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Ph.D. Thesis

Functional Analysis of NEDDylation on the Assembly of Stress Granule

Graduate School of Chosun University

Department of Bio-Materials

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Functional Analysis of NEDDylation on the Assembly of Stress Granule

Stress Granule 형성에 있어 NEDDylation의

기능적 연구

25th August 2016

Graduate School of Chosun University

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Functional Analysis of NEDDylation on the Assembly of Stress Granule

Advisor: Prof. Takbum Ohn

A dissertation submitted to the Graduate School of Chosun University in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Science

April 2016

Graduate School of Chosun University

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국문초록

Stress Granule 형성에 있어 NEDDylation의 기능적 연구

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생물신소재학과

스트레스 과립은 외부환경에서 유래된 다양한 스트레스에 의해 형성되는 세포질 내 RNA-protein (RNP) 응집체이다. 스트레스과립은 번역과정이 저해된 mRNP로서, RNA 결합단백질, 신호전달물질을 포함하고 있으며, 유전자 발현을 선택적으로 조절하여 세포의 운명을 결정하는 중요한 역할을 수행하고 있다. 이러한 스트레스 과립이 최근 연구에서 frontotemporal dementia (FTD), amyotrophic lateral sclerosis (ALS)와 같은 퇴행성 신경질환과 암과 같은 질환과 연관성이 있음이 밝혀졌다.

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그럼에도 불구하고 스트레스과립이 어떠한 분자적 기전에 의해서 형성이 되는지는 알려진 바가 거의 없다. 본 연구에서는 arsenite 스트레스에 반응하여 형성되는 스트레스과립이 NEDDylation 신호전달체계에 의해 조절됨을 밝혔다. NEDDylation 신호전달체계에서 필수적인 역할을 하는 유전자들이 결핍되었을 때 폴리좀 (polysome)의 분해가 저해되고, 스트레스과립의 형성이 억제됨을 확인하였다. Affinity 정제법과 질량분석 기법을 이용하여 NEDD8이 결합되는 타겟 프로테인들을 밝혀내었다. 이러한 단백질들 중에는 기존에 알려진 리보솜 단백질과 다양한 종류의 RNA결합 단백질들이 포함되어 있었으며, 무엇보다도 최근 본연구실에서 그 기능이 밝혀진 SRSF3단백질도 포함되어 있음을 확인하였다. 추가적인 분자기전 연구를 통하여 SRSF3 단백질의 리신 85번이 스트레스에 반응하여 NEDDylation됨을 확인 하였고, 이 과정을 통해 스트레스과립의 형성이 유도됨을 확인하였다. 본 연구를 통해 처음으로 NEDDylation 신호전달체계가 SRSF3를 타겟팅함으로서 스트레스 과립의 형성이 유도됨을 증명하였다.

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I. Introduction

In eukaryotes, translation can be divided into initiation, elongation, termination and ribosome recycling, in which translation initiation is considered as a highly regulated and sophisticated step as it allows rapid and reversible control of gene expression [1]. Translation initiation begins with the assembly of eIF2.GTP.Met-tRNAi ternary complex and, subsequent binding with the small (40S) ribosomal subunit to form 43S pre-initiation complex. The 43S pre-initiation complex is then recruited to the 5' end of mRNA containing various factors such as, eIF4F complex comprising cap binding protein (eIF4E), an RNA helicase (eIF4A) and a large scaffolding protein (eIF4G) and eIF3 and poly (A) binding protein (PABP). The pre-initiation complex then starts to scan in the 5' to 3' direction until it recognizes the initiation codon (AUG) which leads to codon-anticodon base pairing. After the recognition of initiation codon and 48S complex formation, eIF5 and eIF5B promote hydrolysis of eIF2-GTP and releases the Met-tRNA$_i$ into 40S subunit and facilitates joining of large (60S) ribosomal subunit to form 40S.Met.tRNA$_i$.mRNA complex to promote elongation [2].

Stress induced translation arrest is predominantly the cause of eIF2α phosphorylation at serine 51 which converts eIF2 from a substrate to competitive inhibitor of eIF2B, thereby reduces the availability of eIF2.GTP.Met-tRNA$_i$ ternary complex required for translation initiation leads

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to global translation initiation shut down. This causes elongating ribosomes to run off their transcripts and form polyadenylated circularized mRNPs which acts as substrate for SG formation [3]. The adverse condition is sensed by a variety of eIF2α kinases, including **PKR** (Protein Kinase R), a double stranded RNA dependent kinase activated upon viral infection and cold shock [4, 5]; **PERK** (PKR like Endoplasmic Reticulum Kinase) is activated by endoplasmic reticulum (ER) stress when unfolded proteins accumulate in ER lumen [6]; **GCN2** (General Control Non-repressible 2) is activated in response to amino acid starvation and UV irradiation [7]; **HRI** (Heme Regulated Inhibitor) primarily activated under heme deprivation and arsenite - induced oxidative stress and heat shock [8]. These kinases monitor different types of stress and regulate translation initiation through phosphorylation of eIF2α.

Stress granules (SGs) are non-membranous, cytoplasmic aggregates at which translationally stalled messenger ribonucleoprotein (mRNP) complexes are localized in response to various cellular stresses[9]. In cells exposed to adverse conditions, activation of the integrated stress response (ISR) leads to translational arrest, polysome disassembly, and SG assembly [10, 11]. The signature constituents of SGs are non-canonical 48S preinitiation complexes harboring non-translating mRNAs bound to small ribosomal proteins, 5'-cap (7-methyl guanosine, m^7G) proximal initiation factors eIF4E, eIF4G, eIF4A, eIF3s and poly(A)-binding protein (PABP) [12,

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13]. SGs also contain numerous RNA-binding proteins (RBPs) that regulate mRNA translation (e.g., TIA-1, TIAR, SRSF3, hnRNPs, TDP-43) and decay (e.g., Argonautes and XRN1) as well as signal transducers (e.g., TRAF2, G3BP1, RACK1 and TORC1) that modulate various cellular events such as cell growth and apoptosis[14-20].

Several signaling pathways and their associated post-translational protein modifications have been shown to modulate SG assembly and disassembly. The phosphorylation of eIF2 through the ISR is a key initial step to stimulate SG assembly, although inhibition of eIF4A using drugs or lipid mediators have been reported to initiate SG assembly independently of phospho-eIF2 [21, 22]. Phosphorylation of Ras-Gap Binding protein 3 (G3BP) at Serine 149 has been reported to regulate SG assembly[23, 24]. During heat shock, focal adhesion kinase (FAK) also modulates SG assembly through targeting growth factor receptor-bound protein 7 (Grb7). Dual specificity tyrosine-phosphorylation-regulated kinase 3 (DYRK3) has recently shown to modulate SG dynamics through possibly targeting RBPs and proteins downstream of mTORC1 signaling[25]. *O*-GlcNAc modification of ribosomal proteins is known to regulate SG aggregation but not stress-induced translation inhibition and polysome disassembly[10]. Stress-responsive poly(ADP) ribosylation of SG components has also been implicated in SG aggregation via a potential scaffolding function[26]. Although relevant targets and associated mechanisms are unknown, ubiquitination and acetylation are

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likely have important roles in SG assembly[27]. These previous studies suggest that multiple signaling pathways and related molecular targets are crucial to coordinate SG dynamics in cells exposed to various stresses.

NEDD8 (neural precursor cell expressed developmentally downregulated protein 8) is a small ubiquitin-like protein (UBL) that is covalently conjugated to Lys residues on protein substrates in a manner similar to ubiquitin. The NEDD8 conjugation system consists of a single E1 activating enzyme (NAE), a heterodimer of amyloid- precursor protein binding protein 1 (APPBP1) and ubiquitin-activating enzyme 3 (UBA3), and two E2s, UBE2M (also known as UBC12) and UBE2F. NEDD8-specific E3 ligases are not well understood and all currently reported E3s can also function in the ubiquitination system[28]. Neddylation primarily targets Cullin components of Cullin-RING Ligases (CRLs), although there are other known targets of Nedd8, including p53 and Histone H4[29-31].

