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Functional Study of Dihydrocapsaicin on the Assembly of Stress Granule

Graduate School of Chosun University

Department of Biomedical Sciences

Koushitak De

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스트레스 과립의 조립에 대한 디히드로캡사이신의 기능적 연구

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Advisor: Prof. Takbum Ohn

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This is to certify that the Ph.D. dissertation of

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쿠시탁 데

교수: 온탁범 Ph.D.

의생명과학과

조선대학교 대학원

스트레스 과립(SG)은 스트레스에 의해 번역 개시가 교란될 때 형성되는 세포질 생체분자의 응축 물질이다. SG는 단백질-단백질, 단백질-RNA 및 RNA-RNA 상호작용뿐만 아니라 본질적으로 무질서한 도메인 및 RNA 결합 단백질 (RBP) 내의 번역 후 수정을 통해 형성된다. SG는 세포 사멸의 활성화를 지연 시킴으로써, 스트레스로 인한 세포손상을 최소화함으로써 세포 생존에 기여하는 것으로 알려져 있다. 본 연구에서 캡사이신의 유사체인 dihydrocapsaicin(DHC)이 eIF2α의 인산화를 통해 폴리솜 분해와 글로벌 단백질 번역을 억제하여 SG 형성을 유도하는 것을 발견하였다. DHC 매개 SG 형성은 serine 51 위치에서 eIF2α의 인산화에 의해 제어되며, 이는 HRI가 최대 효과를 보이는 4가지 eIF2α stress kinase (HRI, PKR,

PERK 및 GCN2) 에 의해 제어되는 것을 발견하였다. 우리는 DHC가 SG 조립을 유도하고, 폴리솜을 분해하고, eIF2α를 HRI 의존적인 방식으로 인산화하며 전체적 번역을 억제하는 보나파이드 화합물임을 증명하였다. 결론적으로, 본 연구의 결과는 DHC가 새로운 SG 유도체이며 SG 역학을 연구하기 위한 새로운 스트레스임을 시사한다.

I. INTRODUCTION

RNA granules or Stress granules (SGs) are classified as densely aggregated, membraneless organelles, which comprised of protein-mRNA aggregations dynamically recruited in response to various stress conditions such as selenite, arsenite, glucose deprivation, heat shock or viral infection as a response to the translational inhibition and select the cell's survival by contributing important cellular mechanisms [1-3]. The major constituents of SGs are translation initiation factors such as eIF4F complex (constitutes EIF4E, EIF4A and EIF4G) and eIF3, small 40S ribosome and poly(A) binding protein (PABP). Additionally, several RNA binding proteins such as G3BP1, TIAR, TIA-1, SRSF3, RACK1, TRAF2, TORC1 and untranslated mRNAs [4-12]. There are some RNA binding proteins like HuR and FMRP also found to have localized in SGs upon stress [13,14]. A recent study shows that neuregulin-2 (a member of epidermal growth like factor), TDP-43, hnRNPs are stress granule components and believe to modulate cellular functions [15-18]. Interestingly multiple cellular events and post translational modifications of some proteins found to contribute in the promotion of SG dynamics.

Post–translational modification (PTM) including ubiquitination can modulate SG assembly. Methylation of FMRP, O-GlcNAcylation of ribosomal proteins or acetylation of HDAC6 found to modulate SG assembly or disassembly [15,19,20]. Blocking of the ubiquitin-proteasome system can promote SG formation in cells [21]. Neural precursor cell expressed, developmentally down-regulated protein 8 (NEDD8), which is also a small ubiquitin-like (UBL) protein has a role in the formation of SG assembly [22]. Dual specificity tyrosine-phosphorylation regulated kinase 3 (DYRK3) is responsible for the regulation of SG assembly and essential for mTORC1 regulation [23]. 5′-AMP-activated protein kinase (AMPK) alpha,

which is an enzyme found to contribute a significant role in the cellular energy homeostasis, found to regulates SG biogenesis [24]. RNA granules are known to suppress apoptosis by inhibiting the stress-activated mitogen-activated protein kinase (MAPK) pathway [25]. On the other hand, RhoA/ROCK1 localizes in SGs and activates JNK apoptotic pathway, which promotes cell survival [26,27]. Whereas JNK inhibition results in the reduction of SGs and PBs [28]. Processing bodies (PBs) are another type of RNA granule which are primarily composed of translationally repressed mRNAs and proteins related to mRNA decay. PBs are different in composition or function compare to SGs, known to be found in untreated or unstressed cells unlike SG or RNA granule. PBs can expand in quantity upon stress environment by contributing a major role in the mRNA decay [29,30].

The concept of SGs formation and its structural components have been thoroughly elicited in recent decades [31-33]. Upon experiencing to different environmental stresses, cells quickly generate a response by stopping protein translational initiation by the two important pathways. The first checkpoint or pathway includes translational initiation of eIF4F complex, which recognizes the cap (m^7GTP) structure at the 5' end of mRNA monitored by the PI3K-mTOR. During the stress environment, inactivation of mTOR trigger the activation of 4E-BP1 by restraining EIF4F complex following by suppressing the translational initiation [34,35]. The second mechanism involves eIF2/GTP/$tRNA_i^{Met}$ ternary complex formation, which carries initiator $tRNA_i^{Met}$ to the 40S ribosome.

Under various stress environments, predominantly eIF2α stress kinases become stimulated, resulting in the eIF2α phosphorylation of at serine 51 position and inhibition of protein synthesis [36,37]. There are four different types of distinct stress kinases are present: **(1)** EIF2AK1 or Heme regulated initiation factor 2α kinase (HRI) is a well-known kinase,

which introduce phosphorylation of eIF2α in association with oxidative stress (e.g., selenite and arsenite), culminating in depleted formation of ternary complex and impairing translation of mRNA. Although, HRI is well known for its role as a heme sensor in erythroid progenitors, pharmacologic activation of HRI has been demonstrated to have anti-cancer activity across a wide range of tumor sub-types. HRI is expressed most abundantly in erythroid cells, where it is activated by deficiency in heme, the prosthetic group of hemoglobin, through two heme-binding domains. In this manner, HRI coordinates globin mRNA translation with available iron and is required to prevent accumulation of misfolded globin chains in the absence of heme [38]. Due to HRI deprivation, significant amount of globin synthesis occurred during heme shortage by precipitating and creating inclusions ensuing proteotoxicity [39,40]. Moreover, HRI is known to be stimulated by oxidative stress and denatured proteins. Previous investigation has unveiled that eIF2α gets phosphorylated through the activation of EIF2AK1 kinase [41].

(2) EIF2AK2 or Protein Kinase R (PKR) is triggered by the cellular response to several stress signals (e.g., cold shock, viral infection, cytokines, irradiation, lack of nutrients, or mechanical stress). The PKR pathway gets activated by the stimulation of cellular pathways like PP2A, p38, NFkB, JNK and encourage eIF2α to get phosphorylated [42]. Phosphorylation of eIF2α promotes translational repression and stress granule formation. PKR is known to participate through dealing with various inflammatory kinases like eIF2α, JNK, IKK, insulin receptor etc. Such cytosolic complexes are known as metaflammasome or metabolic inflammasome [43]. Additionally, PKR is well known to be involved in the mitochondrial unfolded protein response (UPRmt) via transcription factor AP-1. Thus, PKR has proved to be pertinent to intestinal inflammation [44].

(3) EIF2AK3 or PKR like ER kinase (PERK) is well known for monitoring

unfolded protein and gets activated during endoplasmic reticulum (ER) stress causing to the inactivation of eIF2α, and thus initiate rapid reduction of translational initiation and repression of global protein synthesis. ER stress caused by excess of unfolded proteins leads to inflammatory responses [45]. PERK is claimed to be responsible for repressing global protein translation via phosphorylation of eIF2α can initiate the up-regulation of CHOP/GADD153 (CCAAT/enhancer-binding protein-homologous protein/growth arrest and DNA damage inducible protein) and ATF3 expression [46-49]. Therefore, activation of ER-resident protein kinases, cells respond to ER stress via intracellular signaling pathways to regulate gene expression.

(4) EIF2AK4 or General control non-depressible 2 (GCN2) checks the amino acids levels and activated during the scarcity of amino acid in cells [50-53]. GCN2 known to contribute in the regulation of protein synthesis during various stresses resulting in a nutritional fluctuation. GCN2 firmly inhibit the protein synthesis through eIF2α phosphorylation. Stimulation of any four eIF2α kinases promote inhibition of protein translation through reducing of eIF2-GTP-tRNA-met ternary complex, contributing a major role in the aggregation of SG assembly and polysome disassembly. Stress encourages the translational repression, which permit cells to initiate their whole genome expression and increases the probability of survival.

Capsaicinoids are a pungent chemical which produces a burning sensation in tissues upon contact. Chili peppers are known to be the primary origin of natural capsaicinoids, which includes capsaicin, homocapsaicin, homodihydrocapsaicin, dihydrocapsaicin and nordihydrocapsaicin. The major components such as capsaicin and dihydrocapsaicin constitute 80 to 90 percent of overall capsaicinoids content in the pepper extract [54]. They found to

possess various physiological and pharmacological roles involving biological functions such as anticancer, analgesia, antioxidant, anti-obesity and anti-inflammation. Consequently, capsaicinoids may have the significant role in pain relief, cancer prevention and weight loss [55,56]. DHC (N-[(4-hydroxy-3-methoxyphenyl)methyl]-8-methyl-nonanamide), is an alkaloid and a major constituent of capsaicinoids found in red peppers [57]. Both capsaicin and DHC could arrest in vitro platelet aggregation and the activity of clotting factors VIII and IX, a property which may involves to the prevention of the onset and treatment of CVD and triggers apoptosis in cell and follow Ca^{2+} mediated mitochondrial pathway [58,59]. DHC has multiple physiological or pharmacological effects involving anti-cancer and induces fluctuation of p53 protein translation [60]. DHC is recognized as a downregulator of apoM translation upon suppressing Foxa2 and aiding the LXRα expression in HepG2 cells and can elevate the resistance of LDL to oxidation by slowing the initiation or delaying the rate of oxidation [61,62] . It was also found that DHC has a significant role in the prevention of cardiovascular diseases. Previously it was reported that DHC is a well-known ER stressor, a modulator of PERK kinase [63]. Indeed, ER stress is responsible for promoting SG assembly (e.g., Thapsigargin stress), and given that the DHC stress can results ER stress, we investigated whether DHC stress can trigger SG assembly similar to sodium arsenite (an important SG promoter). We found that DHC treatment promote SG assembly following in a dose-dependent manner. Additionally, we explored that DHC stress can disassemble polysomes at a slow rate but has a great blocking effect on the general translational mechanism– one of the vital events participating in the SG aggregation. Finally, we established that EIF2AK1 or HRI kinase takes part in a major role in DHC induced SG assembly. Overall, our study demonstrates that DHC is an important candidate for the development of SG assembly and a substitute to sodium arsenite to study SG dynamics.

