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Corresponding Author: Takbum Ohn

Authors: Jin Ah Kim[1,#], Aravinth kumar Jayabalan[1,#], Vinoth kumar Kothandan[1], Ramesh Mariappan[1], Younghoon Kee[2], Takbum Ohn[1,*]

Institution: [1]Department of Cellular & Molecular Medicine, Chosun University, [2]Department of Cell Biology, Microbiology, and Molecular Biology, University of South Florida,

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Identification of Neuregulin-2 (NRG2) as a novel Stress Granule component

Jin Ah Kim[1#], Aravinth Kumar Jayabalan[1#], Vinoth Kumar Kothandan[1], Ramesh Mariappan[1], Younghoon Kee[2] and Takbum Ohn[1,*]

[1]Department of Cellular & Molecular Medicine, College of Medicine, Chosun University, Gwangju, 501-759, Republic of Korea

[2]Department of Cell Biology, Microbiology, and Molecular Biology, College of Arts and Sciences, University of South Florida, Tampa, Florida 33620, USA

Running Title: NRG2 is a novel SG component

Key words: Stress granules, RNA granules, Translation, NRG2, Stress signaling

[*]Correspondence:

Takbum Ohn, Ph.D

Department of Cellular & Molecular Medicine, College of Medicine, Chosun University, Republic of Korea, Gwangju, 501-759, Republic of Korea

Phone: +82-62-230-6285; Fax: 82-62-233-6337

Email: tohn@chosun.ac.kr

[#]These authors contributed equally to this work.

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Abstract:

Stress Granules (SGs) are microscopically visible, phase dense aggregates of translationally stalled messenger ribonucleoprotein (mRNP) complexes formed in response to distinct stress conditions. It is generally considered that the SG formation is induced to protect cells from stress. The precise constituents of SGs and how SGs are dynamically regulated in response to stresses are incompletely understood. Hence, it is important to identify proteins which regulate SG assembly and disassembly. Here, we report that NRG2 is a novel SG component whose depletion potently inhibits the SG formation. We show that NRG2 specifically localizes to SGs under various stress conditions. Knockdown of *NRG2* has no effect on stress-induced polysome disassembly suggesting that NRG2 does not influence the early step of SG formation. We also found that reduced expression of NRG2 marginally increases cell survival under arsenite stress.

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INTRODUCTION

Stress granules (SGs) are membrane-less, dense cytoplasmic aggregates formed in response to various environmental stress conditions (1). In general, cells monitor adverse conditions with the help of at least four stress sensing kinases such as (a) PKR (Protein Kinase R) activated by viral infection, heat shock and UV irradiation, (b) PERK (PKR like Endoplasmic Reticulum Kinase) activated by unfolded protein response, (c) GCN2 (General Control Non-derepressible 2) induced by starvation and (d) HRI (Heme Regulated Initiation factor 2 kinase) triggered by sodium arsenite exposure. Activation of either of this kinase causes phosphorylation of eIF2α at serine 51, a component of eIF2/tRNAi/GTP ternary complex which is required for translation initiation. In absence of ternary complex, translation initiation is compromised and forming translationally stalled mRNP complex which eventually accumulate into SGs (2). Thus, SGs mostly harbor translation initiation factors such as eIF3, eIF4A, eIF4G, eIF4E as well as poly(A)-mRNA, PABP and small (40S) but not large (60S) ribosomal proteins. In addition, many RNA binding proteins (RBPs) such as TIA-1, TIAR, G3BP and FMRP and some signaling proteins such as RACK1, TRAF2 are also present in SGs (3). Processing bodies (PBs) are another type of RNA granule which closely resembles SGs but functionally and compositionally different. SGs appear only after sensing stress stimuli, whereas PBs are present in normal (unstressed) cells and proportionally increased upon stress exposure. PB mostly harbors mRNA decay enzymes such as Dcp1 or Hedls, DCP2, etc. Under certain type of stress, SG and PB come close together and dock some proteins and mRNA. By this phenomenon, these two compartments decide cell fate under stress conditions by allowing translation of few transcripts while some are stored (in SGs) or degraded (in PBs).

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Stress-induced translational repression and SG assembly allows cell to reprogram overall gene expression profile and permit selective translation of stress responsive genes. There are two types of SGs, canonical (or universal) and non-canonical, based on their composition, structure or size and number of granules per cell, yet no functional difference has been found between them. SGs usually assembled in minutes after stress encounter, but the disassembling kinetics differs with stress type. There are numerous factors identified to be involved in the regulation of SG assembly such as RNA-binding proteins, post-translational modifications, presence of PRD (prion-related domain), low complexity (LC) or intrinsically disordered (ID) regions in SG nucleating factors (1, 4). Although SG assembly is essential for cell survival mechanism, it has also been implicated in many disease conditions such as cancer, fraxile X syndrome and neurodegenerative diseases such as ALS and AD (5). Most of the SG associated diseases are reported as a consequence of persistent granules which are failed to disassemble even after cells recovered from stress. This could be a result from deregulation of proteins involved in assembly or disassembly kinetics, precisely proteins act in the stress signaling pathway (6, 7). There have been several studies explaining the role of SG formation, components and function, but proteins which modulate SG dynamics is clearly lacking.

