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Hydrogen peroxide induces stress granule formation independent of eIF2α phosphorylation

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ABSTRACT

In cells exposed to environmental stress, inhibition of translation initiation conserves energy for the repair of cellular damage. Untranslated mRNAs that accumulate in these cells move to discrete cytoplasmic foci known as stress granules (SGs). The assembly of SGs helps cells to survive under adverse environmental conditions. We have analyzed the mechanism by which hydrogen peroxide (H_2O_2)-induced oxidative stress inhibits translation initiation and induces SG assembly in mammalian cells. Our data indicate that H_2O_2 inhibits translation and induces the assembly of SGs. The assembly of H_2O_2-induced SGs is independent of the phosphorylation of eIF2α, a major trigger of SG assembly, but requires remodeling of the cap-binding eIF4F complex. Moreover, H_2O_2-induced SGs are compositionally distinct from canonical SGs, and targeted knockdown of eIF4E, a protein required for canonical translation initiation, inhibits H_2O_2-induced SG assembly. Our data reveal new aspects of translational regulation induced by oxidative insults.

1. Introduction

Translational repression triggered by stress occurs as a consequence of reduced translation initiation. This results from reduced assembly of the pre-initiation complexes eIF4F (composed of eIF4E, eIF4G, and eIF4A) and 43S (composed of the small ribosomal subunit in association with several initiation factors) [1]. Assembly of the eIF4F complex is inhibited by eIF4E-binding proteins (4EBPs) that interfere with interactions between eIF4E and eIF4G [2]. Stress-induced inactivation of the PI3 K-mTOR pathway reduces the constitutive phosphorylation of 4EBPs to promote the assembly of inhibitory eIF4E:4EBP complexes [2]. Assembly of the 43S complex is inhibited by stress-induced activation of PKR, PERK, GCN2 and HRI, kinases that phosphorylate eIF2α, a component of the eIF2-GTP-$tRNA^{Met}$ ternary complex essential for 43S assembly [1]. These complementary mechanisms are primarily responsible for the global repression of protein synthesis observed in cells subject to adverse environmental conditions.

Non-translatable mRNAs that accumulate as a result of stress-induced translational repression are frequently compartmentalized into cytoplasmic foci known as stress granules (SGs) [3,4]. SGs are ribonucleoprotein (RNP) complexes composed of abortive translation initiation complexes and a host of RNA binding proteins and signaling proteins involved in various aspects of cellular metabolism. Current evidence suggests that SGs, in concert with a related class of RNA granule known as the processing (P-) body, play important roles in determining the fate of mRNAs in stressed cells [5]. SGs have also been implicated in stress-induced signaling cascades such as inflammatory signaling and stress-induced apoptotic signaling [6,7]. Thus, SGs are thought to promote cell survival under stress conditions by modulating various aspects of cell metabolism.

Reactive oxygen species (ROS) are an important trigger for SG assembly. However, whether hydrogen peroxide (H_2O_2), the most common and stable form of ROS, induces SG assembly has remained controversial. Whereas some studies report that H_2O_2 induces SG assembly [8–10], others do not [6,7,11]. When H_2O_2-induced SGs were observed, they were rapidly disassembled, suggesting that their composition may be different than canonical SGs induced by other stimuli. In the present study, we found that both the mechanism of H_2O_2-induced SG assembly and the composition of H_2O_2-induced SGs are different than that of canonical SGs. We show that H_2O_2 triggers phospho-eIF2α-independent SG assembly by disrupting the eIF4F complex. Unlike phospho-eIF2α-triggered SGs, H_2O_2-induced SGs often lack eIF3b, a key component of the translation initiation machinery. Our results reveal that H_2O_2

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triggers a novel class of SGs that may have unique properties in the regulation of the stress response program.

2. Materials and methods

2.1. Cell culture and drug treatment

U2OS cells, wild type mouse embryonic fibroblasts (MEFs) as well as MEFs expressing mutant eIF2α (S51A) were cultured as described previously [12]. Treatment of cells with sodium arsenite (Sigma), pateamine A (Desmethyldesamino-modified; a gift from Jun Liu, Johns Hopkins University) or emetine (Sigma) was as described in [13]. For H_2O_2 (Sigma) treatment, cells were incubated with the indicated H_2O_2 concentrations in normal growth medium at 37 °C in a CO_2 incubator for 1 or 2 h. N-acetyl Cysteine (NAC) treatment was done as previously described [14].

