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Ph.D Thesis

Functional Study of Calcium-Calmodulin Signaling in the Assembly of Stress Granule

Graduate School of Chosun University

Department of Biomedical Sciences

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Stress granule 형성에 있어 Calcium-Calmodulin
신호전달체계의 기능 연구

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국문초록

Stress Granule 형성에 있어 Calcium-Calmodulin 신호전달체계의 기능 연구

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의과학과

스트레스 과립은 스트레스 환경에서 세포의 기능적 부산물이며 퇴행성 뇌 질환 및 암 세포 내성에 관여하는 것으로 알려져 있다. 현재까지 스트레스 과립에 대한 연구가 활발히 진행되고 있지만 스트레스 과립 형성을위한 신호 전달 시스템에 대해서는 자세히 알려지지 않았다. 또한, 실험 결과는 세포 내 칼슘 신호 전달 매개체 인 칼 모듈 린이 필요함을 시사한다. 또한 스트레스 세포 신호 전달에 중요한 역할을 하는 다양한 포스파타제 효소를 테스트하여 Akt 키나아제가 Calmodulin의 하위 인자로서 결정적인 역할을 한다는 것을 밝혀 냈다. 이 연구를 통해 스트레스 과립 형성에 관여하는 새로운 세포 신호 전달 시스템이 확인되었으며, 향후 질병 연관성 연구에 많은 기여를 할 것으로 기대된다.

I. Introduction

Stress granules are membrane less components composed of non-translating messenger ribonucleoproteins (mRNPs) that rapidly aggregate in mammalian cells in cytoplasmic region when exposed to adverse environmental conditions[1]. Stress granules are regulated by stress induced eIF2α phosphorylation, either by preventing or delaying translational initiation[1]. During various stresses, eIF2α family kinases PERK (PKR-like ER kinase), PKR (protein kinase double-stranded RNA-dependent), GCN2 (general control non-derepressible-2), and HRI (heme-regulated inhibitor), activated and phosphorylates the eIF2α[2]. Not all stresses are eIF2α dependent[3]. P-eIF2α promotes mRNA translation of ATF4[4], ER stress and HRI induced eIF2α phosphorylation activate ATF4[5,6]. In the course of performing a human MAPK assay screening, upon calmodulin knock down and calmodulin inhibitor treatment we observed AKT, JNK and ERK with arsenite treated condition.

Calcium ion is one of the major secondary messengers which regulates various signaling pathways that are involved in the survival of cell, proliferation[7], differentiation, transcription and apoptosis[8-11]. Studying the importance of intracellular[12, 13], extracellular[14], mitochondrial calcium level[15, 16] and calcium dynamics in mammalian cells is more necessary to understand a number of different disease conditions[17]. In plants, association of calcium with SG was confirmed[18] but not in mammalian system. Along with these the stress granules also modify the flow of cellular information by sequestering proteins that are involved in signal transduction[19].

Calmodulin (CaM) is a calcium transducer. It is a ubiquitous, versatile Ca^{2+} binding protein that can bind to and regulate downstream target proteins, thereby affecting many different cellular functions[20]. CaM is regulated by intracellular calcium concentration in many ways[21]. CaM has many roles in cardiac ryanodine receptor (RyR2) control[22] cardiac arrhythmias[23, 24] Cell proliferation[7, 25] and cell survival[26]. However, the molecular mechanism underlying the Ca^{2+}/Calmodulin function in cell survival via stress granule assembly is remain unreported. Protein kinase B (PKB) or AKT, is a serine/threonine kinase and it plays a key role

in the activation of PI3K signaling pathway. AKT also works as an anti-apoptotic factor[27]. Calmodulin mediates AKT in almost every other human breast cancer cell lines [28]. AKT binds to the C-terminal lobe of calmodulin[29].

We attempted to unravel the function of calcium signaling (Ca^{2+}/Calmodulin) in cell survival through HRI/eIF2α/ATF4 pathway mediated SG assembly. We found that AKT is crucial for this process through human P-MAPK assay screening. Moreover, we also found that arsenite induced eIF2α and AKT phosphorylation is affected by calcium chelators and calmodulin antagonist. AKT interaction with eIF2α and it's site specific.

Altogether, these suggest that calcium signaling is necessary for eIF2α mediated SG assembly. AKT playing as a pro-apoptotic factor which is also mediated by SG assembly through HRI/eIF2α/ATF4 dependent manner. Reduced eIF2α phosphorylation, elevation of ATF4 expression upon AKT inhibition on arsenite condition shows the importance of AKT on eIF2α/ATF4 pathways.

II. Materials and Methods

Cell Culture and Transfection

U2OS, HEK293 and HeLa cells were received from Korean cell line bank and maintained in DMEM medium (welgene). Supplemented with 10% FBS (welgene), 1% V/V penicillin and streptomycin (Lonza) at 37^0C in 5% CO2. siRNA Transfections were performed using Lipofectamine 2000 (Invitrogen) at 40 nM final concentration, all siRNA sequences used in this study are listed in (Table no:1). All DNA plasmids were transfected using either PEI (Polysciences) or Fugene 6 (Promega, Madison, WI) with opti-MEM (Serum free media-Welgene) as per manufacture's protocol.

Immuno fluorescence microscopy

U2OS cells were seeded in four well plates as per the experimental requirements. After specific treatment condition, the cells were washed twice with 1X PBS and then fixed with prechilled 4% paraformaldehyde for 15 min and permeabilized with ice cold methanol for 10 min. Cells were again washed twice with 1X PBS later blocked for 1h (5%NHS-PBS - 1% sodium azide). Appropriate SG and P-body markers were used for staining. Image-j and photoshop softwares were used for image arrangements.

Immunoblot Analysis

After treatment cells were rinsed with 1X cold PBS to remove excess media and the cells were scraped with RIPA buffer containing protease inhibitor cocktail (Thermo scientific). The cells were kept on ice for 10 min then centrifuged for 20 min at 4^0C in 13,000 rpm and the supernatant was collected. Protein concentration was measured using the Bradford assay method. Equal amounts of proteins were used for further SDS-PAGE analysis with 2X SDS sample buffer. The samples were boiled at 85^0C for 5 min. After the SDS-PAGE the proteins were transferred to a PVDF membrane using wet transfer system. The membranes were blocked with 5% NHS-PBS-Sodium azide for 1 h and then incubated with primary antibodies overnight, followed by 1X TBST wash for three times. Appropriate horse radish peroxide conjugated secondary antibodies were incubated for an hour then developed using developing solution (Bioshop.inc).

Antibodies

The listed antibodies were used for this study, AKT (1:1000, Cell signaling, #9272), p-AKT(D9E) (Ser473) (1:1000, Cell signaling, #4060), CaM (G-3)(1:200, Santa Cruz, SC-137079), CaM (FL-149)(1:200, Santa Cruz, SC-5537), CaM (1:500, Abcam, ab45689), eIF2α (FL-315)(1:1000, Santa Cruz, SC-11386), p- eIF2α (1:1000, BML-SA405, Enzo life sciences) Anti Beta actin (Ac-15)(Abcam, ac6276).

Knock down

Calmodulin siRNAs (three different siRNAs to block all the three isoforms) from Bioneer were used to knock down the CaM gene. The knock down success was confirmed by immunoblot analysis showing reduction in the endogenous CaM level.

Polysome Analysis

U2OS cells were grown in 150 mm dishes and above-mentioned treatment procedures were followed. After treatment, 100 μg ml−1 cycloheximide was added and incubated for 5 min at RT, washed twice with 1X cold PBS, then lysed with 1 ml of polysome lysis buffer (20 mM HEPES pH 7.4, 5 mM MgCl2, 125 mM KCl, 1% NP-40, 2 mM DTT) supplemented with 100 μg ml−1 cycloheximide (Sigma), protease inhibitor cocktail (EDTA-free; Thermo scientific) and RNAsin (Ambion) in cold room. Cell lysates were tumbled for 15 min at 4 °C and centrifuged at 13,000 r.p.m. for 15 min. The supernatants were fractionated in 17.5–50% linear sucrose gradients by ultracentrifugation (35,000 r.p.m. 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). Fractions were acetone precipitated at −20 °C for overnight and processed for further immunoblot analysis.

Co-Immunoprecipitation Assay

Cells were harvested and lysed in IP buffer (50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Trition X-100) supplemented with protease inhibitor cocktail (Thermo scientific) on ice for 20 min, centrifuged at highspeed for 15 min and the supernatants were collected in fresh tube. For IP,1–2 mg lysate was incubated with 30–50 µl Flag agarose beads (sigma Aldrich) overnight at 4°C. The resulting immunoprecipitants were washed at least three times in IP buffer, before boiling with 4X SDS sample buffer. Samples were used for western blot analysis.

Proximity Ligation Assay (PLA Assay)

To detect the protein – protein interaction with two different antibodies from two different species accurately proximity ligation assay was used. To visualize the close proximity between AKT and Calmodulin in U2OS cells. $4X10^4$ cells were seeded in four well plate and after 6 hours arsenite was treated. After treatment, cells were washed with 1X cold PBS then fixed with 4% paraformaldehyde for 10 min in the dark, again washed twice with 1X cold PBS, permeabilized with 1% triton X-100 for 5 minutes and the dark washing with 1X cold PBS was repeated. Both antibodies were taken equally kept it in RT for 1 h in the dark washed twice with 1X cold PBS, both PLA probes from different species were used. Incubated for 1h at 37°C, washed with buffer A. Diluted ligation buffer was added and incubated for 30 min at 37°C, washed with buffer A again for two times. Polymerase was used for signal amplification and incubated for 100 min at 37°C, washed with 1X buffer B twice mounted with standard DAPI containing mounting medium. Signal intensities were measured by image j analysis software.

Statistical analysis

All the results are presented as mean±SEM. Prism 5.0 was used for data analysis. Student's t-test is used to confirm the statistical significance of the data. Differences were considered statistically significant at values of $p<0.05$.

III. Results

Ca^{2+}/Calmodulin signaling involvement in Stress granule assembly

Calcium is a vital secondary messenger. Maintaining the cellular calcium homeostasis is important for normal cellular functions[30]. Calmodulin is a calcium sensing protein, which carries calcium to regulate intracellular calcium level[31]. Formation of stress granule will be hindered once there is a change in extra and Intracellular calcium levels (Supplementary Fig. 1&2). To further prove this phenomenon, we designed three different siRNAs to target all the three CaM isoforms (CALM1, CALM2 and CALM3) and the stress granule assembly was monitored in a time dependent arsenite treatment using SG marker eIF3b and EIF4G. There was a significant impairment in the stress granule assembly upon the CaM knockdown (Fig 1a). The immunoblot data clearly shows the endogenous CaM knock down (Fig.1a, c). In order to validate this result, CaM antagonist W5 and W7 were pretreated for 30 minutes followed by arsenite treatment in U2OS cells. This inhibits the SG assembly strongly in a dose dependent manner (Fig.1d).

To examine the association of Stress granule assembly with Calcium and Ca^{2+}/Calmodulin pathway, we used calcium chelating agent – EGTA Ethylene glycol tetra acetic acid (EGTA) (chelates calcium at extracellular level) and BAPTA-AM (chelates calcium at intracellular level). To study calcium influx/outflux in intracellular at cellular organelle level, a plasma membrane Ca^{2+} channel blocker Lanthanum Chloride (LaCl3)[32-34] and mitochondrial calcium uniporter inhibitor - Ruthenium red (RuR)[35] were used.

P-MAPK assay Screening

We wanted to rule out what are all the Human P-MAPK kinases involved upon calmodulin inhibitor pretreatment either in the presence or absence of arsenite treatment (Supplementary Fig. 5). Three important kinases such as AKT, JNK and ERK were observed. Knock down of Calmodulin data results were also indicating the same (Supplementary Fig. 6).

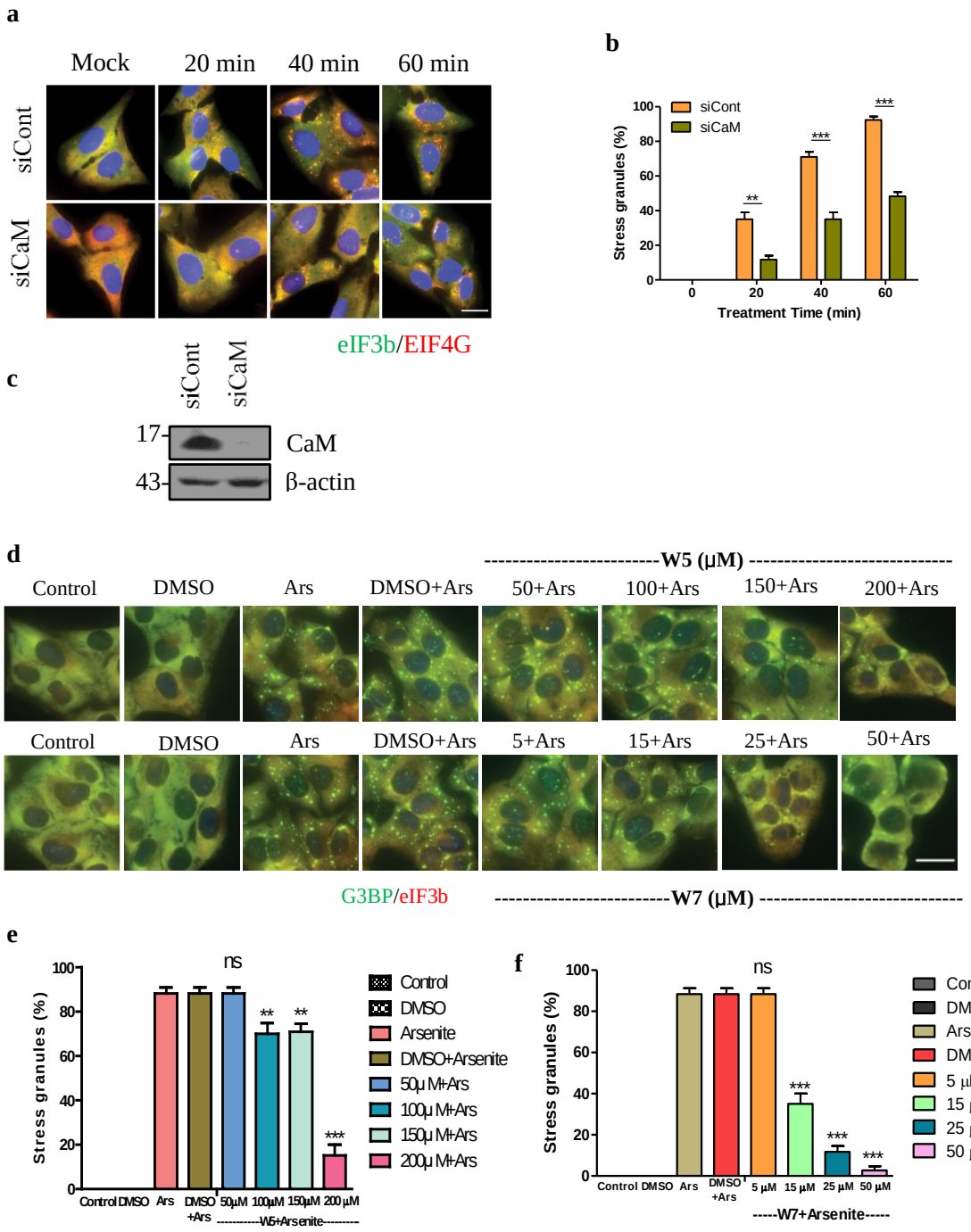


Fig. 1 Ca^{2+}/Calmodulin signaling involvement towards stress granule assembly. (a) U2OS cells transfected with siCONT or siCaM for 72 h were cultured in the absence or presence of arsenite (0.2 mM) for indicated time points, and then immunostained against SG marker eIF3b (green), EIF4G(red). Nuclei are counterstained with Hoechst (Blue) (**b**) Bar graph representing

the percentage of cells bearing SGs. Error bars indicate s.t.d. (*n*=3). ***P*<0.01, ****P*<0.001, Student's *t*-test. **(c)** Knock down efficiency of CaM was assessed by immunoblot analysis. **(d)** U2OS cells were pretreated with CaM antagonist W5 and W7 for 30min subsequently 0.2 mM Arsenite treatment was given for 1h. Immunostained with G3BP(green) and eIF3b(red). **(e & f)** The graphs show the percentage of cells bearing SGs. Error bars indicate s.t.d. (*n*=3). ***P*<0.01, ****P*<0.001, Student's *t*-test. ns means non-significant. Scale bar 20μM.

Calmodulin and AKT are components of stress granules

Co-localization is a rapid way to find the association between two fluorescently labeled proteins[36]. Calmodulin and AKT both were colocalized with global SG marker G3BP (Fig 2, a-d). We used florescence microscopy technique to reveal that CaM and AKT are part of SG by using antibodies against CaM and AKT. Both proteins were localized in cytoplasmic region. In stressed cells CaM and AKT are strongly colocalize with SG marker (Fig 2, a-d). Two different siRNAs were designed to target AKT's coding region and 3'UTR region. We knocked down both AKT siRNAs those inhibit stress granules in the U2OS cells (Fig. 7). Three different stresses were used, such as, Arsenite (Oxidative stress), Thapsigargin (ER Stress) and Clotrimazole (mitochondrial stress). The results from all these experiments propose that calmodulin was active in all the three different stresses.

