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Pioneer round of translation occurs during serum starvation

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Abstract

The pioneer round of translation plays a role in translation initiation of newly spliced and exon junction complex (EJC)-bound mRNAs. Nuclear cap-binding protein complex CBP80/20 binds to those mRNAs at the 5′-end, recruiting translation initiation complex. As a consequence of the pioneer round of translation, the bound EJCs are dissociated from mRNAs and CBP80/20 is replaced by the cytoplasmic cap-binding protein eIF4E. Steady-state translation directed by eIF4E allows for an immediate and rapid response to changes in physiological conditions. Here, we show that nonsense-mediated mRNA decay (NMD), which restricts only to the pioneer round of translation but not to steady-state translation, efficiently occurs even during serum starvation, in which steady-state translation is drastically abolished. Accordingly, CBP80 remains in the nucleus and processing bodies are unaffected in their abundance and number in serum-starved conditions. These results suggest that mRNAs enter the pioneer round of translation during serum starvation and are targeted for NMD if they contain premature termination codons.

Keywords: Pioneer round of translation; Steady-state translation; Nonsense-mediated mRNA decay; Serum starvation; Stress granule; Processing body; CBP80; eIF4E

In mammalian cells, the steps in mRNA processing are highly coordinated events responsible for the production of functional mRNAs; these steps include 5′-end capping, splicing, and 3′-end polyadenylation before the mRNA is transported from the nucleus to the cytoplasm [1,2]. Once the nascent pre-mRNA is capped, the heterodimer of nuclear cap-binding proteins (CBPs) 80/20 binds co-transcriptionally to the cap structure, which is required for the subsequent steps of splicing, translation, and mRNA quality control.

Splicing contributes to the formation of mRNPs from intron-containing pre-mRNAs, depositing exon junction complex (EJC) 20–24 nucleotides upstream of each exon–exon junction. The metazoan EJC consists of four core proteins including eIF4AIII, Y14, Magoh, and Barentsz/MLN51, and other auxiliary proteins (reviewed in Ref. [3–5]). EJCs deposited on newly spliced mRNAs serve to mark mRNAs for downstream processing steps such as mRNA export, translation, and nonsense-mediated mRNA decay (NMD), which is an mRNA surveillance mechanism that recognizes and gets rid of aberrant mRNAs that prematurely terminate translation. EJCs of newly spliced mRNAs sequentially recruit the NMD factors Upf3/3X and Upf2. As such, NMD functions to eliminate production of potentially deleterious truncated proteins.

CBP80/20, which binds at the 5′-end of newly spliced mRNAs, interacts with eukaryotic translation initiation factor eIF4G, which recruits the ribosome, presumably via eIF3, to initiate the first round of translation [6]. This mode of ribosome recruitment occurs during mRNA export after completion of proper mRNA processing in the nucleus and is referred to as the "pioneer round of translation" [7]. During the pioneer round of translation, translating ribosomes along the mRNAs dissociate the bound EJCs from the mRNAs and CBP80/20 is replaced by major cytoplasmic cap-binding protein eIF4E, which directs steady-state translation initiation. Thus, a pioneer

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round of translation differs from steady-state translation as follows [6–9]. First, the pioneer round of translation occurs on newly spliced and CBP80/20-bound mRNAs, whereas steady-state translation occurs on mRNAs bound by eIF4E at the 5′-end. Second, the pioneer round of translation occurs on mRNAs that harbor EJCs. Third, steady-state translation is responsible for the majority of cellular protein synthesis. Notably, NMD restricts to only newly synthesized mRNAs in mammalian cells. That is, NMD targets CBP80/20-bound mRNA during the pioneer round of translation rather than eIF4E-bound mRNA during steady-state translation.