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II. Materials and Methods

Cell culture and transfection

U2OS (human osteosarcoma), HeLa and HEK293T cells were obtained from ATCC and 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 siRNAs were performed using Lipofectamine 2000 (Invitrogen) at 40 nM final concentration, all siRNA sequences used in this study are listed in Supplementary Table-2. All DNA plasmids were transfected using either PEI (Polysciences) or Fugene 6 (Promega) as per manufacture's protocol.

Antibodies

The following antibodies were used for immunoblotting: rabbit polyclonal anti-FANCM (1:1000; (H-300) sc-98710; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Mouse monoclonal anti-FANCD2 (1:1000; (FI17): sc-20022; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Rabbit-polyclonal BRCA1 (1:1000; (C-20): sc-642; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Rabbit-polyclonal BRCA2 (1:2000; A303-434A; Bethyl Laboratories, USA). Mouse monoclonal anti-b actin (1:10,000; (C4): sc-47778; Santa Cruz Biotechnology, Santa Cruz, CA, USA).

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FANCD2 foci was detected by immunofluorescence staining using FANCD2 antibody (AB2187; Abcam, Cambridge, UK) at 1:500 dilution. G-H2AX foci were detected by immunofluorescence staining using g-H2AX mouse monoclonal antibody (JBW301), Upstate Biotechnology, Temecula, CA, USA) at the dilution of 1:200. RAD51 foci were detected by immunofluorescence staining using mouse monoclonal anti-RAD51 (14B4) (ab213; Abcam, Cambridge, UK).

Immunofluorescence microscopy

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 hr. Primary antibodies diluted in blocking solution were added and incubated either at RT for 1hr or overnight at 4℃. Cells were then washed with PBS (three times, 10 min each) and were incubated with respective secondary antibodies (Jackson Immunoresearch ML grade) for 1hr at RT, washed thrice with PBS (10 min each) and were mounted in polyvinyl medium. All images were taken using a Nikon Eclipse 80i fluorescence microscope, processed in Image J and compiled using Adobe Photoshop CS5. Knockdown or overexpression effects on SGs and PBs were assessed by quantifying the number of cells out of at least 100 cells from different fields as percentage.

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Immunoprecipitation

Cells were harvested and lysed in IP buffer (50 mM Tris-Cl (pH7.5), 150 mM NaCl, 1 mM EDTA, 1% Trition X-100) supplemented with proteinase inhibitors 1 mM PMSF, 10 μg/ml aprotonin, 5 μg/ml leupeptin, 0.5 μg/ml pepstatin and 5 mM NaF on ice for 20 min, centrifuged at high speed for 15 min and the supernatant were collected in fresh tube. For immunoprecipitation, 1-2 mg lysates were incubated with 20-30 μl Flag agarose beads overnight at 4^0C. The resulting immunoprecipitates were washed at least three times with IP buffer, before boiling with SDS sample buffer. The resulting eluates were blotted against indicated antibodies.

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.

Polysome profiling analysis

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U2OS cells (2 x 150mm dish) were treated with indicated time and concentration of sodium arsenite. After treatement, 10 µg ml^{-1} cycloheximide was added and incubated for 5 min at RT, washed with cold PBS, then lysed with 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.

Immunopurification of NEDD8 modified proteins

U2OS cells stably expressing Bir-A or FB-NEDD8 (6 x 150mm dish) treated with arsenite (0.5 mM) for 1hr were harvested in polysome lysis buffer and subjected to sucrose gradients as described earlier. To purify nedd8 modified proteins, fraction no 7 and 8 (which are enriched with neddylated proteins) were combined, acetone precipitated overnight at -20^{0}C, centrifuged at high speed for 15 mins and the pellets were air-dried. Air-dried pellets were resuspended in 100µl denaturing buffer (50 mM Tris-Cl (pH7.6), 2 mM

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EDTA, 1% SDS), boiled at 60^0C for 10 min and diluted to 1 ml using dilution buffer (50 mM Tris-Cl(pH7.6), 2 mM EDTA, 150 mM NaCl, 0.1% NP-40) containing protease inhibitor cocktail (Pierce), 5 mM NaF and 10 mM IAA (Sigma). Denatured total proteins were incubated with streptavidin agarose beads at 4℃ overnight. The beads were washed extensively for 4 times using dilution buffer including 0.1% SDS, and the proteins were eluted by boiling with SDS sample buffer for 10 min. The eluted proteins were either resolved in 12% SDS PAGE for immunoblotting or stained with commassie to reveal nedd8 modified proteins.

Enzymatic in-gel digestion

The proteins separated by SDS-PAGE were excised from the gel and the gel pieces containing protein were destained with 50% acetonitrile (ACN) containing 50 mM NH_4HCO_3 and vortexed until CBB was completely removed. These gel pieces were then dehydrated in 100% acetonitrile and Vacuum-dried for 20min with speedVac. For the digestion, gel pieces were reduced using 10 mM DTT in 50 mM NH_4HCO_3 for 45 min at 56℃, followed by alkylation of cysteines with 55mM iodoacetamide in 50 mM NH_4HCO_3 for 30 min in dark. Finally, each gel pieces were treated with 12.5 ng/μL sequencing grade modified trypsin (Promega, Madison, WI) in 50mM NH_4HCO_3 buffer (pH 7.8) at 37℃ for overnight. Following digestion, tryptic peptides were extracted with 5% formic acid in 50% ACN solution at room

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temperature for 20 min. The supernatants were collected and dried with SpeedVac. Re-suspended samples in 0.1% formic acid were purified and concentrated using C18 ZipTips (Millipore, MA) before MS analysis.

Nano-LC-ESI-MS/MS analysis

The tryptic peptides were loaded onto a fused silica microcapillary column (12 cm x 75 μm) packed with C18 reversed phase resin (5 μm, 200 Å). LC separation was conducted under a linear gradient as follows: a 3-40% solvent B (ACN containing 0.1% formic acid) gradient (solvent A; DW containing 0.1% formic acid), with a flow rate of 250 nL/min, for 60 min. The column was directly connected to LTQ linear ion-trap mass spectrometer (Finnigan, CA) equipped with a nano-electrospray ion source. The electrospray voltage was set at 1.95 kV, and the threshold for switching from MS to MS/MS was 500. The normalized collision energy for MS/MS was 35% of main radio frequency amplitude (RF) and the duration of activation was 30 ms. All spectra were acquired in data-dependent scan mode. Each full MS scan was followed by five MS/MS scan corresponding from the most intense to the fifth intense peaks of full MS scan.

Database searching and validation

The acquired LC-ESI-MS/MS fragment spectra was searched in the BioWorksBrowser™ (version Rev. 3.3.1 SP1, Thermo Fisher Scientific Inc.,

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CA) with the SEQUEST search engines against National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) Homo Sapiens database. The searching conditions were trypsin enzyme specificity, a permissible level for two missed cleavages, peptide tolerance; ±2 amu, a mass error of ±1 amu on fragment ions and variable modifications of carbamidomethylation of cysteine (+57 Da) and oxidation of methionine (+16 Da) residues. The delta CN was 0.1; the Xcorr values were 1.8 (+1 charge state), 2.3 (+2), 3.5 (+3); and the consensus score was 10.15 for the SEQUEST criteria.

