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Formation of tRNA granules in the nucleus of heat-induced human cells

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ABSTRACT

The stress response, which can trigger various physiological phenomena, is important for living organisms. For instance, a number of stress-induced granules such as P-body and stress granule have been identified. These granules are formed in the cytoplasm under stress conditions and are associated with translational inhibition and mRNA decay. In the nucleus, there is a focus named nuclear stress body (nSB) that distinguishes these structures from cytoplasmic stress granules. Many splicing factors and long non-coding RNA species localize in nSBs as a result of stress. Indeed, tRNAs respond to several kinds of stress such as heat, oxidation or starvation. Although nuclear accumulation of tRNAs occurs in starved *Saccharomyces cerevisiae*, this phenomenon is not found in mammalian cells. We observed that initiator $tRNA^{Met}$ (Meti) is actively translocated into the nucleus of human cells under heat stress. During this study, we identified unique granules of Meti that overlapped with nSBs. Similarly, elongator $tRNA^{Met}$ was translocated into the nucleus and formed granules during heat stress. Formation of tRNA granules is closely related to the translocation ratio. Then, all tRNAs may form the specific granules.

1. Introduction

Eukaryotes respond to different kinds of stress (e.g. heat, oxidation, and starvation) by undergoing various processes such as cell proliferation, repair, acquisition of stress resistance and other physiological phenomena. Important factors involved in stress response include up-regulation of heat shock proteins, modification of specific proteins and alterations in the metabolism and/or localization of RNA [1–3].

The nuclei of higher eukaryotes are highly compartmentalized and consist of structurally and functionally distinct organelles that contain special components involved in transcription, RNA processing or ribonucleoprotein assembly [4]. These nuclear domains under normal (i.e. unstressed) conditions include the nucleolus, nuclear speckles, paraspeckles, PML bodies and Cajal bodies [5–8].

Recent reports have focused on a different type of cellular organelle that is present under stress conditions [9,10]. In the cytoplasm, P-body and stress granules are formed under stress conditions [11] and are involved in translation inhibition as well as mRNA decay [9,11]. However, critical stress responses also occur in the nucleus where specific granules, known as nuclear stress bodies (nSBs), are formed [9]. nSB is one of these unique nuclear granules. The nSBs also contain heat shock factor 1 (HSF1), heat shock factor 2 (HSF2), SRC-activated during mitosis 68 kDa protein (SAM68), alternative splicing factor/splicing factor 2 (ASF/SF2), long non-coding RNA Satellite III repeats into stable RNAs (SatIII) and heat shock RNA 1 (HSR1) [12–16]. Although nSBs are rarely detectable in unstressed cells, these structures are present during heat stress as well as other kinds of stress such as exposure to heavy metals, protease inhibitors or translation inhibitors [10].

Transfer RNAs (tRNAs) are transcribed by RNA polymerase III, and respond to several kinds of stress [17,18]. Recent studies have reported that nuclear accumulation of tRNAs occurs in stress-induced *S. cerevisiae* [19,20]. By contrast, although tRNAs actively accumulate in the nucleus of etoposide-treated and histidinol-treated human cells, nuclear accumulation of tRNAs is not detected in starved mammalian cells [20]. We have studied the relationship between tRNA degradation and translocation in heat stress-induced HeLa cells. Our data show that initiator $tRNA^{Met}$ (Meti) is specifically degraded and translocated into the nucleus under heat stress conditions (prepared paper; Watanabe et al.). During the course of this research, we established that Meti formed unique granules. Until now, it has been reported that the small tRNAs are derived preferentially from the 5′ halves of mature tRNAs and are recruited to distinctive cytoplasmic granules in nutrient-deprived *Trypanosoma cruzi* [21,22]. However, nuclear tRNA granules have not been reported in any mammalian species. Here, we report that Meti and elongator $tRNA^{Met}$ (Mete) granules are translocated into the nucleus of HeLa cells under hyperthermic conditions. Furthermore, these tRNA granules overlapped with nSB.

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2. Materials and methods

2.1. Preparation of cells

HeLa cells were grown in RPMI1640 medium (Wako Pure Chemicals, Tokyo, Japan) containing 10% fetal bovine serum (FBS) (BioWest) and antibiotic–antimycotic (GIBCO, Grand Island, NY) at 37 °C in under an atmosphere of 5% CO_2 until 70 ~ 80% confluent. The cells were given fresh medium prior to each experiment.