II. Materials and Methods

Cell culture and reagents

U2OS (Human Osteosarcoma), HeLa and Mouse embryonic fibroblasts (MEFs) with /without eIF2α- S51A were maintained in DMEM medium (Welgene) supplemented with 10% inactivated FBS (Welgene). Wild type, ΔHRI, ΔPKR, ΔPERK and ΔGCN2 (HAP-1 cells) were procured from Horizon Discovery and grown in IMDM medium (Welgene). All cell culture medium was supplemented with 10% heat inactivated fetal bovine serum (Welgene), 1% (v/v) penicillin and streptomycin (Lonza) and cells were incubated at 37ºC in 5% CO_2. Dihydrocapsaicin was purchased from Cayman Chemical. Sodium arsenite, Cycloheximide, Hoechst, Emetine dihydrochloride hydrate and Puromycin dihydrochloride were procured from Sigma. The following antibodies have been used in this experiment. Anti-eIF3b (N-20), Anti-TIA-1 (C-20), Anti-G3BP1 (H-10), Anti-EIF4G (H-300), p-eIF2α and eIF2α (FL-315) were obtained from Santa Cruz. β-actin (AC-15) was procured from Abcam. Anti-Puromycin 12D10 was obtained from EMD Millipore. PB marker RCK (anti-DDX6) was purchased from Bethyl. All secondary antibodies have been used in immunofluorescence microscopy or western blot including Cy2/Cy3 or Horseradish peroxide (HRP) conjugated, were obtained from Jackson Immunoresearch Laboratory.

Western blot analysis

Cells were cultured and grown up to ~80% confluency in 6-well plates. After treatment with drug, cells were subjected to cold PBS washing and followed by lysis with RIPA lysis buffer (50 mM Tris-Cl (pH 8.0), 1% NP-40, 150 mM NaCl, 1% Sodium deoxycholate, 0.1%

SDS and 1 mM EDTA containing proteinase inhibitors 5 mM NaF and 1 mM PMSF) up to 15 min in ice and followed by centrifugation at 13,000 rpm for 15 min. Total proteins were quantified using Bradford reagent (Biorad). Equal amounts of protein (20-60μg) were subjected to 8-12% SDS-PAGE, transferred to nitrocellulose membranes. Membranes were blocked with 5% normal horse serum (NHS) dissolved in PBS containing 0.1% sodium azide for 1 h. Then incubated with the indicated primary antibodies overnight in 4°C. Membranes were then briefly washed with TBST (0.1% Tween 20) and incubated with species specific horseradish peroxidase-labeled secondary antibody for 1 h. Immunoblots were developed by using Pico-EPD western blot detection kit (ELPIS-biotech), and densitometry was performed by ImageJ software.

Immunofluorescence microscopy

Cells were grown on coverslips, which were previously incubated with complete growth media for at least 8 h in 5% CO_2 incubator, nearly ~70% confluency, were untreated or treated with DHC or sodium arsenite for indicated doses or time. Then washed three times with PBS (pH 7.4) at RT. All cells were followed by 4% paraformaldehyde fixation for 15 min, then permeabilized with ice-cold methanol for 10 min and blocked with 5% normal horse serum (NHS) in PBS containing 0.1% sodium azide for 1 h. All primary antibodies were diluted in blocking solution and treated to the cells and then followed by overnight incubation at 4°C. All cells were washed with PBS (pH 7.4) at RT for three times (10 min each). Then incubated with respected secondary antibodies for 1 h at RT. Then washed with PBS (pH 7.4) at RT for three times (10 min each). All coverslips were mounted with polyvinyl medium. Fluorescence images were taken with the help of Nikon Eclipse 80i fluorescence microscope (40x). All

fluorescence images were processed using ImageJ software and compiled using Adobe Photoshop CS6.

Ribopuromycylation assay

U2OS cells were grown in 6-well plate until ~70% confluency and treated with indicated concentration of DHC for 1 h, then pulsed with 10μg/ml puromycin 10 min at 37ºC in 5% CO_2 incubator except control. Then cells were washed with cold PBS twice and lysed with RIPA lysis buffer (50 mM Tris-Cl (pH 8.0), 150 mM NaCl, 1% NP-40, 0.1% SDS, 1 mM EDTA and 1% Sodium deoxycholate containing proteinase inhibitors 1 mM PMSF, 5 mM NaF) for 15 min in ice and centrifuged at 13,000 rpm for 15 min. Total proteins were quantified using Bradford reagent (Biorad). Approximately, equal amounts of protein (40μg) were subjected to immunoblot analysis. Ribopuromycylated proteins were detected using Puromycin antibody (1:25,000).

Polysome profiling analysis

U2OS cells were grown on 150 mm dish up to ~90% confluency, were treated with referred concentration and time of DHC. After exposure, cells were followed by 10 μg ml−1 cycloheximide treatment and incubated for 5 min at RT, washed with cold PBS three times, and lysed with 700 μl of polysome lysis buffer (20 mM HEPES (pH 7.6), 125 mM KCl, 5 mM MgCl2, 2 mM DTT and 1% NP-40) accompanied with 100 μg ml−1 cycloheximide, protease inhibitor cocktail (EDTA-free; pierce) in a cold room. Cell lysates were tumbled for 10 min at 4ºC and followed by centrifugation at 13,000 rpm for 15 min. The supernatants were

fractionated in 17.5-50% linear sucrose gradients by ultracentrifugation (30,000 rpm for 3 h 10 min) in a Beckman ultracentrifuge using SW40-Ti rotor. Gradients were eluted with a gradient fractionator (Brandel) and monitored with a UA-5 detector (ISCO).

Statistical analysis

All experimental results are represented as mean ± SEM of at least three independent experiments. Statistical comparisons were carried out using two-tailed paired t-test. Statistical analysis was carried out using Microsoft Excel. *p* value less than or equal to 0.05 (*) is considered as significant. Moreover, p value less than or equal to 0.001 (***) is taken as statistically highly significant.

III. Results

DHC promotes SG formation in eukaryotic cell

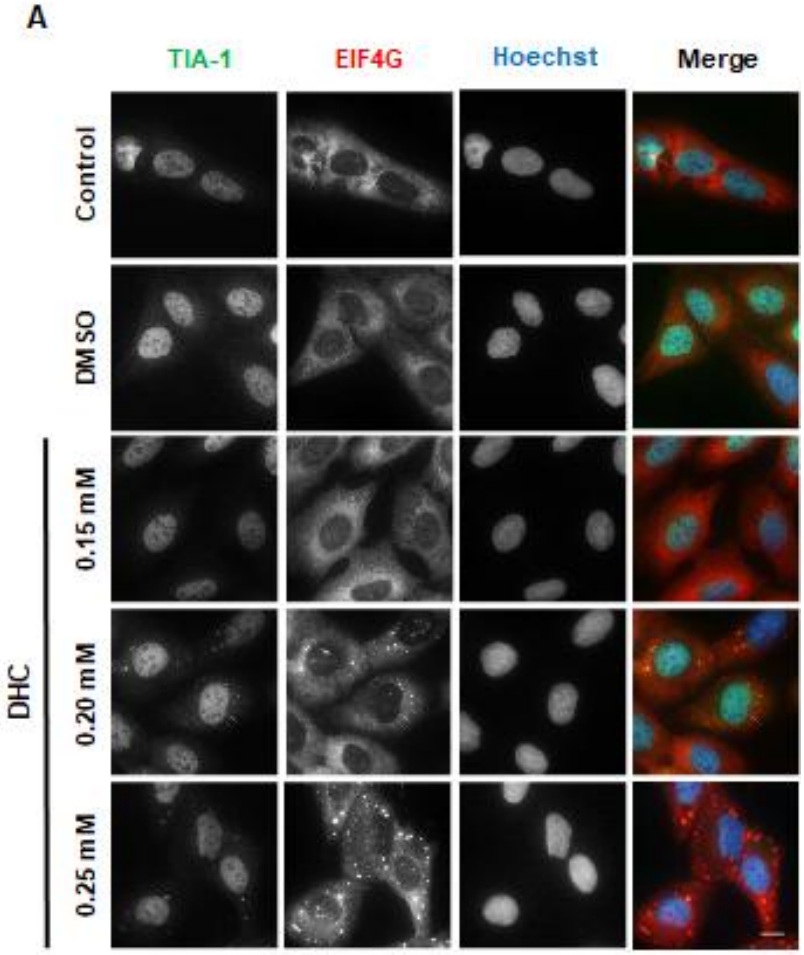
To determine whether cells exposed to DHC trigger SG formation, we treated U2OS cells with different concentrations of DHC for 60 min except for control (untreated), followed by immunostaining with SG markers EIF4G and TIA-1. Cells exposed to 0.25 mM DHC can induce robust SG formation in ~100% of cells after 60 min exposure which is evident from the formation of small granules and co-localization of SG proteins EIF4G and TIA-1 (Fig. 1A, B). Next, we asked whether DHC can promote SG assembly in other cell lines such as HeLa. Both U2OS and HeLa cells were treated with 0.25 mM DHC for indicated time points and experienced a similar outcome as the previous study by using immunostaining with primary antibodies against SG markers eIF3b and G3BP1. We found that both eIF3b and G3BP1 are co-localized in U2OS and HeLa cells. Surprisingly, the quantity of SGs present in U2OS cells are found to increase during the time-dependent experiment contrast to HeLa cells (Supplementary Fig. 1A, B). Western blot data established that DHC stress induces eIF2α phosphorylation alike arsenite stress (Fig. 1C).