Calcium regulates AKT activation

Phosphorylation of AKT is also induced by extracellular and intracellular calcium levels. The presence of Lacl3 and RuR proves it prominently[37, 38]. Increased cytosolic Ca^{+2} increases the sensitivity of cells to apoptosis *via* activation of the calmodulin-dependent kinase II (CaMKII) signaling cascade. $LaCl_3$ affects calcium flow through regulating plasma membrane channel[3-5]. SG assembly was inhibited in the U2OS cells which were treated with $LaCl_3$ followed by arsenite treatment and later (Supplementary Fig. 3) RuR was treated along with arsenite. This also had the same level of SG inhibition showing that the mitochondrial calcium influx/outflux could affect SG formation (Supplementary Fig. 4). In both $LaCl_3$ and RuR pretreated conditions, SG has been inhibited significantly upon arsenite treatment (Fig. 6 b & c).

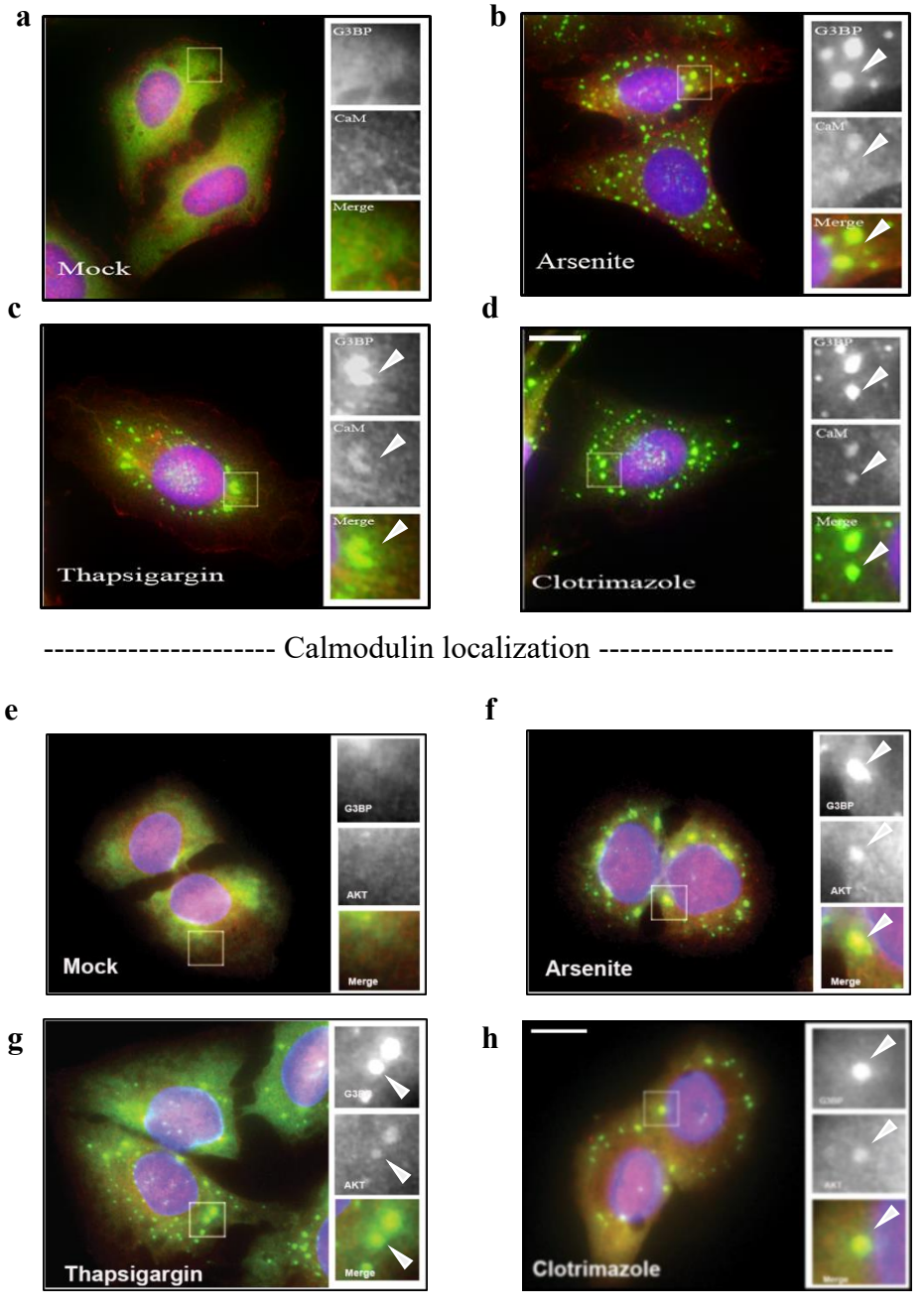


Fig. 2 Calmodulin and AKT are components of SG's. HeLa Cells either **(a)** untreated or treated with **(b)** Arsenite, 0.5mM **(c)** Clotrimazole, 40μM and **(d)** Thapsigargin, 1 μM for 1h were immunostained against SG marker G3BP (Green), CaM (Red) and nuclei (Blue). HeLa cells were either **(e)** untreated or treated with **(f)** arsenite, 0.5mM **(g)** Thapsigargin, 1 μM and **(h)** Clotrimazole, 40μM for 1h were immunostained against SG marker G3BP (Green), AKT

(Red) and nuclei (Blue) Boxed regions are enlarged as both merged and separate colored views. White arrow heads indicating the SGs. Scale bar 20μm.

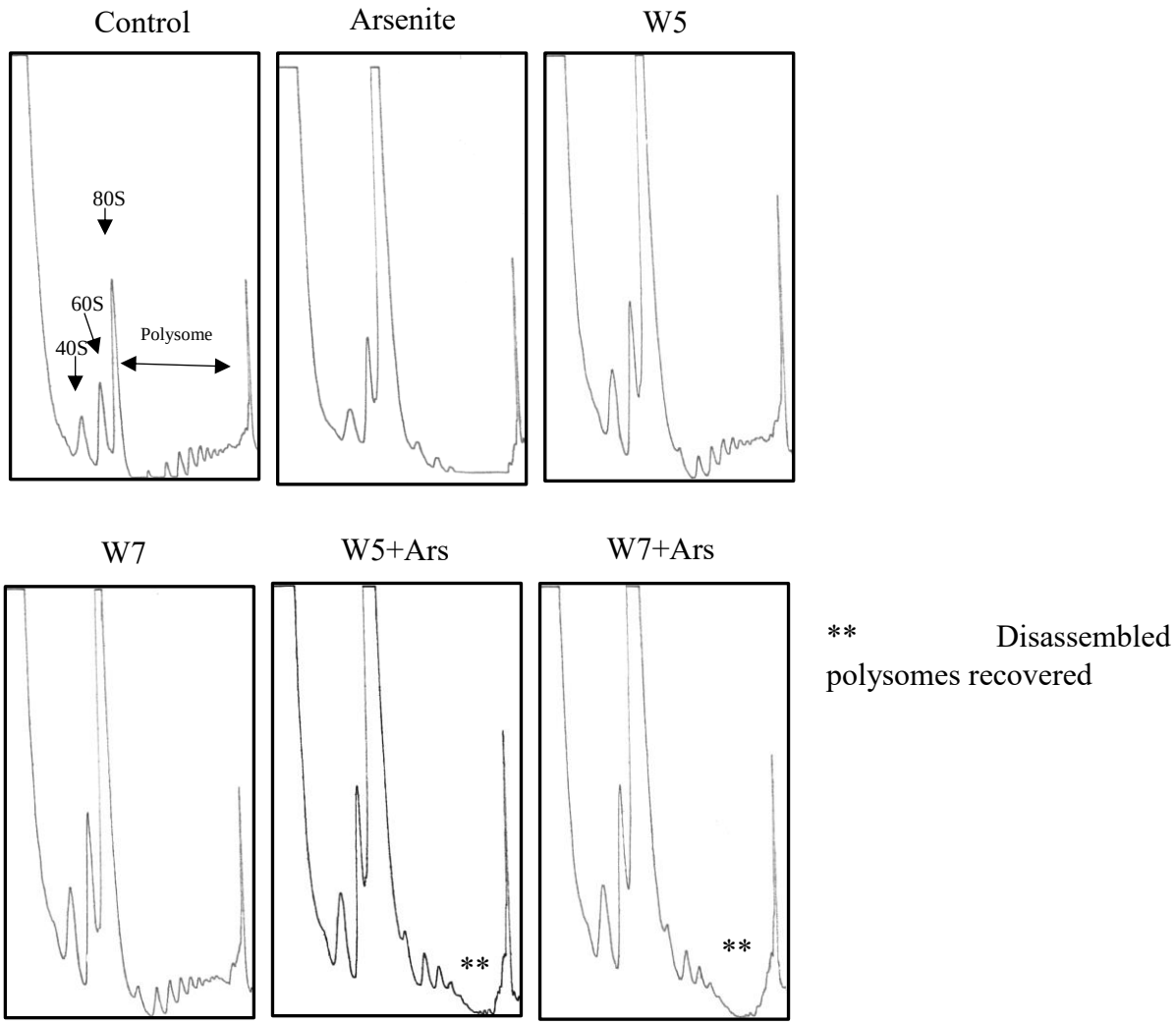


Fig. 3 Arsenite induced polysome disassembly is recovered due to CaM antagonist W5 and W7 pretreatment. U2OS cells untreated /treated with W5 and W7 for 30min with or without 0.2mM Arsenite for 1h.

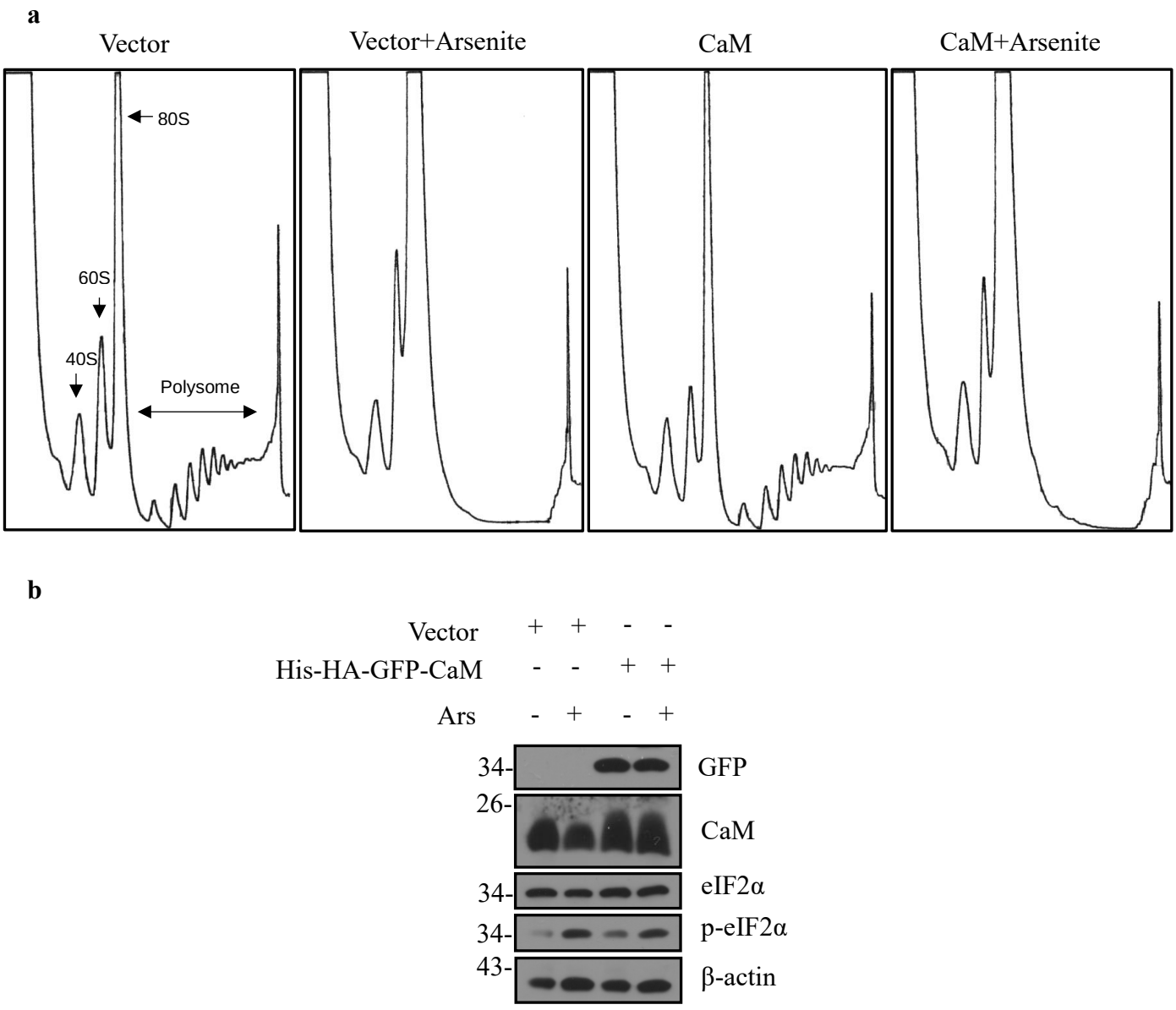


Fig. 4 Overexpression of Calmodulin doesn't affect polysome disassembly either in non-treated/treated condition. (a) U2OS cells were transfected with above mentioned plasmids Polysome Analysis in U2OS cells untreated /treated with W5 and W7 for 30 min with or without 0.2mM Arsenite for 1h. **(b)** Transfection efficiency was assessed by immunoblot analysis.

Ca^{2+}/ Calmodulin complex is needed for eIF2α phosphorylation

Calcium could not function in its free ionic form therefore it needs calmodulin like calcium binding proteins for its proper function[31]. eIF2α phosphorylation and cytosolic Ca^{2+} both were increased simultaneously while cell faces stress [39]. During stressful environment eIF2α phosphorylation is involved in cell survival through preventing apoptosis[40] via activating four various kinases of eIF2α[2]. From our data we found that Calcium dependent Calmodulin is needed for eIF2α phosphorylation (Fig 5, c).

According to Guo, Y. *et al* PERK/eIF2α/ATF4/CHOP pathway is a major requirement for cell survival during ER stress[41]. Based on our results we could deduce that HRI/eIF2α/ATF4 is also involved in cell survival. Other AKT inhibitor results also support the same (Fig. 9).

AKT is an interaction partner of CaM and eIF2α

Next, we wanted to examine AKT's role in cell survival mechanisms in eIF2α mediated SG assembly. We found that AKT interacts with eIF2α and CaM upon arsenite treatment. Results from PLA assay (Proximity Ligation Assay) also highlights the same. AKT and CaM antibody was used for this assay to find the endogenous level of protein-protein interaction. As we stated before AKT is a stress granule component (Fig. 2), prolonged oxidative stress leads to increased eIF2α phosphorylation and AKT activation can also induce SG assembly (Fig. 5a). Because both involve in the prevention of cell apoptosis.

When cells were in unpleasant environment eIF2α get phosphorylated at ser 51 region and as a continuous process polysomes where disassembled. SG assemble at this point to prevent apoptosis[42]. eIF2α interact with AKT upon arsenite treatment (Fig. 8d) but Flag- eIF2α-S51D mutant has no interaction with AKT (Fig. 8e). From this observation we can suggest that AKT is involved in apoptosis by binding eIF2α in Flag- eIF2α-S51A site not in Flag- eIF2α-S51D.