Steady-state translation initiation directed by eIF4E has been reported to allow for an immediate and rapid response to changes in physiological conditions. Such regulation is of particular importance under stress conditions such as heat shock, hypoxia, nutrient deprivation, endoplasmic reticulum (ER) stress, apoptosis, ultraviolet (UV) irradiation, temperature changes, oxidative stress, viral infection, and exposure to various drugs or toxins [10–14].

For the pioneer round of translation, few studies have been reported on its control in response to changes in physiological conditions. In this study, we ask how the pioneer round of translation responds to the cellular stresses, and what happens to mRNAs synthesized during the stress conditions. To answer these questions, we monitor the level of NMD, which restricts to only the pioneer round of translation but not to steady-state translation, in serum-starved conditions where the bulk of cellular translation, that is, steady-state translation initiation, is significantly blocked. We show that both nucleus-associated and cytoplasmic NMD efficiently occur in serum-starved conditions. We also show that a component of major nuclear cap-binding protein, CBP80, is highly localized in nucleus, regardless of serum starvation. Furthermore, serum starvation does not change the cellular abundance and numbers of processing bodies. These results suggest that the pioneer round of translation and its downstream event NMD efficiently work to monitor and degrade aberrant mRNAs, even during stressful conditions, such as serum starvation.

Materials and methods

Plasmid construction. To construct pCDNA3-FLAG-CBP80, which encodes full-length human CBP80 tagged with the FLAG epitope at the N-terminus, pcDNA3-FLAG (Invitrogen) digested with BamHI and HindIII was ligated to a PCR fragment that was also digested with BamHI and HindIII. A PCR fragment containing the CBP80 cDNA sequence was amplified using pMS2-HA-CBP80 [9] as a template and two oligonucleotides: 5′-CGGGATCCGATGTCGCGGCGGCGGCACAGCGACGAG-3′ (sense) and 5′-CCCAAGCTTTTAGGCCTGCAGGGCACAGAACTGCTG-3′ (antisense). The BamHI and HindIII sites are underlined.

Cell culture, transfections, and protein and RNA purification. Human HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Cambrex) containing 10% fetal bovine serum (Cambrex), penicillin (Cambrex), and streptomycin (Cambrex). For experiments to monitor the translational efficiency and NMD efficiency, 2×10^6 HeLa cells were transiently co-transfected with four plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions: (i) 1.0 μg of the pGL3-control plasmid (Promega), (ii) 0.1 μg of the pmCMV-Gl test plasmid (either Norm or Ter), (iii) 0.1 μg of the pmCMV-GPx1 test plasmid (either Norm or Ter), and (iv) 0.1 μg of the phCMV-MUP reference plasmid. All of the plasmids were previously described [15]. Cells were cultured in serum-free DMEM for 24 h prior to cell harvest to induce serum starvation. Total proteins and RNAs were prepared as reported previously [15].

RNA analysis. FLUC, Smg7, β-Gl, GPx1, and MUP mRNAs were amplified as previously described [6,8]. In brief, total RNAs were isolated from serum-stimulated and serum-starved HeLa cells. RT-PCRs of FLUC, Smg7, β-Gl, GPx1, and MUP mRNAs were performed using specific primer sets and α-[^{32}P]-dATP. Labeled PCR products were separated by gel electrophoresis and visualized by autoradiography.

Western blotting. Western blotting was carried out as previously described [15]. Antibodies that were used recognized Upf1, Upf2, Upf3/3X (gifts from Dr. Lynne E. Maquat), CBP80 [16], eIF4AIII [17], Phospho-eIF2α(Ser51) (Cell Signaling), eIF2α (Cell Signaling), eIF4E (Cell Signaling), Phospho-eIF4E-BP1(Ser65/Thr70) (Santa Cruz), eIF4E-BP1 (this antibody recognizes all types of eIF4E-BP1; Cell Signaling), eIF4GI (a gift from Dr. Sung Key Jang of POSTECH), eIF3 (a gift from Dr. John W. Hershey), and β-actin (Sigma).

Luciferase activity assays. Assays were performed as previously described [6,8].