Here we explored that DHC can able to promote SG aggregation in cytoplasm by harbouring RNA binding proteins such as EIF4G, TIA-1, G3BP1 and eIF3b. Next, we inquire whether these RNA binding proteins in SGs can be disappear in response to some drugs (e.g., cycloheximide or emetine), which are well known for reducing translation elongation through the termination of polysome disassembly, consequently blocking the delivery of mRNA into RNA granules or SGs [64]. To prove this theory, 20μg/ml emetine was added to the DHC treated U2OS cells followed by another incubation for 60 min. As mentioned in the

supplementary Fig. 2, mRNA binding proteins such as eIF3b and G3BP1 both moves to cytoplasmic SGs in response to DHC (0.25mM, 60 min) stress. Whether addition of emetine to DHC (0.25mM DHC for 60 min, followed by treating 20μg/ml emetine for additional 60 min) stressed cells promoting SG disassembly, which is evident from the depletion of SG formation (Supplementary Fig. 2). Similar results were found using cycloheximide (50 μg/ml) for additional 60 min incubation to DHC treated cells. In the both cases, complete SG disassembly was achieved increasing the incubation time up to 120 min for each emetine and cycloheximide (data not shown). Here we noticed a significant depletion in the formation of SG in cells. Overall, these results define that DHC is a bona fide SG promoter.

DHC induces PB formation

PBs are well known to be ribonucleoprotein (RNP) granules, which constituted fundamentally of mRNAs in the complex with various proteins in cytoplasmic region related with translation inhibition and 5′-to-3′ mRNA degradation. These cytoplasmic RNP granules are present in eukaryotes and shows similarities to other RNP granules like SGs, nucleoli and cajal bodies, where they rely on complex networks of RNA−protein interactions. Next, we questioned that whether DHC treatment can able to trigger the formation of PB, U2OS cells were exposed with different doses of DHC for 60 min, following by immunostaining with RCK (a PB marker) and G3BP1 (a well-known SG marker). Here we found RCK localization in the cells treated with DHC, suggesting that DHC can promote PB formation (Fig. 1D, E) in cells.



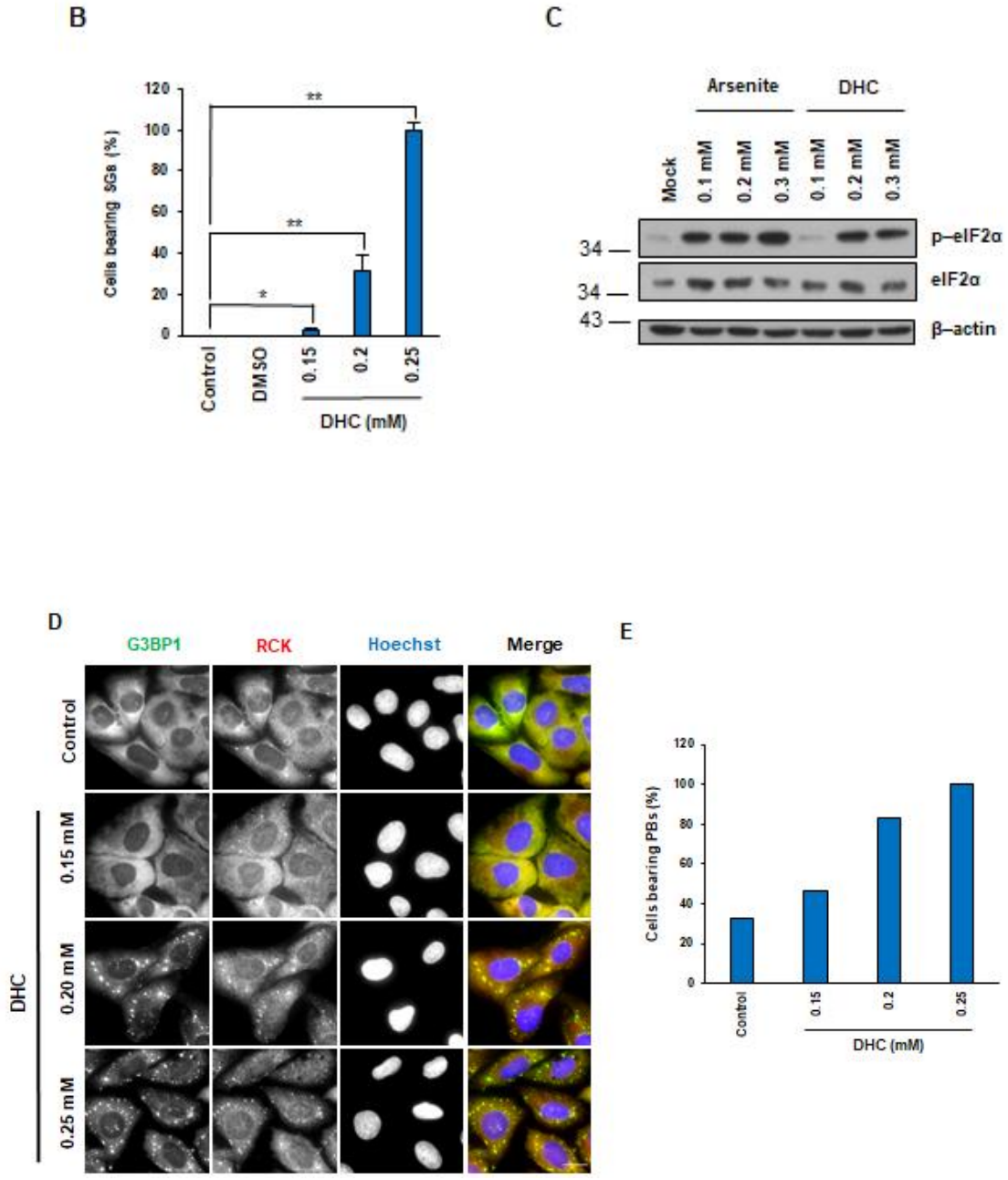


Figure 1. Dihydrocapsaicin promotes stress granule formation. (A) U2OS cells were stressed with indicated concentrations of DHC for 60 min except control and DMSO alone, then stained against SG markers TIA-1 (green), EIF4G (red) and nuclei stain Hoechst (blue). Scale bar, 10 μm. **(B)** SG quantification data showing percentage of cells harboring SGs. Error

bars indicate s.e.m. (n=3). *P<0.05, **P<0.01, Student's t-test. **(C)** Whole cell lysates were subjected to immunoblot assay to check phosphorylation of eIF2α. β-actin was used as loading control. **(D)** U2OS cells were treated with DHC (0.15 mM, 0.20 mM and 0.25 mM) for 60 min except control and then immunostained against eIF3b (green), RCK (red) and nuclei stain Hoechst (blue) respectively. Scale bar, 10 μm. **(E)** Bar graph showing the percentage of cells containing PBs upon DHC stress.

DHC induces polysome disassembly

Stress promoted phosphorylation of eIF2α protein is essential for the induction of polysome disassembly [65]. We found that DHC promotes the phosphorylation of eIF2α in U2OS cells. To know whether DHC supported phosphorylation of eIF2α can induces polysome disassembly, polysome profiling was achieved under various doses of DHC. Next, U2OS cells were exposed with DHC in a dose-dependent manner (0.15 mM, 0.25 mM, and 0.35 mM) for 60 min, followed by lysis under ice and supernatants were subjected to polysome analysis. We noticed a mild disassembly pattern at 0.25 mM stress, whereas 0.35 mM stress can completely disassemble the polysomes (Fig. 2A). Additionally, we carried out polysome profiling in a time-dependent manner (30, 60 and 90 min) using U2OS cells stressed with 0.25 mM concentration of DHC (Fig. 2B) shows that although DHC treatment for 60 min promoted phosphorylation of eIF2α and SGs assembly nearly ~95% cells, it only supports partial polysome disassembly pattern. Interestingly, cells affected by the DHC stress, started rescuing polysomes after 90 min treatment (Fig. 2B).