2.2. Treatment of cells with reagent and heat stress

Cells (1×10^5 cells/ml or 2×10^5 cells/ml treated with rapamycin) were seeded onto a microscope cover slip (Matsunami Glass, Osaka, Japan) on the previous day. When tRNA transcription was inhibited, cells were pre-treated with RNA polymerase III inhibitor (Merck) for 30 min before being exposed to heat shock. The inhibitor was dissolved in DMSO at a stock concentration of 50 μM. The final concentration of inhibitor used in the experiments was 50 nM.

Rapamycin (Sigma–Aldrich, St Louis, MO) was dissolved in DMSO at a stock concentration of 5 μM. Cells were heat treated in the absence or presence of rapamycin (final concentration of 5 nM).

Unless otherwise stated, heat shock was performed at 43 °C using a water bath for 0–6 h before being allowed to recover in an incubator at 37 °C.

2.3. Analysis of the nuclear localization of tRNA by fluorescence in situ hybridization (FISH) using DIG-labeled RNA probes

The tRNA mixture was extracted from *E. coli* using phenol and chloroform and then precipitated with ethanol. After heat treatment and exposure to rapamycin, cells were fixed with 4% paraformaldehyde phosphate buffer solution (Wako Pure Chemicals) for 20 min at room temperature. Fixed cells were rinsed with 1× PBS and permeabilized in 1xPBS containing 0.5% Triton X-100 for 10 min on ice. The cells were incubated with prehybridization solution [2xSSC, 1xDenhardt's solution (Sigma–Aldrich), 50% formamide, 10 mM EDTA, 100 μg/ml *E. coli* tRNA mixture, and 0.01% Tween-20] at 55 °C for 2 h. DNA template for transcription of Meti and Mete RNA probe were prepared by PCR (Meti forward primer; 5′-AGC AGA GTG GCG CAG CGG-3′, Meti reverse primer; 5′-CCA AGC TCT AAT ACG ACT CAC TAT AGG AGC AGA GGA TGG TTT CGA TCC-3′, elongator $tRNA^{Met}$ (Mete) forward primer; GCC TCG TTA GCG CAG TAG GT, Mete reverse primer; 5′-CCA AGC TCT AAT ACG ACT CAC TAT AGC CCG TGT GAG GAT CGA ACT C-3′). Meti and Mete RNA probes were prepared using DIG RNA labeling kit (Roche, Basel, Switzerland) according to the manufacturer's protocols. Prehybridized cells were incubated with hybridization solution (prehybridization solution plus 5% dextran sulfate), containing the DIG-labeled RNA probe, at 55° C overnight. The cells were washed twice with prewarmed wash buffer (2×SSC, 50% formamide, 0.01% Tween-20) at 55° C for 30 min. Excess probes were digested with 10 μg/ml RNase A in NTET buffer [10 mM Tris–HCl (pH 8), 1 mM EDTA, 500 mM NaCl, and 0.1% Tween-20] at 37C for 30 min. The cells were washed twice with buffer (2×SSC, 0.01% Tween-20) at 55° C for 1 h. The cells were then blocked with blocking buffer [10% FBS (BioWest), 1×PBS, 0.01% Tween-20] at room temperature for 1 h and incubated with anti-DIG, antibody (Roche) diluted with blocking buffer for 1.5 h. Unbound antibodies were removed by washing three times in PBST (1×PBS, 0.2% Tween-20) for 15 min. The cells were incubated with Alexa-conjugated secondary antibodies (Invitrogen, Carlsbad, CA) for 1 h. After washing, the cells counterstained with 4′,6-diamidino-2-phenylindole (DAPI).

2.4. Image analysis and statistical analysis of fluorescent intensity

All imaging experiments were performed using a DMI6000B microscope and LAS AF-TCS SP5 system (Leica, Mannheim, Germany). Fluorescent intensity was quantified by LAS AF Lite software (Leica) and the numerical data were analyzed by Student's *t*-test (for localization quantification: $n = 40$, for granule numeration: $n = 400$ as a minimum).