***in vivo* neddylation assay**

Identification of neddylated proteins were performed as previously described[32] with little modification. Briefly, HEK293T cells co-transfected with indicated plasmids for 36 to 40 hours were untreated or treated with arsenite, washed twice with PBS and scraped using 1ml PBS. 10% of cell suspension was centrifuged and the cell pellet was lysed using RIPA buffer for Western blot analysis. The remaining cell suspension was directly added to 6ml Guanidinium buffer (6M guanidinium-HCl, 0.1M Na2HPO4/NaH2PO4, 0.01M Tris-Hcl, pH-8.0) containing 5 mM imidazole, 0.1% Triton X-100 and 10 mM β-mercaptoethanol and lysed for 20 min. 50μl of Ni-NTA- agarose beads were added directly and lysates were incubated for 4hr at RT. After incubation, beads were washed once with 800 μl of

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Guanidinium buffer containing 5 mM imidazole, 0.1% Triton X-100 and 10 mM β-mercaptoethanol, once with 800µl of Urea buffer A (8 M Urea, 0.1 M Na2HPO4/NaH2PO4, 0.01 M Tris-HCl(pH8.0)) containing 5 mM imidazole, 0.1% Triton X-100 and 10 mM β-mercaptoethanol and thrice with 900 µl of Urea buffer B (8 M Urea, 0.1 M Na2HPO4/NaH2PO4, 0.01M Tris-HCl(pH6.3)) containing 5 mM imidazole, 0.1% Triton X-100 and 10 mM β-mercaptoethanol. 6xHis-tagged proteins were eluted by incubating the beads in 50µl elution buffer (5% SDS, 200 mM imidazole, 0.15 M Tric-Cl (pH6.7), 30% glycerol, 0.72 M β-mercaptoethanol, 0.01% Bromophenol Blue) for 20 min. The eluted proteins were directly resolved in SDS-PAGE and blotted to reveal Nedd8 conjugates. For *in vivo* neddylation assay targeting endogenous NEDD8, immunoprecipitation was performed as described in the previous report[33] with little modification. Briefly, cells transfected with indicated plasmids for 36 hours were lysed in denaturing lysis buffer (1% SDS, 20mM Tris-HCl pH 8.0, 5mM EDTA, 10mM iodoacetamide, 250U/ml Benzonase (Santa cruz), protease inhibitors), boiled for 5 mins at 90^0C and diluted 10 fold with dilution buffer (20mM Tris-HCl pH8.0, 150mM NaCl, 1% NP-40 and protease inhibitors). Cell lysates were then immunoprecipitated using flag beads (Sigma) and the precipitates were blotted against anti-NEDD8 antibody.

Generation of FB-NEDD8 stable cell line

U2OS cells transfected with pEF1α-Bir-A were maintained in G418 (neomycin) at 0.5 mg/mL concentration for several days until cells in parental

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U2OS cells (U2OS cells without transfection) were completely dead. Medium was changed for every 40 h to remove dead cells as well as replenish drug sensitivity. Drug resistant cells were trypsinized, diluted, single clone was selected and the expression level was analysed by immunoblot. The selected U2OS cell line stably expressing Bir-A was transfected with pEF1α-FB-NEDD8 (puromycin resistant vector) for 40 h before maintained in a medium containing puromycin at 2 μg/mL concentration. Non-transfected U2OS cells stably expressing Bir-A was used as control to monitor puromycin drug resistant. A single clone stably expressing FB-NEDD8 similar to endogenous NEDD8 was selected and used for further experiments.

Statistical analysis

Data in all experiments are represented as mean ± s.d. Statistical comparisons were carried out using two-tailed paired *t*-test. We considered $p < 0.01$(**) as significant. Analyses were carried out with Prism software (GraphPad) and Excel (Microsoft). Negative correlation of MDC1 expression with pAkt1 levels was assessed using the Pearson correlation test with p value. p values less than or equal to 0.01 were considered statistically significant.

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III. Results

Neddylation pathway regulates SG assembly

In our previous RNAi screen (~7,300 genes) designed to identify genes whose expression is required for arsenite-induced SG assembly, the E2 conjugating enzyme UBE2M was a "hit" [10]. We confirmed that UBE2M knockdown strongly impairs the arsenite-induced SG assembly (Fig. 1).

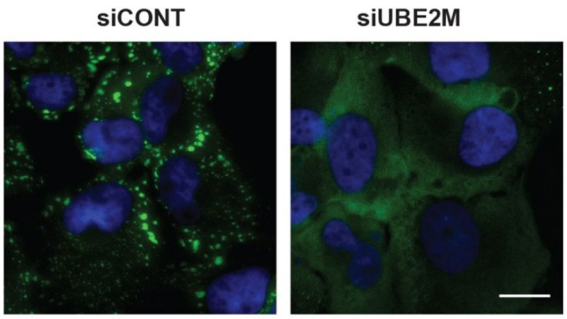

Figure 1. UBE2M knockdown impairs SG assembly.

Since UBE2M mediates protein neddylation, we hypothesized that the neddylation pathway might regulate SG assembly. To test this, we performed UBE2M and NEDD8 knockdown experiments. As shown in Fig. 2, cells depleted of UBE2M and NEDD8 protein have a significant defect in eIF3b-positive SG assembly.

To further explore the impact of neddylation on SG assembly, we used MLN4924 (pevonedistat), a small molecule inhibitor of NAE

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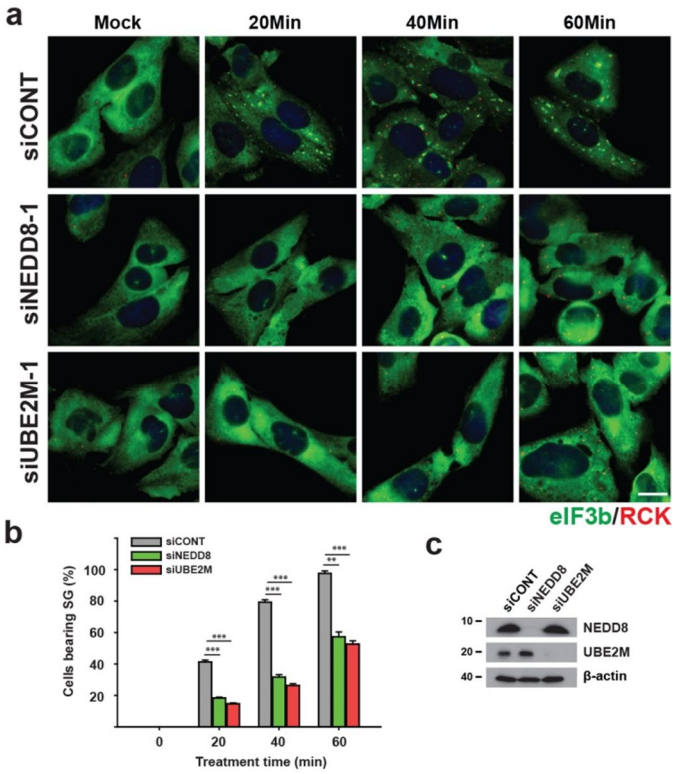

Figure 2. Neddylation pathway regulates SG assembly.

(NEDD8 activating E1 enzyme), to block the cellular neddylation pathway [34]. In a dose-response analysis (Appendix 1), inhibition of SG assembly was observed at a minimum concentration of 1 M. A time course analysis at this concentration revealed MLN4924-mediated inhibition of SG assembly was similar in magnitude to that observed following UBE2M and NEDD8 knockdown (Fig. 3). These results were confirmed using HeLa (Appendix 2) and U2OS-derived EGFP-G3BP stable cell lines (Appendix 3).

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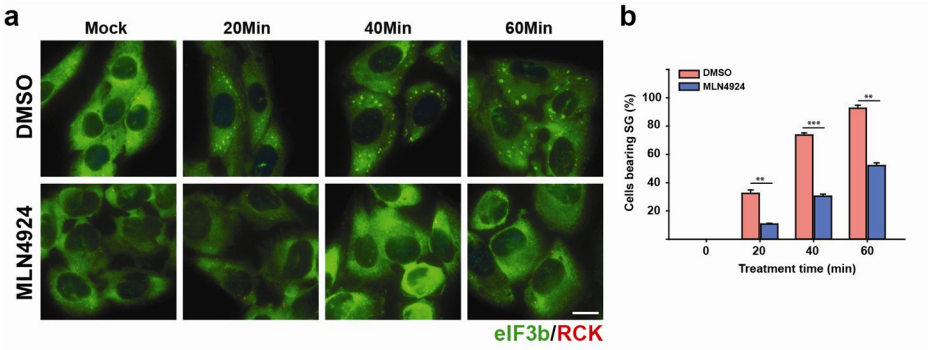

Figure 3. MLN4924 inhibits SG formation.

