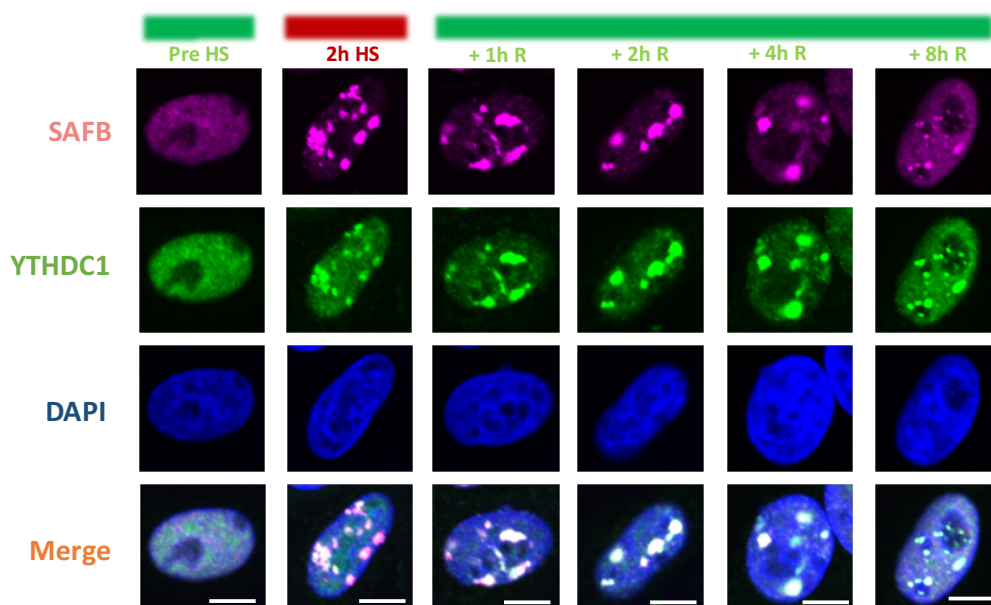


Figure 23. Immunofluorescence of SAFB and YTHDC1. HeLa cells were treated at 42°C for 2 hours (2 hours HS) then allowed to recover at 37°C for 1h, 2h, 4h, or 8h., and before thermal stress (Pre HS). The nuclei were stained with DAPI. Scale bars, 10 μ m.

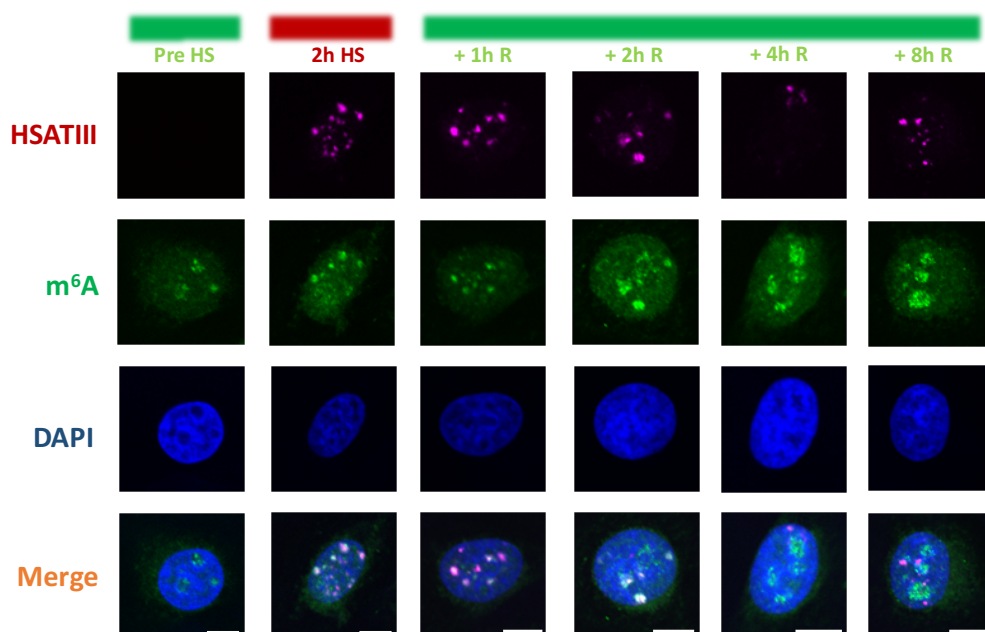


Figure 24. Detection of thermal stress-induced m⁶A methylation in nSBs via HSATIII RNA-FISH and m⁶A immunofluorescence. HeLa cells were treated at 42°C for 2 hours (2 hours HS) then allowed to recover at 37°C for 1 hour, 2 hours, 4 hours, or 8 hours., and before thermal stress (Pre HS). The nuclei were stained with DAPI. Scale bars, 10 µm.