3. Results

3.1. Formation of tRNA granules under heat stress conditions

We studied nuclear translocation of Meti under heat stress conditions by fluorescence *in situ* hybridization (FISH) experiments. After nuclear translocation of Meti, the formation of Meti granules was detected (Fig. 1A and C). Because Meti was specifically degraded under hyperthermic conditions in our previous study, it was difficult to detect Meti granules in the presence of RNA polymerase III inhibitor. Here, we attempted to examine whether other undegraded tRNAs formed granules. We selected Mete as a target for comparison with Meti. Although both Meti and Mete transport methionine to the ribosome, Meti and Mete play distinct roles in the translation process. Meti is a translation initiation factor and forms a specific complex with the initiation factor eIF2α. Meanwhile, Mete is a translation elongation factor and forms a ternary complex with elongation factor eEF1 [23]. Our FISH experiments showed that Mete was also translocated into the nucleus and formed granules in the same way as Meti (Fig. 1B and C). Meti and Mete granules tend to form once the ratio of nuclear to cytoplasmic tRNAs reaches 70% (Fig. 1C and D). However, Meti with Mete granules form at different times (Fig. 1C). Specifically, Meti granules began to form after 3 h whereas the formation of Mete granules began after only 1 h (Fig. 1C). The nuclear translocation ratio of Mete was higher than that of Meti under normal conditions (Fig. 1D). Moreover, degradation of Meti inhibited its nuclear accumulation. Thus, Mete granules probably form earlier than Meti granules.

In addition, Mete and Meti tRNA granules formed close to the nucleolus, and a weak signal for Mete could be detected in the nucleolus (Fig. 1A and B). Interestingly, nSB is adjacent to the nucleolus under hyperthermic conditions [24]. Because tRNAs were expected to be recruited into nSBs, HSF1 was used as the marker protein for nSB. HSF1 is an important stress-responsive transcriptional activator that is essential for the inducible expression of heat shock proteins (HSPs) [25]. HSF1 proteins and tRNA granules were observed by immunofluorescence (IF) and FISH experiments. After HSF1 foci were formed, Meti and Mete granules were generated and overlapped with HSF1 foci (Fig. 1A and B). Therefore, tRNAs may be one of the components of nSBs.

3.2. Formation of tRNA granules under heat stress conditions in the presence of RNA polIII inhibitor

We used RNA polymerase III inhibitor in order to evaluate whether formation of tRNA granules depended on transcription or occurred by translocation from the cytoplasm to the nucleus. We found that Meti and Mete granules were formed when transcription of tRNAs was inhibited by an RNA polymerase III inhibitor (Fig. 2A–C). These results were consistent with those described in the previous section. Meti granules reached the maximum value in 5 h (Fig. 2A and B). Meti degradation was promoted by inhibiting