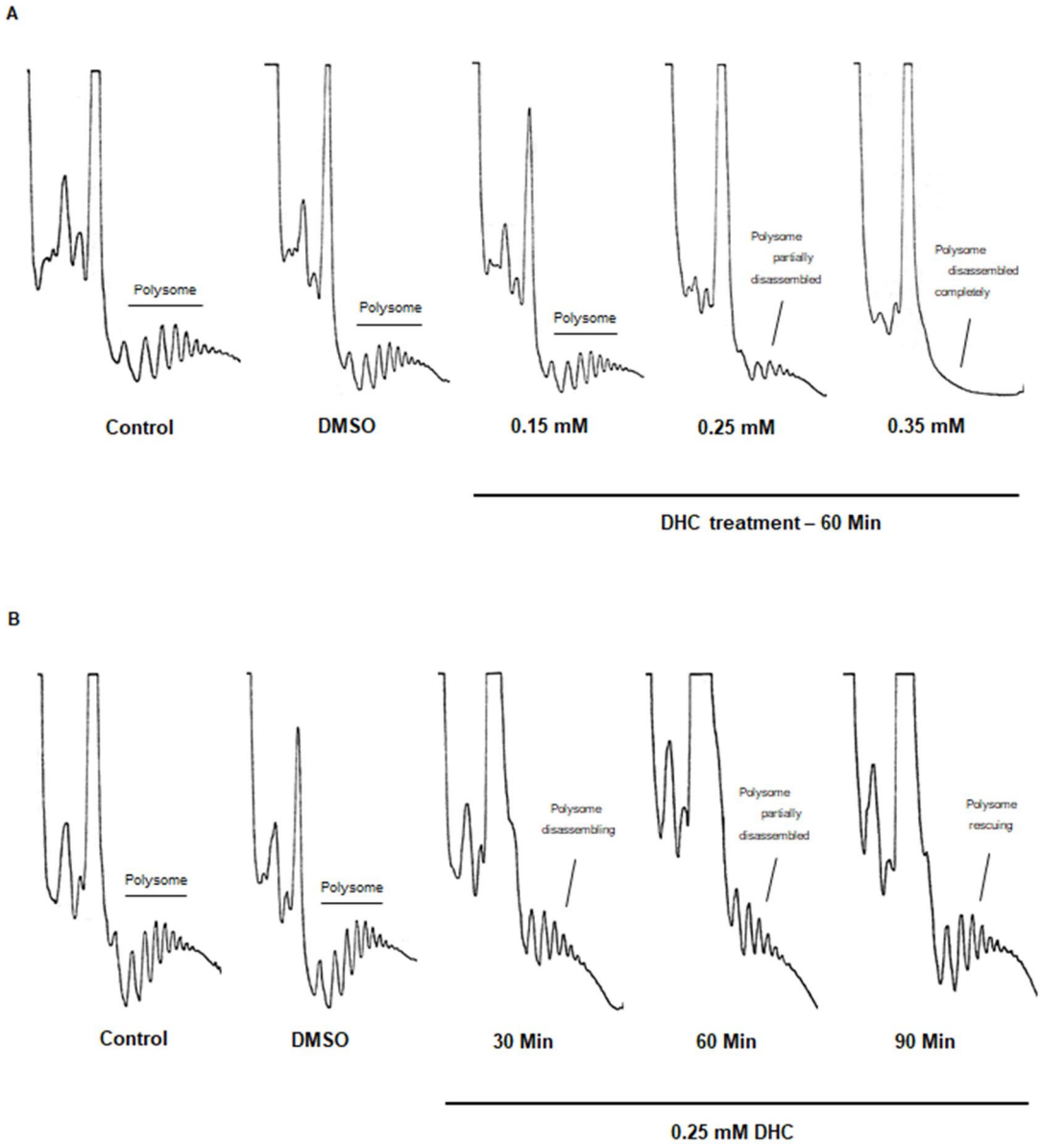


Figure 2. Dihydrocapsaicin induces polysome disassembly. (A) Polysome profile obtained from U2OS cells were treated with dihydrocapsaicin (0.15 mM, 0.25 mM and 0.35 mM) for 60 min except control and DMSO. After treatment, all cells were incubated with 100µg ml−1 cycloheximide for 5 min at RT, lysed with polysome lysis buffer and subjected to polysome profile analysis using gradient fractionator (Brandel) and monitored with a UA-5

detector (ISCO) **(B)** Similarly, U2OS cells were grown in 150 mm petri dishes, treated with 0.25 mM DHC for indicated time points, lysed with polysome lysis buffer and polysome profile generated (see materials and methods).

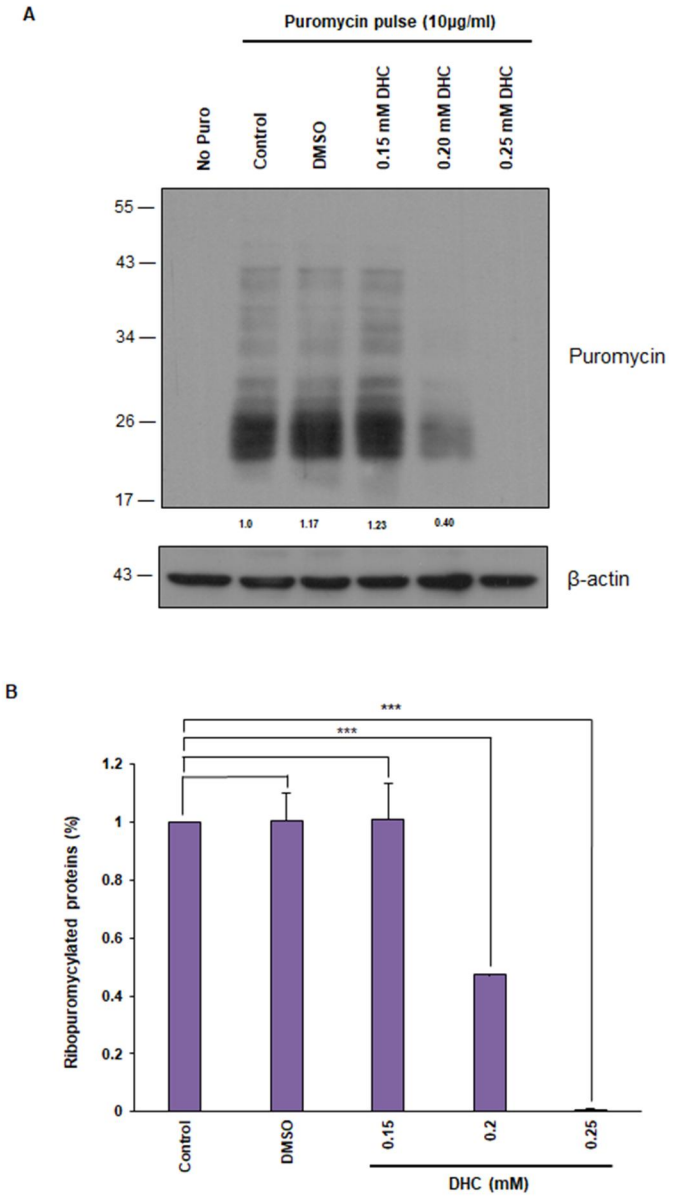


Figure 3. Dihydrocapsaicin represses global protein synthesis in U2OS cells. (A) U2OS cells were grown on 6-well plate, stressed with DHC (0.15 mM, 0.20 mM and 0.25 mM) except one control, no puro and DMSO for 1 h, then pulsed with puromycin (10μg/ml) for 10 min except no puro (untreated) sample. Cells were lysed with RIPA lysis buffer in ice and then immunoblotted against puromycin antibody (see materials and methods). β-actin was used as

loading control. Ribopuromycylated western bands were quantified with ImageJ software. **(B)** Graphical data represents the percentage of global protein translation in DHC treated U2OS cell. Error bars indicate s.e.m. (n=3). ***P<0.001, Student's t-test.

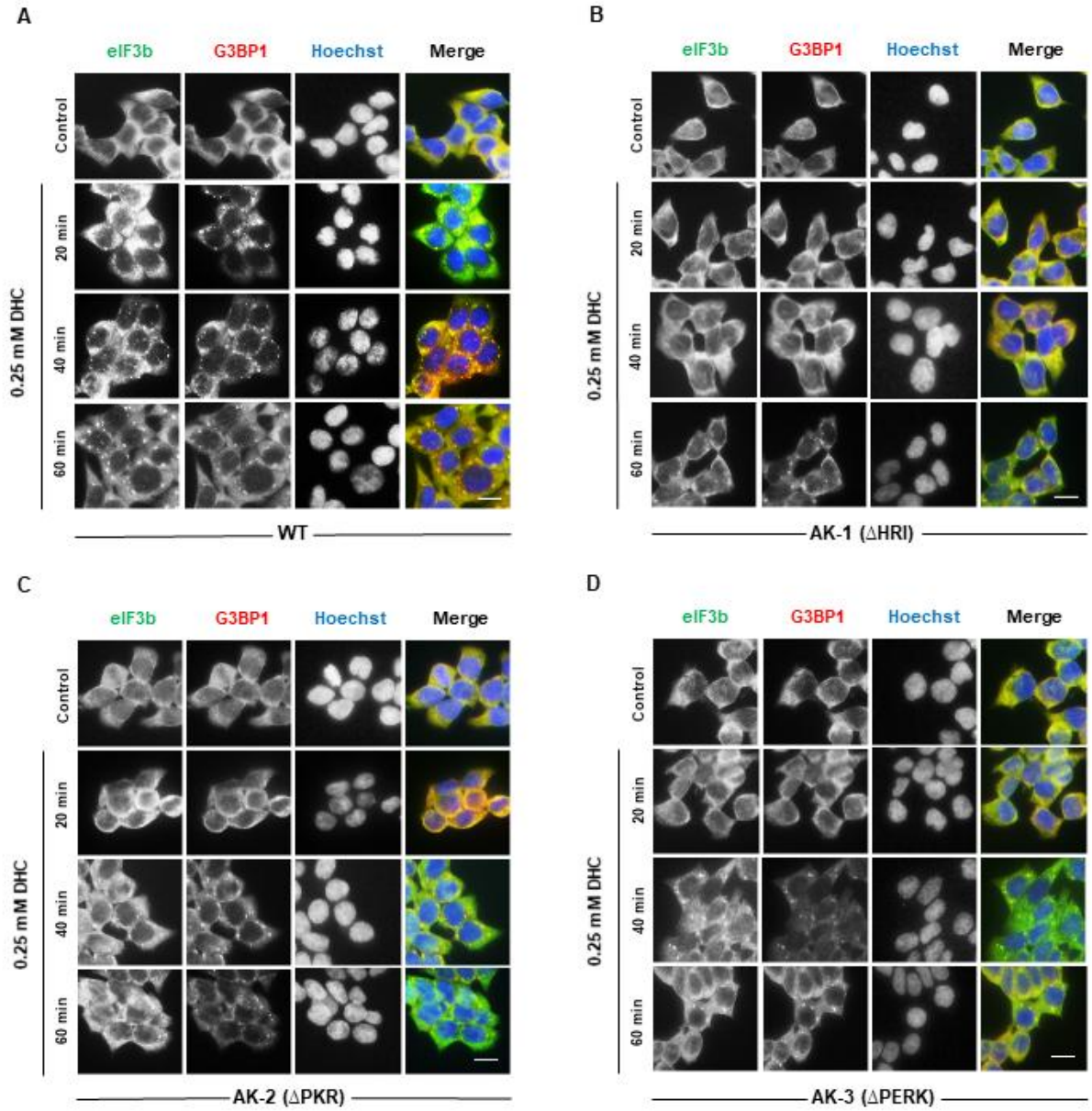
DHC arrests global protein translation in cells

Inhibition of protein translation is depending on the external stress which triggers SG formation and polysome disassembly. To test whether DHC treatment inhibits translation, we treated U2OS cells with puromycin, a Tyr-tRNA mimetic that incorporates into the ribosome, after DHC treatment. Immunoblot analysis against puromycin antibodies showed that global protein translation was reduced at 0.20 mM compared to 0.15 mM DHC treatment. Notably, the low molecular weight proteins were still translating whereas high molecular weight proteins stopped translating which is evident from the absence of puromycin intensity. The complete termination of protein synthesis was observed at 0.25 mM DHC stress (Fig. 3A, B). This result shows that DHC stress can arrest a subset of protein translation at a lower concentration.