Immunofluorescence. For immunofluorescence microscopy, cells were fixed with 2% paraformaldehyde (Merck) in PBS for 10 min and permeabilized with 0.5% Triton X-100 in PBS for 10 min. Cells were incubated with blocking buffer (1.5% BSA in PBS) for 1 h, and primary antibodies (anti-eIF4E antibody, BD Bioscience; anti-FLAG antibody, Sigma) for 1 h. The primary antibodies were detected with rhodamine-conjugated secondary antibodies (Pierce). Nuclei were stained with DAPI (Molecular Probes). Cells were observed with a ZEISS confocal microscope (LSM510 META). When indicated, HeLa cells were transiently transfected with 2 μg of pCDNA3-FLAG-Dcp1a [18] or pCDNA3-FLAG-CBP80 using Lipofectamine 2000 (Invitrogen) two days before cell fixation.

Results

Steady-state translation (overall translation) of eIF4E-bound mRNAs, but not the pioneer round of translation of CBP80-bound mRNAs, is drastically blocked during serum starvation

To test the effect of serum starvation on steady-state translation (overall translation) and the pioneer round of translation, we used firefly luciferase (FLUC) and NMD reporters to monitor the efficiencies of steady-state translation and the pioneer round of translation, respectively. HeLa cells were co-transfected with four plasmids: (i) the pGL3-control reporter plasmid, which allows monitoring of the efficiency of steady-state translation; (ii) a pmCMV-Gl test plasmid that was either nonsense-free (Norm) or contained a premature termination codon (PTC) at the position of the 39th amino acid (39Ter) of the globin gene (Gl), which together provide an assay for nucleus-associated NMD; (iii) the pmCMV-GPx1 test plasmid, either Norm or 46Ter which provides an assay for cytoplasmic NMD; and (iv) the phCMV-MUP reference plasmid, which controls for variations in the efficiencies of cell transfection and RNA recovery. One day after DNA transfection, serum starvation was induced by culturing the transfected cells in serum-free media. After one additional day, total-cell proteins and RNAs were purified,

and then luciferase assays and the semi-quantitative RT-PCR analyses were performed to determine the abundance of FLUC mRNAs and the NMD efficiency of Gl and GPx1 mRNAs.

The results indicate that serum starvation had no significant effect on the abundance of FLUC mRNAs (Fig. 1A). In contrast, the translation efficiency of FLUC mRNAs was drastically inhibited by 4-fold under serum-starved conditions relative to serum-stimulated conditions (Fig. 1C), which is consistent with previous reports indicating that serum starvation blocks the bulk of translation, presumably via modification of translation initiation factors [19–21].

Using the same total RNAs as in the above experiment, NMD efficiencies of globin mRNAs and GPx1 mRNAs were monitored using semi-quantitative RT-PCR analyses (Fig. 2). In serum-stimulated conditions, the abundances of globin mRNAs harboring 39Ter and GPx1 mRNAs harboring 46Ter were reduced to about 7% and 20% of the normal mRNAs, respectively. Importantly, both the NMDs of globin mRNAs and GPx1 mRNAs were still active in serum-starved conditions (Fig. 2A for globin NMD and Fig. 2B for GPx1 NMD). Globin mRNAs and GPx1 mRNAs have been shown to be subject to nucleus-associated NMD and cytoplasmic NMD, respectively. Notably, both types of NMD restricts to only the pioneer round of translation in mammalian cells. Taken together, these results indicate that the pioneer round of translation occurs and, hence, both types of NMD are functionally active under serum-starved conditions where steady-state translation is drastically compromised.