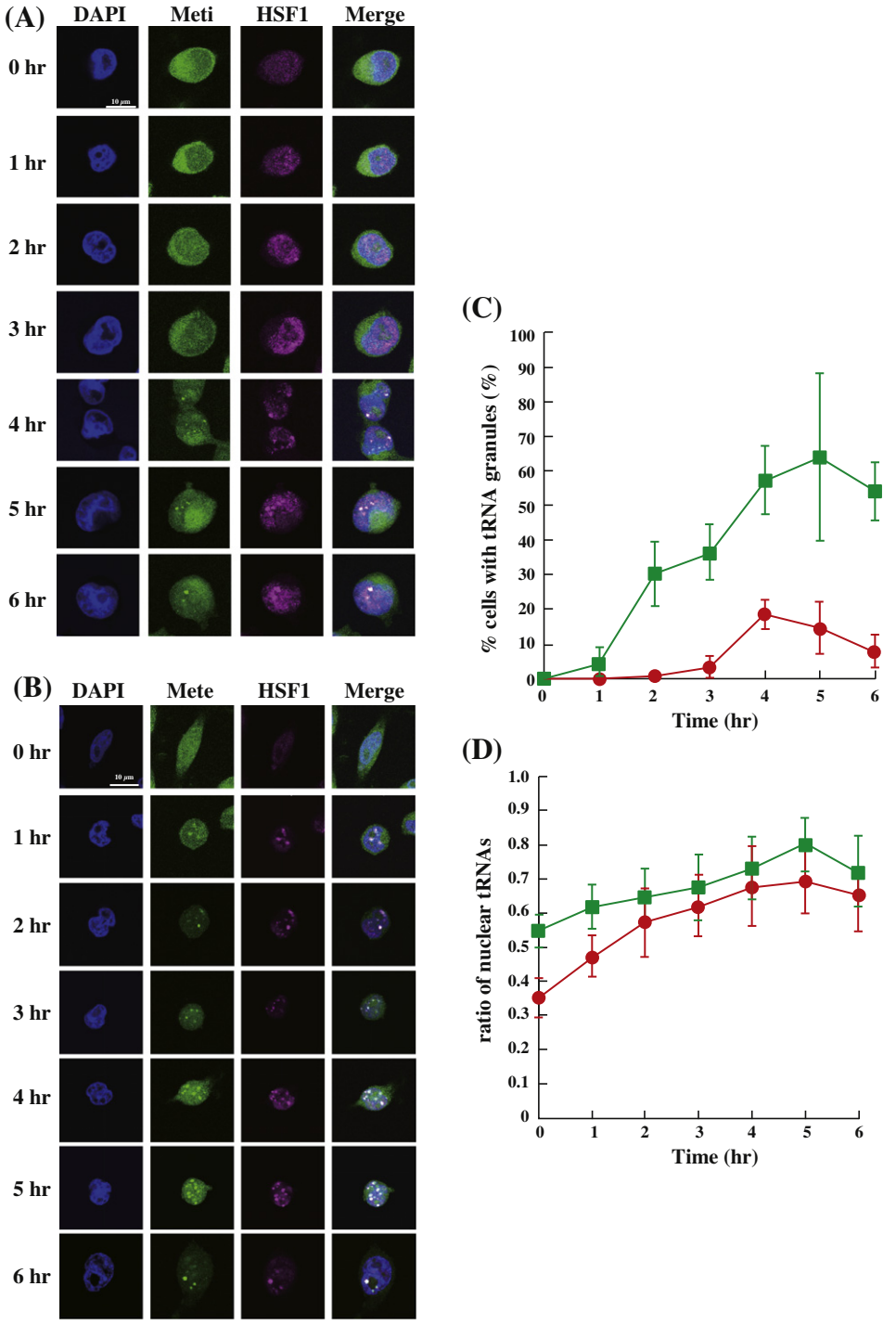

Fig. 1. Meti and Mete granules formation under heat stress conditions. Nuclear translocation and granule formation of Meti (A), Mete (B) were monitored by FISH and IF experiments in HeLa cells at 43 °C for 0–6 h. Nuclear DNA was stained with DAPI. (C) The ratio of cells with Meti granules (red), Mete granules (green) is shown (n = more than 400, $p < 0.05$). (D) The ratio of nuclear Meti (red), Mete (green) is shown ($n = 40$, $p < 0.05$).

transcription. Thus, Meti translocation and/or accumulation into the nucleus was probably delayed, which may explain why Meti granules tended to form later. These results suggest that formation of both Meti and Mete granules occur by translocation from the cytoplasm to the nucleus. tRNA granules were formed when the ratio of nuclear to cytoplasmic tRNAs reached 70% (Fig. 2C and D). These results are consistent with the findings shown in Fig. 1. The ratio of nuclear translocation of tRNAs to the formation of tRNA granules also showed some correlation.

3.3. Effect of rapamycin on tRNA granules

The mammalian target of rapamycin (mTOR) plays an important role in a complex intracellular signaling pathway that helps mediate multiple processes including cell growth, cell proliferation and autophagy. The first identified inhibitor of mTOR is rapamycin [26]. Recent studies have shown that rapamycin promotes the nuclear accumulation of tRNAs in *S. cerevisiae* [20]. Indeed, our latest data demonstrate that rapamycin facilitates the nuclear