2.4.4 TDP43 is a possible modulator of m⁶A methylation in nSBs

Expansion of GGAAU repeat in the intron of brain expressed associated with NEDD4-1 gene (BEAN1) is a pathological hallmark of spinocerebellar ataxia type 31 (SCA31) disease. UGGAA expression induces toxicity in vivo and accompanied with RNA foci accumulation. A recent report has identified TAR DNA-binding protein 43 kDa (TDP43), fused in sarcoma (FUS), and heterogeneous nuclear ribonucleoprotein A2/B1 (HNRNPA2/B1) as UGGAA-binding proteins which exhibit RNA chaperone activity by inhibiting UGGAA RNA misfolding. UGGAA repeat is identical to the m⁶A consensus we identified in HSATIII lncRNAs. In addition, we detected TDP43, FUS, and HNRNPA2B1 enrichment in nSBs by ChIRP-MS. So we questioned whether these UGGAA-binding proteins might have a role in regulating the thermal stress-induced nSBs-m⁶A methylation. To assess that, our laboratory has established TDP43 knockout (KO) HAP1 cell lines using CRISPR/Cas9 (Figure 26) then checked the nSBs-m⁶A enrichment upon TDP43 depletion.

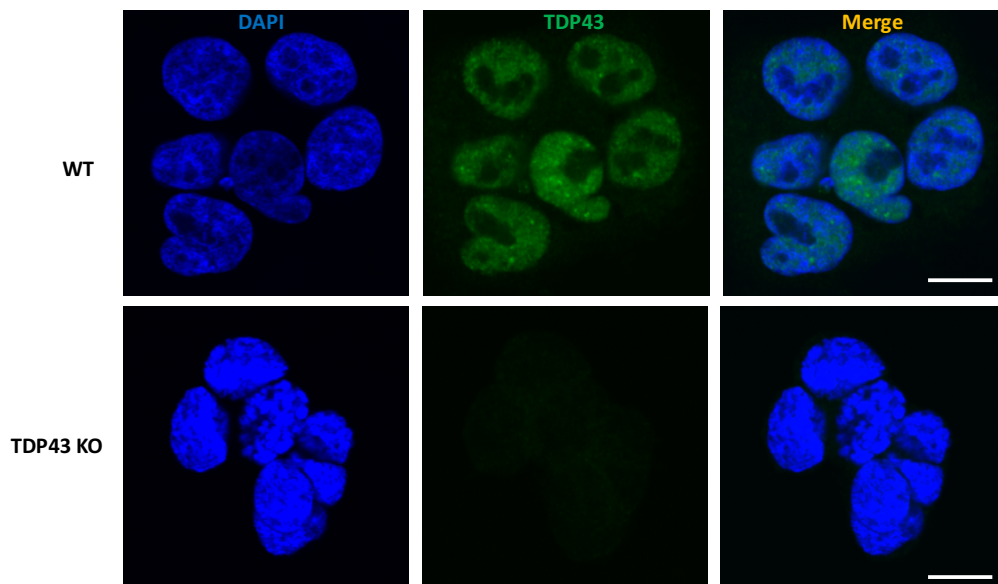


Figure 26. Immunofluorescence of TDP43 in HAP1 WT and WTAP KO cell lines. The nuclei were stained with DAPI. Scale bars, 10 µm.

Surprisingly, we found that nSB-m⁶A enrichment is dramatically inhibited upon TDP43 depletion compared to WT cells (Figure 27 and 28). We noticed that TDP43 KO cells grow with slower rate compared to HAP1 WT. To exclude the possibility that this nSBs-m⁶A inhibition might be cell growth-related, we compared nSBs-m⁶A enrichment in RBM14 KO, which exhibit slower cell growth rate compared to TDP43 KO, along with another TDP43 KO#2 colony. As shown in Figures 27 and 28, we found that nSBs-m⁶A is specifically inhibited in TDP43 KO cell lines compared to either HAP1 WT or RBM14 KO cell lines. nSBs-m⁶A downregulation upon TDP43 depletion suggests a novel regulatory role of TDP43 in thermal stress-induced nSBs-m⁶A RNA methylation.