The abundance of cellular factors involved in the pioneer round of translation and NMD does not differ between serum-starved and serum-stimulated conditions

For more comprehensive understanding of the pioneer round of translation and NMD in serum-starved cells, we assessed the levels of cellular proteins involved in these processes. These included a component of nuclear cap-binding protein CBP80, a core EJC component eIF4AIII, and the key NMD factors, Upf1, Upf2, and Upf3/3X. Endogenous β-actin served as loading control (Fig. 3A). Western blotting revealed no significant differences in the abundance of any of the tested proteins between serum-stimulated and serum-starved conditions (Fig. 3A), which is consistent with the efficient NMD during serum starvation (Fig. 2).

Eukaryotic translation initiation factors involved in steady-state translation were also assessed. These included eIF4E, eIF4GI, eIF4AI, eIF3, eIF2α, and eIF4E-BP1. Notably, it has been reported that eIF4GI interacts with CBP80 and that it is presumably involved in the pioneer round of translation and NMD [6]. Western blotting showed no significant differences in their abundance between the serum-stimulated and serum-starved condition (Fig. 3B).

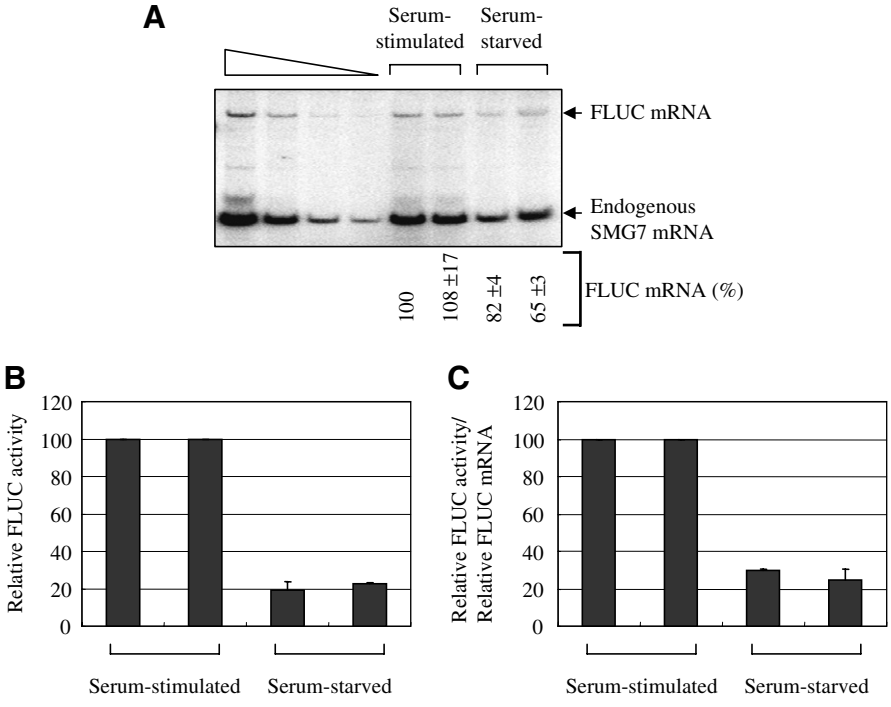

Fig. 1. Serum starvation decreases general translation efficiency. (A) HeLa cells were transiently transfected with plasmid expressing firefly luciferase (FLUC) for luciferase activity and then serum starvation was induced by culturing the transfected cells in serum-free media during 24 h. The numbers below the panel represent the level of FLUC mRNA after normalization to the level of endogenous SMG7 mRNA. (B) Relative FLUC luciferase activity decreased about 80% in serum-starved condition. (C) The level of general translation efficiency was assessed from FLUC activity after normalization to the level of total protein per FLUC mRNA after normalization to the level of endogenous SMG7 mRNA.