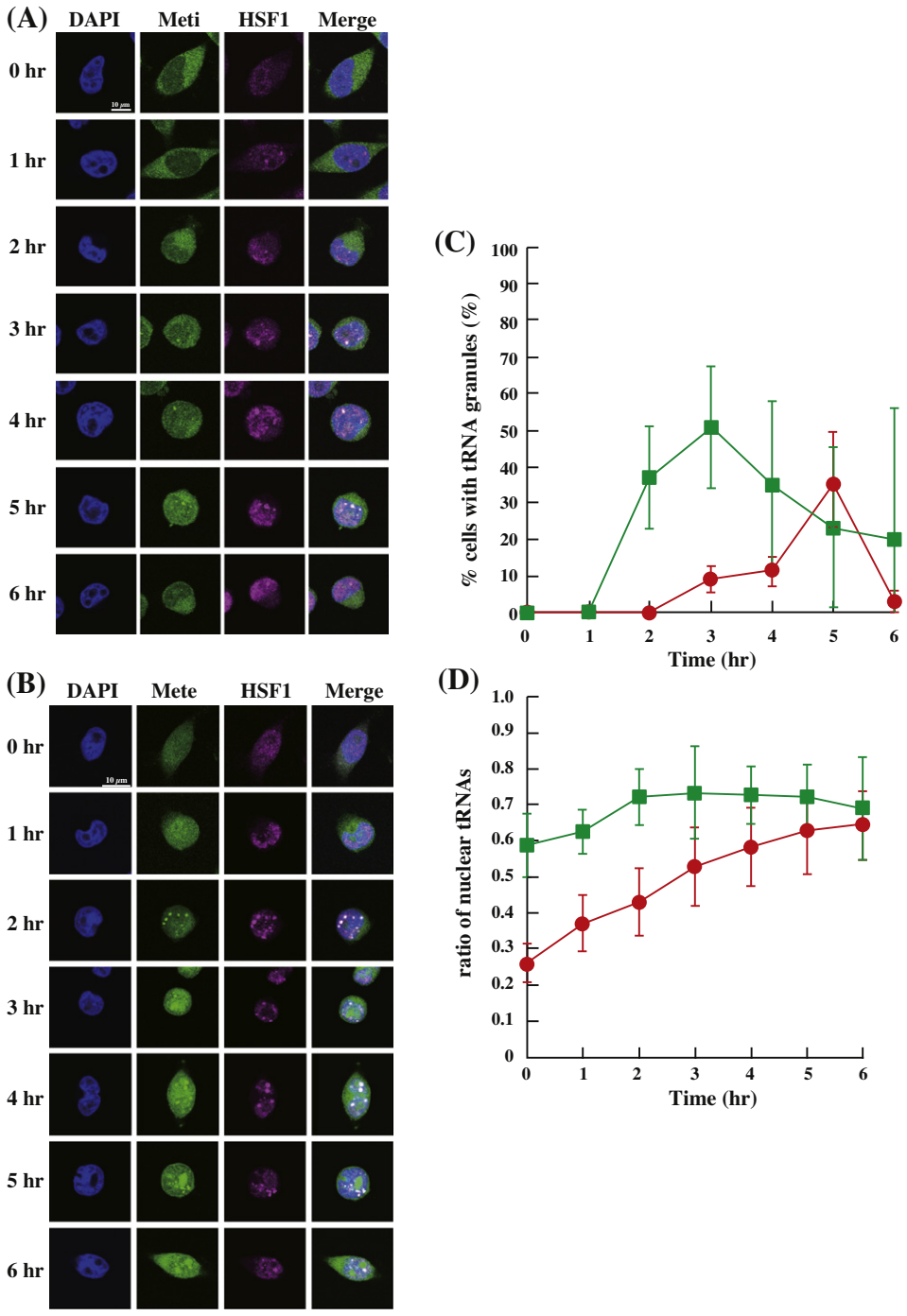

Fig. 2. Meti and Mete granule formation under heat stress conditions in the presence of RNA polymerase III inhibitor. Nuclear translocation and granule formation of Meti (A), Mete (B) were monitored in the presence of RNA polymerase III inhibitor. (C) The ratio of cells with Meti granules (red), Mete granules (green) is shown (n = more than 400, $p < 0.05$). (D) The ratio of nuclear Meti (red), Mete (green) is shown ($n = 40$, $p < 0.05$).