Arsenite-induced phosphorylation of eIF2 causes translational arrest, polysome disassembly and SG assembly[35]. We used sucrose gradient analysis to compare polysome profiles in cells expressing reduced levels of UBE2M and NEDD8. In siCONT transfected cells, arsenite-induced translational arrest results in the collapse of polysome profiles and accumulation of monosomes and individual ribosomal subunits (Fig. 4a, upper panels). Knockdown of both NEDD8 and UBE2M modestly increases the accumulation of non-translating ribosomal peaks but largely have no effect on polysomal peaks in the absence of stress (Fig. 4a, lower left panel).

In contrast, arsenite-induced polysome disassembly is partially inhibited in stressed cells with reduced NEDD8 and UBE2M expression (Fig. 4a, lower right panel). The phosphorylation of eIF2 is an indicative of polysome collapse and translation repression[36].

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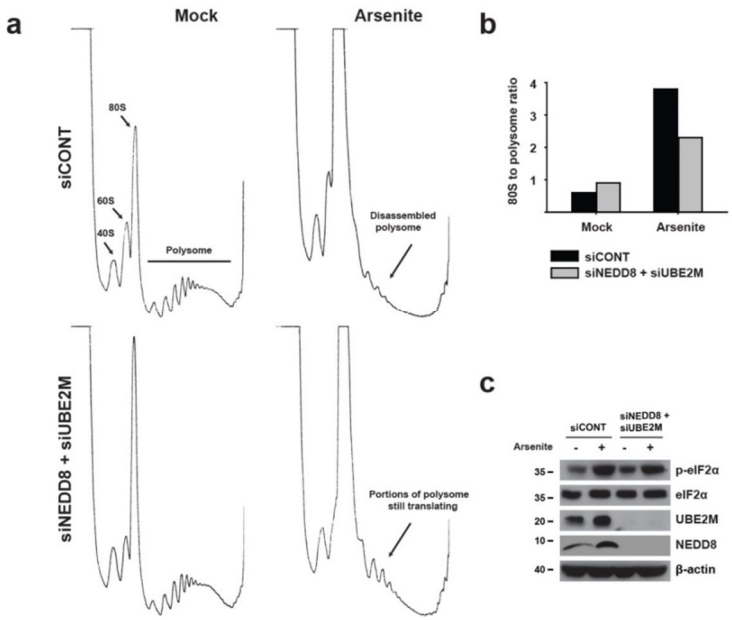

Figure 4. Blocking neddylation pathway slows down polysome disassembly.

Interestingly, the basal levels of eIF2 phosphorylation in a non-stressed condition are marginally increased in NEDD8 or UBE2M knockdown cells that are likely reflected by marginally increased 80S monosome peaks. The extent of arsenite-induced phosphorylation of eIF2 in NEDD8 or UBE2M knockdown cells are comparable to that of control knockdown cells suggesting that the neddylation pathway acts downstream of eIF2 phosphorylation (Fig. 4c). Similarly, a time-course analysis of polysome profiles in cells treated with or without MLN4924 reveals a delay in arsenite-induced polysome disassembly (Appendix 4). These results argue that

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neddylation promotes polysome disassembly after ISR-induced translational arrest.

UBE2M and NEDD8 are integral components of SG

Because altered expression of SG components generally affects SG dynamics, we next examined whether UBE2M and NEDD8 are present in SGs induced with arsenite (oxidative stress), clotrimazole (mitochondrial stress) or thapsigargin (endoplasmic reticulum stress) by employing indirect immunofluorescence microscopy using antibodies against UBE2M and NEDD8. In unstressed cells, UBE2M is diffusely distributed throughout the cell (Fig. 5), while NEDD8 is concentrated in the nucleus (Fig. 6).

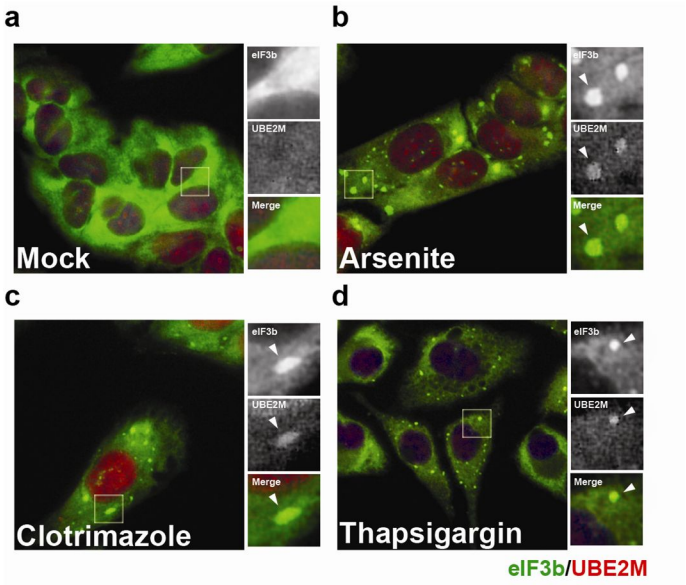

Figure 5. UBE2M is a component of SGs.

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In stressed cells, spots of both UBE2M and NEDD8 became visible in SGs that are positive with eIF3b (Fig. 5b-d) and G3BP-containing SGs (Fig. 6b-d), respectively. Thus, we conclude that UBE2M and NEDD8 are integral components of SGs. Knockdown of NEDD8 or UBE2M also strongly inhibited clotrimazole or thapsigargin-induced SG formation (Appendix 5), suggesting that the role of the neddylation system is not limited to the arsenite-induced stress.

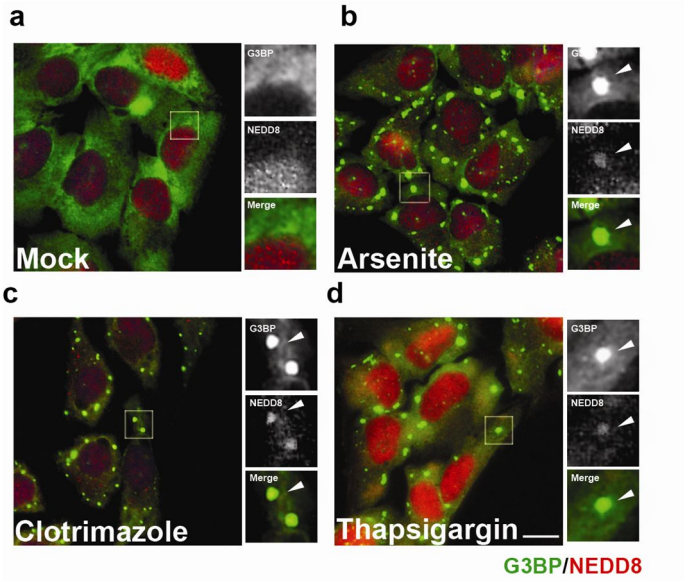

Figure 6. NEDD8 is a component of SGs.

Inhibition of the neddylation does not affect PB assembly

P-bodies (PB) are a second class of cytoplasmic RNA granule whose composition and function are distinct from SGs[37, 38]. To test whether perturbation of the neddylation pathway affects the PB assembly, PBs were

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visualized using antibodies reactive with RCK (DDX6) and S6K1 in cells treated with control, UBE2M and NEDD8 targeting siRNAs. As shown in Fig. 7, neither UBE2M nor NEDD8 are required for constitutive or arsenite-induced PB assembly. MLN4924 treatment also does not alter the kinetics of PB assembly. These results suggest that neddylation pathway is required for the efficient assembly of SG, but not PB.