2.2. Antibodies

Mouse monoclonal antibodies to G3BP, HuR, p70 S6 kinase (SK1-hedls), eIF4E, 4E-BP1, 4E-T and PABP, rabbit polyclonal antibody to eIF4G, goat polyclonal antibodies to eIF3b, TIAR, TIA-1, or FXR1 were purchased from Santa Cruz Biotechnology. Rabbit polyclonal phospho-specific anti-eIF2α was from Assay Designs, rabbit polyclonal anti-RCK was from Bethyl, mouse monoclonal anti-HA was from Covance and mouse monoclonal antibody to β-actin was from Chemicon. Secondary antibodies conjugated with horseradish peroxidase (HRP) were from GE Healthcare. Cy2-, Cy3-, and Cy5-conjugated secondary antibodies were purchased from Jackson Immunoresearch Labs.

2.3. DNA plasmids, siRNAs, and cell transfection

Plasmids pMT2-HA, which expresses either wild type eIF2α or a non-phosphorylatable eIF2α S51A mutant were described previously [15]. Control siRNA (D0) was designed as described previously [16]. siGENOME SMART pool siRNA for HRI [17] and eIF4E were purchased from Thermo Scientific. 4E-BP1 siRNA (5′TGGGAACTCACCTGTGACCAA3′) was purchased from Qiagen. Transfections with DNA plasmids or siRNAs were done as previously described [12].

2.4. Immunoblot analysis

Immunoblotting was done as described previously [12]. Briefly, cells were solubilized in lysis buffer (5 mM MES (Sigma) pH 6.2 and