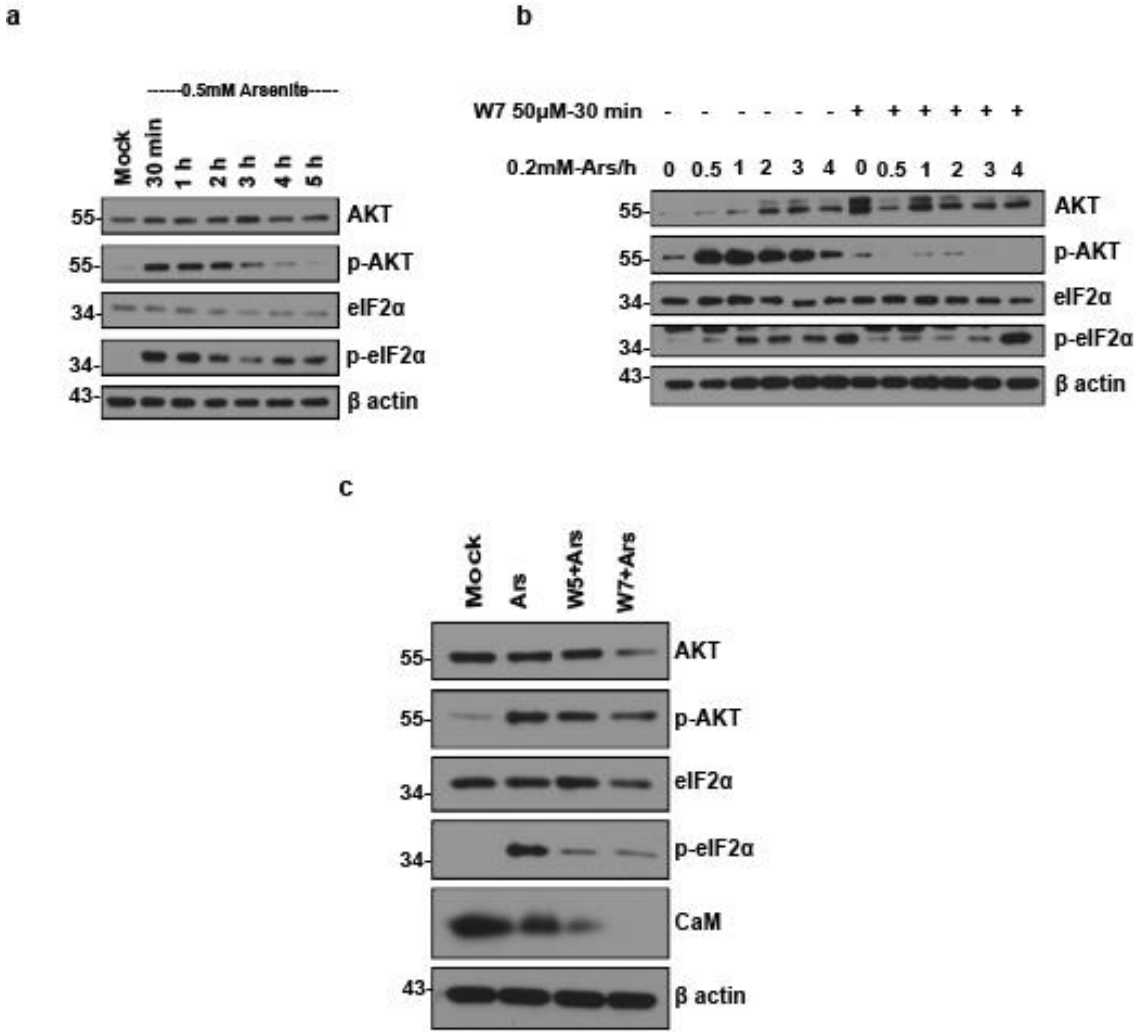


Fig. 5 Calmodulin is required for AKT activation and eIF2α phosphorylation. (a) U2OS cells were treated with arsenite in time dependent manner. **(b)** U2OS cells were treated with arsenite in time dependent manner then CaM inhibitor was treated as indicated. **(c)** As in (b) cells were pretreated with CaM antagonist (W5 & W7 for 30 min) followed by Arsenite treatment for 0.5mM-1 h.

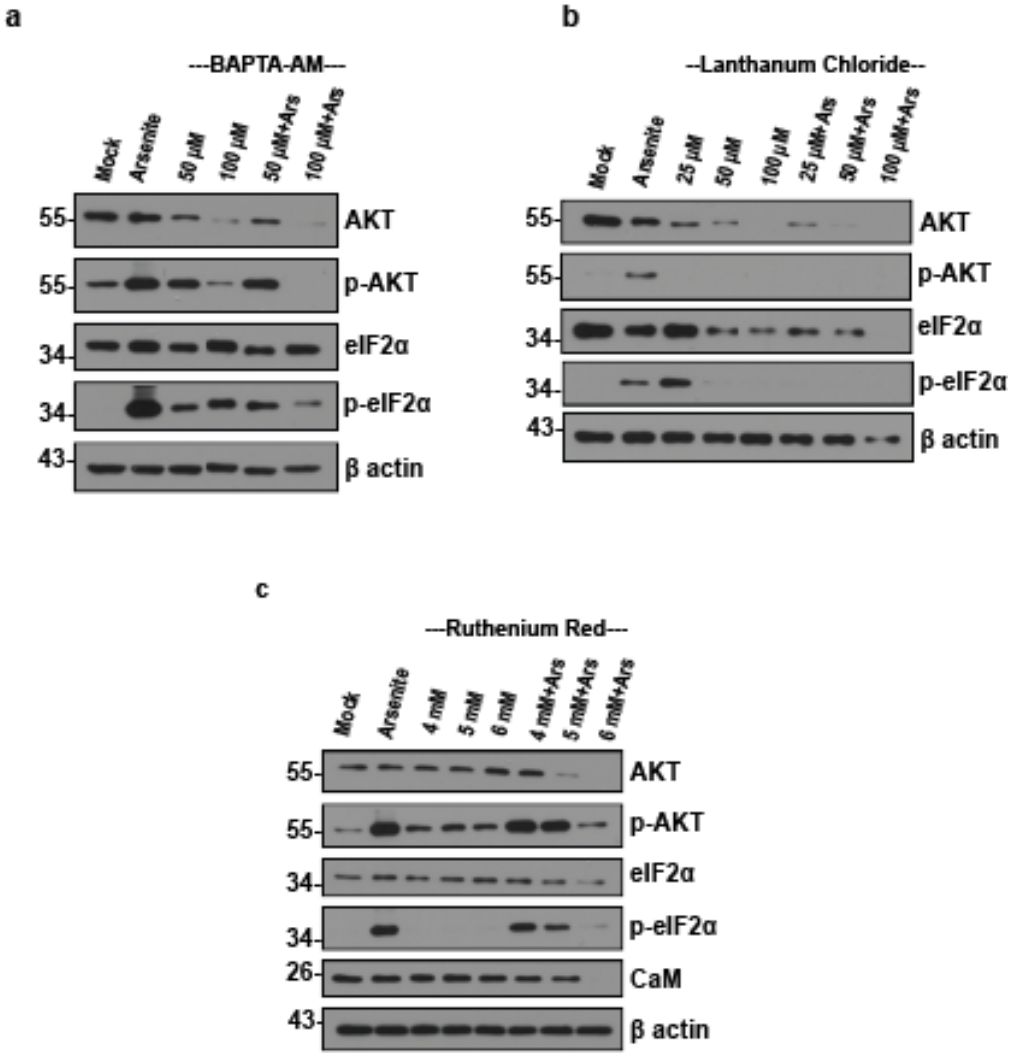


Fig. 6 Calcium is required for AKT activation and eIF2α phosphorylation. (a) U2OS cells were pretreated with BAPTA-AM for 12 h with or without arsenite (0.5mM -1 h). **(b)** U2OS cells were pretreated with Lanthanum chloride ($LaCl_3$) for 5 h with or without arsenite treatment **(c)** U2OS cells were pretreated with Ruthenium Red (RuR) for 5 h with or without arsenite treatment (0.5mM -1 h).

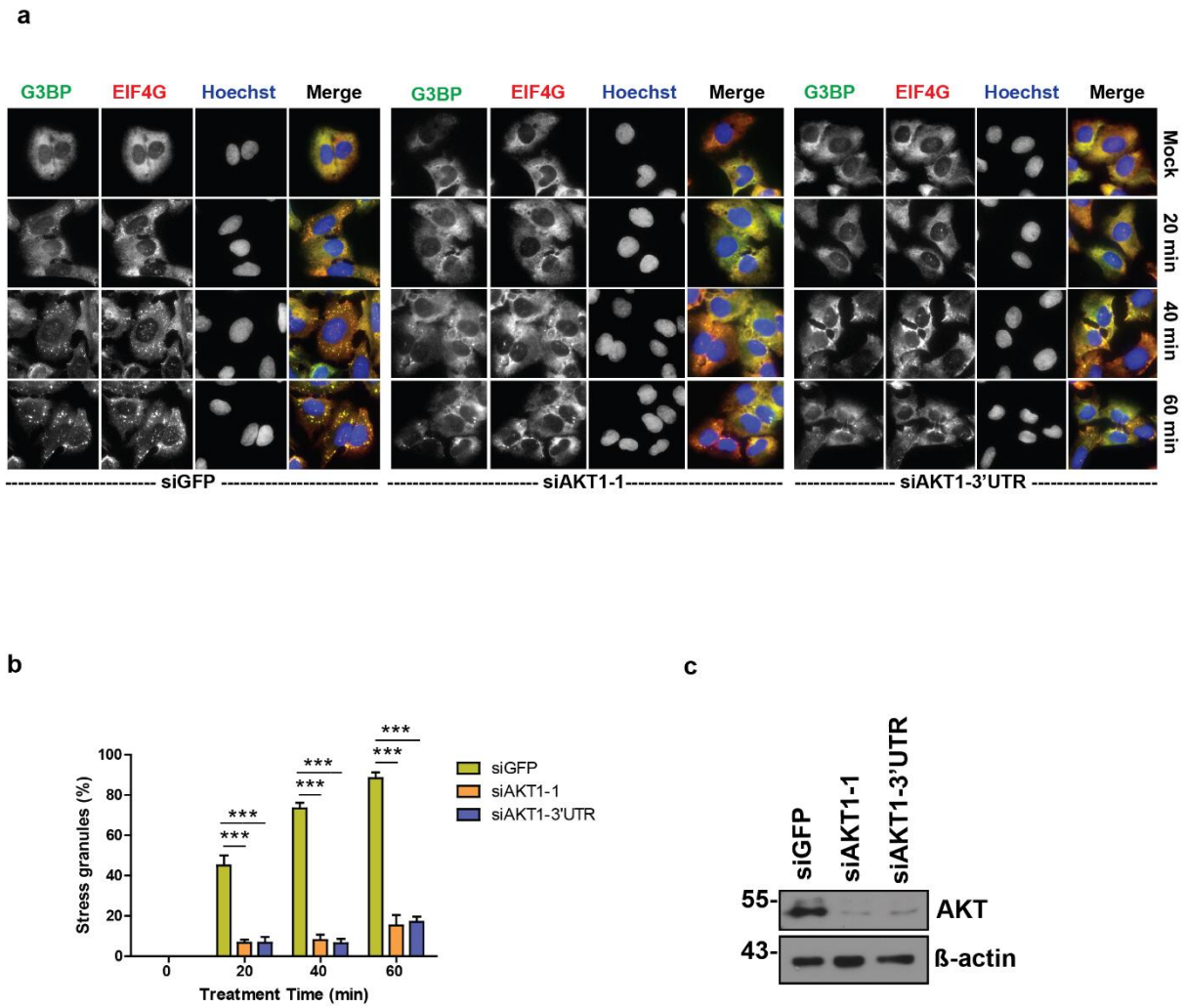


Fig. 7 AKT knock down impairs stress granule assembly. (a) U2OS cells transfected with siCONT, siAKT1-1 and siAKT1-3'UTR for 72 h were cultured in the absence or presence of arsenite (0.2 mM) for indicated time points, and then immunostained against SG marker G3BP (green) and EIF4G(red). Nuclei are counterstained with Hoechst (Blue) (**b**) Bar graph representing the percentage of cells bearing SGs. Error bars indicate s.t.d. (n=3). **P<0.01, ***P<0.001, Student's t-test. **(c)** Knock down efficiency of AKT1-1 and AKT1-3'UTR was assessed by immunoblot analysis.

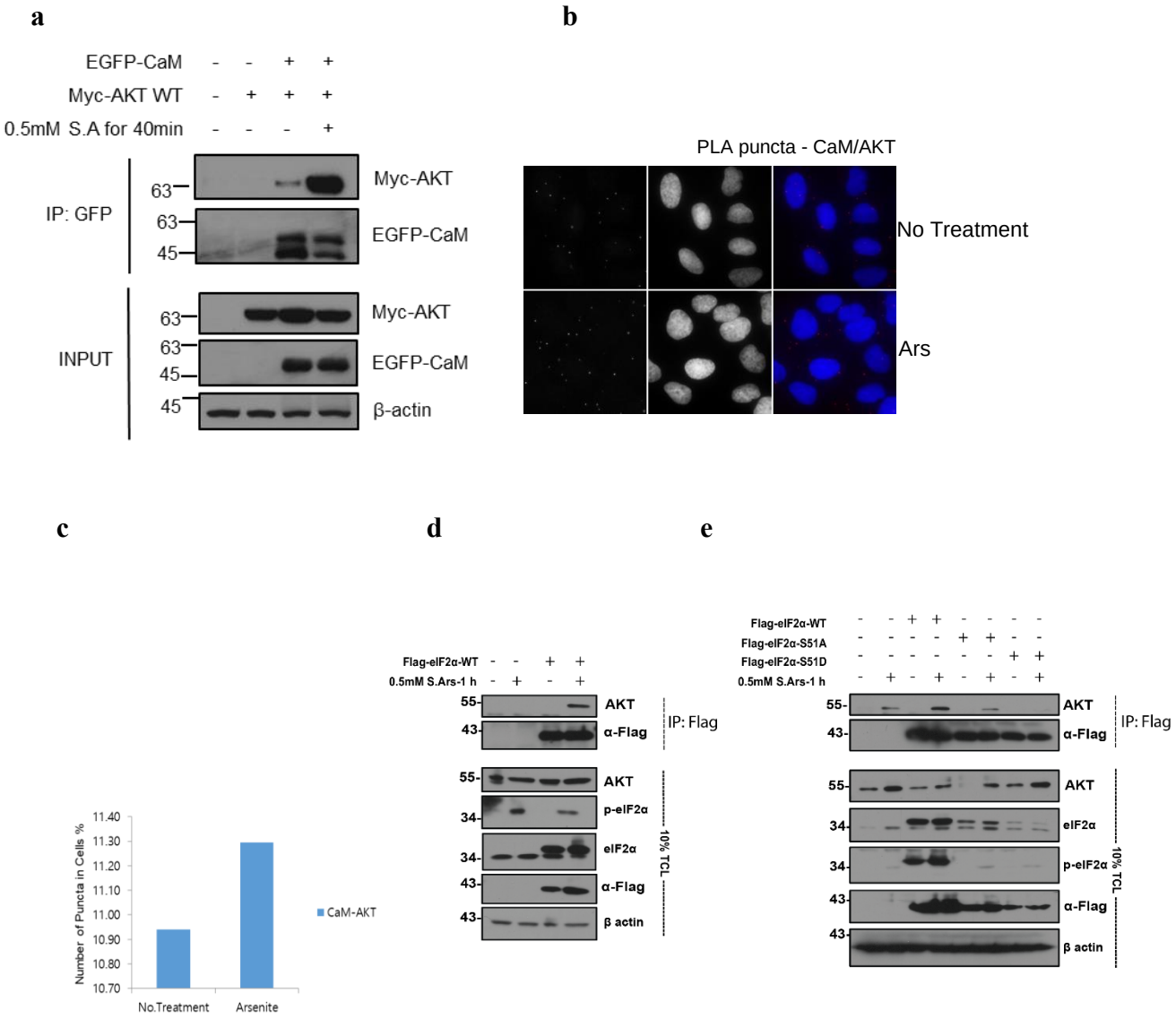
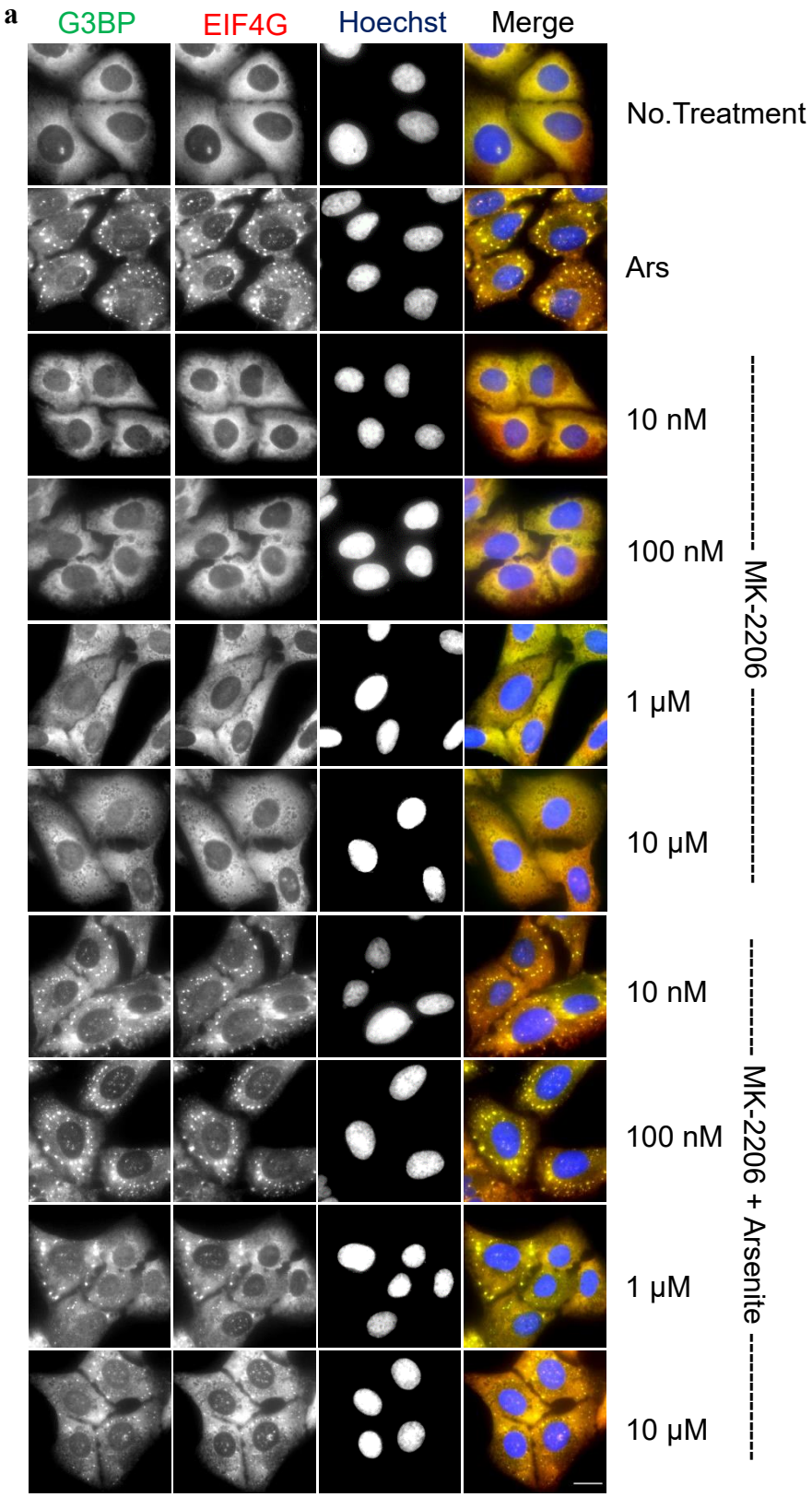


Fig. 8 AKT interacts with Calmodulin and eIF2α upon arsenite treatment.