translocation of Meti in HeLa cells (prepared paper; Watanabe et al.). We selected rapamycin in our experiments to examine whether formation of tRNA granules depends on the ratio of translocation. Nuclear translocation of Meti was facilitated in the presence of rapamycin, resulting in accelerated formation of Meti granules (Fig. 3A, C and D). Likewise, the ratio of Mete translocation also increased at an earlier stage in the presence of rapamycin, thereby accelerating formation of Mete granules (Fig. 3B–D). Indeed, the data shown in Fig. 3C and D is consistent with the findings shown in Figs. 1 and 2. The ratio of nuclear translocation of tRNAs is correlated with the trend in the formation of tRNA granules. Additionally, this result was closely-matched with the phenomena observed in cells under heat stress in the absence of rapamycin. It was likely that this '70%' ratio is key to the formation of tRNA granules. Moreover, because rapamycin is an inhibitor of mTOR, it is possible that mTOR is responsible for nuclear translocation of both Meti and Mete.

3.4. Recovery from hyperthermic stress

In order to determine the period of recovery of tRNA granules, cells were allowed to recover for 1–6 h at 37 °C after 4-h (Meti)

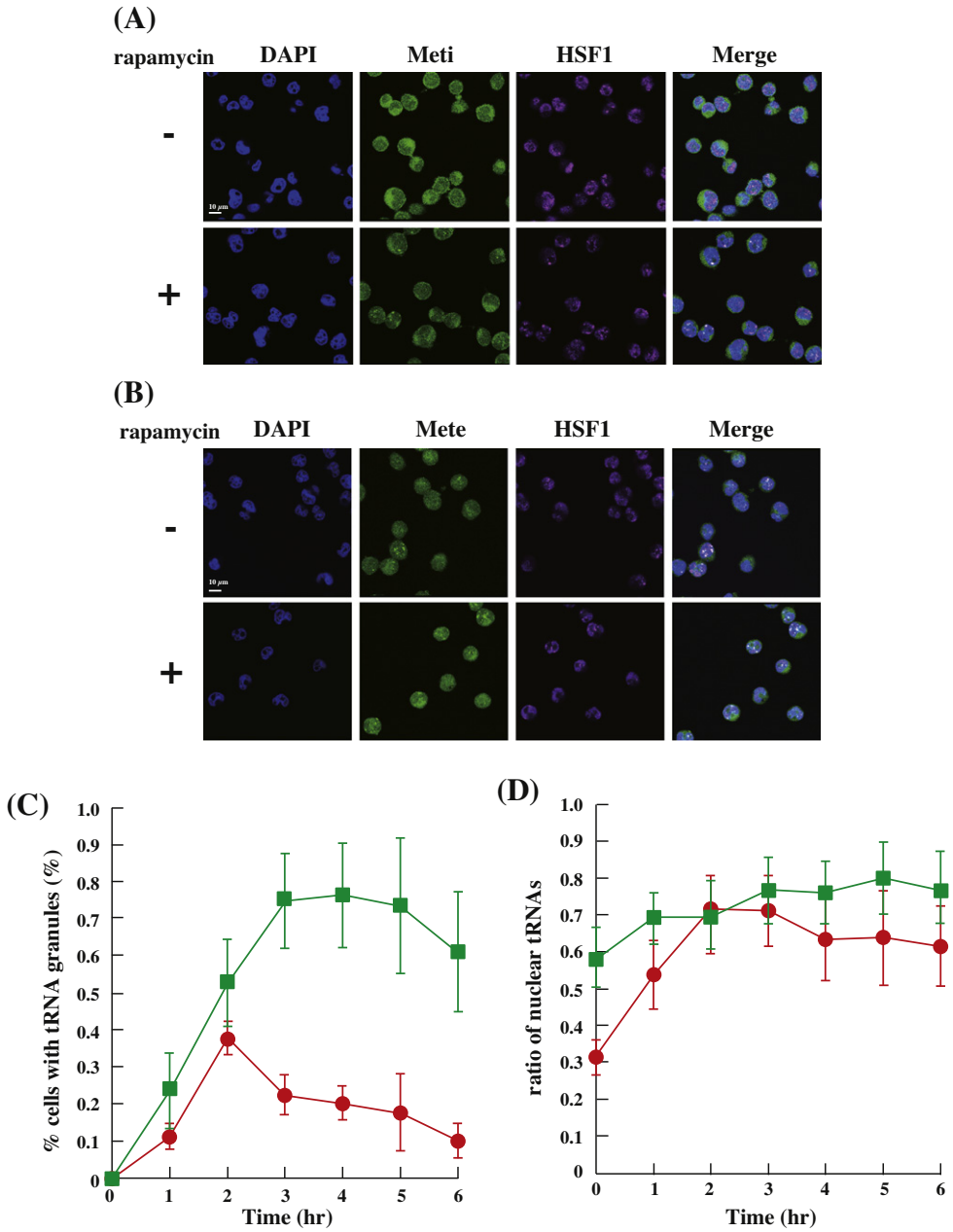

Fig. 3. Facilitation of Meti and Mete granule formation by rapamycin. Nuclear translocation and granule formation of Meti (A), Mete (B) were monitored in the presence of rapamycin. Cells that were heat treated for 2 h were shown. (C) The ratio of cells with Meti granules (red), Mete granules (green) is shown (n = more than 400, $p < 0.05$). (D) The ratio of nuclear Meti (red), Mete (green) is shown ($n = 40$, $p < 0.05$).