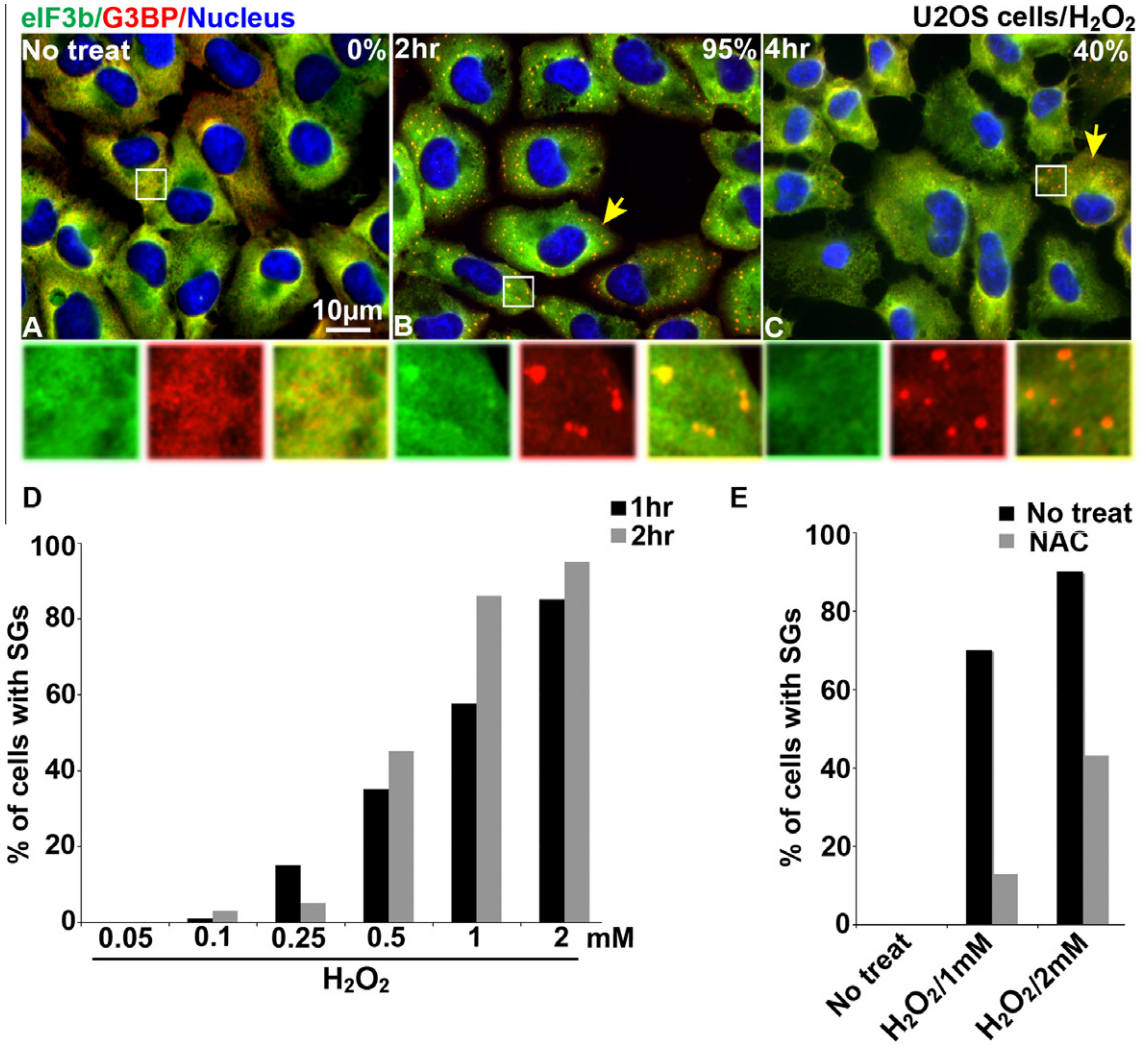

Fig. 1. H_2O_2 induces SGs in U2OS cells. Immunofluorescence microscopy of U2OS cells that were left untreated (A) or treated with 1 mM H_2O_2 for 2 h (B) or 4 h (C) and then stained with SG markers [eIF3b (green) and G3BP (red)]. Nuclei are stained with Hoechst. Insets show enlarged views of individual and merged channels. Yellow arrows point out cells containing SGs. (D) Percentage of U2OS cells with G3BP-positive SGs after 2 h treatment with 0.05 mM, 0.1 mM, 0.25 mM, 0.5 mM, 1 mM, and 2 mM H_2O_2. (E) The effect of NAC on H_2O_2-induced SG assembly. U2OS cells were treated with 50 mM of NAC for 1 h then treated with 1 mM or 2 mM H_2O_2 for 2hr before processing for immunofluorescence microscopy.

2% SDS), and equal amounts of protein were separated by 4–20% SDS–PAGE before transfer to nitrocellulose filter membranes. After 1h of blocking in 5% normal horse serum (NHS) in TBS, membranes were incubated with primary antibodies overnight at 4 °C. Subsequently, membranes were incubated with appropriate secondary antibodies and proteins were detected using the Super Signal chemiluminescent detection system (Pierce).

2.5. 7-Methyl GTP sepharose chromatography

Assembly of eIF4E-containing complexes from untreated U2OS cell lysates or lysates treated with H_2O_2 (1 mM, 2 h) or SA (100 μM, 2 h) was performed as described [18].

2.6. Immunofluorescence microscopy

Immunofluorescence was done as previously described [11,13]. Briefly, cells were fixed in 4% para-formaldehyde, permeabilized using 100% chilled methanol, and incubated with blocking buffer (5% NHS in PBS). Cells were then incubated with primary antibodies, followed by staining with appropriate secondary antibodies and Hoechst 33258 dye (Molecular probes) to reveal the nuclei. Cover slips were mounted in polyvinyl mounting medium and the cells were viewed and photographed with an Eclipse E800 (Nikon) equipped with a digital camera (CCD-SPOT RT; Diagnostic Instrument) using 60X oil immersion objective. The images were merged and analyzed using Adobe Photoshop (v. 10).

2.7. Quantification of stress granules

Cover slips were coded for each independent experiment and all quantifications were done blindly in ~10 separate fields. The percentage of cells with SGs was quantified by counting 200 cells/experiment.

3. Results

To conclusively determine whether H_2O_2 induces SG assembly, U2OS cells were first treated with 1 mM H_2O_2 for 0, 2, and 4 h before staining for the SG markers eIF3b and G3BP. As shown in Fig. 1A–C, discrete SGs containing G3BP, but not eIF3b, were induced in cells treated with H_2O_2 (Fig. 1B and C, yellow arrows).