We previously identified many genes involved in the regulation of SG and processing body based on an RNAi screen (8). In quest of signaling protein involved in SG assembly, we reviewed our data and found NRG2 was one of the hit from the screen. Neuregulins (NRGs) are a member of epidermal growth like (EGF-like) factor which stimulate ErbB-receptor tyrosine phosphorylation and mediate distinct biological processes. NRG2, a splice variant of NRG1 is a transmembrane protein helps to regulate cell proliferation, differentiation, survival, whereas its deregulation results in diseases such as cancer (9, 10). Activation of NRG2 signal by ErbB

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receptors can elicit different downstream signaling pathways such as MAPK, PI3, Protein Kinase C, JAK-STAT pathway (11). But, how these signals provoke specific cellular responses such as proliferation or survival is limited, except one report which identified an intracellular signaling component MEMO (Mediator of ErbB2-driven cell motility) integrates ErbB signal and controls cell motility (12). It has also been reported that NRG2 crosstalk with broad range of intracellular signaling networks in which ErbB receptor act as a scaffold in transmitting the information to downstream cellular pathways (13).

In this report, we identify neuregulin-2 (NRG2) as a novel component of SGs. Depletion of NRG2 strongly inhibits SG aggregation, but do not affect stress-induced polysome disassembly. Consistently, NRG2 localized to SGs under multiple stress condition as well as in different cell types, arguing its functional importance in SG dynamics. Moreover, reduced expression of NRG2 marginally increases cell survival under arsenite stress suggesting that it may negatively regulate stress-mediated cell death.

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RESULTS

SiRNA mediated *NRG2* knockdown impairs SG assembly

Previous RNAi-mediated loss-of-function screen has identified NRG2 as a potential regulator of SGs (8, 14). To test whether NRG2 indeed is a component and / or regulator of SG assembly, we first depleted endogenous NRG2 using two different siRNA's and evaluated the SG assembly kinetics. SiCONT (non-targeting) and siNRG2 knocked-down cells were cultured in the absence or presence of sodium arsenite to induce SG assembly in time dependent manner (30 and 60 mins). Immunofluorescence (IF) microscopic analysis was then performed with universal SG marker (eIF3b) and SG/PB marker (RCK) to visualize the presence of SG and PB, respectively. As shown in Fig. 1A, depletion of NRG2 strongly impaired SG formation after exposure to arsenite compared to siCONT cells. Immunoblot analysis confirmed that the expression of NRG2 was significantly reduced (Fig. 1B). Conversely, the percentage of RCK-positive cells remains unchanged in the *NRG2* knockdown cells, suggesting that the PB assembly is not affected by NRG2 depletion. Our results show that the percentage PB in basal (around 63% unstressed cells) and after stress (98%), following similar assembly pattern comparable to earlier reports (15, 16). We tested this phenomenon in different cell lines including U2OS stably expressing EGFP-G3BP (Fig. 1C), HEK293T, HeLa and obtained consistent results (data not shown). Moreover, siNRG2 treated cells had about 70% inhibition of SGs even after 60 mins of exposure to arsenite with respect to siCONT (Fig. 1D), suggesting NRG2 plays a direct potential role at the early stage of SG assembly (8).

NRG2 localizes to SGs upon arsenite stress

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The proteins involved in the regulation of SG assembly such as translation initiation factors, RNA binding proteins, kinases or stress signaling molecules are generally localized to the SG after stress stimuli. NRG2 is a transmembrane protein with N-terminal sequence present in extracellular region, while the C-terminal portion is protruding through the cytoplasm. Thus, we investigated whether NRG2 re-localizes to SGs under arsenite stress (10). In brief, immunofluorescence microscopy was employed for U2OS cells treated with 0.5 mM sodium arsenite and stained with antibodies against eIF3b (SG marker), NRG-2 and p70S6K (PB marker). As shown in Fig. 2A, NRG2 strongly localized to SGs (eIF3b) after arsenite treatment, whereas it did not localize to PBs (p70S6K). This result is consistent with the observation that *NRG2* knockdown specifically affected SG formation, but not the PB assembly (Fig. 1) (17). Localization of NRG2 to SGs was further verified by counter-staining with other well characterized SG markers TIA-1 and G3BP (Fig. 2B) (18). Localization of NRG2 is also observed in HeLa (Fig. 2C) and HEK293T (data not shown). SGs generally recruit specific proteins at different stages of aggregation, for instance, eIF3b and G3BP are readily accumulated to SG at primary stage as soon as cells encounter stress whereas other proteins such as RACK1, TRAF2 localize to SG at secondary aggregation stage (2, 19, 20). Our assays show that NRG2 becomes localized to the SGs as early as 15 mins after stress induction (data not shown) similar to TIA-1/TIAR suggesting that NRG2 may have a role in the initial aggregation of SGs (19). Consistently, *NRG2* knockdown impacted the initial formation of SGs. This implies two possible roles for NRG2, either it might act as a stress signaling protein for granule assembly or it has a role in the primary aggregation stage. Collectively, these data suggest that NRG2 is a *bonafide* component as well as a regulator of the SG assembly.