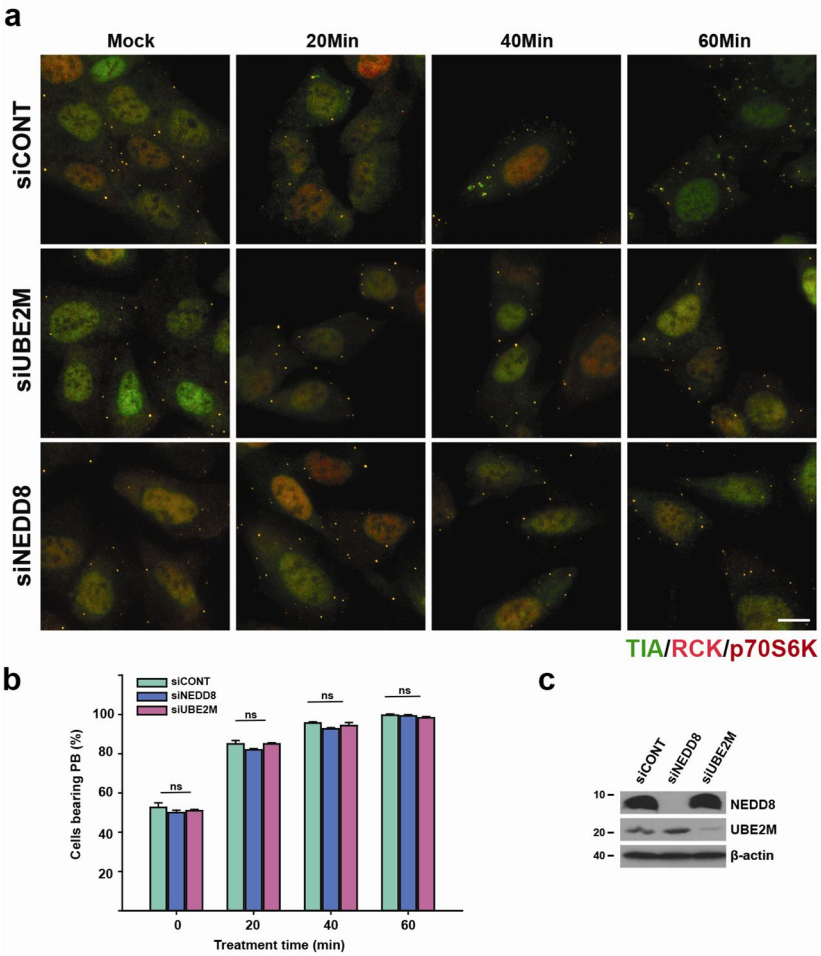

Figure 7. Neddylation pathway does not regulate PB assembly.

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Proteomics identifies neddylated proteins

In light of the above findings, we sought to identify proteins whose neddylation is required for SG assembly. To identify specific neddylated proteins involved in SG assembly, we first analyzed sucrose gradient fractions obtained from control versus arsenite-treated cells.

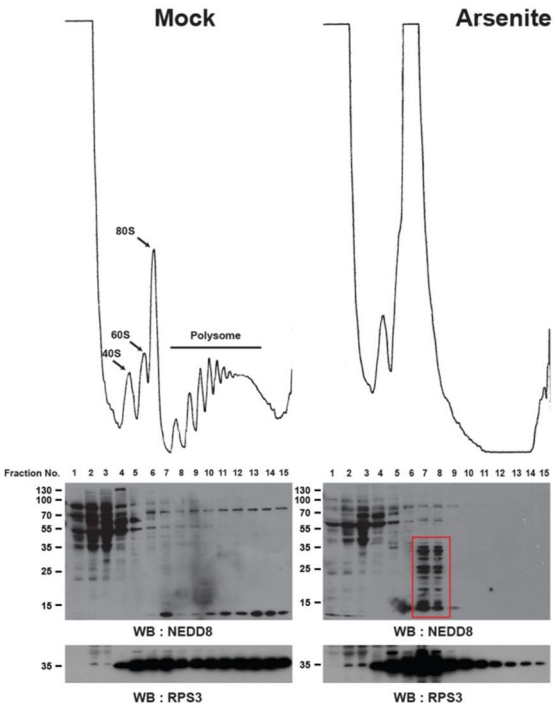

Figure 8. Neddylated proteins accumulate at 80S monosome fractions.

Interestingly, Western blotting with an anti-NEDD8 antibody reveals the arsenite-induced neddylation of low-molecular-mass (10-40 kDa) proteins that sediment together with monosomes and untranslated mRNPs (Fig. 8). To

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affinity-purify those proteins, we employed an *in vivo* biotinylation system[39] that has been successfully used in previous studies[40]. For the first step, bacterial biotin ligase (BirA) was stably expressed in U2OS cells and clonal selection was performed using G418 (Fig. 9).

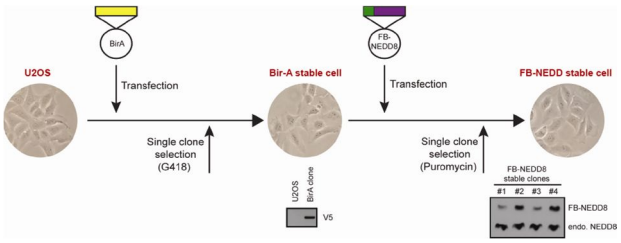

Figure 9. Schematic representation for generation of FB-NEDD8 stable cell line.

Next, Flag-biopeptide- (FB-) tagged NEDD8 was stably expressed in BirA stable cells and clonal selection was performed using puromycin (see Methods). Of the four clones selected, we chose clone #2 which expresses a low level of FB-NEDD8 compared to endogenous NEDD8 for affinity purification (Fig. 9, Western blot panel). The morphology and growth phenotype of FB-NEDD8 stable cells are similar to those of parental U2OS. More importantly, the level of arsenite-induced SG assembly in FB-NEDD8 stable cells is similar to that of U2OS parental cells (Appendix 6). Western blotting of arsenite-treated ribosomal fraction samples with streptavidin-HRP conjugate displays similar pattern to that of immunoblot detected with

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NEDD8 antibody (Fig. 10a, compare boxed regions in Fig 8a and Fig 10). These fractions were precipitated with acetone, boiled in 1% SDS to disrupt protein or mRNP complexes completely, then diluted and affinity-purified with streptavidin beads (see methods for details). Neddylated proteins resolved on PAGE were revealed by staining with Coomasie blue (Fig. 10b). A total of 17 distinct bands were excised and subjected to mass-spectrometry (see methods), which identified a large number of ribosomal proteins that are previously known neddylation targets[41].

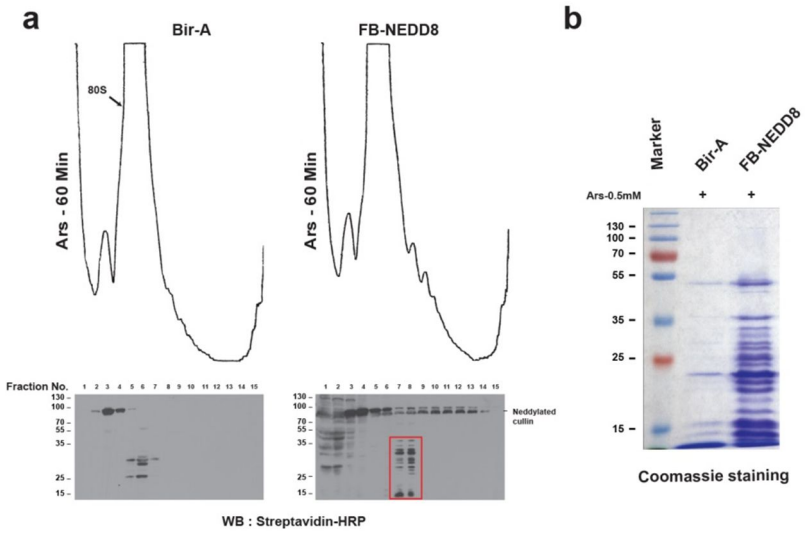

Figure 10. Immunopurification of stress induced neddylated proteins.