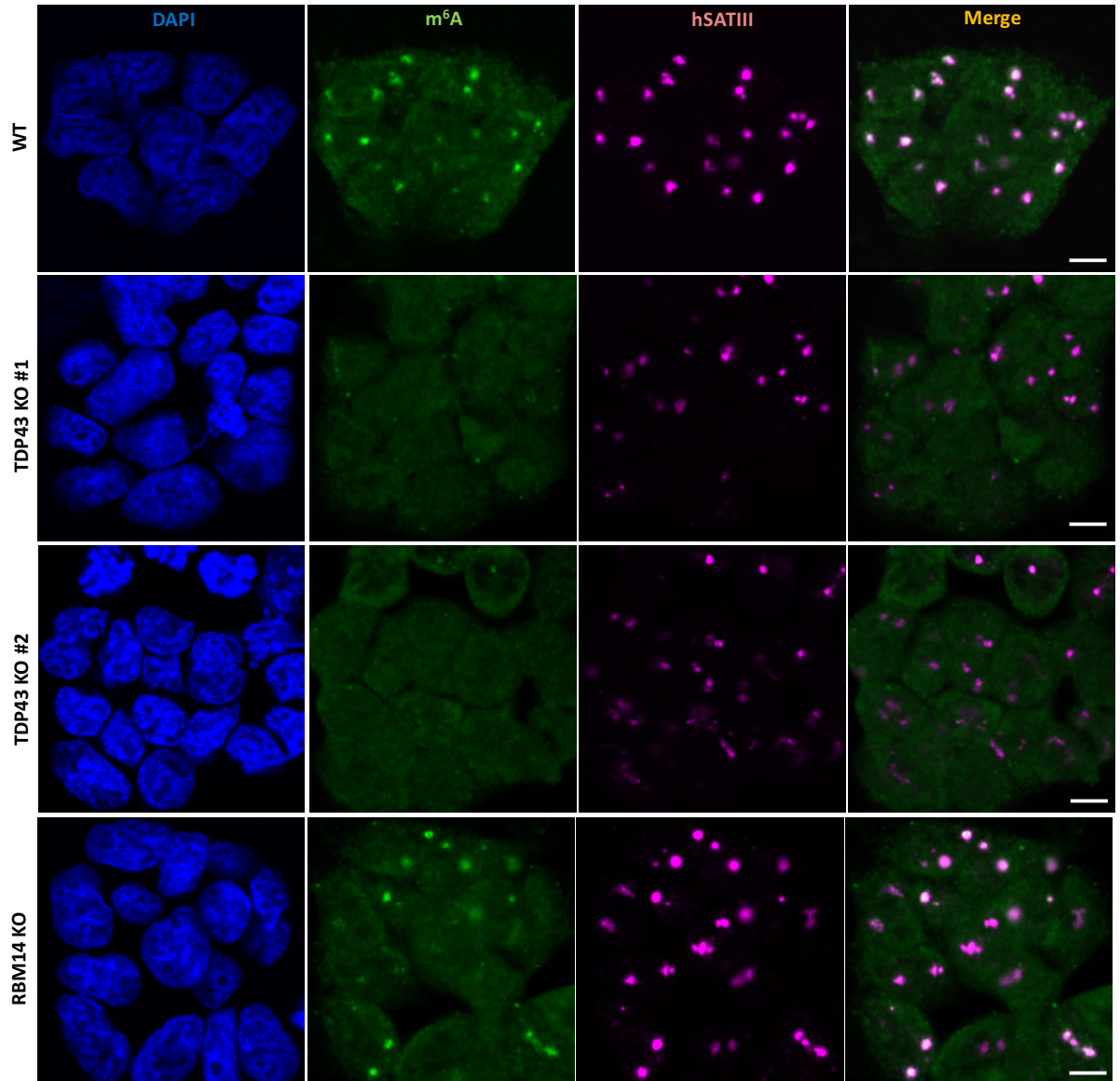


Figure 27. Detection of thermal stress-induced m⁶A methylation at nSBs via HSATIII RNA-FISH and m⁶A immunofluorescence in HAP1 WT, TDP43 KO, and RBM14 KO. Cells were treated at 42°C for 3 hours, followed by incubation at 37°C for 1 hour. The nuclei were stained with DAPI. Scale bars, 10 μ m.