Activation of EIF2AK1 or HRI kinase is essential for DHC mediated SG formation

Stress induced translational suppression due to stress stimulation is primarily depending on the four eIF2α stress kinases. SGs are aggregated in response to reduction of translational mechanism in cell and demand for either phosphorylation of eIF2α or mTOR inactivation to promote SGs assembly. To enquire whether DHC-triggered SG aggregation is depending on the phosphorylation of eIF2α, we used non-phosphorylatable knock-in mutation Ser51Ala (S51A MEF) and mouse embryonic fibroblast (MEF) wild-type and stressed with 0.25 mM DHC for indicated time durations. Here we noticed that DHC induces SG formation in WT-MEFs cells but absent in S51A-MEF cells demonstrating that phosphorylation of eIF2α protein is essential for DHC-triggered SG assembly (Supplementary figure 1C). Next, we investigated

which eIF2α stress kinase is responsible for the promotion of eIF2α phosphorylation and SG formation upon DHC treatment. To test this, we have used the HAP-1 wild-type cell line with four eIF2α kinases knockout such as HRI, PKR, PERK and GCN2. All cell lines including HAP-1 wild-type and four knockout cells were stressed with 0.25 mM DHC for 20 min, 40 min, and 60 min, except control in a time-dependent manner and then immunostained with SG markers eIF3b and G3BP1. We found that all cell lines induced SGs; albeit cells bearing SGs were less in quantity at 40 min and 60 min DHC treatment when compared to WT. (Fig. 4 A-E). Interestingly, the number of cells harbouring SGs was significantly depleted in the HRI KO cell line (AK1) (Fig. 4F). Finally, these results suggest that DHC promoted SGs assembly is dependent on the activation of eIF2α stress kinases and HRI kinase is involved in DHC-triggered SG aggregation.



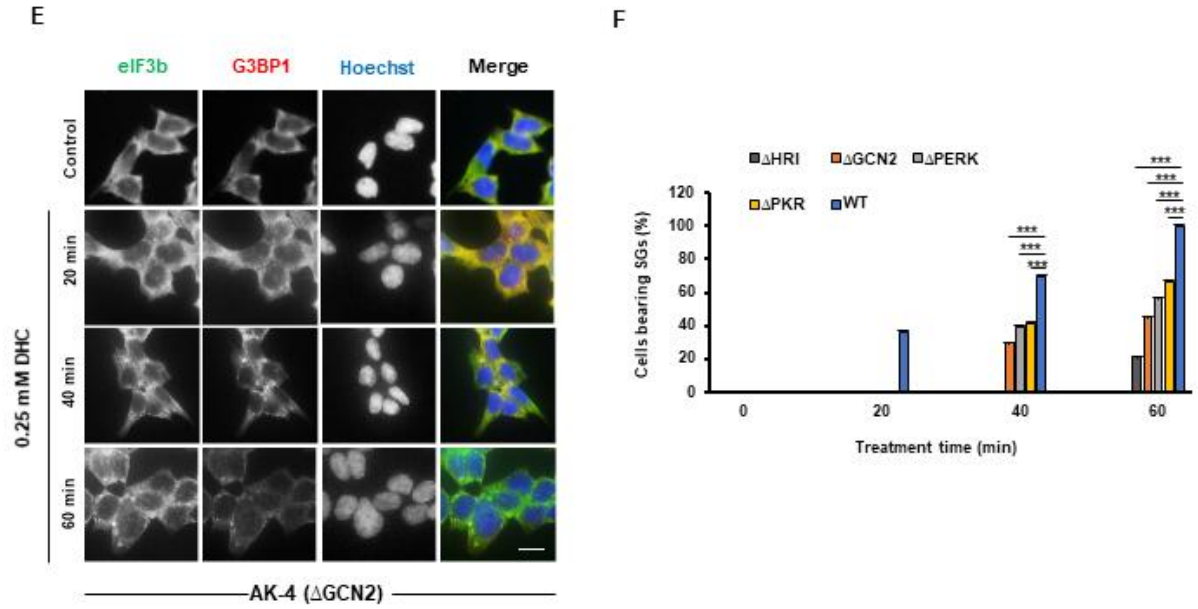


Figure 4. Dihydrocapsaicin promotes HRI kinase activation through oxidative stress. **(A-E)** HAP-1 cells (kinase mutant) were seeded equally on coverslips (preincubated with IMDM medium) in 4-well plates**,** grown and treated with 0.25 mM DHC for indicated time points. Cells were immunostained with eIF3b (green), G3BP1 (red) and nuclei stain Hoechst (blue) and then proceed for immunofluorescence microscopy (see materials and methods). Scale bar, 10 μm. **(F)** Graphical representation shows percentage of cells containing SGs. Error bars indicate s.e.m. (n=4), ***P<0.001, Student's t-test.

IV. Discussion

Here we report that DHC is a strong inducer for the promotion of SG assembly and polysome disassembly. Our data reveal that SGs are formed in ~100% of cells at 0.25 mM DHC concentration after 60 min treatment. The SG components TIA-1 and EIF4G are co-localized upon DHC stress in a dose-dependent manner (Fig. 1A). We confirmed these findings using multiple SG markers such as eIF3b and G3BP1 in multiple cells lines such as U2OS and HeLa cells suggesting DHC-mediated SG assembly occur in a variety of cell lines (Supplementary Figure 1A). We also observed that prolonged stress with DHC (0.25 mM) above 90 min or more than 0.35 mM DHC stress for 1 h can induce cell death rapidly (data not shown) bearing such prolonged stress environment exert cytotoxic effect. Previously, it was discovered that the eIF2α phosphorylation is one of the major incidents for the activation of SG formation and encouraging the translational inhibition in cells. We also noticed that DHC is assisting the phosphorylation of eIF2α and triggering SG formation in cells (Fig. 1C). PBs are known to be another dynamic structure, which retain RNA decay proteins. PBs are found to juxtapose to SGs and trafficking mRNA between them [66]. Interestingly, stress rises the number of PBs in cells. We noticed that PB formation was consistently increased at a higher DHC dose (Fig. 1D, E). Translational repression and polysome disassembly are closely associated with the SG formation in eukaryotic cells. Both eIF2α dependent and independent factors are known to activate SG aggregation through the translational suppression [7,67]. This data explained that the mRNAs are quitting from polysomes in order to transfer into SGs during DHC stress. Interestingly, translational blockers such as cycloheximide or emetine known to stall elongation and hamper polysome disassembly in arsenite treated cells [64]. To test this hypothesis, we incubated with emetine (20μg/ml) in DHC treated U2OS cells for 60 min and

observed a significant reduction of SG formation compared to the DHC alone. Also, similar results were achieved using cycloheximide (50μg/ml) incubation in DHC treated cells for 60 min (Supplementary Fig. 2). We noticed that both emetine and cycloheximide were able to dissolve pre-existing SGs as well as preventing further SG formation. Intriguingly, complete SG disassembly was observed by increasing emetine or cycloheximide incubation up to 120 min (data not shown).

Recent studies have unraveled different types of SGs assemblies depending on their mechanism, formation and physiological functions. Canonical SGs are also known as Type I SGs, which are induced by treating with arsenite in cells, leading to the phosphorylation of eIF2α protein and depletion of eIF2-GTP-tRNA-met ternary complex. Interestingly, eIF2/5 is known to be absent in Type I SGs, which assists cells to endure during stress conditions [68]. Type II SGs are assembled in cells by treating with selenite resulting to the suppression of eIF4A activity and can be activated independently of eIF2α phosphorylation [2]. Additionally, another type of SGs (Type III) are caused by xenobiotic stress, known for the absence of eIF3 [69]. Further inquiries are needed to examine whether DHC triggered SG assembly is type I, II or III.

To our understanding, SG initiation, translational repression and polysome disassembly are related to each other [37,50]. Therefore, we planned to further investigate the role of DHC stress on polysome assembly in U2OS cells. We noticed polysome disassembly pattern in a dose-dependent manner due to DHC stress. Intriguingly, 0.25 mM DHC treatment triggered polysomes to disassemble and a complete disassembly pattern was noticed at 0.35 mM (Fig. 2A). Interestingly, polysome started rescuing after 90 min with 0.25 mM DHC treatment, confirming SG disassembly (Fig. 2B). Protein synthesis is a process which spends energy and

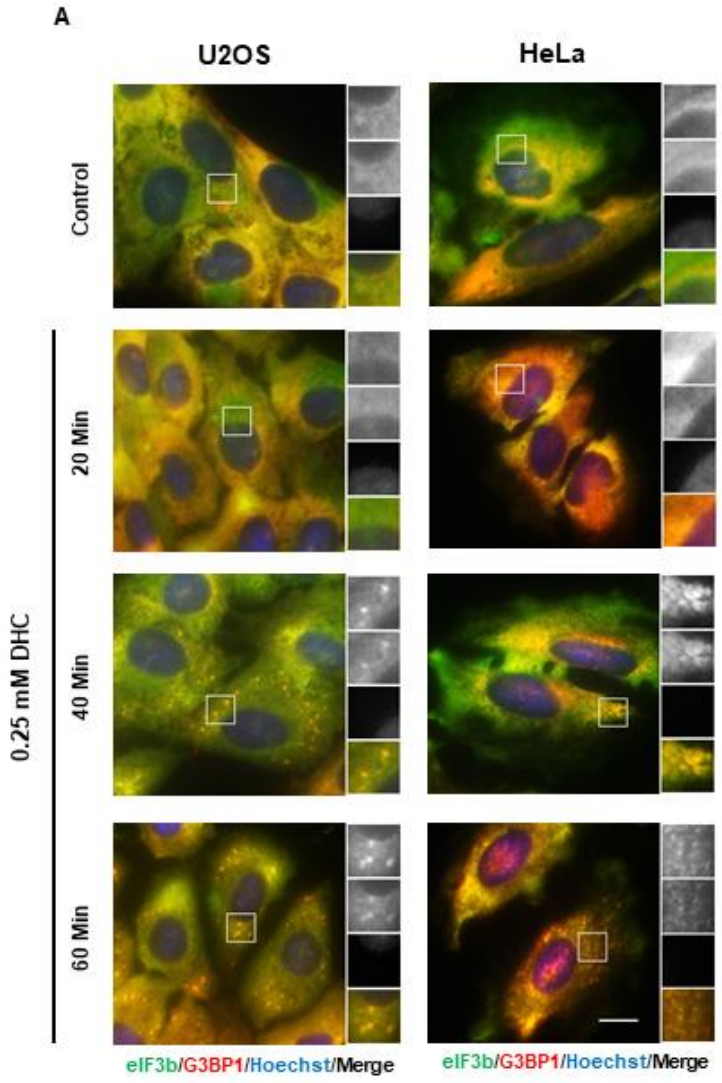
cells experiences translational inhibition under stress condition to retain energy for the restoring of stress induced damage [70]. As previously stated, global protein translation and polysome disassembly have a considerable link, we postulated that DHC-mediated stress could suppress protein translation. Using ribopuromycylation assay, we observed that although DHC treatment the inhibited majority of the protein translation, the low molecular weight proteins were still translating. Interestingly, 0.25 mM DHC treatment showed complete inhibition of translation; however, we did not observe complete polysome disassembly at this concentration. Further studies such as polysome-based proteomics needed to distinguish which proteins are synthesizing upon DHC treatment and a more sensitive assay to recognize translation repression is required to conclude this interesting finding.