Depletion of NRG2 does not affect the stress induced polysome disassembly

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It is well known that the stress-induced phosphorylation of eIF2α acts as an initial stimulus for the SG assembly (19). The phosphorylated eIF2α eventually causes translational arrest and polysome disassembly. To examine whether NRG2 has a role in polysome disassembly upon arsenite stress, we depleted endogenous NRG2 and performed the polysome profiling analysis. U2OS cells transfected with siCONT or siNRG2, were treated with or without 0.2 mM arsenite for 30 mins. Cells were subsequently lysed and the extracted proteins were subjected to ribosomal fractionation. Unexpectedly, the control and *NRG2* knockdown cells did not show any differences in the disassembly profile arsenite treatment (compare b and d). This result is a reminiscence of previous finding that *O*-GlcNAc depletion did not affect polysome disassembly but only affected SG aggregation (21). Altogether, this data suggests that NRG2 acts downstream of p-eIF2α and may act as a nucleation factor for the SG assembly.

NRG2 depletion enhances cell survival under stress

The major functional role for SG formation is to protect cells from adverse stress stimuli by temporarily halting energetically costly translation process and activate survival program. This allows cells to conserve much cellular resources, and thereby ease them to recover from stress (22, 23). The polysome profile data suggested that NRG2 acts independently or at a level downstream of the eIF2α phosphorylation. Recent report identified inhibition of SG assembly induces stress-mediated apoptosis regardless of p-eIF2α activation. As we shown earlier (Fig. 1), *NRG2* knockdown strongly impaired SG assembly, so we sought to determine to what extent this phenotype affects cell viability rate under arsenite stress. To investigate this, we carried out the cell survival assay with siCONT, siNRG2-1 and siNRG2-2 transfected cells following 0.5 mM arsenite treatment at different time points. To our little surprise, the NRG2-depleted cells were modestly resistant to the arsenite treatment compared to control cells (Fig. 4). Particularly, this

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phenomenon was more evident after 2 hrs of arsenite (0.5mM) exposure, the dose and time at which stress-induced apoptotic pathway triggers (24, 25). This result suggests that NRG2 promotes cell death upon arsenite stress. Indeed, some reports suggest that NRGs are involved in apoptosis (26, 27). Based on these results, we propose a model that NRG2 is recruited to SGs at initial stage to prevent stress-induced apoptotic pathway under mild stress (until 2 hrs), whereas with prolonged stress (after 2 hrs) NRG2 eventually re-localizes from SGs to participate in stress mediated apoptosis.

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DISCUSSION

Stress induced translation arrest and reprogramming of gene expression is a surveillance mechanism adopted by cells to keep them alive as well as save energy to cope up stress recovery. Recruitment of untranslated housekeeping but not stress-responsive mRNAs into SGs with the help of numerous RNA binding proteins, signaling molecules and subsequent docking with PBs are critical to decide cell fate and maintain RNA triage. Here we report NRG2 (neuregulin-2) as a novel component of SGs. We showed that NRG2 depletion impaired the SG assembly dynamics, without affecting the PB assembly. Our further analysis showed NRG2 does not affect the polysome disassembly suggesting either it acts independently or immediate downstream of eIF2α phosphorylation likely at the aggregation stage similar to *O*-GlcNAc modification (8).

NRG2 is also known to mediate the ErbB receptor tyrosine kinase signaling (10). Whereas eIF2α phosphorylation is critical for translation arrest and ribosome run-off, SG components such as RNA binding proteins (TIA, HuR, etc) predominantly act as nucleating (or aggregation) factor. Recently, it was revealed that many signaling proteins which are recruited to SGs typically harbor prion related (PR), intrinsically disordered (ID) or low complexity domain (LCD). These structurally disordered domains which fail to fold into active tertiary structure are shown to be involved in phase separation and formation of membrane-less compartments inside cell. Thus, we checked whether NRG2 resides any disordered region using the webserver (http://iupred.enzim.hu/), and surprisingly most of the C-terminal region is completely disordered (28). It confirms our hypothesis that NRG2 acts as an aggregation factor to facilitate SG assembly after p-eIF2α (Fig 3). This cytoplasmic C-Terminal domain is also involved in trafficking and proteolytic processing by dimerization suggesting this could involve in phase separation after stress signaling.