(a) HEK293 cells were transiently co-transfected with indicated plasmids. After36 h, cells were treated with either No treatment or arsenite, lysed using IP buffer protein A affinity resin was used (see Methods). The precipitates were then blotted against anti-GFP antibody. **(b)** U2OS cells were used **(c)** Graph represents the percentage of PLA

punctas in untreated/treated condition in U2OS cells. **(d)** eIF2α interact with AKT upon arsenite treatment. HEK293 cells transfected with empty vector or Flag– eIF2α were treated with arsenite and immunoprecipitated using Flag agarose beads (see Methods). **(e)** Flag-eIF2α-S51D has no interaction with AKT upon arsenite treatment. HEK293 cells transfected with mentioned plasmids for 36 h, cells were treated with arsenite and pull down with Flag-agarose beads.



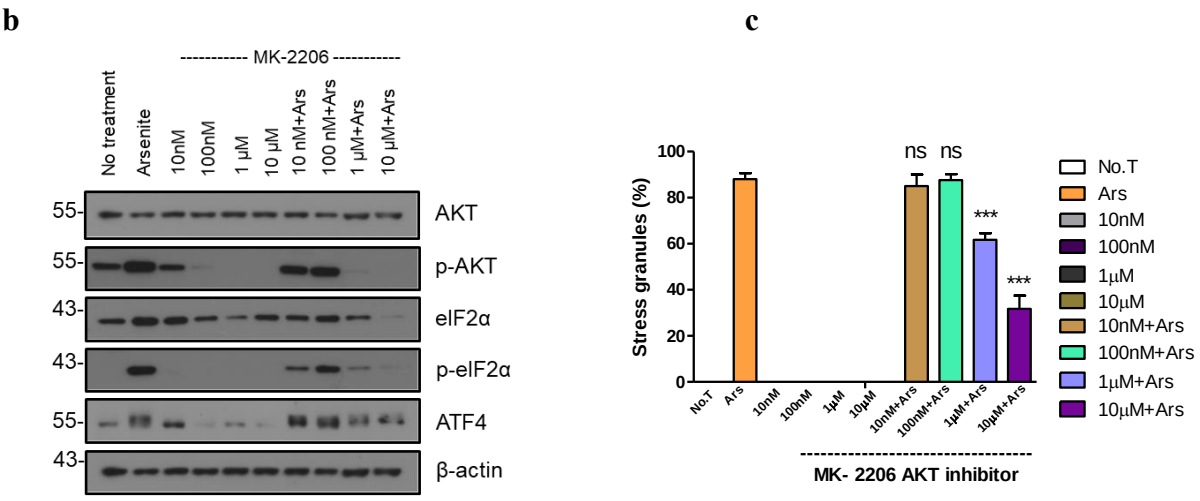


Fig. 9 AKT is necessary for eIF2α/ATF4 signaling pathway. (a) U2OS cells pretreated with MK-2206 inhibitor (which inhibits AKT specifically) for 48 h followed by 0.2mM Arsenite for 1 h. Stained with G3BP(green) and EIF4G(red). **(b)** AKT inhibition affects eIF2α phosphorylation and upregulates ATF4 expression upon arsenite treatment. **(c)** Bar graph showing the percentage of cells bearing SGs. Error bars indicate s.t.d. (n=3). **P<0.01, ***P<0.001, Student's t-test. ns - non-significant. Scale bar 20μm.

IV.Discussion

As per our previous study we came to know that calcium signaling pathway's involvement towards stress granules (SG) assembly upon arsenite treatment[43]. We show that Ca^{2+}/Calmodulin acts an important role in the assembly of stress granules. Previously few studies have been done in plants regarding the importance of exogenous and endogenous calcium levels upon various stresses[44,18]. Several forms of cell death such as necrosis, apoptosis and autophagy are also regulated by calcium (Ca^{2+})[45]. Diverse functions of calcium regulations can be seen in cardiac muscles contraction, neuroprotection, cellular responses to ionizing radiation[17, 46, 47] and the list goes on. From our experiments we found that Ca^{2+}/Calmodulin signaling pathway is essential for Stress granule assembly (Fig.1a - f) and the targeted depletion of calmodulin inhibits the arsenite-induced assembly of SG (Fig.1a - c). This advocates the necessity of CaM in the RNA granule assembly. Cellular functions were maintained by strictly controlling calcium homeostasis[48].

Activation of eIF2α and its phosphorylation upon various stresses have been widely studied in the past. Its role in numerous cellular processes like cell cycle, cell survival, translation and regulation have also been reported[49-51]. eIF2α plays a major role in translation by inhibiting the formation of stress granules which are required for the polysome disassembly.

Of note CaM inhibitor delays polysome disassembly, this indirectly shows the relationship between translation initiation factor eIF2α and calcium binding protein CaM on oxidative stress (Fig. 3). AKT activation was gradually increased upon the increase in arsenite stress level (Fig.5 a), whereas, absence of CaM disturbs the expression of eIF2α and AKT phosphorylation on stressful circumstances (Fig.5 b & c). CaM antagonist (W5 and W7) immunostaining results also shows the inhibition of SG (Fig.1, d-f).

PI3K/AKT signaling is activated in cancers and controls tumor initiation and progression[52]. The AKT response towards various stresses is poorly understood. Here we identify CaM as an essential regulator for AKT activation and eIF2α phosphorylation by arsenite stress (Fig. 5 b & c). AKT phosphorylates in two different sites Thr408 and Ser473.

Phosphorylation at Ser473 is required for the full activation of AKT, it then phosphorylates numerous downstream target proteins to regulate various biological processes which are necessary for cell survival, growth, proliferation, angiogenesis and metabolism[53]. Comparatively AKT-CaM interaction[29] was well studied than AKT- eIF2α interaction. Though both AKT and eIF2α phosphorylation involved in cell survival.

AKT knock down indicate that it is a component of SG (Fig. 7). MK-2206 dihydrochloride, an allosteric AKT1, AKT2 and AKT3 specific inhibitor which inhibits AKT phosphorylation, downregulates eIF2α phosphorylation and upregulates ATF4 on Stressful condition. Immunocytochemistry image analysis clearly shows the inhibition of SGs during the treatment of MK-2206 along with arsenite treatment (Fig. 9, a). In future it is essential to study the interactions between eIF3B versus AKT and AKT versus GSK3β in molecular biology level to confirm the role of Ca^{2+}/Calmodulin in eIF2α phosphorylation and the activation of AKT.

In conclusion, calmodulin knock down impairs stress granule assembly. Arsenite treated polysome disassembly was delayed upon CaM inhibition. Simultaneously, overexpression of CaM hasn't changed the polysome distribution. CaM and AKT are components of Stress granules. Ca^{2+}/Calmodulin is essential for AKT activation and eIF2α phosphorylation in response to arsenite treatment. By treating AKT inhibitor (MK-2206) eIF2α phosphorylation was down regulated, meanwhile Activating Transcription Factor (ATF4) translation was upregulated during oxidative stress. We found AKT's interaction between CaM and eIF2α during the oxidative stress condition. Calcium signaling mediate the AKT pathway to act in cell survival mechanisms through HRI/eIF2α/ATF4 pathway. Understanding the role of AKT in cell survival mechanism will help us to drive it towards therapeutic aspects.

Supplementary figure-1

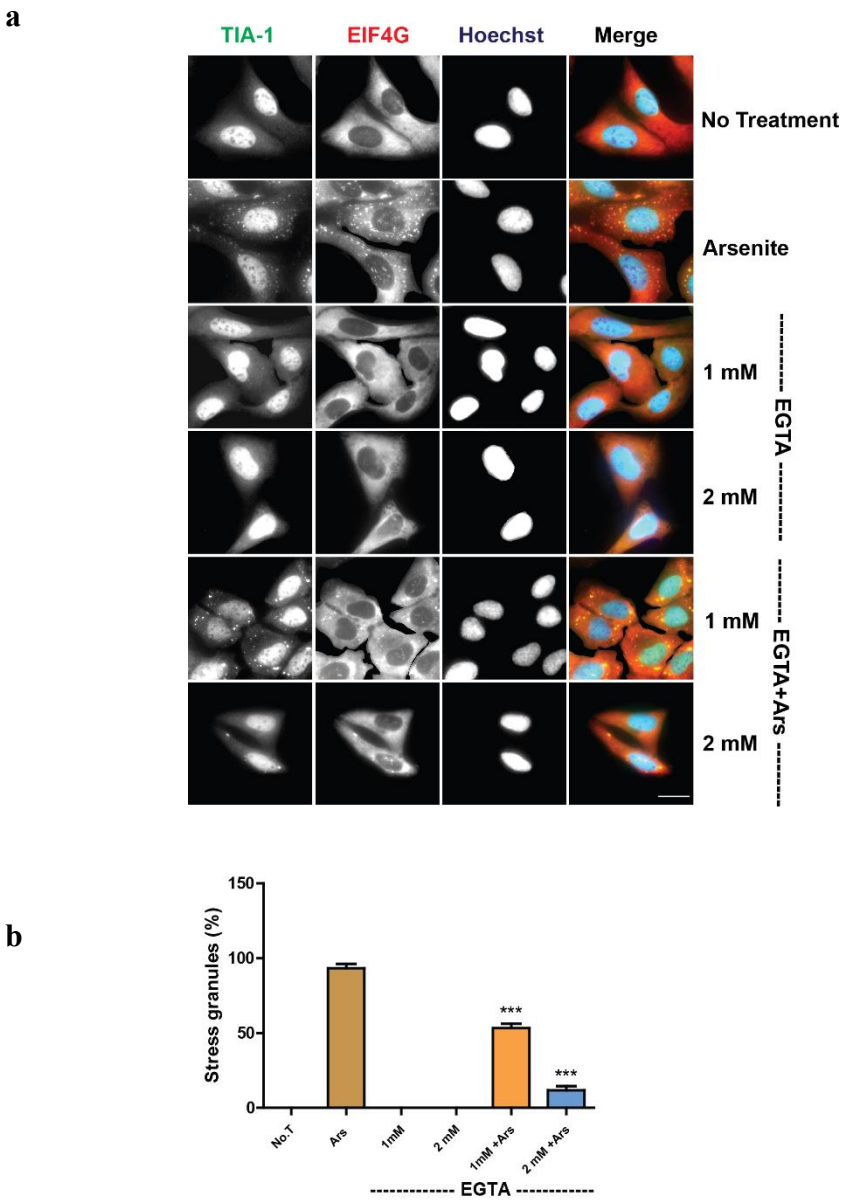


Fig. 1 Calcium chelator EGTA inhibits stress granule assembly. (a) U2OS cells pretreated with EGTA (which chelates calcium in extracellular level) for 12 h followed by 0.2mM Arsenite for 45 min. TIA-1(green) and EIF4G(red) was used for immunostaining. **(b)** Bar graph showing the percentage of cells bearing SGs. Error bars indicate s.t.d. (n=3). $^{**}P<0.01$, $^{***}P<0.001$, Student's t-test. Scale bar 20μm.

Supplementary figure-2

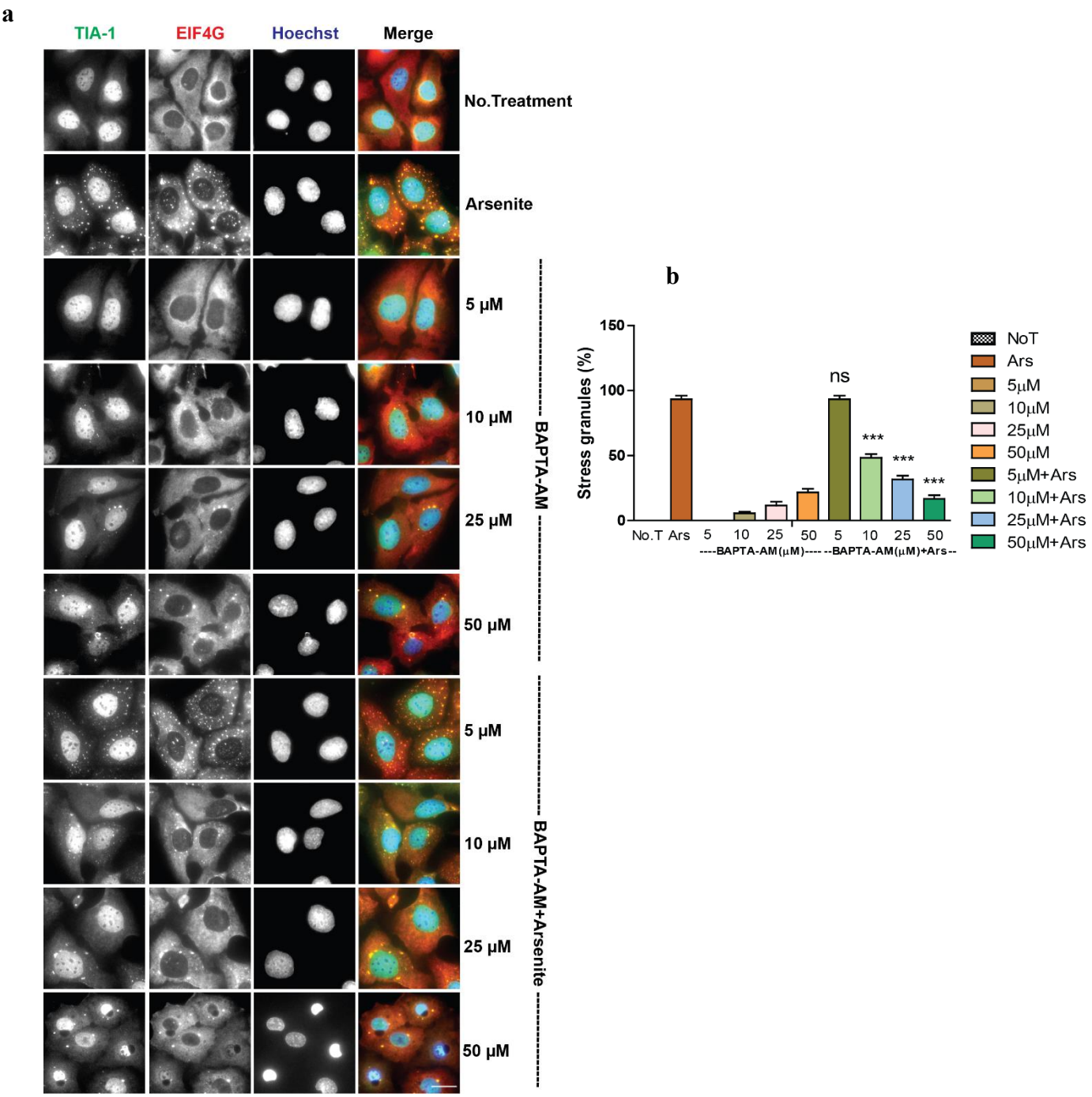


Fig. 2 Calcium chelator BAPTA-AM inhibits stress granule assembly. (a) U2OS cells pretreated with BAPTA-AM for 12 h followed by 0.2mM Arsenite for 45 min. TIA-1(green) and EIF4G(red) was used for immunostaining. **(b)** Bar graph showing the percentage of cells bearing SGs. Error bars indicate s.t.d. (n=3). $^{**}P<0.01$, $^{***}P<0.001$, Student's t-test. Scale bar 20μm.