or 5-h (Mete) exposure to heat stress. In this rescue experiment, the cells were exposed to heat for 4 or 5 h because formation of Meti granules peaked at 4 h (Fig. 1C) and Mete granules peaked at 5 h (Fig. 1C). As soon as the heat-stressed cells were returned to 37 °C, the ratio of nuclear tRNAs slowly reduced to almost the same level as the ratio of unstressed control cells (Fig. 4A–F). Meti granules gradually decreased and most of the Meti granules disappeared within 3 h of recovery at 37 °C (Fig. 4A and B). Furthermore, most of Mete granules disappeared within 5 h of recovery (Fig. 4D and E). These results show that nuclear translocation of tRNAs is important for the formation of tRNA granules. Moreover, both nuclear translocation of tRNAs and formation of tRNAs are induced by heat stress.

4. Discussion

In heat stress-induced HeLa cells, Meti and Mete were preferentially translocated into the nucleus and tRNA granules that overlapped with nSBs were formed. Furthermore, tRNA granule formation was facilitated when the cells were treated with rapamycin, which accelerated nuclear translocation. In cells recovering from heat stress, tRNA granules disappeared and nuclear tRNAs returned from the nucleus to the cytoplasm. As a result, with an increase in the ratio of Meti and Mete translocation, Meti and Mete granules were formed and overlapped with nSBs.

Recent reports have proposed the curious concept of an RNA thermometer in prokaryotes [16,27,28]. The RNA thermometer is