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It is stated that depletion of key components of SGs concomitantly reduces the survival rate after stress exposure. Typically, this phenomenon is due to the inhibition of SG assembly but, in contrast we observed a marginal increase in survival rate with *NRG2* knockdown. It is also known that SGs inhibit stress induced apoptosis by recruiting signaling molecules such as RACK1, TRAF2 under mild stress, but these proteins localize to SG at later stage. In fact, it is clear that LCD regions present in NRG2 can act in the nucleation process at the primary aggregation stage and eventually localized to SGs within minutes after stress exposure. One likely reason for late localization of RACK1 to SGs is that, RACK1 is completely devoid of any disordered region. This suggests that the disordered region present in NRG2 is an advantageous criterion for SG assembly, unless further experiments addressing the role of NRG2 deletion mutants and its localization to SG is confirmed. This finding is parallel to the report in which SG suppresses stress responsible MAPK pathway by actively recruiting RACK1 under mild stress conditions. Moreover, this data adds NRG2 in the previously proposed model of SGs as multi-cellular signaling hub where many signaling proteins orchestrate stress induced response (1). Together, our data outlines the functional role for the localization NRG-2 into SGs.

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MATERIALS AND METHODS

Cell culture and transfections

U2OS (human osteosarcoma), HeLa and HEK293T cells were maintained in DMEM medium (Welgene) supplemented with 10% inactivated FBS (Welgene), 1% (v/v) penicillin and streptomycin (Lonza) at 37℃ in 5% CO_2. Transfection of siRNA was performed using Lipofectamine 2000 (Invitrogen) at 40 nM final concentration as per manufacturer's instruction. siRNA sequences were as follows: siCONT, 5`-GCATTCACTTGGATAGTAA-3`; siNRG2-1, 5`-GGAACAGCCCTTAGTCTTT-3`; siNRG2-2, 5`-GGTCGGGTGGCGTTGGTAA-3`.

Western blot analysis

Cells were lysed in RIPA buffer (50 mM Tris-Cl (pH 8.0), 150 mM NaCl, 0.1% SDS, 1% NP-40, 1 mM EDTA, 1% Sodium deoxycholate, containing proteinase inhibitors 5mM NaF, 1mM PMSF) for 15 min in ice and centrifuged at 13,000 rpm for 15 min. Proteins were quantified using Bradford reagent. Total proteins (20-50 µg) were subjected to SDS-PAGE, transferred to nitrocellulose membranes and detected with respective antibodies. Western blot was performed using ECL detection system. Primary antibodies were purchased as follows: Anti β-actin from Abcam (Cambridge, MA, USA); p-eIF2α from Biomol (Farmingdale, NY, USA); eIF2α from Santa Cruz Biotechnology and anti - NRG2 from Millipore.

Immunofluorescence analysis

Cells grown on coverslips were untreated or treated with drugs, rinsed twice with PBS (pH7.4), fixed with paraformaldehyde for 15 min, permeabilized with cold methanol for 10 min and blocked in 5% normal horse serum in PBS containing 0.02% sodium azide for 1 h. Primary

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antibodies diluted in blocking solution were added and incubated either at RT for 1h or overnight at 4℃. Cells were then washed with PBS (three times, 10 min each) and were incubated with respective secondary antibodies for 1h at RT, washed thrice with PBS (10 min each) and were mounted in polyvinyl medium. Fluorescence microscopic images were taken using a Nikon Eclipse 80i fluorescence microscope (40X). All images were processed in Image J and compiled in adobe photoshop CS5. Primary antibodies for ICC were purchased as follows: eIF3b, G3BP, TIA-1 and p70S6 kinase from Santa Cruz; RCK from Bethyl laboratories; NRG2 from Millipore. Cy2-, Cy3-, Cy5- conjugated secondary antibodies were purchased from Jackson Immunoresearch labs.

Polysome profiling

U2OS cells (2 x 150mm dish) were treated with indicated time and concentration of sodium arsenite. After treatment, 10 µg ml^{-1} cycloheximide was added and incubated for 5 min at RT, washed with cold PBS, then lysed in 1ml of polysome lysis buffer (20 mM HEPES (pH7.6), 5 mM $MgCl_2$, 125 mM KCl, 1% NP-40, 2 mM DTT) supplemented with 100 µg ml^{-1} cycloheximide (sigma), protease inhibitor cocktail (EDTA-free; pierce) and RNAsin (Ambion) at cold room. Cell lysates were tumbled for 15 min at 4 ^{0}C and centrifuged at 13,000 rpm for 15 min. The supernatants were fractionated in 17.5 - 50% linear sucrose gradients by ultracentrifugation (35,000 rpm for 2 h 40 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 ^{0}C for overnight and processed for further analysis.