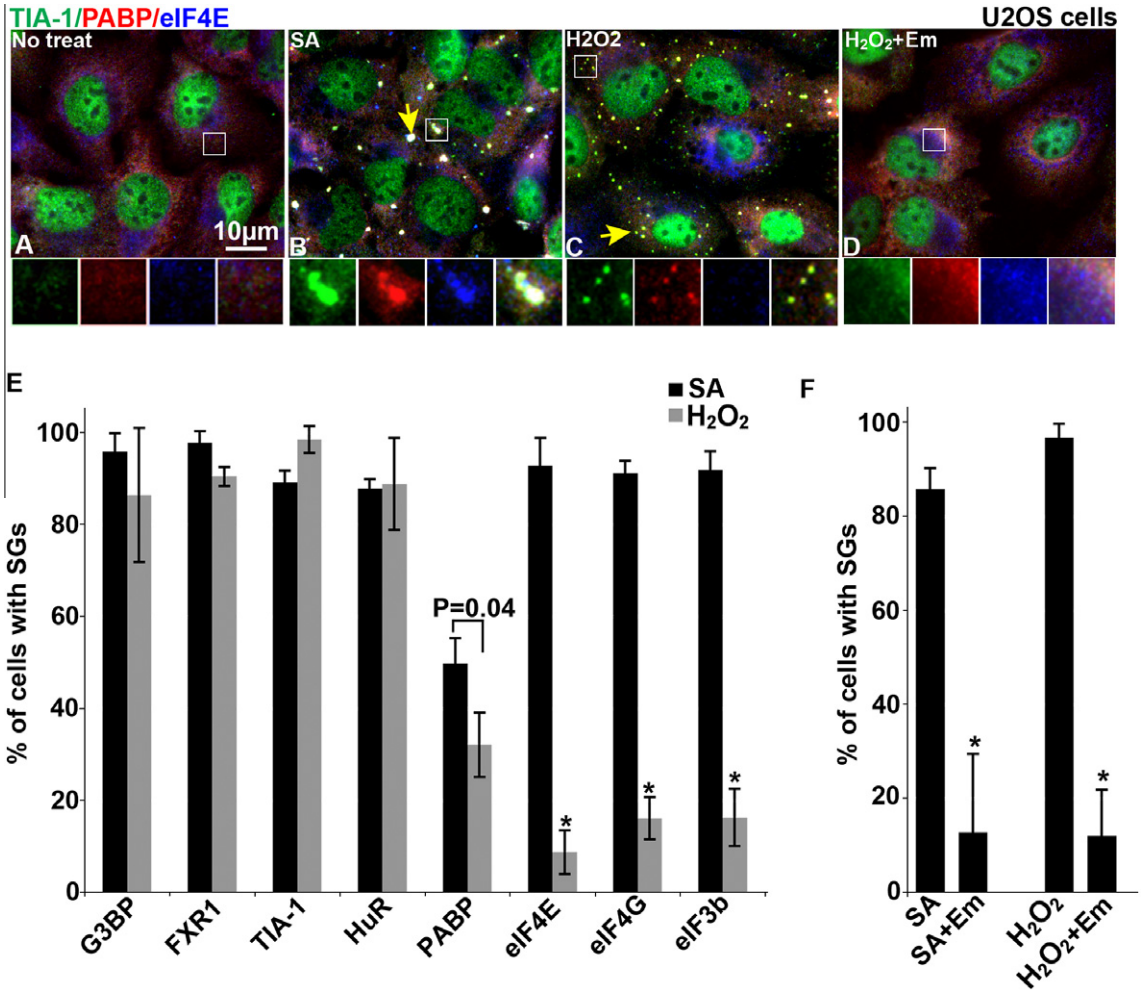

Fig. 2. H_2O_2 induces the assembly of non-canonical SGs. Immunofluorescence microscopy showing non-treated U2OS cells (A; No treat), cells treated with 250 μM sodium arsenite (B; SA) or 2 mM H_2O_2 (C; H_2O_2), or cells treated with H_2O_2 followed by emetine treatment (D; H_2O_2 + Em). Yellow arrows indicate SGs stained with SG markers [TIA-1 (green), PABP (red) and eIF4E (blue)]. Insets show enlarged views of individual and merged channels. (E) Percentage of cells with SA- or H_2O_2-induced SGs that contain the indicated protein markers. The average percentage of cells with SGs is shown ($n = 3$). Error bars indicate the standard deviation. $* = p$ Values, that were calculated by comparing the percentage of cells with SGs in SA- and H_2O_2-treated cells (PABP, $p = 0.04$; eIF4E, $p = 0.0006$; eIF4G, $p = 0.002$; and eIF3b, $p = 0.004$). No other protein markers (G3BP, FXR1, TIA-1, and HuR) showed statistically significant differences. (F) Percentage of cells with SA- and H_2O_2-induced SGs before and after treatment with emetine. Error bars indicate the standard deviations of the mean ($n = 3$). $^*p$ Value < 0.005 when comparing the percentages of SGs in SA- or H_2O_2-treated cells with those treated with emetine.

The induction of SGs was transient, as the percentage of cells positive for these G3BP-enriched SGs decreased after 2 h of treatment. H_2O_2 induced SGs in a dose-dependent manner with a minimal effective concentration of 0.1 mM (Fig. 1D).

Since H_2O_2 is a known inducer of ROS, we tested the effect of N-acetyl-L-cysteine (NAC), a highly potent ROS scavenger [19], on the ability of H_2O_2 to induce SG assembly. U2OS cells were treated with H_2O_2 (1 mM or 2 mM for 2 h) after pretreatment with NAC for 1 h. As shown in Fig. 1E, NAC significantly inhibited SG assembly by H_2O_2, suggesting that ROS induction by H_2O_2 treatment is responsible for SG assembly.