Interestingly, eukaryotic translation initiation (eIF2 , eIF3g, eIF3m, eIF3i, eIF3h, eIF4AII, eIF6, CTIF) and elongation (eEF1 . factors were identified - many of these proteins are previously identified but unconfirmed neddylation targets[42]. Additionally, many heterogeneous nuclear ribonucleoproteins

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(hnRNPs) and two serine/arginine (SR)-rich splicing factors (SRSF1, SRSF3) that function in a wide range of RNA processing and regulation events were identified. The ribosome-associated protein RACK1 that was identified as an *O*-GlcNAc modification target is also in the list from Mass Spec Data. Because translation factors and RBPs are well-known regulators of SG assembly, these findings suggest that neddylation of these proteins might promote SG assembly.

It was interesting for us to find SRSF3 from the proteomic study, because we recently reported that SRSF3 is a novel and necessary SG component and when depleted, SGs are potently disrupted[10, 14]. The knockdown effect of SRSF3 on SG assembly was confirmed with different siRNAs in U2OS cells (Appendix 7). Endogenous and Flag-tagged SRSF3 staining also revealed its presence in SGs (Appendix 8). Based on this evidence, we selected SRSF3 to explore the possible mechanistic link between the neddylation pathway and SG assembly.

SRSF3 is neddylated in cells subjected to arsenite stress

To confirm whether SRSF3 is indeed neddylated, we undertook a neddylation assay under denaturing conditions using HEK293-T cells[32]. Flag-SRSF3 was transfected with either His-NEDD8 or His-NEDD8ΔGG (a non-conjugatable NEDD8), then mock or arsenite-treated cells were harvested under denaturing conditions, and affinity pull-down assay was performed

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followed by Western blotting analysis (see Methods). We detected neddylated Flag-SRSF3 at approximately 40 kDa consistent with di-neddylation in cells treated with arsenite (Fig. 11).

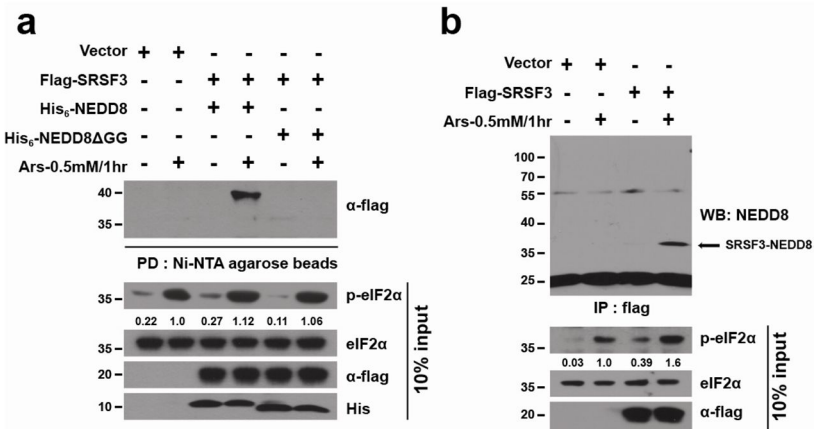

Figure 11. SRSF3 is neddylated under arsenite stress.

None of the experiments utilizing empty vector or His-NEDD8ΔGG display the corresponding band suggesting that SRSF3 is conjugated with NEDD8 in response to stress. We also confirmed that endogenous NEDD8 is conjugated to Flag-SRSF3 under arsenite stress. The stress-induced neddylation of SRSF3 is dose- and time-dependent, and the effect of arsenite is reversible; the neddylated species disappears upon recovery from arsenite (Appendix 9). The neddylation of SRSF3 was dependent on neddylating enzymes NAE1 and UBE2M, as inhibition of NAE1 by treatment of MLN4924 (Fig. 12), or knockdown of UBE2M effectively inhibited the formation of Nedd8-SRSF3 (Fig. 13a).

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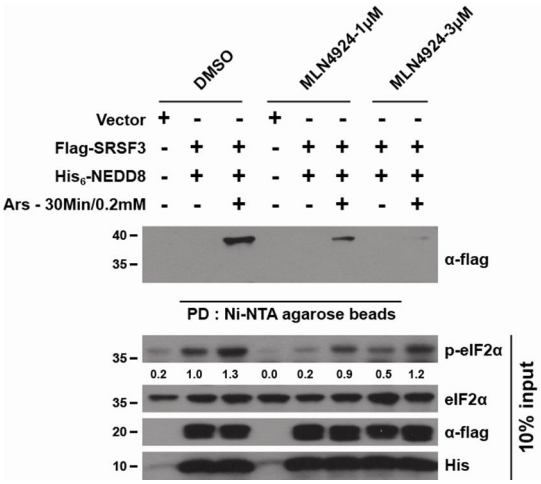

Figure 12. MLN4924 inhibit SRSF3 neddylation in dose dependent manner.

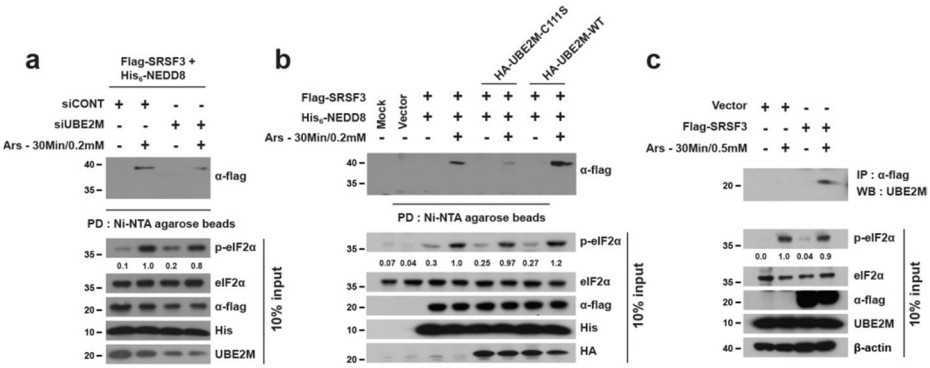

Figure 13. UBE2M is the E2 conjugating enzyme for SRSF3 neddylation.

Overexpressing a catalytically inactive UBE2M (C111S), but not the wild type, led to a decrease in the NEDD8-SRSF3 species, implying its dominant negative effect (Fig. 13b). Co-immunoprecipitation analysis suggests that

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Flag-SRSF3 interacts with UBE2M, particularly when cells were treated with arsenite (Fig. 13c). Finally, to further confirm if the stress-induced upshifted Flag-SRSF3 is indeed NEDD8 conjugated form, NEDP1 (also known as DEN1 or SENP8), a NEDD8 specific Cys protease, was coexpressed.

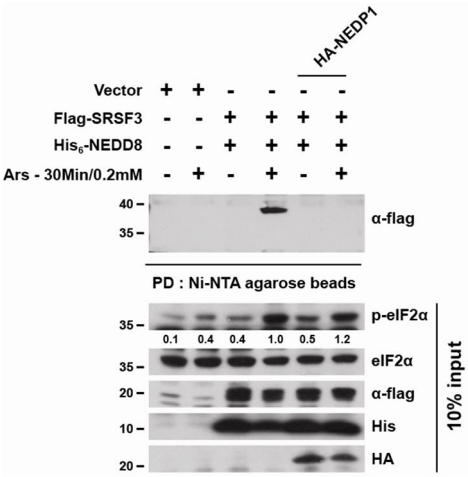

Figure 14. NEDP1 is the NEDD8 iso-peptidase for SRSF3 neddylation.

As shown in Fig. 14, while expression of Flag-SRSF3 in the presence of His-NEDD8 results in appearance of modified Flag-SRSF3 species, these species were not detected when HA-NEDP1 is co-expressed. We also found that the SG formation is effectively inhibited when the HA-UBE2M mutant (C111S) or HA-NEDP1 is overexpressed (Appendix 10), which is consistent with our model that the neddylation of SRSF3 is necessary for the SG formation. Collectively, these data strongly support that neddylation of SRSF3 is induced

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by arsenite stress through NAE-UBE2M axis of neddylation system and this may lead to SG formation.

SRSF3 is neddylated at Lys85

SRSF3 is the smallest member of the highly conserved Serine-Arginine (SR)-rich splicing factor family and is composed of one structured RNA recognition motif (RRM) at the N-terminus and one disordered RS domain at the C-terminus[43].