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Cell viability assay

Cell viability was measured using MTT [3(4,5-cimethylthiazol-2-yl)-2,5-diphenil tetrazolium bromide] (Sigma). After treatment, cells were washed with PBS twice and incubated with 0.5 g/ml MTT for 2 hours at 37°C until the formation of dark formazan crystals. After lysis with DMSO, absorbance was measured at 570 nm. All measurements were done in triplicate wells.

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FIGURE LEGENDS

Figure 1. *NRG2* knockdown impairs SGs assembly. (A) U2OS cells transfected with siCONT, siNRG2-1 and siNRG2-2 for 90 hours were grown on coverslips and treated with 0.2 mM arsenite for indicated time points. Cells were then stained against universal SG marker eIF3b (green), SG/PB marker RCK (red) and nuclei stain Hoechst (blue). (B) Whole cell lystaes were subjected to immunoblot assay to determine knockdown efficiency of NRG2-1 and NRG2-2 and induction of p-eIF2alpha. Actin was used as loading control. Quantified western blot results were represented below. (C) U2OS derived EGFP-G3BP stable cell line was transfected with different siRNAs and arsenite treatment was given as in Fig.1A. Presence of SG was then visualized using GFP fluorescence and PB using antibody reactive to p70S6K. (D) SG quantification data showing percentage of cells with SGs. Data are means ± s.d. of atleast three independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, Student's t-test. Scale bar, 10µm.

Figure 2. NRG2 localizes to SGs under arsenite stress. (A) U2OS cells grown on coverslips were treated with 0.5 mM arsenite for 1 hour before processing for immunostaining against eIF3b (green), NRG2 (red), PB marker S6K (far red) and nuclei stain Hoechst (blue). (B) U2OS cells were treated with 0.5mM arsenite for 1 hour and stained against established SG markers TIA (green), NRG2 (red), G3BP (far red) and nuclei stain Hoechst (blue). (C) HeLa cells were grown on coverslips and treated with 0.5 mM arsenite for 1 hour prior to immunostaining against eIF3b (green), NRG2 (red), S6K (far red) and nuclei stain Hoechst (blue). Scale bar, 10µm. Enlarged views of boxed areas show individual channels (green, red and far-red). Arrow head represents SGs. Data presented represents atleast three independent experiments.

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Figure 3. NRG2 depletion doesn't affect stress induced polysome disassembly. U2OS cells seeded at low density in 150 mm dish were knocked-down twice with siCONT or siNRG2-1 for 90 hours. Cells were then untreated (control) or treated with 0.2 mM arsenite for 30 mins to induce polysome disassembly. After treatment, cells were lysed in polysome lysis buffer, subjected to sucrose gradient and polysome profile analysis. See methods for detailed procedure.

Figure 4. *NRG2* knockdown increases cell survival rate upon oxidative stress. U2OS cells transfected with control siRNA or two different siRNAs targeting NRG2 for 90 hours were seeded in 96 well plate. Next day, cells were untreated or treated with 0.5 mM arsenite at different time point (0, 1, 2, 3 and 4 hours). Cells were then subjected to cell survival assay as described in Methods. The results are mean ± S.D of atleast three independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, Student's t-test.