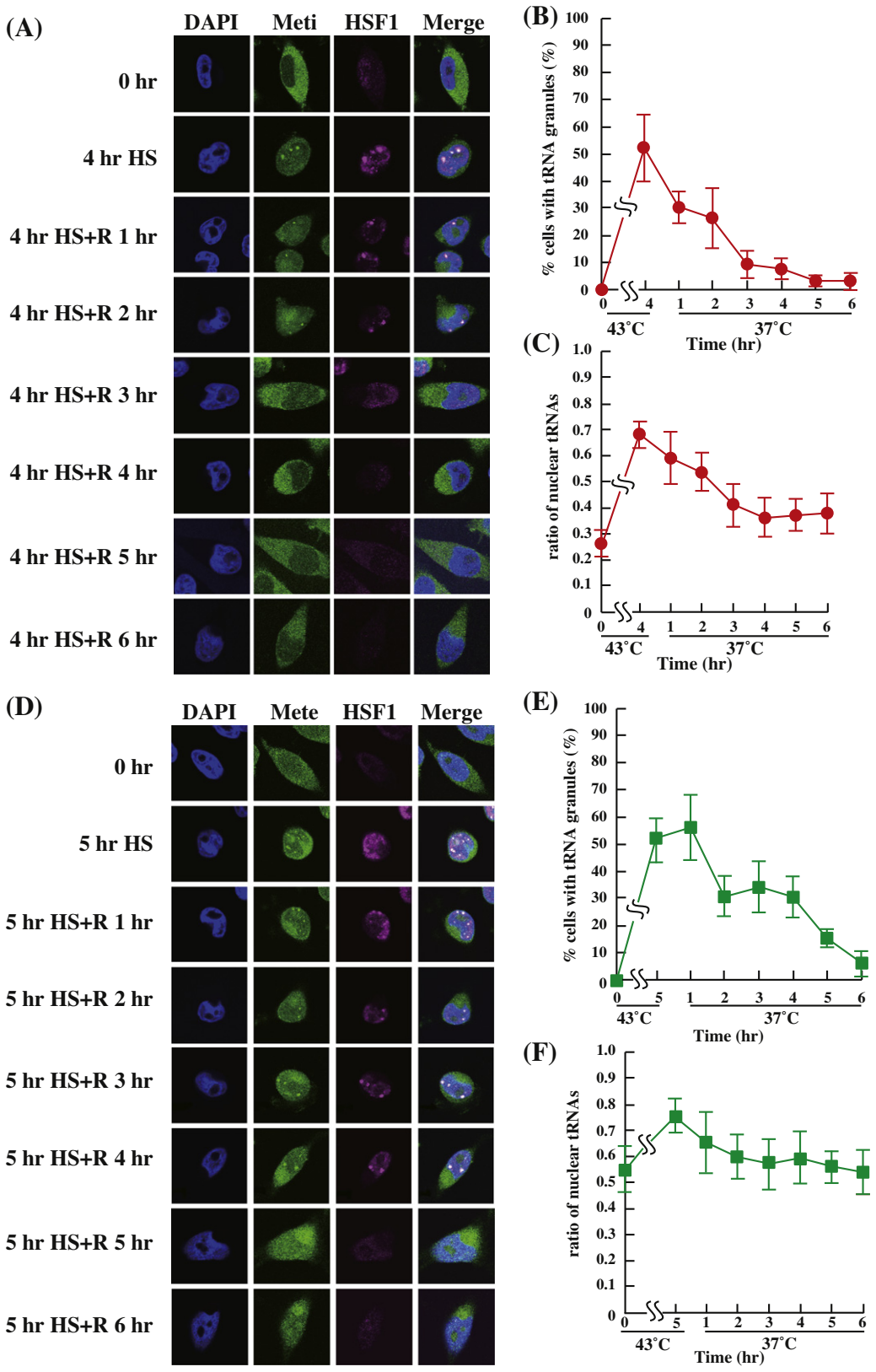

Fig. 4. Recovery after exposure to heat stress. (A) HeLa cells were incubated at 43 °C for 4 h and then allowed to recover at 37 °C for 1–6 h. Nuclear translocation and granule formation of Meti were monitored. HS: heat stress, R: rescue. The ratio of cells with Meti granules (B) and Mete granules (E) is shown (n = more than 400, $p < 0.05$). The ratio of nuclear Meti (C) and Mete (F) is shown ($n = 40$, $p < 0.05$). (D) HeLa cells were incubated at 43 °C for 5 h and then allowed to recover. Mete granules were monitored.

a temperature-sensitive non-coding RNA molecule that regulates the expression of genes required for the heat shock or cold shock response as well as other stress responses [29]. In humans, HSR1 that is a potential RNA thermometer has been reported [16]. HSR1, which is an important factor for nSB formation, activates HSF1 and induces proteins that prevent the cells from overheating

[16]. tRNA is a primitive non-coding RNA that is relatively abundant in the cell. Like HSR1, tRNAs may play a role as a thermosensor when localized into nSGs.

Additionally, SatIII recruits several transcription and splicing factors such as ASF/SF2 to form nSBs [24,30]. It has been reported that SatIII associated-nSB formation affects chromatin remodeling (10). Indeed, tRNAs in concert with SatIII is likely to play a role in chromatin remodeling. Moreover, another splicing factor recruited to nSBs is SAM68 [10]. nSBs including SAM68 have roles in cell cycle progression, mRNA export and splicing [31–33]. When tRNAs are localized into nSBs, tRNAs may have an important function in these phenomena e.g. RNA-mediated alternative splicing by cooperating with these splicing factors in nSBs. Furthermore, another report suggests that SAM68 forms part of the RNA-mediated stress response e.g. alternative splicing triggered by DNA damage [14]. A recent report shows that heat stress causes DNA damage [34]. Hence, heat-induced tRNA granules may also play a role as sensors for DNA damage.

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