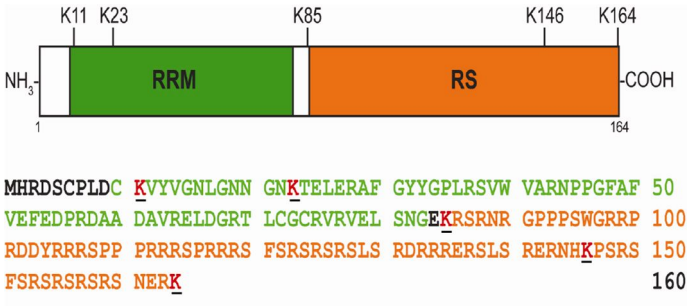

Figure 15. Schematic representation of SRSF3 protein domains.

The protein is composed of 164 amino acids, about half of which belong to the RS domain (Fig. 15). There are 5 lysine residues that may be targeted for neddylation: two reside in the RRM domain, two in the RS domain, and one in between.

To identify residue(s) that are neddylated in response to arsenite we constructed a series of K-R mutants (K11R, K23R, K85R, K146R, K164R; double mutants, K11/23R, K23/85R, K146/164R; all lysine mutant, KO). *In*

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vivo neddylation assays with these mutants revealed that mutants with K85R selectively lack arsenite-induced SRSF3 neddylation (Fig. 16).

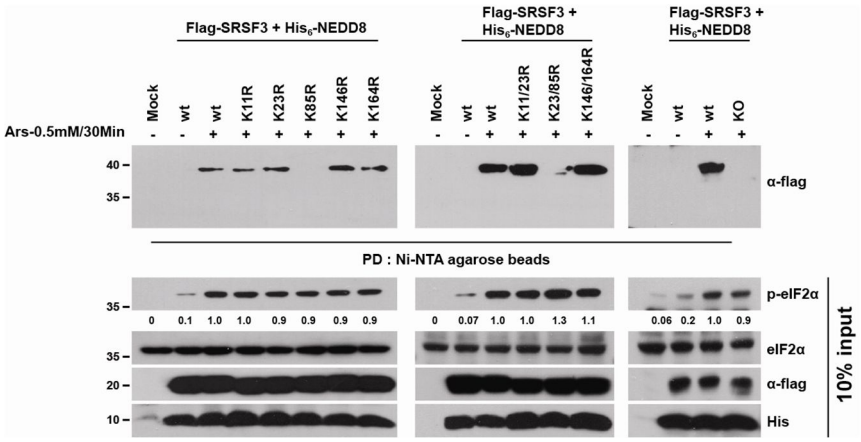

Figure 16. SRSF3 is neddylated at Lys85.

SRSF3 K85R mutation impairs arsenite-induced SG assembly

In order to test the physiological role of neddylation of SRSF3 at K85, we stably expressed either wild type or SRSF3 (K85R) in U2OS cells and monitored the dynamics of SG assembly. Interestingly, we observed that Flag-SRSF3-K85R expressing cells display a significant delay in SG assembly that phenocopies knockdown or inhibition of neddylation components, whereas Flag-SRSF3-WT cells exhibit normal SG assembly (Appendix 11). To convincingly show the overexpression effects on SG assembly in Flag-SRSF3-WT or Flag-SRSF3-K85R stable cells, we depleted endogenous SRSF3 utilizing siRNA targeting 3'-UTR of SRSF3 mRNA and monitored SG dynamics in cells expressing WT or K85R protein over time. In

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siCONT transfected cells, the results of SG assembly dynamics are similar to those in Appendix 11 (Fig. 17 a,b). In siSRSF3-3'UTR transfected cells, SG assembly are normally rescued only in cells expressing Flag-SRSF3-WT, but not in cells expressing Flag-SRSF3-K85R that displays a slightly more inhibitory effect on SG assembly when compared with those transfected with siCONT (Fig. 17 b,c).

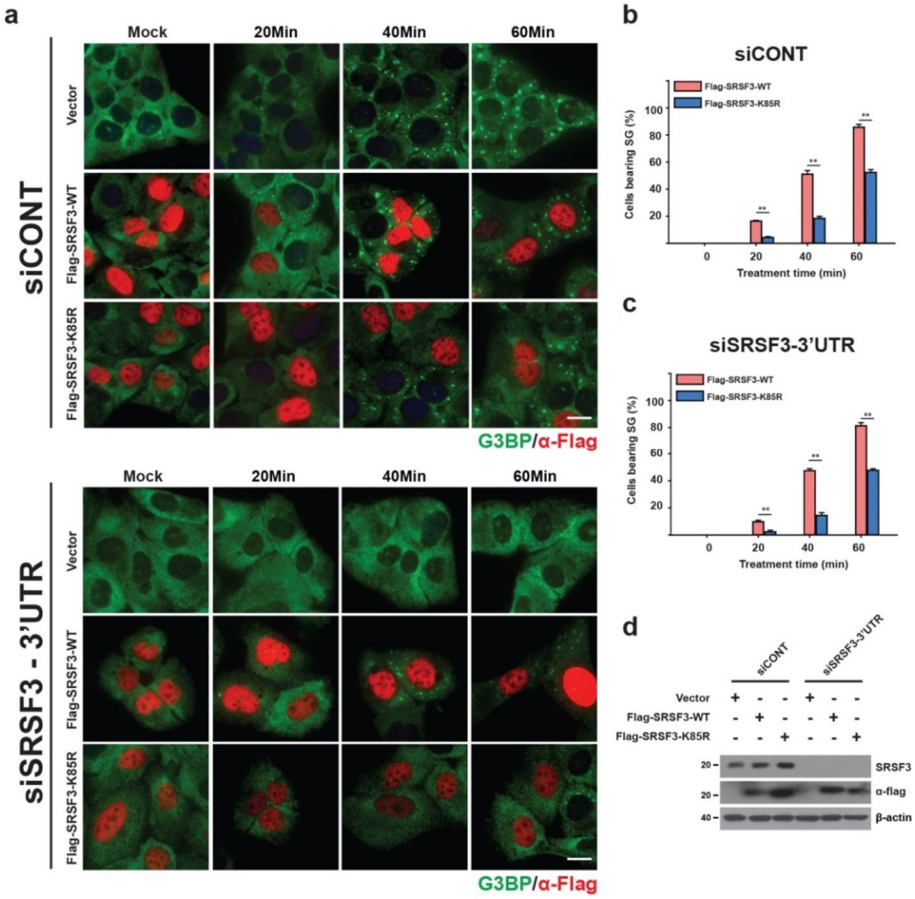

Figure 17. SRSF3-K85R fails to rescue SG assembly.

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Interestingly, the impairment of Flag-SRSF3-K85R localization to SG was not observed under different stresses such as clotrimazole and thapsigargin. Moreover, the neddylation of Flag-SRSF3 could not be detected under those stresses suggesting that the promotion of SG formation through SRSF3 neddylation is arsenite-specific event (Appendix 12).

We also checked the effects of other single or double lysine mutants on SG assembly and found that those are comparable to that of WT except that K146/164R mutations led to marginal inhibitory effects (Appendix 13). The slight inhibitory effect of K146/164R mutant is possibly due to its location in unstructured RS domain such as prion-like or low-complexity domains are crucial for mRNP aggregation[44].

Given that knockdown of SRSF3 abrogate both SG and PB assembly, we tested whether expressing the SRSF3-K85R mutant has an impact on PB assembly using S6K1 as a PB marker. As shown in Appendix 14, both WT and K85R mutant display a comparable PB assembly pattern in the presence or absence of arsenite indicating that the arsenite-induced SRSF3 neddylation selectively modulates SG assembly.

SRSF3 K85R has defects in association with SG components

Recently, we reported that SRSF3 functions as a translation repressor of PDCD4 mRNA by binding its 5'-UTR region[45]. Several translation factors including nuclear cap-binding protein (NCBP1), eukaryotic translation

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initiation factor 2A (EIF2A), eukaryotic translation initiation factor 2 subunit 3 (EIF2S3), eukaryotic initiation factor 4A-1 (EIF4A1) and polyadenylate-binding protein 1 (PABP1) were found to interact with SRSF3.