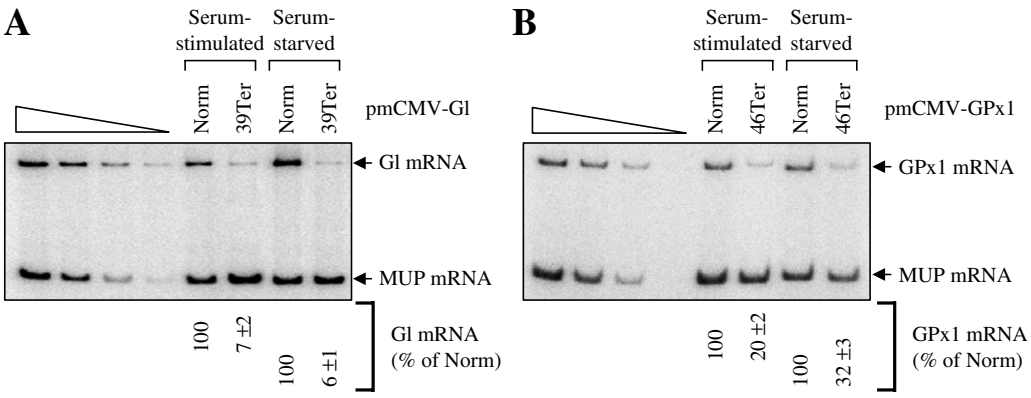

Fig. 2. NMD occurs during serum starvation. (A) As in Fig. 1, except that HeLa cells were transiently transfected with a pmCMV-Gl test plasmid (either Norm or Ter), a pmCMV-GPx1 test plasmid (either Norm or Ter), and the phMUP reference plasmid. The level of Gl mRNA was normalized to the level of MUP mRNA. (B) RT-PCR analysis of GPx1 mRNA.