The surprising lack of eIF3b in H_2O_2-induced SGs led us to survey the composition of H_2O_2-induced SGs. H_2O_2-induced SGs were probed using the established SG markers TIA-1, PABP, eIF4E (Fig. 2C), HuR, FXR1, RCK (Fig. S1C), G3BP, eIF4G, and eIF3b (Fig. S1G). Interestingly, with all markers tested, we found that H_2O_2-induced SGs (yellow arrows) (Fig. 2C, Fig. S1C, and G) were smaller and more numerous than SA-induced SGs (Fig. 2B, Fig. S1B, and F). Although some proteins were recruited to SGs induced by both H_2O_2 and SA, others were only weakly recruited to H_2O_2-induced SGs as opposed to SA (Fig. 2E). The recruitment of G3BP, FXR1, TIA-1, and HuR to H_2O_2-induced SGs was as efficient as SA-induced SGs, whereas translation initiation factors, eIF4E, eIF4G, eIF3B and PABP showed significantly weaker recruitment to H_2O_2-induced SGs compared to those induced by SA (Fig. 2E). These results indicate that SGs induced by H_2O_2 have different characteristics than those induced by SA.

To confirm that H_2O_2-induced SGs are *bona fide* SGs, we tested the effect of emetine, a drug that inhibits SG assembly by preventing polysome disassembly [20]. Treatment with emetine effectively inhibits the assembly of H_2O_2-induced SGs, similar to arsenite-induced SGs (Fig. 2F, Fig. S1D, and H). Collectively, these results indicate that H_2O_2 induces *bona fide* SGs whose size, number, and protein composition are different than SA-induced SGs.

Several reports have shown that H_2O_2 and SA are both oxidative stressors that induce the phosphorylation of eukaryotic translation initiation factor 2α (eIF2α) [21–24]. We confirmed that H_2O_2 induces the phosphorylation of eIF2α in U2OS cells at levels comparable to that induced by SA (Fig. 3A). Since eIF2α phosphorylation was shown to be essential for SG assembly in response to several stresses [25], we determined whether eIF2α phosphorylation is required for H_2O_2-induced SG assembly. To this end, we used mouse embryonic fibroblasts (MEFs) in which the endogenous eIF2α is replaced with a non-phosphorylatable mutant (eIF2α S51A) [26]. Whereas SA treatment does not trigger SG assembly in these knock-in (S51A) MEFs (Fig. 3G), pateamine A, which induces SGs in a phospho-eIF2α independent manner, does (Fig. 3H). As shown in Fig. 3E and I, H_2O_2 induces SGs in both wild-type (WT) and S51A MEFs, demonstrating that eIF2α phosphorylation is dispensable for H_2O_2-induced SG assembly. We also found that overexpression of a non-phosphorylatable mutant of eIF2α, but not wild-type eIF2α, does not affect H_2O_2-induced SG assembly (Fig. S2), thus confirming that H_2O_2-induced SG assembly occurs in a phospho-eIF2α - independent manner. We also found that siRNA-mediated depletion of heme-regulated inhibitor (HRI), an eIF2α kinase that is responsible for SA-induced eIF2α phosphorylation [16], affects neither SG assembly nor eIF2α phosphorylation induced by H_2O_2 (Fig. S3). These results further support the above observations, and indicate that although both SA and H_2O_2 induce oxidative stress, they activate different eIF2α kinases.