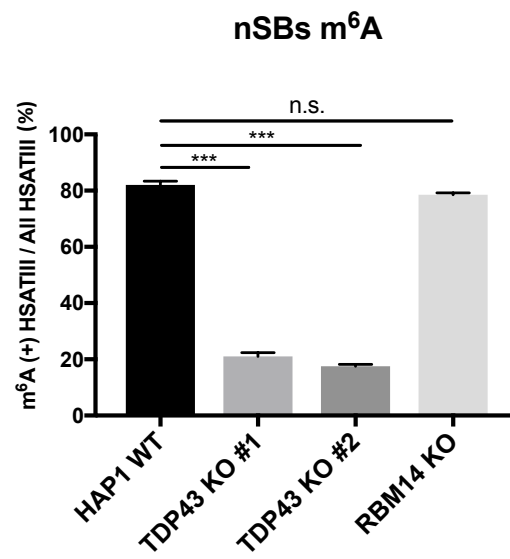


Figure 28. nSBs-m⁶A enrichment in HAP1 WT, TDP43 KO, and RBM14 KO cell lines. (All HSATIII) is HSATIII positive foci and (m⁶A+ HSATIII) is m⁶A RNA foci localize to HSATIII foci. Data are mean $\pm$ s.e.m. from three independent experiments. *P* values were determined by unpaired *t*-test (****P*<0.001). n.s, not significant.

2.5 Discussion

The reversible m⁶A RNA modification has gained a great concern during the past few years due to its critical roles in regulating a bunch of biological processes that refined our understanding of the substantial role of “epitranscriptomic regulation” in modulating gene expression. However, m⁶A epitranscriptomic role in stress response events is still poorly understood. Here, we report a previously unrecognized thermal stress-induced recruitment of m⁶A machinery to the HSATIII lncRNA-based nSBs. Immunofluorescence using the specific anti-m⁶A antibody combined with RNA FISH revealed that m⁶A RNAs localize to most HSATIII thermal stress-induced foci indicating that m⁶A machinery is recruited to nSBs upon stress to install m⁶A modification at nSBs-RNAs. This finding was further confirmed by m⁶A dot blot that has shown the enrichment of m⁶A in the purified nSBs-RNAs. To further investigate the target RNAs of the m⁶A machinery in nSBs, we re-purified HSATIII using biotinylated antisense probes then checked its methylation state by m⁶A dot blot. m⁶A dot blot has shown that HSATIII lncRNAs are enriched with m⁶A modification. We further investigated to identify m⁶A sites within HSATIII transcripts by RNA-MS. The RNA-MS has identified the third A of GGAAU in the highly repetitive HSATIII sequence as the major m⁶A sites. In addition, RNA-MS has identified a small population of HSATIII m⁶A consensus that exhibit two adjacent m⁶A modification at AA of GGAAU. The known consensus motif of m⁶A sites is GGACU which contains one base mismatch with m⁶A sites in HSATIII, GGAAU lacking the C residue after A. The C residue in the m⁶A consensus has been reported to be essential for m⁶A deposition (Dominissini et al., 2012; Liu et al., 2014; Meyer et al., 2012; Wei et al., 1976; Wei and Moss, 1977). Hence, shifting m⁶A consensus motif from GGACU to GGAAU might be thermal stress-dependent. Meanwhile, we cannot rule out the possibility that m⁶A machinery components and/or mechanism of action upon stress is distinct from normal condition. Elucidating mechanism of action of the m⁶A machinery upon stress requires the understanding of the nature of nSBs themselves, the host of m⁶A machinery upon stress. However, our knowledge about nSBs nature remains very limited. Hence, we screened the dynamics of nSB components at different stress/recovery stages using ChIRP-MS. Surprisingly, we found that nSBs are highly dynamic bodies in which some nSBs components strictly enriched under stress and released once stress is recovered (Figure 19). Other group of nSBs components is strictly enriched upon stress removal (Figure 19). A third group of nSBs components are enriched late recovery (Figure 19). These findings elucidate the importance of studying and understanding the dynamics of nSBs as a model of stress