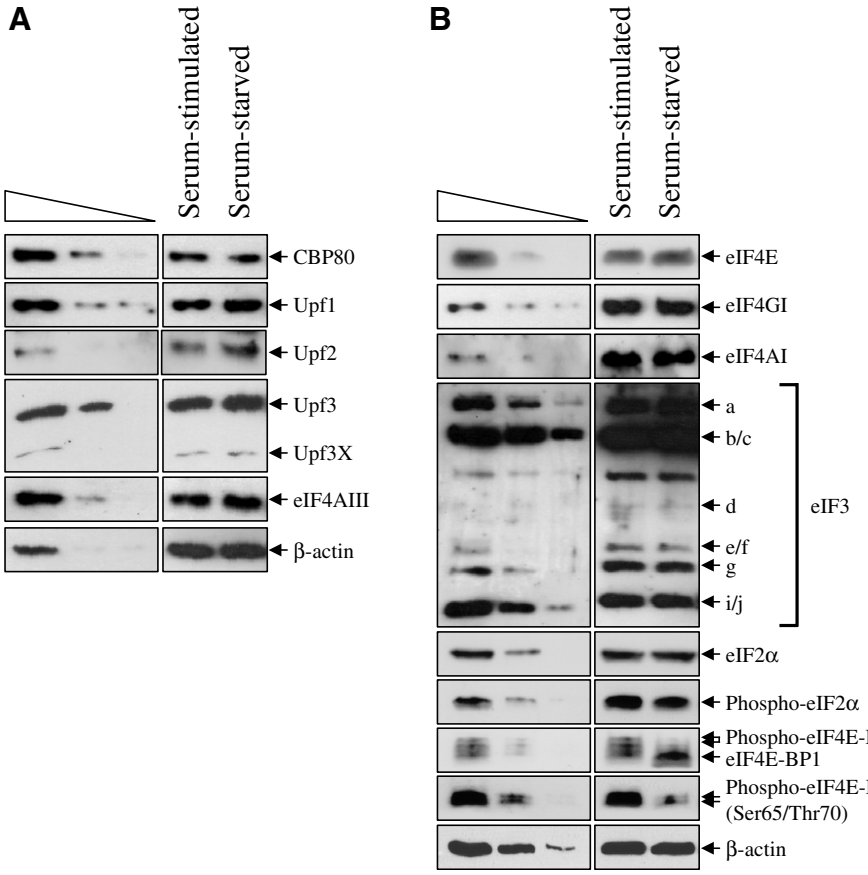

Fig. 3. Serum starvation does not significantly alter the levels of cellular factors involved in NMD and the pioneer round of translation. As in Fig. 1, except that total proteins were analyzed by SDS–PAGE. Western blotting was performed using the specific antibodies as indicated. β-Actin served as a loading control. The three leftmost lanes, in which threefold serial dilutions of cellular proteins were analyzed, demonstrate that the conditions used for Western blotting are semi-quantitative. (B) As in Fig. 3A, except that translation initiation factors involved in steady-state translation were analyzed.

It has been reported that serum starvation induces dephosphorylation of eIF4E, eIF4E-BP1, and eIF4G1 [19–21]. To confirm this in our system, we assessed the levels of phosphorylation of eIF4E-BP1 and eIF2α, which is phosphorylated by stress-activated kinases [14]. Western blotting revealed that all of the tested proteins except for eIF4E-BP1 in our study were unchanged in their abundance during serum starvation (Fig. 3B). However, it is noted that serum starvation triggered the dephosphorylation of eIF4E-BP1 which is an active form to compete with eIF4G for binding to eIF4E, as previously reported [20].

All together, we conclude that serum starvation preferentially inhibits steady-state translation, but not the

pioneer round of translation. As a consequence, PTC-harboring mRNAs that have undergone the pioneer round of translation are targeted for NMD during serum starvation.

The intracellular distribution of CBP80 and the number and size of processing bodies are unchanged during serum starvation

Exposure of mammalian cells to environmental stress (e.g., heat, oxidative conditions, UV irradiation, and hypoxia) triggers the formation of discrete cytoplasmic foci, which is referred to as stress granules. By so doing, eukaryotic cells reprogram mRNP composition to adapt to stressful conditions. Previous work showed that stress granules are highly dynamic cytoplasmic foci where stalled mRNA, TIA-1, TIAR, PABP1, and 40S ribosomal subunit transiently accumulate in response to stress-induced translational inhibition ([22] and references therein). These stress granules also contain many components of steady-state, cap-dependent translation initiation, including eIF4E, eIF3, eIF4G, and small ribosomal subunit.

We asked if CBP80 is redistributed into stress granules during serum starvation. HeLa cells were transfected with plasmid pCMV-FLAG-CBP80 to express FLAG-tagged CBP80. One day later, serum starvation was induced by culturing the transfected cells in serum-free media. After one additional day, the intracellular locations of FLAG-CBP80 and endogenous eIF4E were determined using anti-FLAG and anti-eIF4E antibodies, respectively, with confocal microscopy.

Endogenous eIF4E was mainly localized in cytoplasm under serum-stimulated conditions (Fig. 4A, upper). The small fraction of endogenous eIF4E detected in nucleus is consistent with previous reports [23,24]. Upon serum starvation, endogenous eIF4E highly accumulated at discrete stress granules in the cytoplasm. Interestingly, a large fraction of endogenous eIF4E was redistributed into the nucleus and accumulated in nuclear body-like structures (Fig. 4A, lower; [24]). On the other hand, FLAG-CBP80 was highly enriched in the nucleus, regardless of serum treatment (Fig. 4B).

Next, we explored the regulation of abundance and number of processing bodies (P-body) under stress conditions. P-body has been reported to contain many RNA processing and degrading enzymes including decapping enzymes Dcp1 and Dcp2, a 5′-to-3′ exoribonuclease Xrn1, and Lsm proteins (reviewed in Refs. [25,26] and references therein). The mRNAs destined to be degraded by NMD machinery have been shown to move to the P-body. In our study, FLAG-tagged Dcp1a, which is a marker protein for P-body, was used to determine the effect of serum starvation on P-body formation. In the presence of serum, HeLa cells contained more than 10 independent P-bodies (Fig. 4C), as previously reported [25,26]. Notably, serum starvation had no significant effect on P-body formation. The overall number and size of P-bodies per cell in serum-starved conditions were almost equal to those in serum-stimulated conditions.