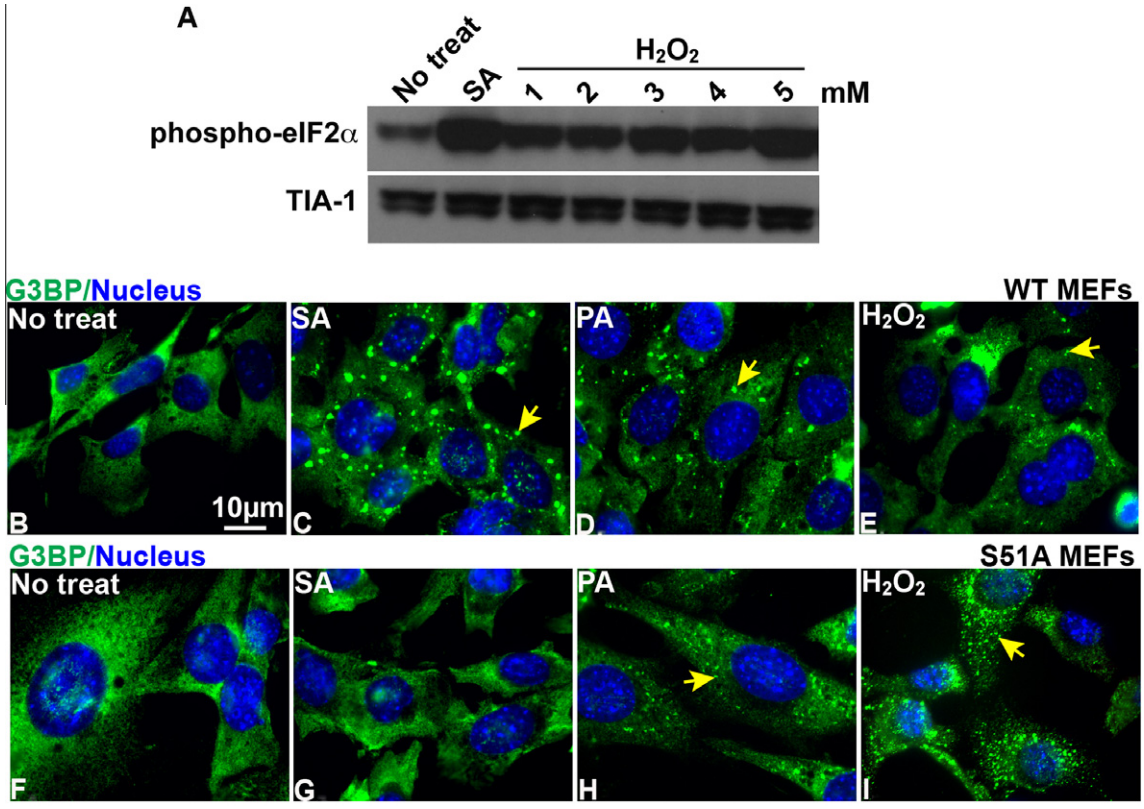

Fig. 3. H_2O_2 induces SGs independent of eIF2α phosphorylation. (A) Western blot analysis of phospho-eIF2α (upper panel) in U2OS cells treated with different concentrations of H_2O_2 (1, 2, 3, 4, and 5 mM) as well as 250 μM sodium arsenite (SA). TIA-1 (lower panels) was used as a loading control. (B-I) Immunofluorescence microscopy showing SG assembly induced by H_2O_2 in wild type (WT MEFs) and S51A eIF2α mutant MEFs (S51A MEFs). Yellow arrows indicate representative G3BP + SGs (green) in untreated U2OS cells (B and F), or cells treated with 200 μM sodium arsenite (SA) (C and G), 50 nM pateamine A (PA) (D and H), and 2 mM H_2O_2 (E and I). Nuclei are stained with Hoechst.