Supplementary figure-3

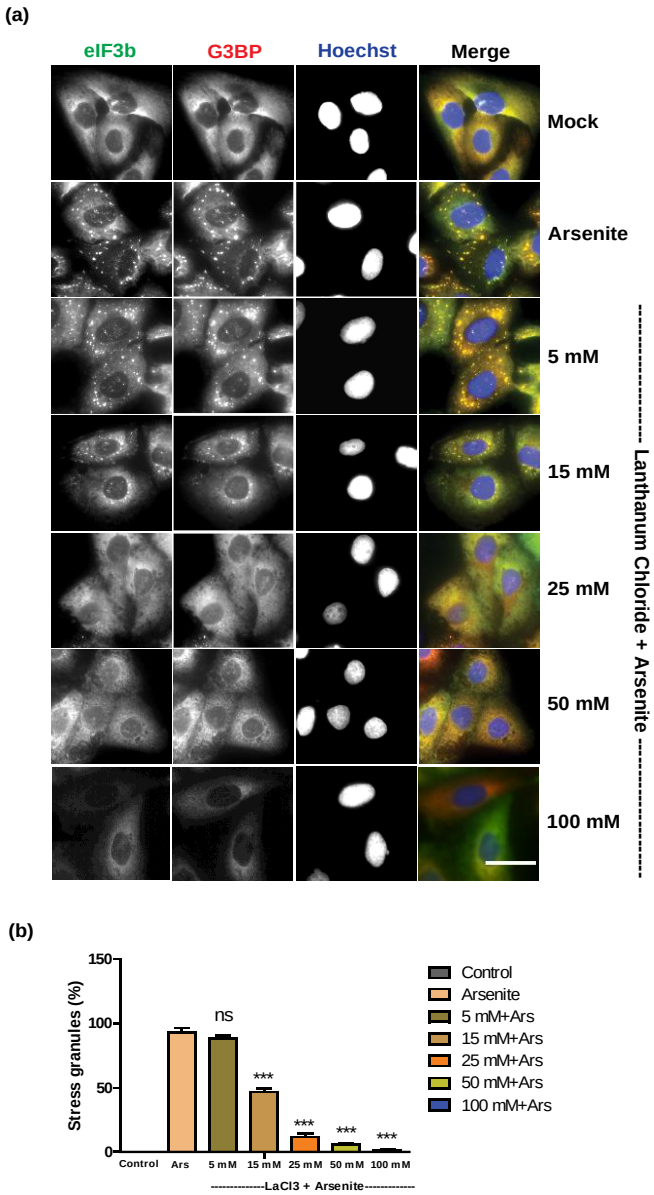
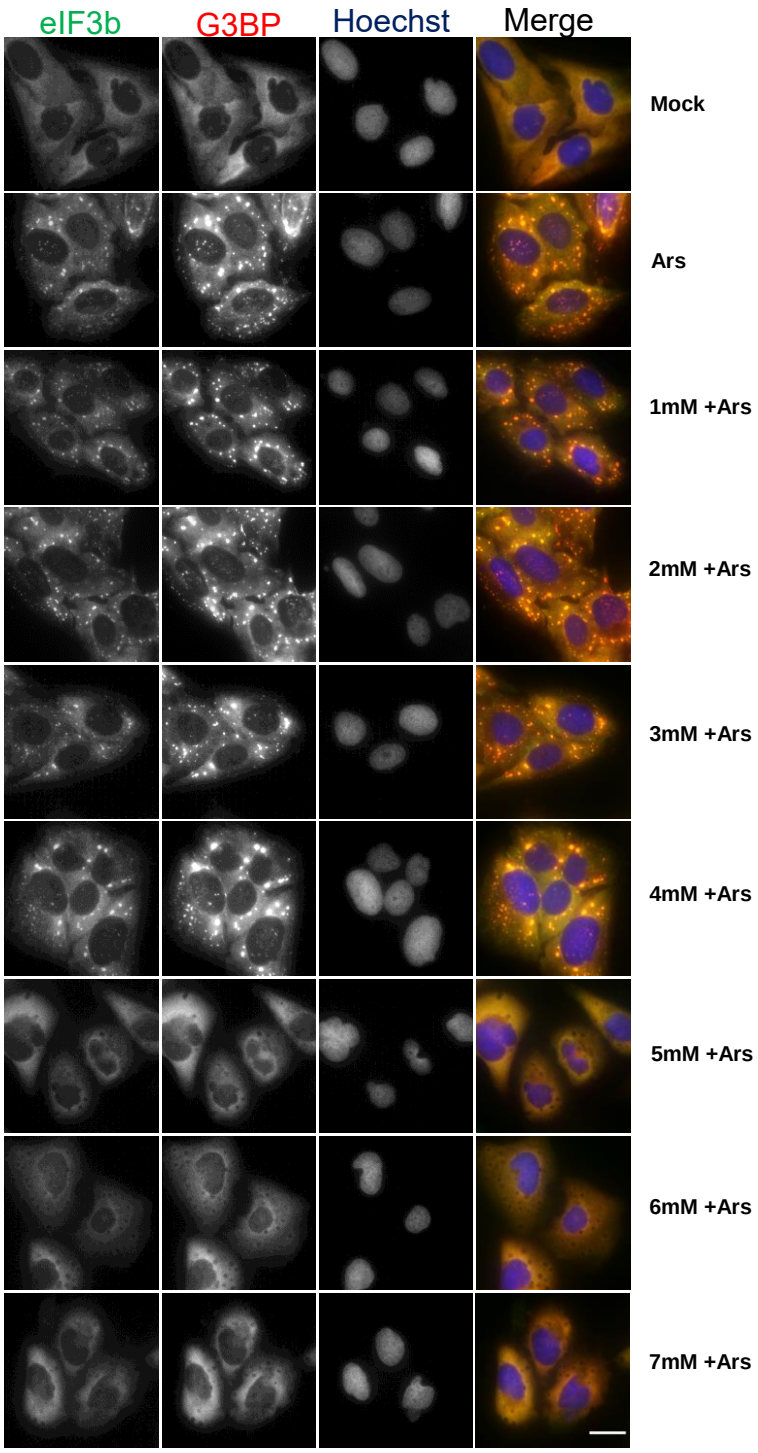


Fig. 3 Blocking of Calcium channels by LaCl3 inhibits SG formation upon Arsenite Treatement. (a) U2OS cells were treated with LaCl3 for 4h 30min prior to Arsenite treatment (0.2mM- 1h). **(b)** Bar graph shows the percentage of cells having SGs. Error bars indicate s.t.d. (n=3). **P<0.01, ***P<0.001, Student's t-test. Scale bar, 20 μm.

Supplementary figure-4

a



b

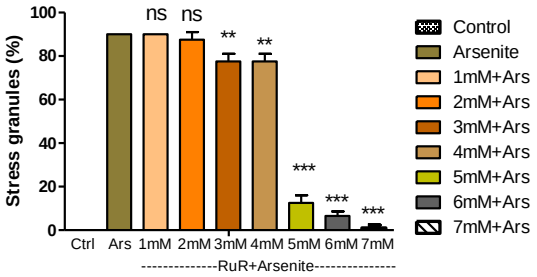
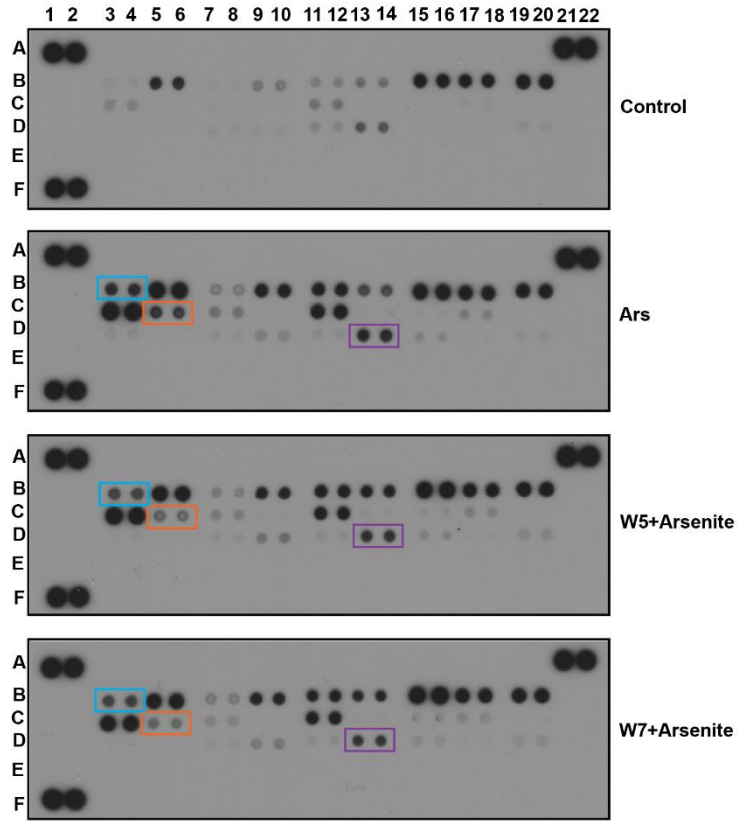


Fig. 4 Arsenite induced Stress granules was inhibited by Ruthenium Red (RuR) treatment. **(a)** U2OS cells were treated with RuR for 4h 30min prior to Arsenite treatment (0.2mM- 1h). **(b)** Bar graph shows the percentage of cells bearing SGs. Error bars indicate s.t.d. (n=3). **P<0.01, ***P<0.001, Student's t-test. Scale bar, 20 μm.

Supplementary figure-5

a



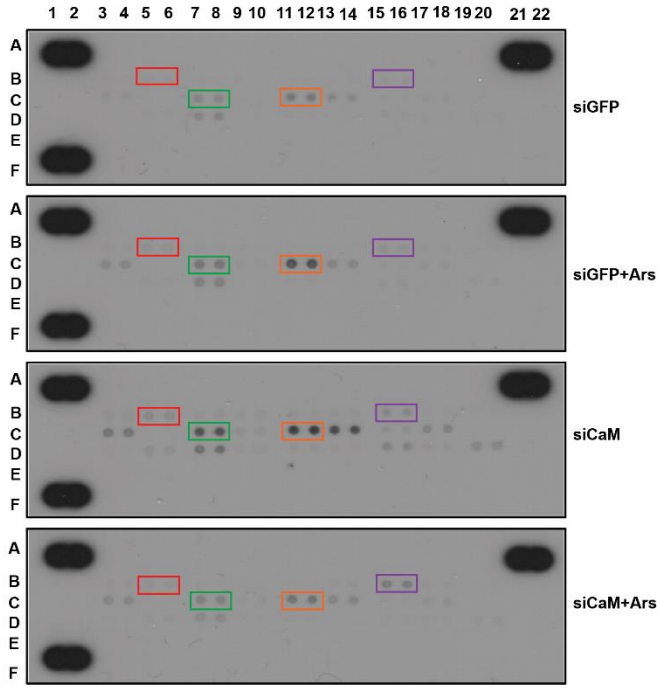
b

No	Co.Ordinate No	Control Vs Ars	Ars Vs W5+Ars	Ars Vs W7+Ars
1	B3 & B4(AKT1)	Increased	Decreased	Decreased
2	C5 & C6(JNK1)	Increased	Decreased	Decreased
3	D13 & D14(ERK1)	Increased	Decreased	Decreased

Fig. 5 The Human Phospho-MAPK Array shows the effect of Calmodulin inhibitors on specific pathways. (a) Human U2OS cells were untreated or treated with CaM antagonist W5 - 200 μM and W7 - 50 μM for 30minutes followed by 0.2mM Arsenite Treatment for an hour. **(b)** The table shows the result of differences in the kinase expression. All array were incubated with 200 μg of lysate.

Supplementary figure-6

a



b

No	Co.Ordinate No	siGFP Vs siGFP+Ars	siGFP+Ars Vs siCaM	siCaM Vs siCaM+Ars
1	B5 & B6(AKT1)	Increased	Increased	Decreased
2	C7 & C8(JNK)	Increased	Increased	Decreased
3	C11 & C12(JNKpan)	Increased	Increased	Decreased
4	B15 & B16(ERK)	Increased	Increased	Increased

Fig.6 The Human Phospho-MAPK Array shows the knock down efficiency of Calmodulin on specific pathways. (a) U2OS cells were transfected with control (siGFP) or siCaM for 72 h were cultured in the absence or presence of arsenite (0.2 mM) for 1h. (b) The table shows the result of differences in the kinase expression. All array were incubated with 200 μg of lysate.

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ABSTRACT

Functional Study of Calcium-Calmodulin signaling in the assembly of Stress granule

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Stress granules are functional by-products of cells in stressful environments and are known to be involved in degenerative brain disease and cancer cell tolerance. To date, studies on stress granules have been actively progressing, but the signal transduction system for stress granule formation are not known in detail. In addition, the experimental results indicate that Calmodulin, in a mediator of intracellular calcium signaling, is required. Furthermore, we tested a variety of phosphatase enzymes that play an important role in stress cell signaling, revealing that Akt kinase plays a decisive role as a sub-factor of Calmodulin. Through this study, a new cellular signal transduction system involved in the formation of stress granules was identified and it is expected to contribute a lot to future disease association studies.

Analysis of MeCP2 protein in Stress Granule Assembly

CONTENTS

CHAPTER-2

LIST OF FIGURES FOR CHAPTER-2

국문초록

스트레스 과립 집합체에서 MeCP2 단백질 분석

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의과학과

Ribonucleoprotein (RNP) 과립은 세포질에 위치한 막이 없는 RNA 결합 단백질(RBP)이다. DNA 메틸화는 스트레스 과립 형성의 조립 및 분해를 제어할 수 있는 번역 후의 변형 중 하나입니다. 추가적인 연구를 위해 나는 메틸 CpG 결합 단백질 2 (MeCP2)와 관련된 실험을 설계했다. 나는 MeCP2가 SG의 새로운 구성 요소로서 MeCP2의 녹다운이 SG 형성을 강하게 억제한다는 것을 발견했다. 또한 나는 MeCP2가 다양한 스트레스 조건 하에서 특별히 SG 에 위치한다는 것을 입증했다. HAP1 세포에서 MeCP2의 녹아웃은 스트레스 유발 Polysome 분해에 영향을 미치지 않지만, 번역 개시 인자 (eIF2α)의 발현 수준 차이를 볼 수 있다. 스트레스 과립 형성에 대한 MeCP2의 역할의 기능적 및 비기능적 도메인을 면역형광 현미경으로 평가하고 그 Polysome 분포도 측정했다. 이 연구를 통해 핵 응축 단백질인 MeCP2가 SG 구성요소 (시토졸 구획)로 확인되었다. 번역 및 mRNA 관리에서 MeCP2의 더 많은 기능을 더 연구해야 한다. 이 단백질과 그 도메인을 이해하면 레트 증후군과 같은 신경 장애에 대한 치료법을 찾을 수 있다.

I. Introduction

Stress granules (SGs) are membrane less, microscopically visible, stress induced granules which are accumulation of RNA binding proteins (RBP), poly (A) binding proteins and translational initiation factors.

SGs are present in both nucleus and on the cytoplasm and it exist in liquid phase. Nucleoli, cajal bodies and paraspeckles are present in nucleus whereas SG, P- and GW- bodies, mt RNA and neuronal germ granules present in cytoplasm[54]. Several SG components were reported in the past and there are still more which are yet to be reported. Nuclear abundant protein MeCP2 was localized in cytoplasm upon various stresses (Fig. 1 a-d).

MECP2 gene encodes methyl CpG binding protein 2, is a multifunctional epigenetic reader which binds to methylated regions of DNA to control transcription[55] and organize chromatin architechture[56]. Knock down of MeCP2 causes changes in the cell proliferation[57] siRNA- based knock down of MeCP2 inhibits Stress granule assembly (Fig. 2 a-c). Most of the MeCP2 related studies had been nuclear based. MeCP2 domains were HMGD1, MBD, HMGD2, TRD and CTD (Fig. 12). First functional domain is methyl CpG binding domain MBD[58], second MeCP2 domain to be characterized was the transcription repression domain TRD and other structural and intrinsically disordered domains of MeCP2 are carboxyl terminal domain CTD, N-terminal domain named the HMGD1 and the second HMG-like domain HMGD2. Schematic representation of MeCP2 domains was shown in Figure 12. Immunocytochemistry analysis shows the spontaneous SGs on overexpressed Flag-MeCP2-HMGD1-MBD and Flag-MeCP2-HMGD2-MBD domains and intrinsically disordered domains of HMGD1 inhibits SGs upon arsenite treatment (Fig.10).