All of these data suggest that, during serum starvation, eIF4E accumulates in stress granules and in the nucleus, resulting in inhibition of steady-state translation initiation. In contrast, the distribution of CBP80 is unaffected by serum starvation. Thus, the pioneer round of translation is active under stress conditions and NMD occurs as a consequence of the pioneer round of translation.

Discussion

In this work, we present strong evidence to indicate that the pioneer round of translation occurs during stressful conditions. Consequently, NMD, which is a downstream event that restricts to only the pioneer round of translation, can still monitor and degrade aberrant transcripts harboring PTC. In stressful conditions, Gl mRNAs and GPx1 mRNAs harboring PTC are efficiently degraded by NMD (Fig. 2), even if steady-state translation is inhibited (Fig. 1). Many cellular factors involved in NMD are unaffected in their abundance during serum starvation (Fig. 3). Furthermore, evenly dispersed eIF4E throughout the cytoplasm in serum-stimulated conditions is redistributed into stress granules in cytoplasm and speckles in nucleus in serum-starved conditions, whereas the localization of CBP80, which is mainly within the nucleus, is not changed by serum starvation (Fig. 4).

Previous reports suggest that assembly of stress granules require phosphorylation of eIF2α, which arrests translation initiation by limiting the amount of ternary complex eIF2-GTP-$tRNA_i^{Met}$ that is needed to initiate steady-state translation as well as pioneer round of translation [27]. However, serum starvation in our condition does not trigger the phosphorylation of eIF2α, even if eIF4E-BP1 is drastically dephosphorylated. Based on these findings, we propose that there might be another pathway to trigger the formation of stress granules independently of phosphorylation of eIF2α, which is previously suggested by several groups [28,29].

Our data highlight the important role of the pioneer round of translation and NMD in mRNA surveillance under stressful conditions. Various cellular machineries that are in charge of RNA processing tend to be modulated with respect to their efficiencies during stress. For instance, it is well known that splicing of pre-mRNAs is generally repressed by heat shock. Exposure of cells to heat shock at 42 °C leads to accumulation of pre-mRNAs containing unspliced introns [30]. This inhibition of splicing has been also observed in a variety of species from yeast to humans [30–33]. Impaired splicing machinery tends to generate aberrant transcripts containing PTC via inefficient or incorrect splicing, which is one of the major causes to generate transcripts targeted for NMD. In this situation, the pioneer round of translation may monitor the fidelity of transcripts. As a consequence, NMD may degrade aberrant transcripts