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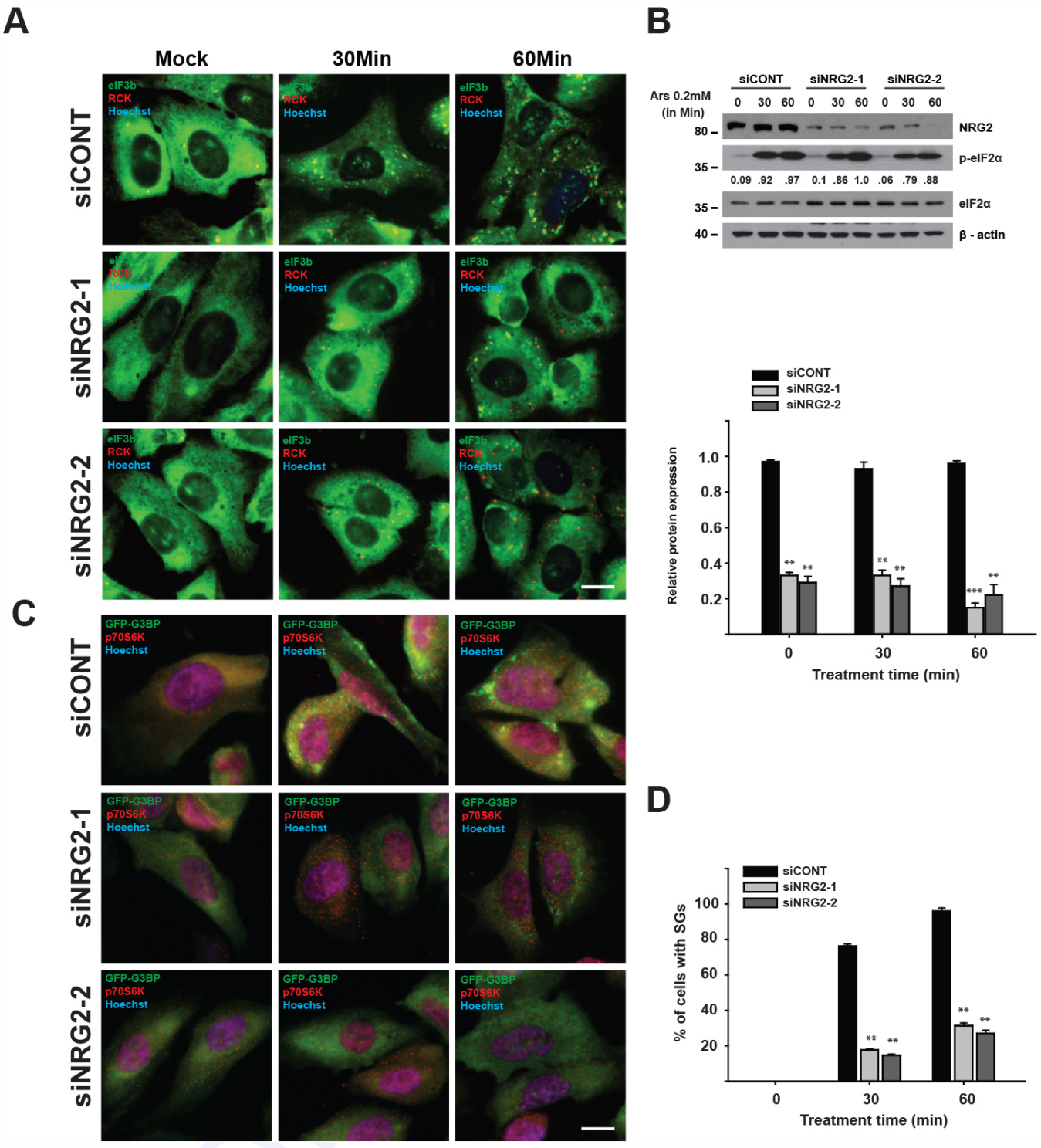
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60Min
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RCK
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Ars 0.2mM
(in Min)
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35 –
NRG2
p-eIF2α
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eIF2α
40 –
β - actin
Relative protein expression
1.0
0.8
0.6
0.4
0.2
**
***
0
30
60
Treatment time (min)
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Hoechst
D
% of cells with SGs
100
80
60
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Fig. 1