Here, we focus on MeCP2 and its functional and non-functional domain's role in SG assembly. Mutations in MeCP2 genes leads to neurodevelopmental disorders like Rett

syndrome (RTT) [59, 60] by affecting the chromatin organization[61].

To confirm the presence of MeCP2 in the cytoplasmic region we have done subcellular fractionation (Cytoplasmic extract (CE), nuclear extract (NE) and Total extract (TE)) by centrifugation method with digitonin containing RSB buffer (Fig. 3).

In this study, we describe MeCP2 as an essential component of SGs. Depletion of MeCP2 strongly inhibits SG aggregation. We also found that MeCP2 localized to SGs under various stress condition. Its expression in the cytoplasm was confirmed by subcellular fractionation method in different cell lines. Arsenite induced SG response was observed after transfecting Flag-tagged MeCP2 domains through immunofluorescence microscopy analysis.

II.MATERIALS AND METHODS

Cell Culture and Transfections

U2OS, HEK293 and HeLa cells were received from Korean cell line bank Supplemented with 10% FBS (welgene), 1% V/V penicillin and streptomycin (Lonza) at 37ºC in 5% CO2. SW480, HCT116 and SH-SY5Y cells were received from ATCC and HAP-1 cells from Horizon. For SW480 and HCT116 cell culture conditions are similar to that of U2OS cells, but for SH-SY5Y, along with DMEM - F12 media was added additionally. siRNA based knock down was performed using Lipofectamine 2000 (Invitrogen) at 50 nM final concentration, siRNA sequence details was attached in separate Table 1. All DNA plasmids were transfected using Fugene 6 (Promega, Madison, WI) as per manufacture's protocol.

Immuno fluorescence microscopy

U2OS cells were seeded in four well plates as per the experimental requirements. After specific treatment condition, the cells were washed twice with 1X PBS and then fixed with prechilled 4% paraformaldehyde for 15 min and permeabilized with ice cold methanol for 10 min. Cells were again washed twice with 1X PBS later blocked for 1h (5%NHS-PBS - 1% sodium azide). Appropriate SG markers were used for staining. Image-j and photoshop software were used for image arrangements.

Immunoblot Analysis

After treatment cells were rinsed with 1X cold PBS to remove excess media and the cells were scraped with RIPA buffer containing protease inhibitor cocktail (Thermo scientific). The cells were kept on ice for 10 min then centrifuged for 20 min at 4^0C in 13,000 rpm and the supernatant was collected. Protein concentration was measured using the Bradford assay method. Equal amounts of proteins were used for further SDS-PAGE analysis with 2X SDS sample buffer. The samples were boiled at 85^0C for 5 min. After the SDS-PAGE the proteins were transferred to a PVDF membrane using wet transfer system. The membranes were blocked with 5% NHS-PBS-Sodium azide for 1 h and then incubated with primary antibodies

overnight, followed by 1X TBST wash for three times. Appropriate horse radish peroxide conjugated secondary antibodies were incubated for an hour then developed using developing solution (Bioshop.inc).

Knock down

MeCP2 siRNAs from Bioneer were used for knock down. The knock down success was confirmed by immunoblot analysis showing reduction in the endogenous MeCP2 level.

Cell fractionation protocol

For subcellular fractionation analysis, cells were harvested with 1X cold PBS. Half of them were used for Total cell extract and rest of them were taken as pellet after a short spin. Lysed with RSB buffer (10 mM Tris Hcl; pH -7.4, 2.5 mM MgCl2, 100 mM NaCl) which has digitonin. After centrifugation at 13,000 rpm for 6 min, the supernatant was transferred to a new tube (cytosolic extracts). The remaining pellet was washed five times with the same RSB buffer containing digitonin and lysed with RIPA buffer, nuclear extracts were isolated by centrifugation at 13,200 rpm for 20min.

Antibodies

MeCP2 (1:1000, Cell signaling, #3456), eIF2α (FL-315)(1:1000, Santa Cruz, SC-11386), p-eIF2α (1:1000, BML-SA405, Enzo life sciences) Anti Beta actin (Ac-15)(Abcam, ac6276). eIF3b(N-20)(1:500, Santa Cruz, Sc-16377), Anti-Lamin B1 (1:1000, abcam, ab16048). α-Flag (1:1000, Sigma Aldrich, F3165), G3BP1 (1:500, Santa Cruz, Sc-365338), α-tubulin (1:1000, Santa Cruz, Sc- 8035).

Polysome Analysis

U2OS and HEK293 cells were grown in 150 mm dishes and transfection was done at two different timings (12 h and 24h), followed by the above-mentioned treatment procedures. After treatment, 100 μg ml−1 cycloheximide was added and incubated for 5 min at RT, washed twice with 1X cold PBS, then lysed with 1 ml of polysome lysis buffer (20 mM HEPES pH 7.4, 5 mM MgCl2, 125 mM KCl, 1% NP-40, 2 mM DTT) supplemented with 100 μg ml−1 cycloheximide (Sigma), protease inhibitor cocktail (EDTA-free; Thermo scientific) and RNAsin (Ambion) in

cold room. Cell lysates were tumbled for 15 min at 4 °C and centrifuged at 13,000 r.p.m. for 15 min. The supernatants were fractionated in 17.5–50% linear sucrose gradients by ultracentrifugation (35,000 r.p.m. 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). Fractions were acetone precipitated at −20 °C for overnight and processed for further immunoblot analysis.

Immunoprecipitation Assay

Cells were harvested and lysed in IP buffer (50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Trition X-100) supplemented with protease inhibitor cocktail (Thermo scientific) on ice for 20 min, centrifuged at highspeed for 15 min and the supernatants were collected in fresh tube. For IP,1–2 mg lysate was incubated with 30–50 µl Magnetic beads (Invitrogen) overnight at 4°C. The resulting immunoprecipitants were washed at least three times in IP buffer, before boiling with 4X SDS sample buffer. Samples were used for western blot analysis.

IDR/IDP Analysis with IUPred2A tool

https://iupred2a.elte.hu/ It's a bioinformatic tool used to analyze intrinsically disordered or structured domain.

Statistical analysis

All the results are presented as mean±SEM of at least three independent experiments. Prism 5.0 was used for data analysis. Student's t-test is used to confirm the statistical significance of the data. Differences were considered statistically significant at values of $p<0.05$.

III. Results and Discussion

MeCP2 is a component of Stress granules

SG formation is regulated by proteins which are involved in translation, post translational modification, intrinsically disordered domain containing proteins, during the time when cells are being exposed to various stresses. To investigate methyl CpG binding protein (MeCP2) role in Stress granule formation, we first knocked down the endogenous MeCP2 using siRNA for 72 h. Knock down efficiency was measured by western blot analysis (Fig.2 a,b). Simultaneously stress granule dynamics was assessed using Immunofluorescence (IF) microscopic image analysis after treating arsenite in time dependent manner (20,40 and 60 min). The cells were stained with universal SG marker eIF3b (green), G3BP (red) and nuclear stain Hoechst (blue). As shown in Fig. 2 a, knock down of MeCP2 specifically affects SG formation. MeCP2 is an abundant nuclear protein ie., present in pericentromeric heterochromatin, which encompasses a large fraction of all genomic 5-methylcytosine[62]. We observed that, upon various stresses MeCP2 localize with SGs in cytoplasmic region in U2OS cells (Fig.1 a-d) as well as in neuroblastoma cell line SH-SY5Y cells (Fig. 12). We have checked whether it colocalize with p-bodies by using RCK (p-body marker). The results showed that it was in fact located adjacent to SG and not colocalized (data not given).

Subcellular fractionation to see the existence of MeCP2 in Cytosol

MeCP2 is an abundant nuclear protein that was initially recognized as the founding member of the methyl-DNA binding proteins family[62]. To confirm the presence of MeCP2 in the cytoplasmic region we have chosen six different cell lines such as U2OS, HEK293, HeLa, SH-SY5Y, HCT-116 and SW-480. The cells were Subcellular fractionated (Cytoplasmic extract (CE), nuclear extract (NE) and Total extract (TE) by centrifugation method (Fig. 3, a). Rabbit MeCP2 antibody was used to check the MeCP2 immunoprecipitation assay in Cytoplasmic extract of SH-SY5Y cells (Fig.3 b). It was precipitated successfully. The results supports the results of the previous subcellular fractionation experiments which we mentioned in the Figure

3. Similar experiments have to be performed to prove MeCP2 function in translational level. SH-SY5Y cells were used for polysome fractionation MeCP2 expression was found in both monosomes as well as polysome distribution upon untreated condition (Fig. 4).

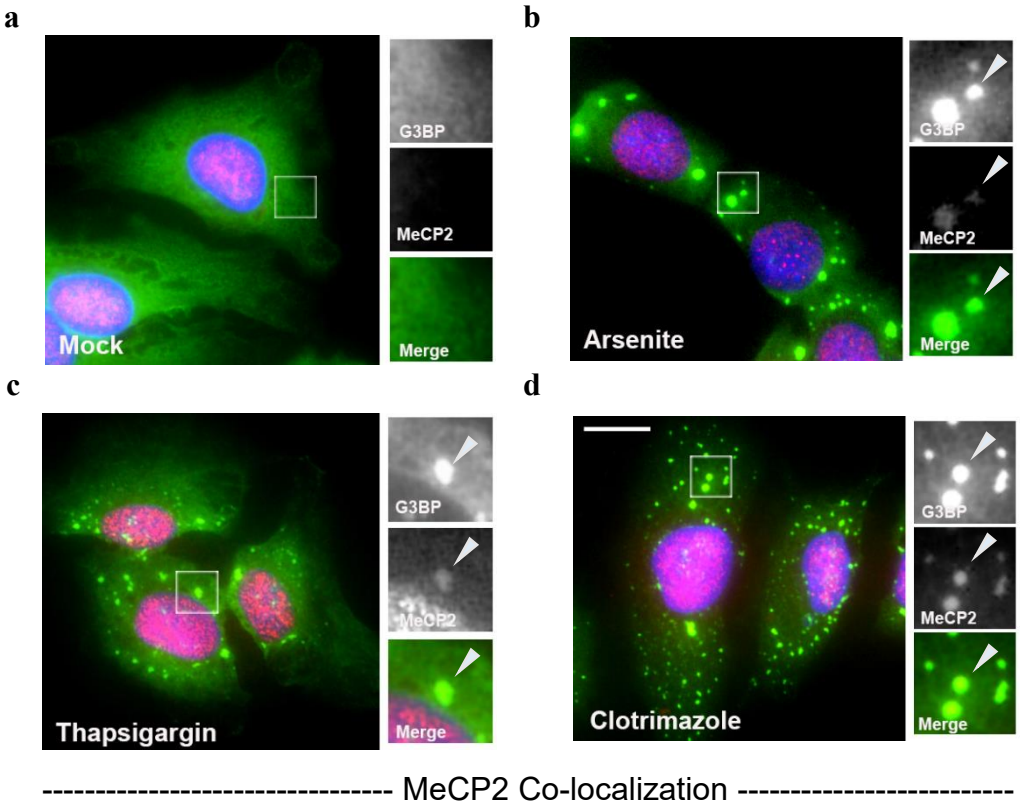


Fig. 1 MeCP2 proteins are components of stress granules. U2OS cells either (**a**) untreated or (**b**) treated with arsenite,0.5 mM (**c**) thapsigargin, 2mM and **(d)** Clotrimazole, 40μM for 1 h were immunostained against SG marker G3BP (green) and MeCP2 (red). Scale bar, 20μm. Boxed regions are enlarged as both merged and separate colored views. White arrow head indicates the stress granule.

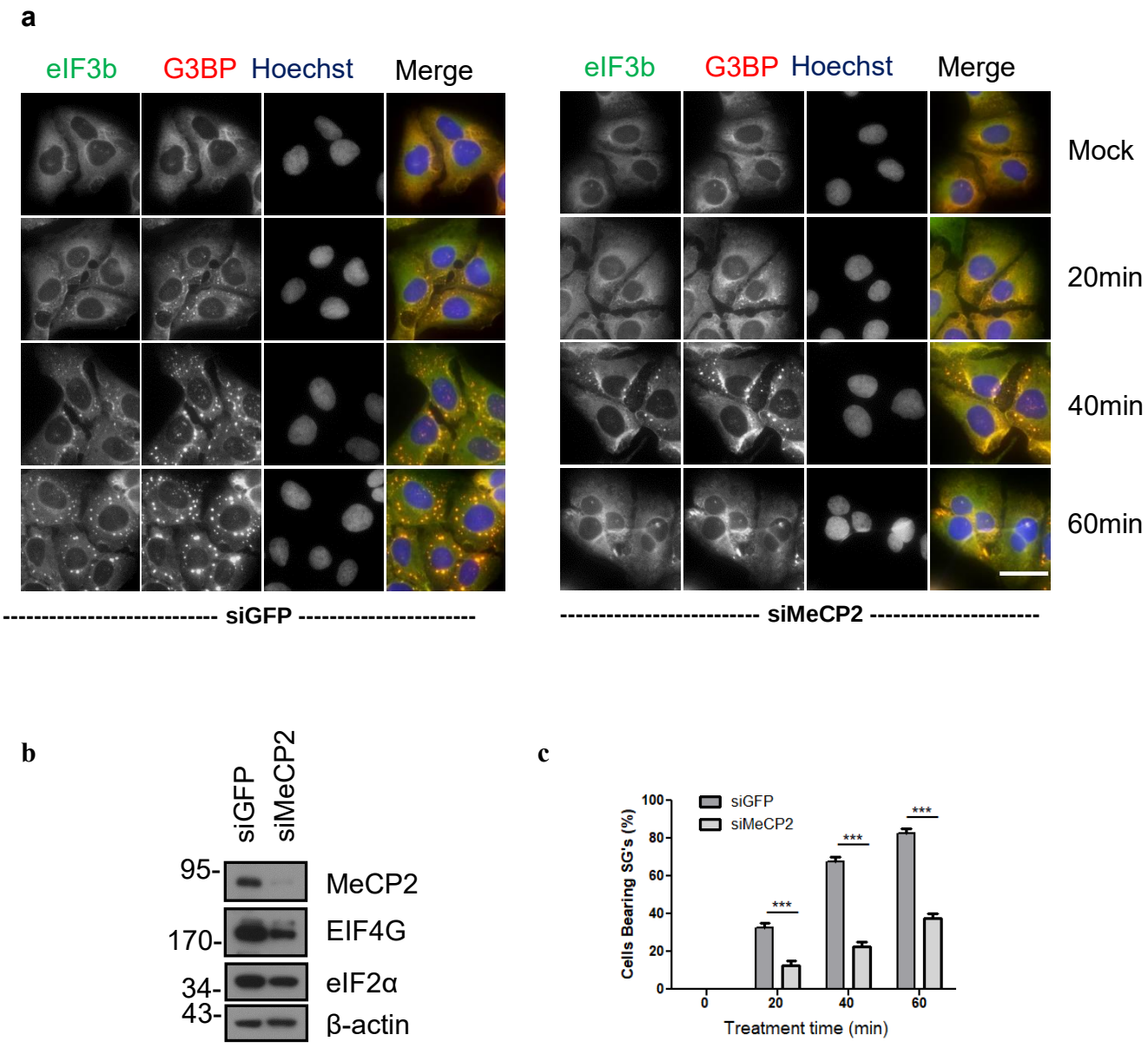
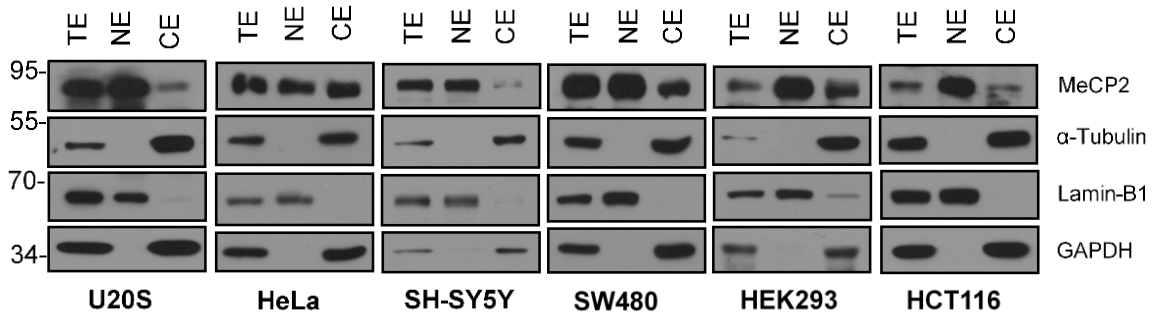


Fig. 2 MeCP2 proteins knock down affect stress granule assembly. (a) U2OS cells transfected with siGFP, siMeCP2 for 72 h were either untreated or treated with arsenite (0.2mM) for indicated time points. SG marker eIF3b (green) and G3BP (red) was used for Immunostaining **(b)** Western blot results showing knock down efficiency of endogenous MeCP2. (**c**) Statistical data represents the percentage of cells bearing SG's. Scale bar, 20μm. Data are means ± s.t.d. of atleast three independent experiments. *p<0.05; **p<0.01;***p<0.0001.

a



b

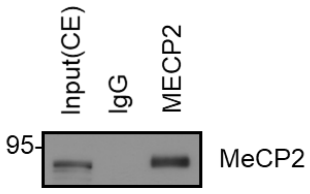


Fig. 3 MeCP2 exist in cytoplasmic extracts of different cell lines. (a) All the cell lines were lysed in RSB buffer with digitonin and RIPA buffer (see methods). Subcellular fractions were separated based on centrifugation method. Nuclear and Cytoplasmic marker were used such as Lamin-B1 and α-tubulin. **(b)** Cytoplasmic extract of SH -SY5Y cell line was used for Co -IP experiment. Rabbit antibody was used to precipitate the cytosolic MeCP2. Chicken MeCP2 antibody was used to detect the precipitated MeCP2.