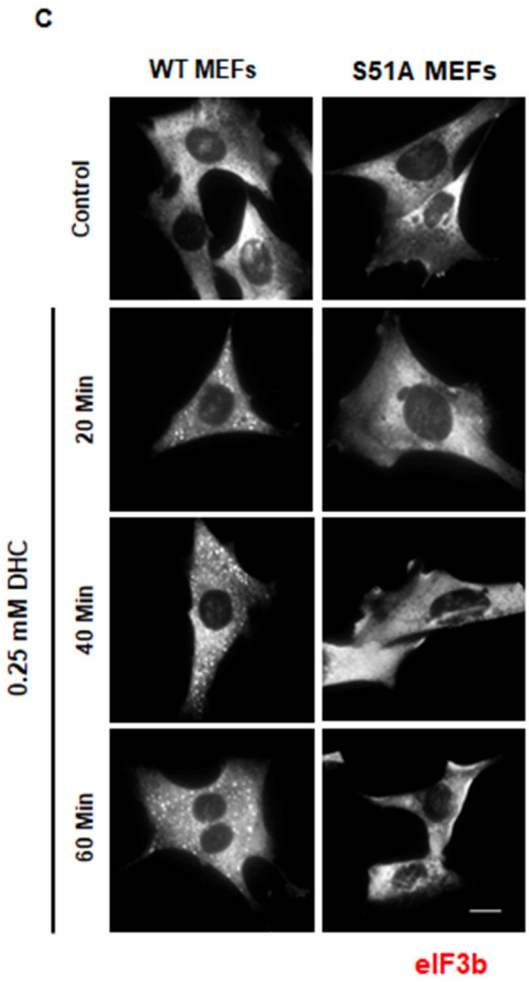
Recent decades several findings have shed lights on the mechanism of SG aggregation and translation repression. Translational initiation is strictly monitored by two major checkpoints such as 1) delivery of eIF4F complex to the 5'cap of mRNA is an essential translation checkpoint under strict monitoring of mTOR; 2) translation repression induces phosphorylation of serine at 51 positions of eIF2α protein following by the activation of stress kinases (HRI, PKR, PERK and GCN2). The phosphorylation of eIF2α is an essential stimulus for SG aggregation and translational inhibition. Although, DHC stress is also able to activate PERK (EIF2AK3) kinase. We observed that all eIF2α stress kinases were stimulated by DHC treatment in HAP-1 cells. Surprisingly, the HRI KO cell line (EIF2AK1) induced a very smaller number of SGs compared to the other cell lines (Fig. 4F; Fig. 4 A-E).

In conclusion, DHC can modulate formation of SG, polysome disassembly and translational inhibition. SGs are essential for cellular adaptation to several physiological and environmental stresses and increases the rate of survival for cells. DHC stress induces

polysome disassembly and bringing down the protein synthesis through the p-eIF2α dependent pathway following the activation of HRI (EIF2AK1) kinase. Although, DHC stress regulates SG formation, there are several questions such as whether these are canonical SGs, promote cell survival, recruit similar mRNAs as seen in arsenite-induced SGs, DHC acts upstream or downstream of polysome disassembly are yet to be identified. Further studies are needed to answer these interesting questions and establish possible pharmacological and therapeutic effects of DHC.

Supplementary Figure-1

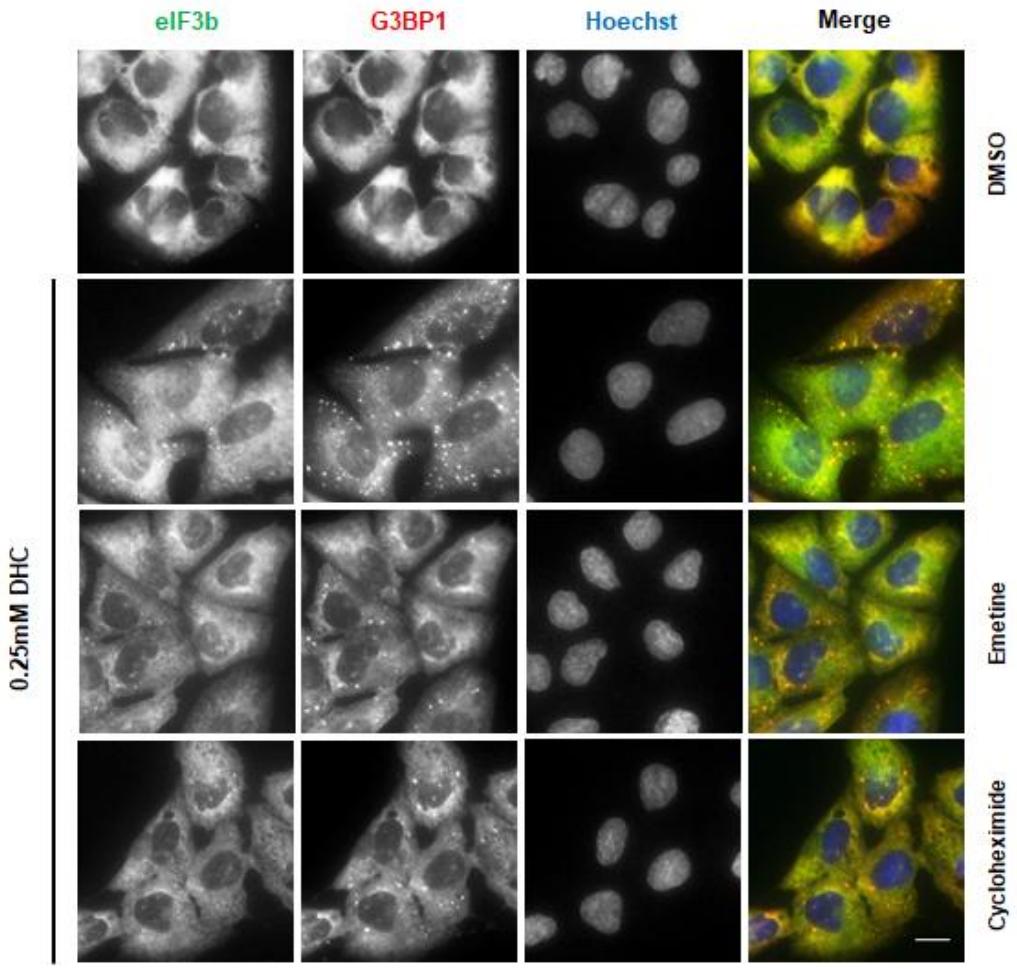




Supplementary Figure 1. (A) Localization of SG protein eIF3b and G3BP1 upon DHC stress in U2OS and HeLa Cells. Both U2OS and HeLa cells were grown on coverslips (preincubated with DMEM medium) in 4-well plates, treated with 0.25 mM DHC for indicated time points, immunostained with eIF3b (green), G3BP1 (red) and nuclei stain Hoechst (blue) and then proceed for immunofluorescence microscopy (see materials and methods). Boxed regions are shown enlarged as both merged and separate channel views indicating SG foci. Scale bar, 10 μm. **(B)** Statistical data represents the percentage of cells bearing SGs. Error bars indicate s.e.m. (n=3). ***P*<0.01, Student's t-test. **(C)** MEFs Wild type and MEFs bearing S51A

mutant eIF2α were treated with 0.25mM DHC for indicated time points, stained with SG marker eIF3b (red). Control represents no treatment. Scale bar, 10 μm.

Supplementary Figure-2



Supplementary Figure 2. Emetine and cycloheximide dissolves DHC induced SG assembly in U2OS cells. U2OS cells were stressed with 0.25mM concentration of DHC for 60 min except for DMSO alone, and incubated in the presence or absence of 20μg/ml emetine

and 50 μg/ml cycloheximide for another 60 min, then stained against SG markers eIF3b (green), G3BP1 (red), and nuclei stain Hoechst (blue). Scale bar, 10 μm.

V. REFERENCES

[1] J.R. Wheeler, T. Matheny, S. Jain, R. Abrisch, R. Parker, Distinct stages in stress granule assembly and disassembly, Elife 5 (2016). 10.7554/eLife.18413.

[2] K. Fujimura, A.T. Sasaki, P. Anderson, Selenite targets eIF4E-binding protein-1 to inhibit translation initiation and induce the assembly of non-canonical stress granules, Nucleic Acids Res 40 (2012) 8099-8110. 10.1093/nar/gks566.

[3] F. Palangi, S.M. Samuel, I.R. Thompson, C.R. Triggle, M.M. Emara, Effects of oxidative and thermal stresses on stress granule formation in human induced pluripotent stem cells, PLoS One 12 (2017) e0182059. 10.1371/journal.pone.0182059.

[4] N. Kedersha, S. Chen, N. Gilks, W. Li, I.J. Miller, J. Stahl, P. Anderson, Evidence that ternary complex (eIF2-GTP-tRNA(i)(Met))-deficient preinitiation complexes are core constituents of mammalian stress granules, Mol Biol Cell 13 (2002) 195-210. 10.1091/mbc.01-05-0221.

[5] N. Kedersha, G. Stoecklin, M. Ayodele, P. Yacono, J. Lykke-Andersen, M.J. Fritzler, D. Scheuner, R.J. Kaufman, D.E. Golan, P. Anderson, Stress granules and processing bodies are dynamically linked sites of mRNP remodeling, J Cell Biol 169 (2005) 871-884. 10.1083/jcb.200502088.

[6] P. Anderson, N. Kedersha, Stress granules: the Tao of RNA triage, Trends Biochem Sci 33 (2008) 141-150. 10.1016/j.tibs.2007.12.003.

[7] N.L. Kedersha, M. Gupta, W. Li, I. Miller, P. Anderson, RNA-binding proteins TIA-1 and

TIAR link the phosphorylation of eIF-2 alpha to the assembly of mammalian stress granules, J Cell Biol 147 (1999) 1431-1442. 10.1083/jcb.147.7.1431.