response events in order to understand how our cells manage and respond to environmental stresses. Within the high dynamics of 141 nSBs components at stress/recovery stages, we looked at m⁶A machinery components to elucidate its mechanism of action upon stress. Remarkably, we found that m⁶A machinery has a unique recruitment pattern to nSBs, in which m⁶A machinery is recruited during stress and deposit m⁶A modification at HSATIII with relative enrichment in the early post-stress recovery stage then released from nSBs by 4 hours post-stress recovery stage. With exception of YTHDC1, all recruited m⁶A factors to nSBs follow the same recruitment pattern including METTL3, WTAP, SPEN, RBM14, and HNRNPA2B1. YTHDC1 showed prolonged enrichment at nSBs until the late recovery stage after 16 hours post-stress recovery. In addition of being m⁶A reader, YTHDC1 prolonged enrichment might be due to additional non-m⁶A related function in nSBs during the late recovery stage.

Since all the m⁶A machinery, with exception of YTHDC1, show a unique and specific enrichment pattern in nSBs, we looked for additional nSB components that imitate the same enrichment pattern of the known m⁶A factors because it is still possible that alternative factors in the m⁶A machinery might be trapped at nSBs during stress. Out of 141 nSBs proteins identified by ChIRP-MS, we found that 33 nSBs proteins mimic the enrichment pattern of the known m⁶A machinery, which are the most enriched in the early post-stress stage then released from nSBs by the late post-stress stage. It is possible that these proteins are either cofactors of the m⁶A machinery or participate in the events related to m⁶A modification in nSBs. These candidates include some DEAD-Box RNA helicases, heterogenous ribonucleoproteins, Paraspeckle proteins, the other RNA-binding proteins, and others. It is worth mentioning that after we proposed these candidates as potential cofactors of the m⁶A machinery, it was reported that ZC3H13, one of the 33 m⁶A machinery candidates, serves as a regulator of nuclear RNA m⁶A methylation (Knuckles et al., 2018; Wen et al., 2018). ZC3H13 is trapped at nSBs and follows the similar enrichment pattern to the m⁶A machinery in nSBs. This finding strengthens the possibility that some of the 33 candidates might be involved in nSBs-m⁶A RNA methylation.

In addition, a previous report has shown that TDP43 specifically bind UGGAA in SCA31 disease and exhibit RNA chaperone activity by inhibiting UGGAA RNA misfolding (Ishiguro et al., 2017). This sequence is identical to the m⁶A consensus motif we identified GGAAU, the highly repetitive sequence of HSATIII. Surprisingly, when we depleted TDP43, nSBs-m⁶A was dramatically downregulated. Hence, we add TDP43 as a novel key player in the regulation of stress-induced m⁶A modification. TDP43 is a canonical RNA binding protein

and it unlikely contains any methylase catalytic motifs. Therefore, it raises the intriguing possibility that TDP43 acts as the adaptor to link the m⁶A regulatory factors to HSATIII lncRNAs with GGAAU repeat sequences under stressed condition.

Conclusion

- The nSBs components SAFB and HNRNPM exhibit adjacent localizations in response to thermal stress.
- Both SAFB-foci and HNRNPM-foci utilize HSATIII lncRNA as architectural scaffold.
- SAFB-foci (nSB-S) and HNRNPM-foci (nSB-M) sequester two distinct sets of RNA-binding proteins. SLTM and NCOA5 are strictly recruited to nSB-S while HNRNPA1 and HNRNPH1 are strictly recruited to nSB-M.
- m⁶A RNA modification writers and readers' machineries are sequestered to nSBs upon thermal stress.
- m⁶A methylated RNAs are localized at HSATIII-based nSBs.
- HSATIII lncRNAs transcripts are the target substrates of m⁶A machineries at nSBs.
- The m⁶A consensus within HSATIII is the highly repetitive sequence GGAAU that is distinct from the global GGACU m⁶A consensus.
- With exception of YTHDC1, all m⁶A factors follow a consistent recruitment pattern at nSBs where they are gradually sequestered during thermal stress (2 hours HS) and enriched at early recovery stage (1 hour post-stress) then rapidly released from nSBs at late recovery stage (4 hours post-stress)
- TDP43 is a possible modulator of m⁶A methylation in nSBs.

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Disclosure of Conflict of Interests

We declare no conflict of interest.

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