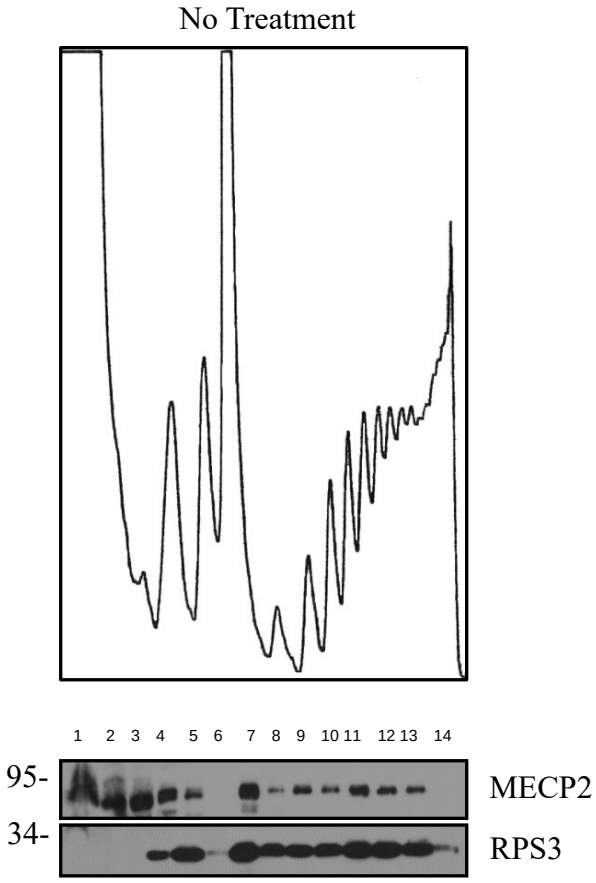


Fig. 4 Polysome Analysis with SH-SY5Y cells.

(a) SH-SY5Y cells were lysed and subjected to polysome fractionation using sucrose gradients (17–50%). A total of 14 fractions were collected, acetone precipitated, and the sample was air dried and dissolved in 1X SDS sample buffer, used for western analysis.

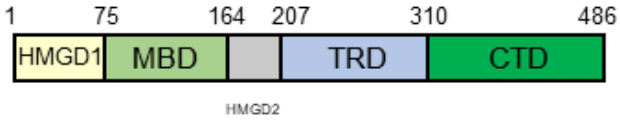
Intrinsically disordered protein (IDP) profile of MeCP2

Intrinsically disordered proteins (IDPs) or Intrinsically disordered regions (IDRs) execute important biological functions[63]. An IDP lacks a fixed or organized three-dimensional structure[64-66]. IDPs are either fully unstructured or partially structured and they include some oddities like random coils, (pre-)molten globules, and large multi-domain proteins which are connected by flexible linkers. They are one of the main types of proteins along with globular, fibrous and membrane proteins[67].

Numerous identifiable disorder-based DNA-binding sites, protein-binding sites and post-translational modification sites located in the intrinsically disordered regions, and DNA demethylation deficiency point mutations in the IDPRs could change the local disorder propensity of these proteins[68]. IDRs contain proteins implicated in transcription, cell signaling and chromatin remodeling functions[69, 70]. Disordered proteins have the binding affinity with their receptors controlled by post-translational modification (PTM), therefore it has been suggested that the flexibility of these disordered proteins facilitates the different conformational requirements for binding the modifying enzymes as well as their receptors[71].

IUPred is one of the widely used bioinformatic tool for predicting IDPs and IDRs based on a biophysics-based model[72]. Amino acid sequence of a particular protein is required to find the IDR or IDP details. IUPred assumes a score between 0 and 1 for each residue, corresponding to the probability of the given residue being part of a disordered region. IDR profile of MeCP2 was analyzed with IUPred bioinformatic tool (Fig. 5). Uniport MeCP2 – Human protein ID (P51608) was used for IDP/IDR prediction. MeCP2 proteins contain extensive disordered regions based on the IUPred result, which is represented in the red colored line in the graph in figure 5.

a



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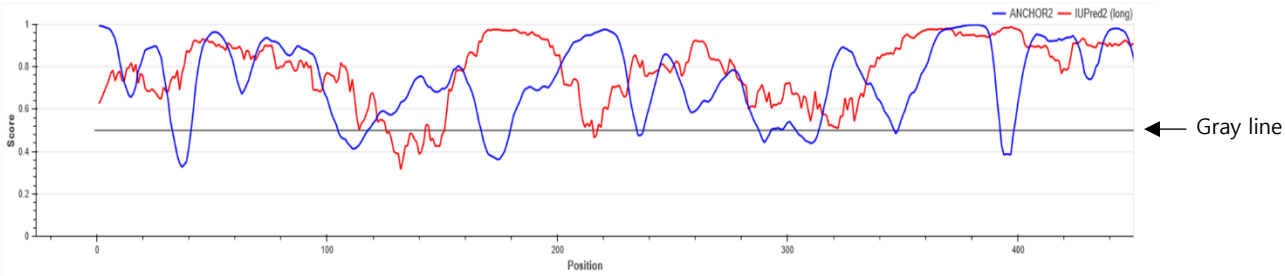

Fig. 5 IDR prediction profile of MeCP2

(a) Schematic representation of MeCP2 domain. **(b)** IUPred was used to find out MeCP2 intrinsic disorder region. Below the gray color line shows the structured domains and above the gray line indicates intrinsically disordered/intrinsically unstructured regions. Based on Figure (a) Except MBD domain all the domains were belongs to intrinsic regions.

Association of MeCP2 and its domains in polysome distribution

MeCP2 WT and Knock out HAP1 cells that are used for polysome analysis, doesn't find any changes in the polysome disassembly after arsenite treatment (Fig. 6). MeCP2 –KO cell lines shows substantial level reduction of eIF2α both in non-treated and treated condition (Fig. 6 b), but knock out cells did not show any SG inhibition on stressful circumstances. This is assessed by SG markers such as eIF3b, G3BP, EIF4G and TIA-1 (Fig. 7) Knock down of MeCP2 affects translation, it was assessed by Ribopuromycylation assay (data not shown). All the five domains of MeCP2 and its mutants were subjected to ribosome analysis in U2OS cells (Fig. 8) and HEK-293 cells (Fig. 9). Strong MeCP2 distribution was observed in SH-SY5Y cells even in non-overexpressed state (Fig. 4), therefore further experiments of polysome profiling with MeCP2 and its domains were performed with SH-SY5Y cells instead of cancer cell lines.

Intrinsically disordered proteins promote SG aggregation[73-76]. MeCP2 is an intrinsically disordered protein, composed of 6 distinct domains namely HMGD1, MBD, HMGD2, TRD and CTD - α and β[77]. MBD and TRD are well characterized functional domains with more functions[78].

MeCP2 and its domains exhibit both impaired SG nucleation and recruitment to SGs in U2OS cells

As per immunocytochemistry results, N-terminal (HMGD1 and HMGD2) and MBD domain was overexpressed in U2OS cells producing spontaneous SGs in untreated condition. HMGD1, CTD and △HMGD1 were overexpressed in U2OS cells that inhibit SGs upon arsenite exposure. Domain specific localization shift was observed (Fig. 10), Each domain has specific roles in transcriptional and translational level that has to be studied elaboratively in future studies.

In this study, we report *MeCP2* (methyl CpG binding protein 2) as a novel component of SGs, knock down of MeCP2 strongly inhibits SG formation. We also demonstrate that *MeCP2* specifically localizes to SGs under various stress conditions. Knockout of *MeCP2* in

HAP1 cells has no effect on stress-induced polysome disassembly, but we could see the expression level difference in translation initiation factor (eIF2α). Functional and non-functional domains of MeCP2 role on stress granules was assessed by immunofluorescence microscopy and its Polysome distribution also measured.

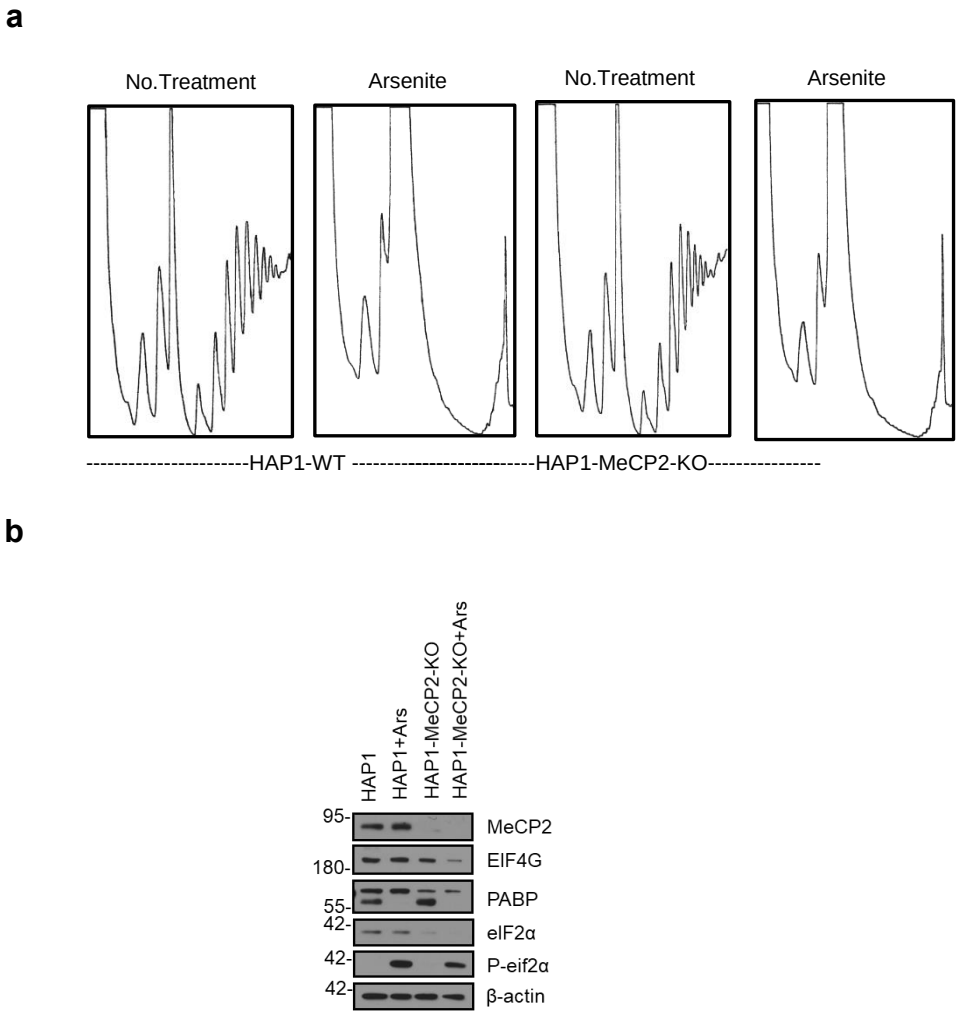

Fig. 6 Knock out of MeCP2 doesn't show any changes in polysome disassembly. (a) HAP1-MeCP2-WT and HAP1-MeCP2- KO cells non-treated or treated with 0.5mM arsenite for 1 h. **(b)** Knock out of MeCP2 and eIF2α-p status was assessed by immunoblot analysis.

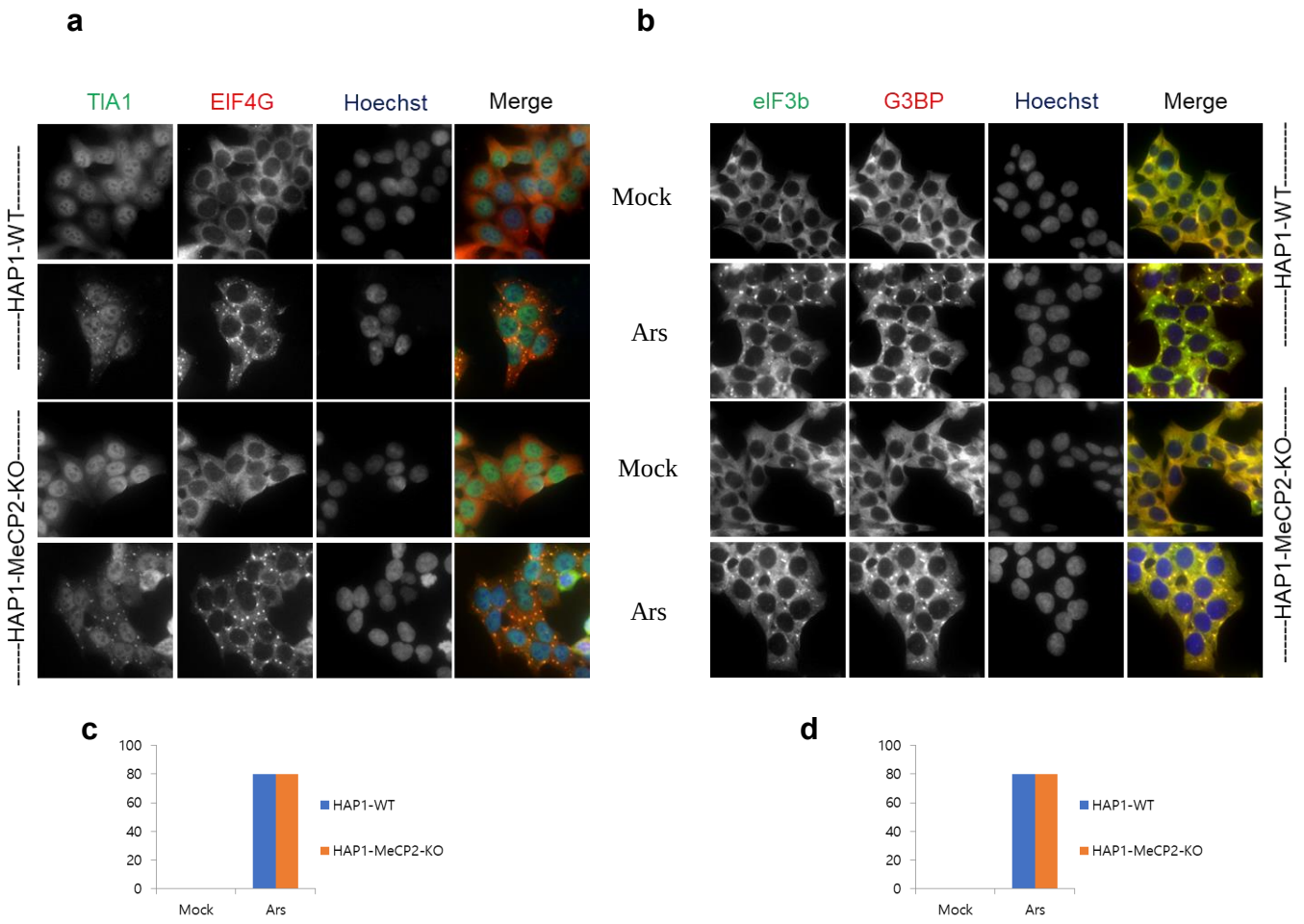

Figure 7. Stress granule inhibition was not seen in HAP1-MeCP2-KO cell line on Arsenite condition.

(a) HAP1-WT-MeCP2 and HAP1-MeCP2-KO cells were untreated or treated with 0.5mM Ars for 1 h stained with SG marker TIA-1 (green), (EIF4G) and Hoechst (blue). In Figure **(b)** same cell lines were stained with eIF3b (green), G3BP (red) and Hoechst (blue). **(c)** and **(d)** SG was indicated in percentage. The experiment was done only once.