[8] H. Tourriere, K. Chebli, L. Zekri, B. Courselaud, J.M. Blanchard, E. Bertrand, J. Tazi, The RasGAP-associated endoribonuclease G3BP assembles stress granules, J Cell Biol 160 (2003) 823-831. 10.1083/jcb.200212128.

[9] S.P. Yoon, H.H. Kim, J. Kim, R.Y. Park, T. Ohn, Regulation of cellular RNA nano-particle assembly by splicing factor SRp20, J Nanosci Nanotechnol 13 (2013) 184-187. 10.1166/jnn.2013.6951.

[10] W.J. Kim, S.H. Back, V. Kim, I. Ryu, S.K. Jang, Sequestration of TRAF2 into stress granules interrupts tumor necrosis factor signaling under stress conditions, Mol Cell Biol 25 (2005) 2450-2462. 10.1128/MCB.25.6.2450-2462.2005.

[11] K. Thedieck, B. Holzwarth, M.T. Prentzell, C. Boehlke, K. Klasener, S. Ruf, A.G. Sonntag, L. Maerz, S.N. Grellscheid, E. Kremmer, R. Nitschke, E.W. Kuehn, J.W. Jonker, A.K. Groen, M. Reth, M.N. Hall, R. Baumeister, Inhibition of mTORC1 by astrin and stress granules prevents apoptosis in cancer cells, Cell 154 (2013) 859-874. 10.1016/j.cell.2013.07.031.

[12] T. Takahara, T. Maeda, Transient sequestration of TORC1 into stress granules during heat stress, Mol Cell 47 (2012) 242-252. 10.1016/j.molcel.2012.05.019.

[13] I.E. Gallouzi, C.M. Brennan, M.G. Stenberg, M.S. Swanson, A. Eversole, N. Maizels, J.A. Steitz, HuR binding to cytoplasmic mRNA is perturbed by heat shock, Proc Natl Acad Sci U S A 97 (2000) 3073-3078. 10.1073/pnas.97.7.3073.

[14] R. Mazroui, M.E. Huot, S. Tremblay, C. Filion, Y. Labelle, E.W. Khandjian, Trapping of

messenger RNA by Fragile X Mental Retardation protein into cytoplasmic granules induces translation repression, Hum Mol Genet 11 (2002) 3007-3017. 10.1093/hmg/11.24.3007.

[15] T. Ohn, N. Kedersha, T. Hickman, S. Tisdale, P. Anderson, A functional RNAi screen links O-GlcNAc modification of ribosomal proteins to stress granule and processing body assembly, Nat Cell Biol 10 (2008) 1224-1231. 10.1038/ncb1783.

[16] J.A. Kim, A.K. Jayabalan, V.K. Kothandan, R. Mariappan, Y. Kee, T. Ohn, Identification of Neuregulin-2 as a novel stress granule component, BMB Rep 49 (2016) 449-454. 10.5483/bmbrep.2016.49.8.090.

[17] K.K. McDonald, A. Aulas, L. Destroismaisons, S. Pickles, E. Beleac, W. Camu, G.A. Rouleau, C. Vande Velde, TAR DNA-binding protein 43 (TDP-43) regulates stress granule dynamics via differential regulation of G3BP and TIA-1, Hum Mol Genet 20 (2011) 1400-1410. 10.1093/hmg/ddr021.

[18] H.J. Kim, N.C. Kim, Y.D. Wang, E.A. Scarborough, J. Moore, Z. Diaz, K.S. MacLea, B. Freibaum, S. Li, A. Molliex, A.P. Kanagaraj, R. Carter, K.B. Boylan, A.M. Wojtas, R. Rademakers, J.L. Pinkus, S.A. Greenberg, J.Q. Trojanowski, B.J. Traynor, B.N. Smith, S. Topp, A.S. Gkazi, J. Miller, C.E. Shaw, M. Kottlors, J. Kirschner, A. Pestronk, Y.R. Li, A.F. Ford, A.D. Gitler, M. Benatar, O.D. King, V.E. Kimonis, E.D. Ross, C.C. Weihl, J. Shorter, J.P. Taylor, Mutations in prion-like domains in hnRNPA2B1 and hnRNPA1 cause multisystem proteinopathy and ALS, Nature 495 (2013) 467-473. 10.1038/nature11922.

[19] N. Dolzhanskaya, G. Merz, J.M. Aletta, R.B. Denman, Methylation regulates the intracellular protein-protein and protein-RNA interactions of FMRP, J Cell Sci 119 (2006) 1933-1946. 10.1242/jcs.02882.

[20] S. Kwon, Y. Zhang, P. Matthias, The deacetylase HDAC6 is a novel critical component of stress granules involved in the stress response, Genes Dev 21 (2007) 3381-3394. 10.1101/gad.461107.

[21] R. Mazroui, S. Di Marco, R.J. Kaufman, I.E. Gallouzi, Inhibition of the ubiquitin-proteasome system induces stress granule formation, Mol Biol Cell 18 (2007) 2603-2618. 10.1091/mbc.e06-12-1079.

[22] A.K. Jayabalan, A. Sanchez, R.Y. Park, S.P. Yoon, G.Y. Kang, J.H. Baek, P. Anderson, Y. Kee, T. Ohn, NEDDylation promotes stress granule assembly, Nat Commun 7 (2016) 12125. 10.1038/ncomms12125.

[23] F. Wippich, B. Bodenmiller, M.G. Trajkovska, S. Wanka, R. Aebersold, L. Pelkmans, Dual specificity kinase DYRK3 couples stress granule condensation/dissolution to mTORC1 signaling, Cell 152 (2013) 791-805. 10.1016/j.cell.2013.01.033.

[24] H. Mahboubi, R. Barise, U. Stochaj, 5'-AMP-activated protein kinase alpha regulates stress granule biogenesis, Biochim Biophys Acta 1853 (2015) 1725-1737. 10.1016/j.bbamcr.2015.03.015.

[25] K. Arimoto, H. Fukuda, S. Imajoh-Ohmi, H. Saito, M. Takekawa, Formation of stress granules inhibits apoptosis by suppressing stress-responsive MAPK pathways, Nat Cell Biol 10 (2008) 1324-1332. 10.1038/ncb1791.

[26] P.P. Ongusaha, H.H. Qi, L. Raj, Y.B. Kim, S.A. Aaronson, R.J. Davis, Y. Shi, J.K. Liao, S.W. Lee, Identification of ROCK1 as an upstream activator of the JIP-3 to JNK signaling axis in response to UVB damage, Sci Signal 1 (2008) ra14. 10.1126/scisignal.1161938.

[27] N.P. Tsai, L.N. Wei, RhoA/ROCK1 signaling regulates stress granule formation and apoptosis, Cell Signal 22 (2010) 668-675. 10.1016/j.cellsig.2009.12.001.

[28] T. Wasserman, K. Katsenelson, S. Daniliuc, T. Hasin, M. Choder, A. Aronheim, A novel c-Jun N-terminal kinase (JNK)-binding protein WDR62 is recruited to stress granules and mediates a nonclassical JNK activation, Mol Biol Cell 21 (2010) 117-130. 10.1091/mbc.E09-06-0512.

[29] D. Teixeira, U. Sheth, M.A. Valencia-Sanchez, M. Brengues, R. Parker, Processing bodies require RNA for assembly and contain nontranslating mRNAs, RNA 11 (2005) 371-382. 10.1261/rna.7258505.

[30] Y. Luo, Z. Na, S.A. Slavoff, P-Bodies: Composition, Properties, and Functions, Biochemistry 57 (2018) 2424-2431. 10.1021/acs.biochem.7b01162.

[31] J.R. Buchan, R. Parker, Eukaryotic stress granules: the ins and outs of translation, Mol Cell 36 (2009) 932-941. 10.1016/j.molcel.2009.11.020.

[32] D.S.W. Protter, R. Parker, Principles and Properties of Stress Granules, Trends Cell Biol 26 (2016) 668-679. 10.1016/j.tcb.2016.05.004.

[33] S. Jain, J.R. Wheeler, R.W. Walters, A. Agrawal, A. Barsic, R. Parker, ATPase-Modulated Stress Granules Contain a Diverse Proteome and Substructure, Cell 164 (2016) 487-498. 10.1016/j.cell.2015.12.038.

[34] R.J. Jackson, C.U. Hellen, T.V. Pestova, The mechanism of eukaryotic translation initiation and principles of its regulation, Nat Rev Mol Cell Biol 11 (2010) 113-127. 10.1038/nrm2838.

[35] N. Sonenberg, A.G. Hinnebusch, Regulation of translation initiation in eukaryotes: mechanisms and biological targets, Cell 136 (2009) 731-745. 10.1016/j.cell.2009.01.042.

[36] M.D. Panas, P. Ivanov, P. Anderson, Mechanistic insights into mammalian stress granule dynamics, J Cell Biol 215 (2016) 313-323. 10.1083/jcb.201609081.

[37] R.C. Wek, Role of eIF2alpha Kinases in Translational Control and Adaptation to Cellular Stress, Cold Spring Harb Perspect Biol 10 (2018). 10.1101/cshperspect.a032870.

[38] J.J. Chen, I.M. London, Regulation of protein synthesis by heme-regulated eIF-2 alpha kinase, Trends Biochem Sci 20 (1995) 105-108. 10.1016/s0968-0004(00)88975-6.

[39] A.P. Han, C. Yu, L. Lu, Y. Fujiwara, C. Browne, G. Chin, M. Fleming, P. Leboulch, S.H. Orkin, J.J. Chen, Heme-regulated eIF2alpha kinase (HRI) is required for translational regulation and survival of erythroid precursors in iron deficiency, EMBO J 20 (2001) 6909-6918. 10.1093/emboj/20.23.6909.

[40] A.P. Han, M.D. Fleming, J.J. Chen, Heme-regulated eIF2alpha kinase modifies the phenotypic severity of murine models of erythropoietic protoporphyria and beta-thalassemia, J Clin Invest 115 (2005) 1562-1570. 10.1172/JCI24141.

[41] L. Lu, A.P. Han, J.J. Chen, Translation initiation control by heme-regulated eukaryotic initiation factor 2alpha kinase in erythroid cells under cytoplasmic stresses, Mol Cell Biol 21 (2001) 7971-7980. 10.1128/MCB.21.23.7971-7980.2001.