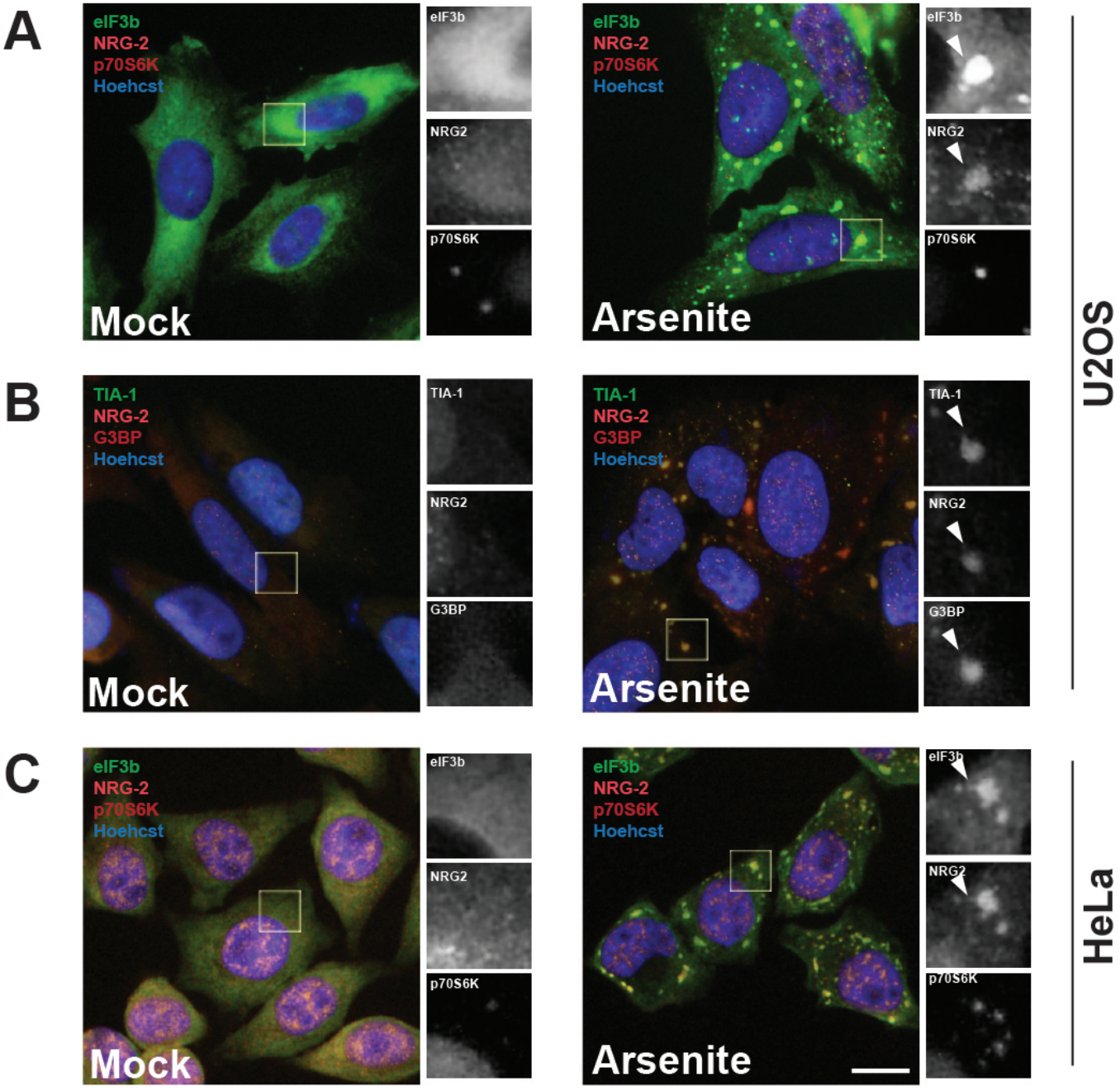

Fig. 2

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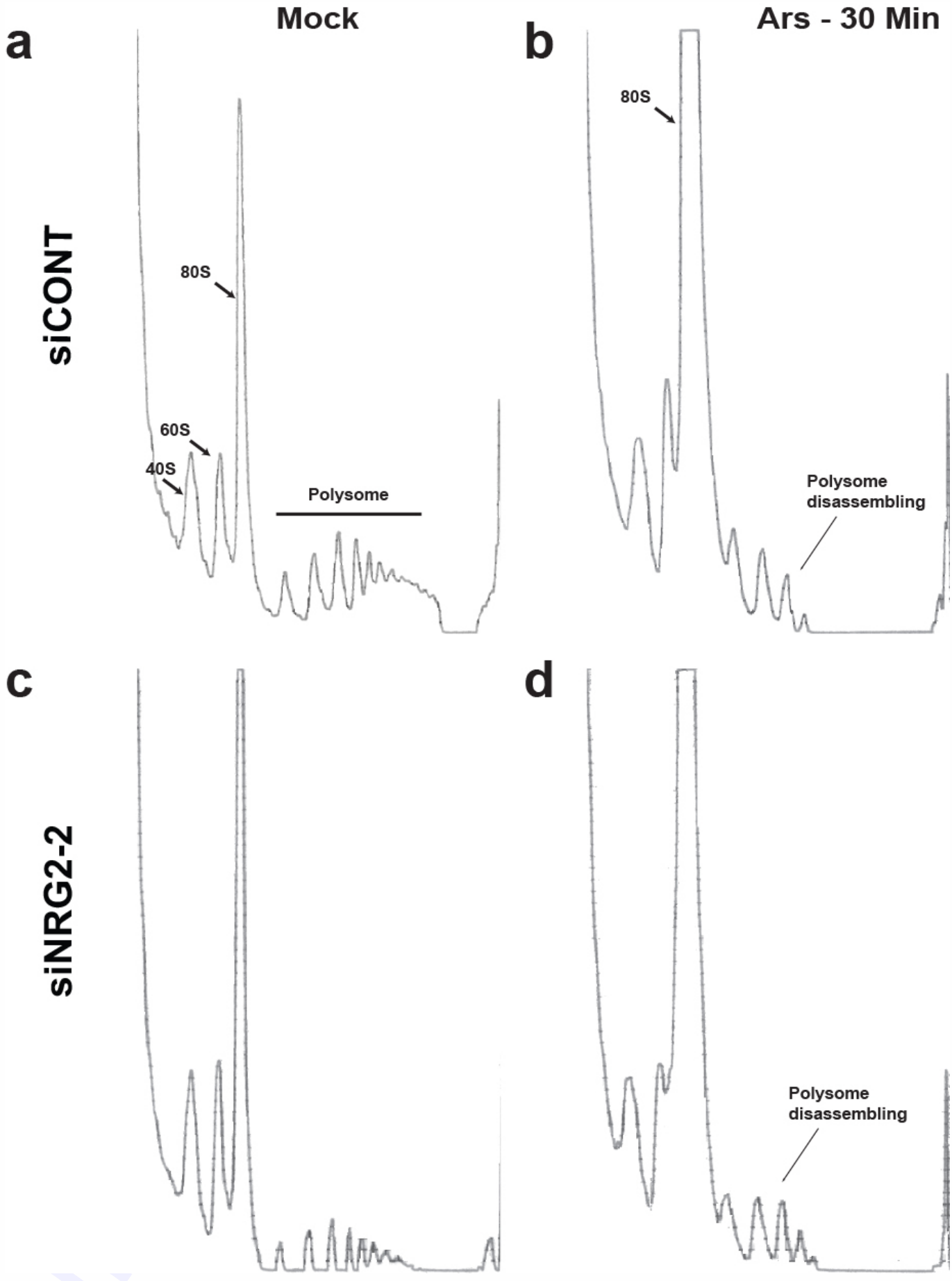
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Mock
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Fig. 3