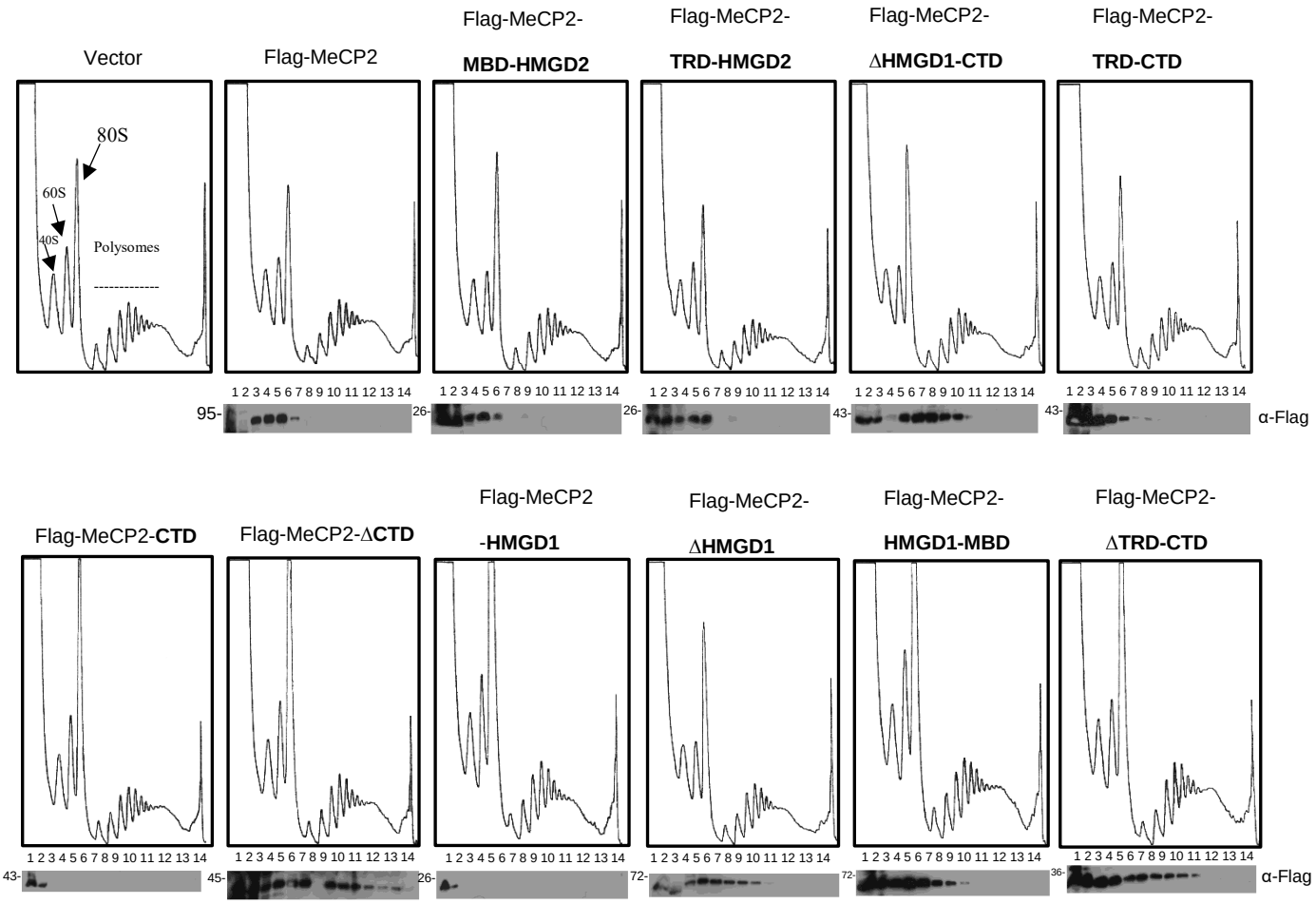

Fig. 8 Polysome association of MeCP2 and its domains were analysed

Distribution of the mentioned Flag-tagged MeCP2 proteins expressed in U2OS cells was monitored by western blotting. Fraction numbers were indicated below the polysome profiles. Monosomes (40S, 60S and 80S) and polysomes were indicated. The same experiment repeated twice.

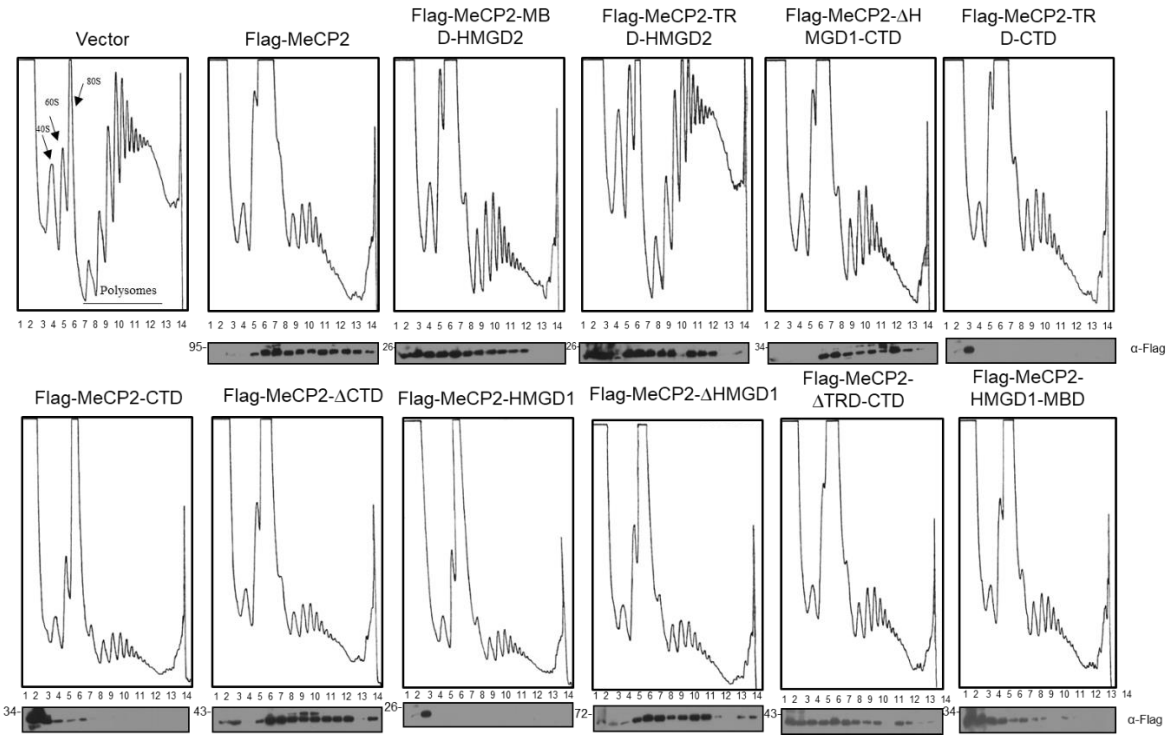

Fig. 9 Polysome association of MeCP2 and its domains were analyzed

Distribution of the mentioned Flag-tagged MeCP2 proteins expressed in HEK 293 cells was monitored by western blotting. Fraction numbers were indicated below the polysome profiles. Monosomes (40S, 60S and 80S) and polysomes were indicated. The same experiment repeated twice.

a

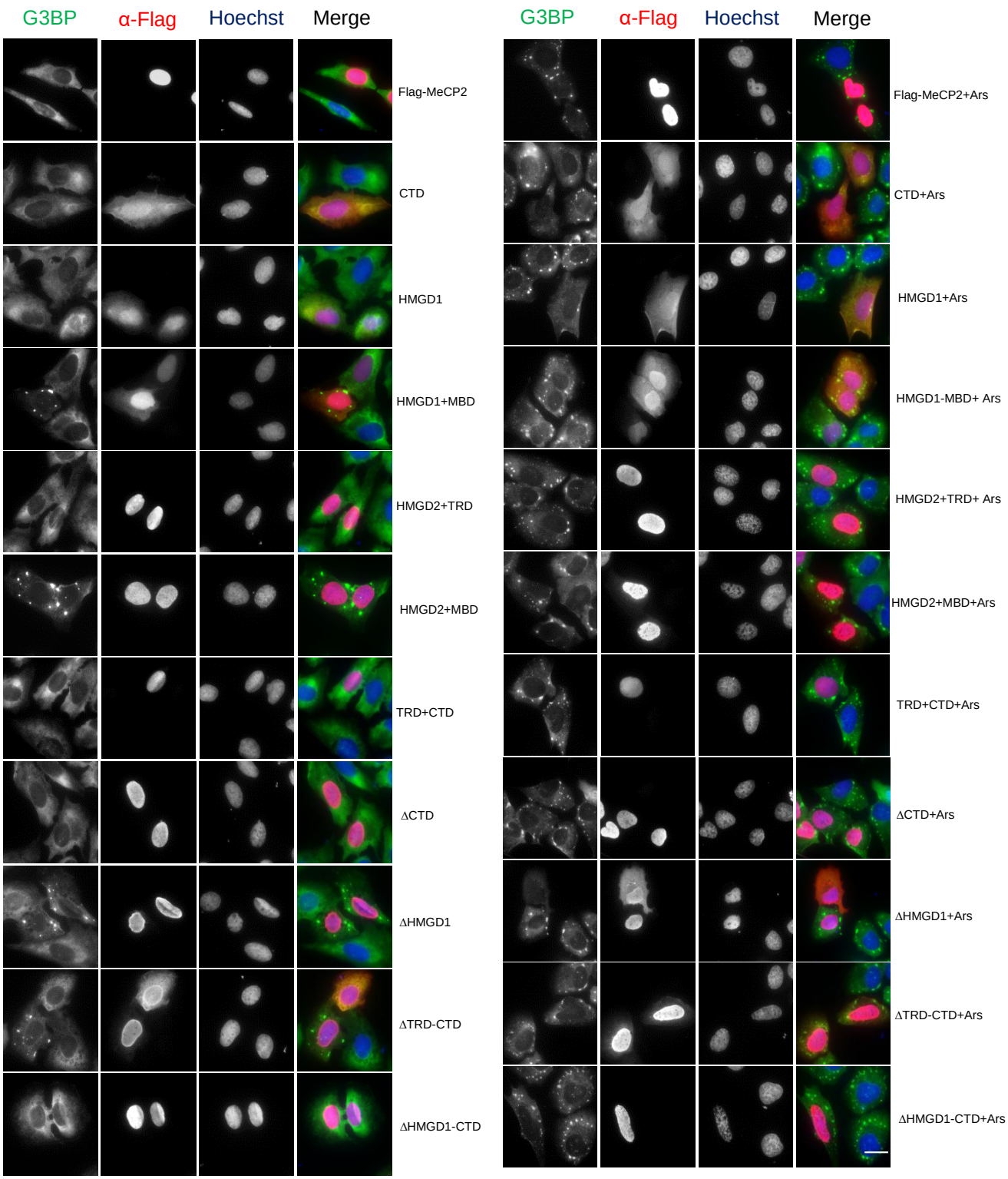

Fig. 10 MeCP2 and its domains exhibit both impaired SG nucleation and recruitment to SGs in U2OS cells. (a) MeCP2-WT and other domain constructs transfected into U2OS cells untreated (Mock) or treated (0.5 mM Sodium arsenite, 1 h), stained with G3BP (green) or α-Flag (red), and nuclei stained with Hoechst (blue). Data represents at least three independent experiments. Scale bar, 20 μm.

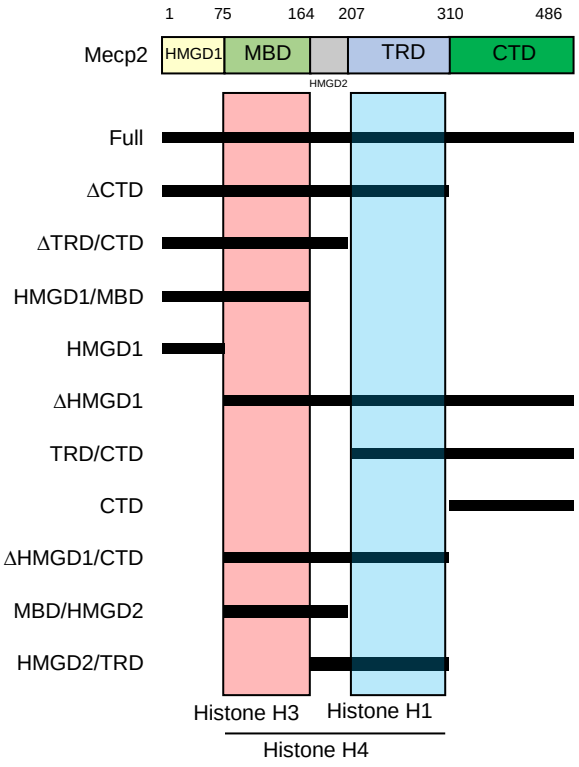

Fig. 11 Schematic representation of MeCP2 domain constructs

a

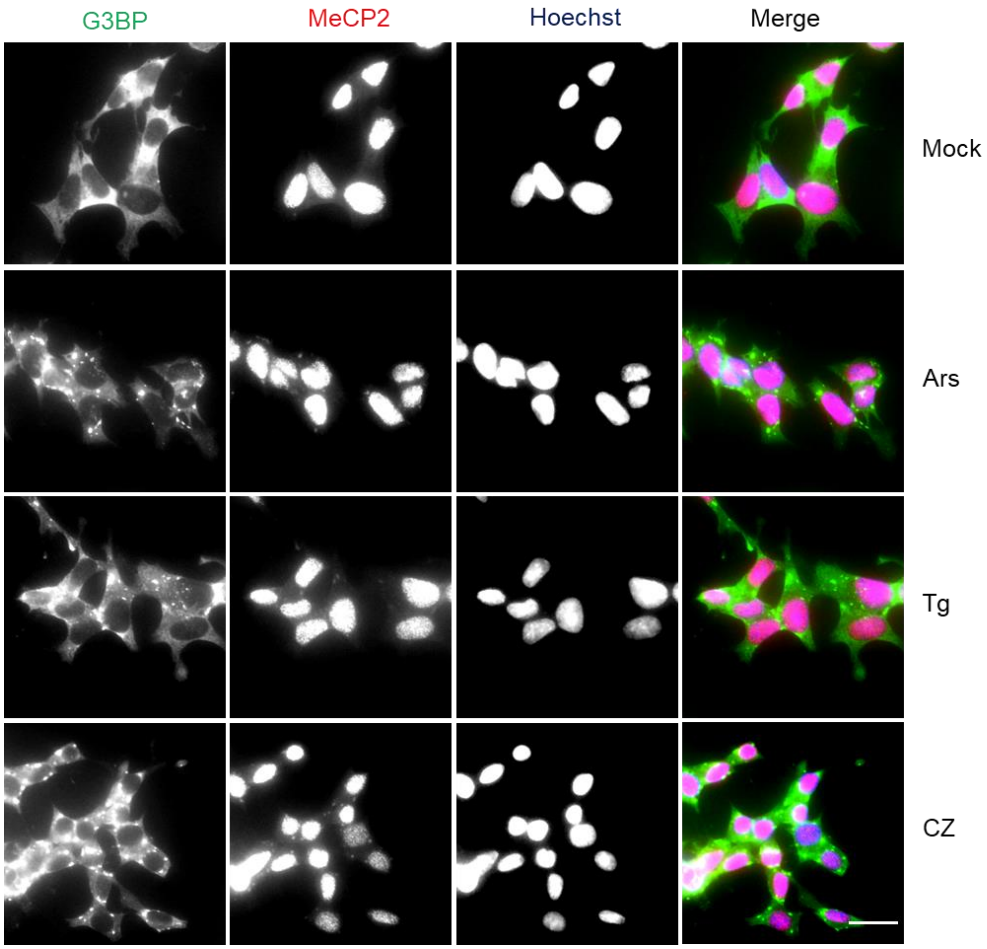

Fig. 12 MeCP2 Co-localize with SG marker G3BP in SH-SY5Y cells.

SH-SY5Y cells either (**a**) untreated or treated with arsenite,0.5 mM, thapsigargin, 2 mM and Clotrimazole, 40 μM for 1 h were immunostained against SG marker G3BP (green) and MeCP2 (red). Scale bar, 20μm.

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V. ABSTRACT IN ENGLISH

Analysis of MeCP2 protein in Stress granule assembly

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Ribonucleoprotein (RNP) granules are membrane-less, RNA-binding proteins (RBPs) localized in cytoplasm. DNA methylation is one of the Post translational modifications that can control the assembly and disassembly of Stress granule formation. To study further we designed experiments related with methyl CpG binding protein 2 (MeCP2). We found that MeCP2 as a novel component of SGs and knock down of MeCP2 strongly inhibits SG formation. We also demonstrate that MeCP2 specifically localizes to SGs under various stress conditions. Knockout of MeCP2 in HAP1 cells has no effect on stress-induced polysome disassembly, but we could see the expression level difference in translation initiation factor (eIF2α). Functional and non-functional domains of MeCP2's role on stress granule formation was assessed by immunofluorescence microscopy and its Polysome distribution also measured. Through this study, a nuclear condensed protein, MeCP2 was identified as SG component (Cytosolic compartment). More functions of MeCP2 in translation and mRNA governance has to be studied further. Understanding of this protein and its domains will give a clue to find cure for neurological disorder like Rett syndrome.

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Dedication

I dedicate my thesis to my parents, brother, grandmother, to all my teachers and Professors.