[42] M.A. Garcia, J. Gil, I. Ventoso, S. Guerra, E. Domingo, C. Rivas, M. Esteban, Impact of protein kinase PKR in cell biology: from antiviral to antiproliferative action, Microbiol Mol Biol Rev 70 (2006) 1032-1060. 10.1128/MMBR.00027-06.

[43] G.S. Hotamisligil, Endoplasmic reticulum stress and the inflammatory basis of metabolic disease, Cell 140 (2010) 900-917. 10.1016/j.cell.2010.02.034.

[44] E. Rath, E. Berger, A. Messlik, T. Nunes, B. Liu, S.C. Kim, N. Hoogenraad, M. Sans, R.B. Sartor, D. Haller, Induction of dsRNA-activated protein kinase links mitochondrial unfolded protein response to the pathogenesis of intestinal inflammation, Gut 61 (2012) 1269-1278. 10.1136/gutjnl-2011-300767.

[45] H.P. Harding, Y. Zhang, D. Ron, Protein translation and folding are coupled by an endoplasmic-reticulum-resident kinase, Nature 397 (1999) 271-274. 10.1038/16729.

[46] C.R. Prostko, M.A. Brostrom, C.O. Brostrom, Reversible phosphorylation of eukaryotic initiation factor 2 alpha in response to endoplasmic reticular signaling, Mol Cell Biochem 127-128 (1993) 255-265. 10.1007/BF01076776.

[47] C.O. Brostrom, M.A. Brostrom, Regulation of translational initiation during cellular responses to stress, Prog Nucleic Acid Res Mol Biol 58 (1998) 79-125. 10.1016/s0079-6603(08)60034-3.

[48] H.P. Harding, I. Novoa, Y. Zhang, H. Zeng, R. Wek, M. Schapira, D. Ron, Regulated translation initiation controls stress-induced gene expression in mammalian cells, Mol Cell 6 (2000) 1099-1108. 10.1016/s1097-2765(00)00108-8.

[49] H.Y. Jiang, S.A. Wek, B.C. McGrath, D. Lu, T. Hai, H.P. Harding, X. Wang, D. Ron, D.R. Cavener, R.C. Wek, Activating transcription factor 3 is integral to the eukaryotic initiation factor 2 kinase stress response, Mol Cell Biol 24 (2004) 1365-1377. 10.1128/MCB.24.3.1365-1377.2004.

[50] E. McEwen, N. Kedersha, B. Song, D. Scheuner, N. Gilks, A. Han, J.J. Chen, P. Anderson, R.J. Kaufman, Heme-regulated inhibitor kinase-mediated phosphorylation of eukaryotic translation initiation factor 2 inhibits translation, induces stress granule formation, and mediates survival upon arsenite exposure, J Biol Chem 280 (2005) 16925-16933. 10.1074/jbc.M412882200.

[51] S.P. Srivastava, K.U. Kumar, R.J. Kaufman, Phosphorylation of eukaryotic translation initiation factor 2 mediates apoptosis in response to activation of the double-stranded RNA-dependent protein kinase, J Biol Chem 273 (1998) 2416-2423. 10.1074/jbc.273.4.2416.

[52] H.P. Harding, Y. Zhang, A. Bertolotti, H. Zeng, D. Ron, Perk is essential for translational regulation and cell survival during the unfolded protein response, Mol Cell 5 (2000) 897-904. 10.1016/s1097-2765(00)80330-5.

[53] S.A. Wek, S. Zhu, R.C. Wek, The histidyl-tRNA synthetase-related sequence in the eIF-2 alpha protein kinase GCN2 interacts with tRNA and is required for activation in response to starvation for different amino acids, Mol Cell Biol 15 (1995) 4497-4506. 10.1128/MCB.15.8.4497.

[54] G.A. Cordell, O.E. Araujo, Capsaicin: identification, nomenclature, and pharmacotherapy, Ann Pharmacother 27 (1993) 330-336. 10.1177/106002809302700316.

[55] N.P. Gannon, E.L. Lambalot, R.A. Vaughan, The effects of capsaicin and capsaicinoid analogs on metabolic molecular targets in highly energetic tissues and cell types, Biofactors 42 (2016) 229-246. 10.1002/biof.1273.

[56] X.J. Luo, J. Peng, Y.J. Li, Recent advances in the study on capsaicinoids and capsinoids,

Eur J Pharmacol 650 (2011) 1-7. 10.1016/j.ejphar.2010.09.074.

[57] S.H. Oh, Y.S. Kim, S.C. Lim, Y.F. Hou, I.Y. Chang, H.J. You, Dihydrocapsaicin (DHC), a saturated structural analog of capsaicin, induces autophagy in human cancer cells in a catalase-regulated manner, Autophagy 4 (2008) 1009-1019. 10.4161/auto.6886.

[58] L. Xie, G.H. Xiang, T. Tang, Y. Tang, L.Y. Zhao, D. Liu, Y.R. Zhang, J.T. Tang, S. Zhou, D.H. Wu, Capsaicin and dihydrocapsaicin induce apoptosis in human glioma cells via ROS and Ca2+mediated mitochondrial pathway, Mol Med Rep 14 (2016) 4198-4208. 10.3892/mmr.2016.5784.

[59] M.J. Adams, K.D. Ahuja, D.P. Geraghty, Effect of capsaicin and dihydrocapsaicin on in vitro blood coagulation and platelet aggregation, Thromb Res 124 (2009) 721-723. 10.1016/j.thromres.2009.05.001.

[60] M. Halme, M. Pesonen, H. Salo, M. Soderstrom, M. Pasanen, K. Vahakangas, P. Vanninen, Comparison of in vitro metabolism and cytotoxicity of capsaicin and dihydrocapsaicin, J Chromatogr B Analyt Technol Biomed Life Sci 1009-1010 (2016) 17-24. 10.1016/j.jchromb.2015.11.042.

[61] J.Y. Zhao, Y.W. Hu, S.F. Li, Y.R. Hu, X. Ma, S.G. Wu, Y.C. Wang, J.J. Gao, Y.H. Sha, L. Zheng, Q. Wang, Dihydrocapsaicin down-regulates apoM expression through inhibiting Foxa2 expression and enhancing LXRalpha expression in HepG2 cells, Lipids Health Dis 13 (2014) 50. 10.1186/1476-511X-13-50.

[62] K.D. Ahuja, D.A. Kunde, M.J. Ball, D.P. Geraghty, Effects of capsaicin, dihydrocapsaicin, and curcumin on copper-induced oxidation of human serum lipids, J Agric Food Chem 54

(2006) 6436-6439. 10.1021/jf061331j.

[63] S.H. Oh, S.C. Lim, Endoplasmic reticulum stress-mediated autophagy/apoptosis induced by capsaicin (8-methyl-N-vanillyl-6-nonenamide) and dihydrocapsaicin is regulated by the extent of c-Jun NH2-terminal kinase/extracellular signal-regulated kinase activation in WI38 lung epithelial fibroblast cells, J Pharmacol Exp Ther 329 (2009) 112-122. 10.1124/jpet.108.144113.

[64] N. Kedersha, M.R. Cho, W. Li, P.W. Yacono, S. Chen, N. Gilks, D.E. Golan, P. Anderson, Dynamic shuttling of TIA-1 accompanies the recruitment of mRNA to mammalian stress granules, J Cell Biol 151 (2000) 1257-1268. 10.1083/jcb.151.6.1257.

[65] N.G. Farny, N.L. Kedersha, P.A. Silver, Metazoan stress granule assembly is mediated by P-eIF2alpha-dependent and -independent mechanisms, RNA 15 (2009) 1814-1821. 10.1261/rna.1684009.

[66] S.L. Moon, T. Morisaki, A. Khong, K. Lyon, R. Parker, T.J. Stasevich, Multicolour single-molecule tracking of mRNA interactions with RNP granules, Nat Cell Biol 21 (2019) 162-168. 10.1038/s41556-018-0263-4.

[67] Y. Dang, N. Kedersha, W.K. Low, D. Romo, M. Gorospe, R. Kaufman, P. Anderson, J.O. Liu, Eukaryotic initiation factor 2alpha-independent pathway of stress granule induction by the natural product pateamine A, J Biol Chem 281 (2006) 32870-32878. 10.1074/jbc.M606149200.

[68] V.M. Advani, P. Ivanov, Stress granule subtypes: an emerging link to neurodegeneration, Cell Mol Life Sci 77 (2020) 4827-4845. 10.1007/s00018-020-03565-0.

[69] P. Ivanov, N. Kedersha, P. Anderson, Stress Granules and Processing Bodies in

Translational Control, Cold Spring Harb Perspect Biol 11 (2019). 10.1101/cshperspect.a032813.

[70] L.M. Lindqvist, K. Tandoc, I. Topisirovic, L. Furic, Cross-talk between protein synthesis, energy metabolism and autophagy in cancer, Curr Opin Genet Dev 48 (2018) 104-111. 10.1016/j.gde.2017.11.003.

VI. ABSTRACT (IN ENGLISH)

Functional Study of Dihydrocapsaicin on the Assembly of Stress Granule

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Stress granules (SGs) are cytoplasmic biomolecular condensates that are formed against a variety of stress conditions when translation initiation is perturbed. SGs form through the weak protein-protein, protein-RNA, and RNA-RNA interactions, as well as through the intrinsically disordered domains and post-translation modifications within RNA binding proteins (RBPs). SGs are known to contribute to cell survivability by minimizing the stress-induced damage to the cells by delaying the activation of apoptosis. Here, we find that dihydrocapsaicin (DHC), an analogue of capsaicin, is a SG inducer that promotes polysome disassembly and reduces global protein translation via phosphorylation of eIF2α. DHC-mediated SG assembly is controlled by the phosphorylation of eIF2α at serine 51 position and is controlled by all four eIF2α stress kinases (i.e., HRI, PKR, PERK, and GCN2) with HRI showing maximal effect. We demonstrate that DHC is a bonafide compound that induces SG assembly, disassembles polysome, phosphorylates eIF2α in an HRI dependent manner, and thereby arrest global translation. Together, our results suggest that DHC is a novel SG inducer and an alternate to sodium arsenite to study SG dynamics.

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"I dedicated this thesis to my Parents, Sister, Brother-in-Law and Professor Takbum Ohn"