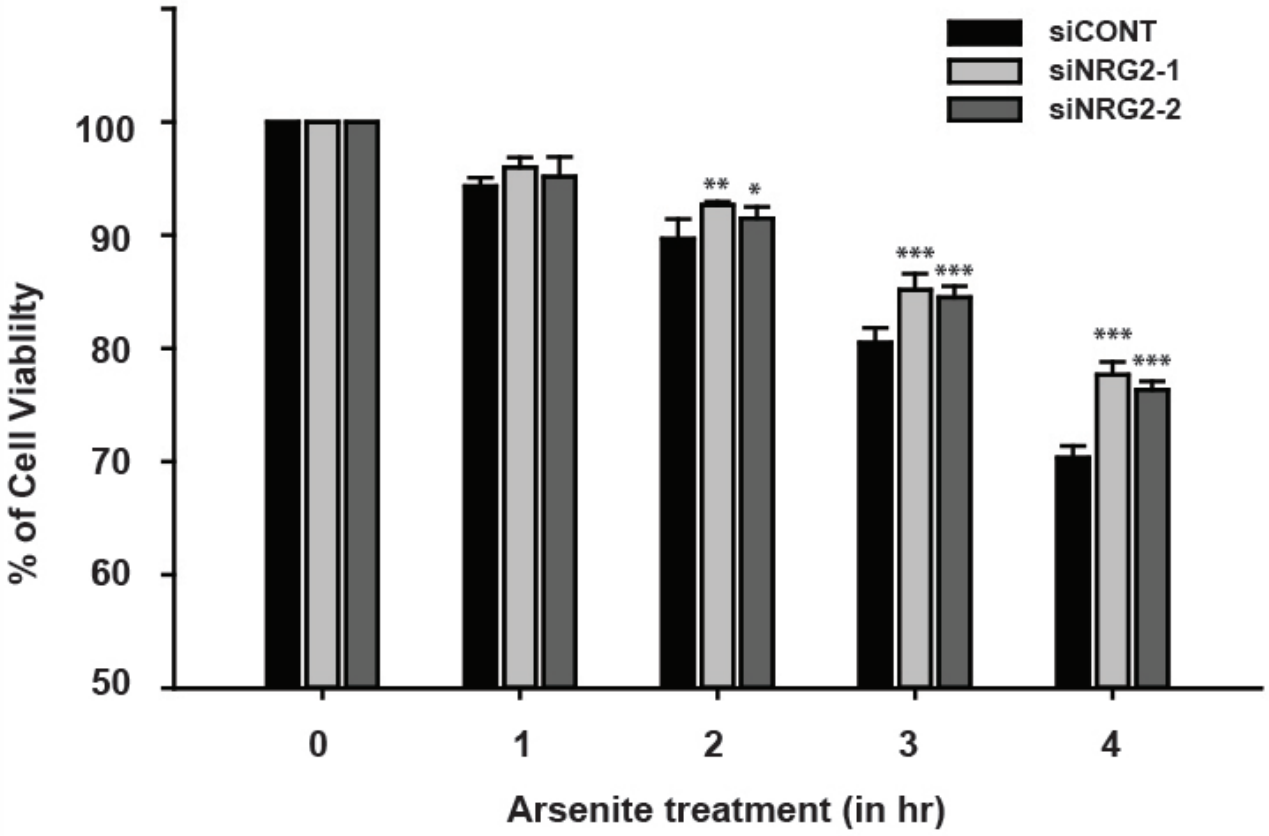

Fig. 4

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