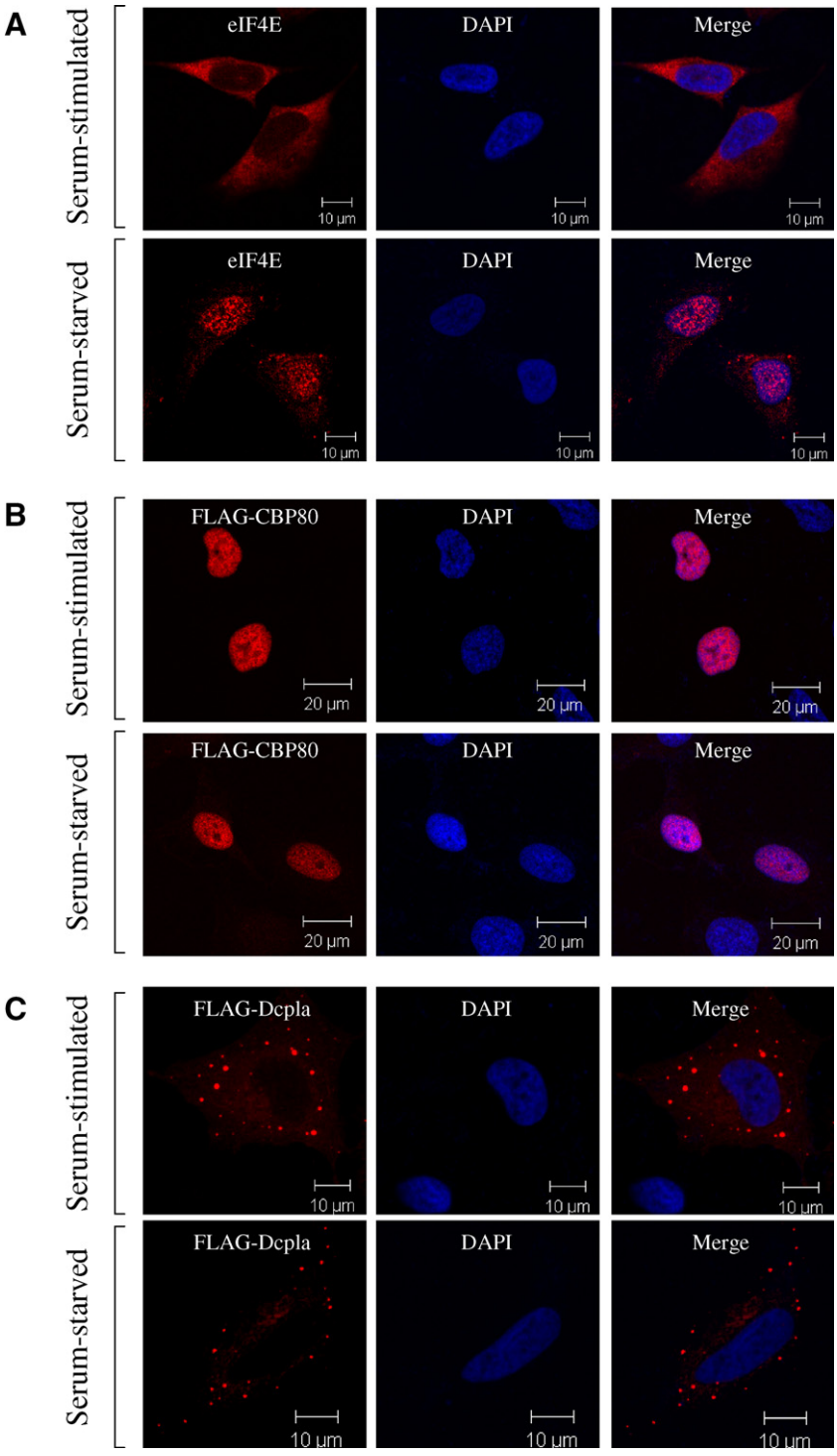

Fig. 4. Serum starvation does not affect the intracellular distribution of CBP80 and P-bodies. (A) The distribution of endogenous eIF4E in HeLa cells was visualized by anti-eIF4E antibody and rhodamine-conjugated secondary antibody. Nuclei were stained by DAPI. (B) As in Fig. 4A, except that pCDNA3-FLAG-CBP80 was transfected into HeLa cells and FLAG-CBP80 was visualized by anti-FLAG antibody and rhodamine-conjugated secondary antibody. (C) As in Fig. 4A, except that pCDNA3-FLAG-Dcp1a was transfected into HeLa cells and FLAG-Dcp1a was visualized by anti-FLAG antibody and rhodamine-conjugated secondary antibody.

generated during stressful conditions, ensuring cell survival during or after stress.

Several issues regarding the relationship between NMD and stress still remain. Does the pioneer round of translation always occur regardless of the type of stress? Alternatively, is the efficiency of the pioneer round of translation and NMD regulated under certain conditions? Why is the pioneer round of translation resistant to stress, unlike steady-state translation? What factor recruits the ribosome under stress conditions? Clearly, we are only just beginning

to understand mRNA surveillance mechanism of NMD under stressful conditions.

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