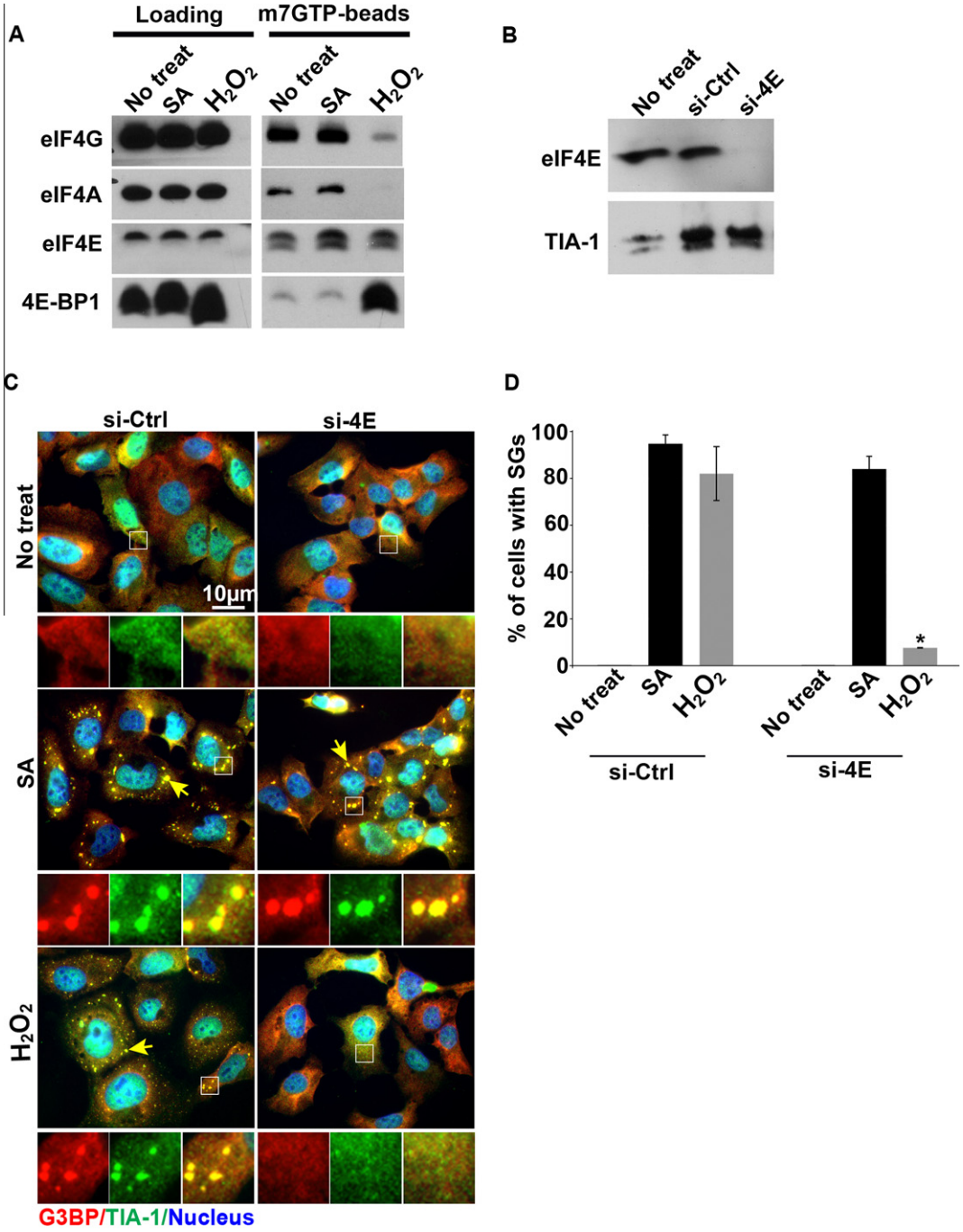

Fig. 4. H_2O_2 induces SGs by targeting eIF4F. (A) H_2O_2 causes eIF4F complex disruption and enhances eIF4E:4E-BP1 interactions. U2OS cells without (No treat) or with drug treatment (sodium aresenite (SA) and H_2O_2) were assembled on m^7GTP-Sepharose as described in [18]. Loading control (Input) and the m^7GTP-bound proteins (m^7GTP-beads) were analyzed by Western blotting using antibodies against eIF4G, eIF4A, eIF4E and 4E-BP1. (B) Western blot analysis showing the knockdown efficiency of eIF4E in U2OS cells transfected with control siRNA (si-Ctrl) or eIF4E-specific siRNA (si-4E). TIA-1 was used as a loading control. (C) Immunofluorescence microscopy showing SG assembly in U2OS cells after eIF4E depletion. Cell treated with the indicated siRNAs were left untreated (No treat, upper panels) or treated with 250 μM SA (middle panels), or 2 mM H_2O_2 (lower panels) and then stained with SG markers G3BP (red) and TIA-1 (green). Nuclei are stained with Hoechst. Insets show enlarged views of individual and merged channels. (D) Percentage of cells with SGs after eIF4E depletion. Error bars indicate the standard deviations of the mean (n = 3, *p = 0.00036).

Inhibition of translation initiation correlates well with SG assembly in cells subjected to different types of stresses [3,4]. Since H_2O_2 inhibits global protein synthesis [22–24,27] in different types of cells primarily at the level of translation initiation [23,24,28] it is likely that H_2O_2 targets the translation initiation machinery and thus induces SG formation. To test this hypothesis, we pulled down the eIF4F complex from lysates of SA or H_2O_2- treated U2OS cells using m^7GTP-Sepharose (Fig. 4A). Although SA had no effect on the assembly of the eIF4F complex, remarkably, H_2O_2 significantly disrupted the eIF4F complex. This analysis revealed that H_2O_2 displaces eIF4G and eIF4A from eIF4E (Fig. 4A), and at the same time, H_2O_2 promotes interactions between 4E-BP1 and eIF4E.

It has been well documented that hypophosphorylated 4E-BP1 isoforms binds to eIF4E to displace eIF4G and inhibit translation initiation [29,30]. Since H_2O_2 causes hypophosphorylation of 4E-BP1 in several cell lines [23,24,28], it was of our interest to determine

whether 4E-BP1 is also hypophosphorylated under our treatment conditions. Western blot analysis done on U2OS cell lysates revealed that H_2O_2 treatment significantly increases levels of hypophosphorylated 4E-BP1 (Fig. S4A) by blocking 4E-BP1 phosphorylation compared to that observed in untreated cells (Fig. S4A, lane 1, asterisk). Furthermore, we found that H_2O_2 (but not SA) re-localizes eIF4E, and its transporter protein 4E-T, from the cytoplasm to the nucleus (Fig. S4B and C). Taken together, these results suggest that H_2O_2 induces hypophosphorylation of 4E-BP1 to increase its association with eIF4E and inhibit translation initiation. This, in turn, results in the assembly of a unique class of SGs.

To determine whether eIF4E:4E-BP1 interaction is essential for H_2O_2-induced SG assembly, U2OS cells were transfected with control or eIF4E-directed siRNAs and then left untreated or treated with H_2O_2 or SA. Western blot analysis verified that siRNAs targeting eIF4E downregulated eIF4E protein levels (Fig. 4B). We found that depletion of eIF4E resulted only in a minor decrease in SG assembly induced by SA (Fig. 4C). In contrast, eIF4E depletion strongly inhibited H_2O_2-induced SG assembly (~80%) (Fig. 4C and D). Knockdown of 4E-BP1 also affected H_2O_2-induced SG assembly, resulting in smaller, less discrete SGs (Fig. S5). These data suggest that eIF4E:4E-BP1 complexes play an essential role in the H_2O_2-induced assembly of SGs.

4. Discussion

SG assembly results from dynamic re-modeling of global translation in cells subjected to a variety of environmental stresses [3,4]. Although SG assembly is a conserved phenomenon in a wide range of eukaryotes, the assembly and composition of SGs clearly demonstrate species-specific and stress-specific differences. Mammalian SGs assembled in response to stress-induced phosphorylation of eIF2α are composed of 40S ribosomal subunits in association with the cap-binding complex (eIF4E, eIF4A and eIF4G), PABP and subunits of eIF3 [3,4]. In *Saccharomyces cerevisiae*, different stresses assemble SGs with distinct compositions. Exposure to the metabolic poison sodium azide induces the assembly of SGs whose composition resembles that of mammalian SGs [31]. In contrast, glucose deprivation induces the assembly of granules that contain eIF4E, eIF4G and PABP (so-called "EGP-bodies) but lack 40S ribosomal subunits and eIF3 subunits [32]. Our results reveal that in mammalian cells, SG composition also differs in a stress-dependent manner.

Although arsenite-induced oxidative stress is the best-characterized inducer of SGs in mammalian cells, the ability of H_2O_2 to trigger SG assembly has been controversial. Whereas some studies show that H_2O_2 induces SG assembly [8–10], other studies report that H_2O_2 does not trigger SG assembly [6,7,11]. Here, we demonstrate that in U2OS cells H_2O_2 clearly induces SGs that are different from canonical SGs. First, H_2O_2-induced SGs are formed transiently and they are morphologically smaller and more abundant than SA-induced granules. Second, H_2O_2-induced SGs have significantly reduced amounts of eIF3, eIF4E and eIF4G indicating that these granules are different from both yeast EGP-bodies and canonical mammalian SGs. Third, although H_2O_2 induces eIF2α phosphorylation similarly to SA, the phospho-eIF2α itself is not required for H_2O_2-induced SGs in contrast to SA-induced SGs. In this regard, H_2O_2 resembles the xenobiotic compounds pateamine A (Pat A) and hippuristanol, the anti-inflammatory lipid mediator 15d-PGJ2 and stress-induced tRNA-derived RNAs (tiRNAs) that all trigger eIF2α-independent SG assembly [12,33–35]. Whereas PatA, hippuristanol, and 15d-PGJ directly bind and inhibit eIF4A [33,34,36] and tiRNAs displace eIF4A:eIF4G from cap-bound eIF4E [18], H_2O_2 indirectly promotes binding of 4E-BP1 to eIF4E by promoting 4E-BP1 hypo-phosphorylation. This raises the possibility that mTOR which regulates 4E-BP1 phosphorylation might be involved in H_2O_2-induced SG assembly. Moreover, in contrast to SA-induced SG assembly, eIF4E itself is required for H_2O_2-induced SG assembly suggesting that the eIF4E/4E-BP1 complex may directly promote the aggregation of untranslated mRNAs.

In summary, our data suggest that mammalian cells can assemble different types of SGs utilizing different mechanisms. These different routes of assembly are stress-specific and dictate recruitment of selective SG constituents. What are the possible functions of different classes of SGs? In analogy to amino acid starvation-induced SGs that selectively sequester mRNAs bearing 5′-terminal oligopyrimidine tracts [37,38], we propose that different classes of SGs selectively recruit specific mRNAs from translating ribosomes. In turn, this selective mRNA re-localization causes stress-specific changes in protein translation allowing adaptation to stress conditions.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.06.033.

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