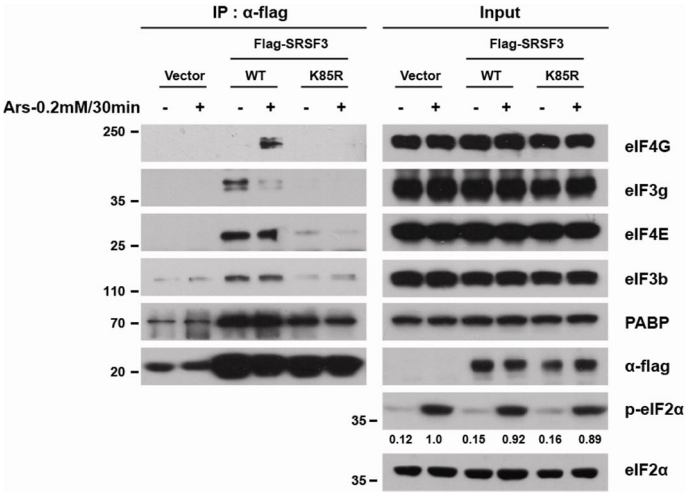

Figure 18. SRSF3-K85R impairs interaction with translation initiation factors.

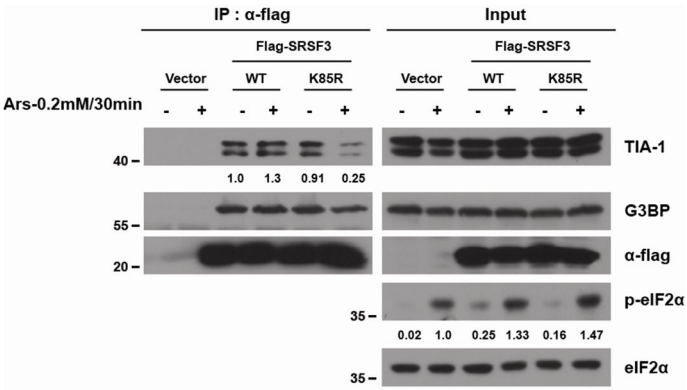

Figure 19. SRSF3-K85R impairs interaction with TIA-1 under arsenite stress. .

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To determine whether SRSF3 neddylation modulates the interaction with these factors, we compared the ability of WT and mutant (K85R) SRSF3 to immunoprecipitate translation factors from lysates prepared from cells cultured in the absence or presence of arsenite. Interestingly, while WT and the K85R mutant are associated with PABP at a similar level, the levels of eIF4E, eIF4G and eIF3 associated with the K85R mutant is noticeably lower compared to WT (Fig. 18).

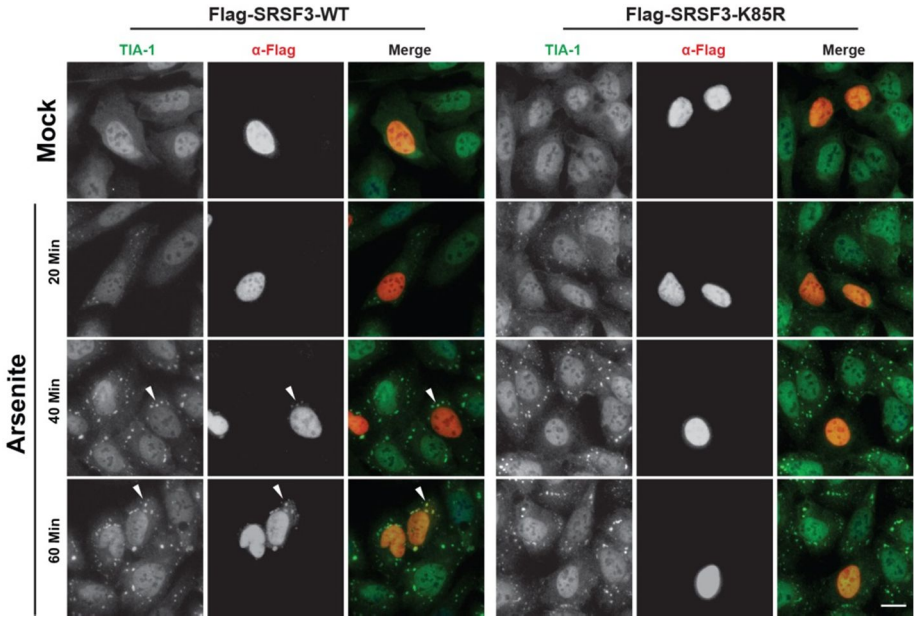

Figure 20. SRSF3-K85R fails to recruit TIA-1 in SGs.

It has been well characterized that several proteins such as Tia-1 and G3BP1, which contain the aggregation prone domain, mediate the SG aggregation and overexpression of these causes abnormal SG formation[23, 46]. Hence, we assessed if the SRSF3-K85R mutant also have defects in associating with

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these proteins under mock or arsenite stressed condition. As shown in Fig. 19, association of Tia-1 with the K85R mutant is much reduced compared to the WT under arsenite stress while G3BP1 displays a comparable association compared to WT. The association defect seen only in the arsenite condition may imply that mRNP remodeling for the SG aggregation is defective with the K85R mutant. Consistently, the recruitment of Tia-1 in SG under arsenite stress is strongly abrogated in cells expressing the K85R mutant, whereas the SRSF3-WT expressing cells displays Tia-1 positive SGs that is colocalized with Flag-SRSF3-WT (Fig. 20). These results indeed support that the neddylation of SRSF3 is critical for interacting with and facilitating the recruitment of Tia-1 to SG. Finally, because inhibiting neddylation delays stress-induced polysome disassembly (Appendix 15), we wanted to determine to what extent the SRSF3 neddylation is responsible for this phenotype. Polysome profiles of cells expressing the wild type or the K85R SRSF3 showed similar pattern (Appendix 15). This result suggests that delayed polysome disassembly shown in the cells defective in neddylation is not due to an inhibition of the SRSF3 neddylation, rather it could be caused by neddylation of unknown proteins. Because eIF2 phosphorylation is prerequisite for arsenite-induced polysome disassembly, we additionally tested whether knockdown of HRI, the kinase responsible for the arsenite-induced eIF2 phosphorylation[36], dampen the neddylation of SRSF3. We found that knockdown of HRI effectively inhibited the eIF2

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phosphorylation under arsenite as previously reported, but this does not affect the arsenite-induced neddylation of SRSF3 (Fig. 21). This observation suggests that the neddylation of SRSF3 at K85 is important for the aggregation process of SG, which is a separate event from the eIF2 phosphorylation and polysome disassembly.

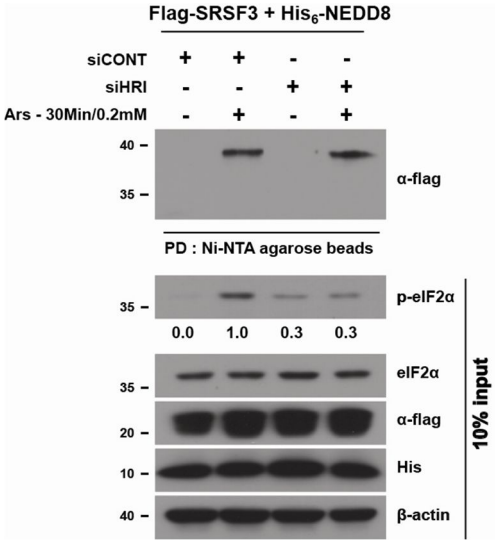

Figure 21. SRSF3 neddylation is independent of p-eIF2α.

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IV. Discussion

Here we report that neddylation of SRSF3 is required for it to promote SG assembly. Our results reveal that: 1) SRSF3 neddylation is induced by arsenite stress, 2) the neddylation and deneddylation of SRSF3 following the application and removal of stress stimuli correlates with the assembly and disassembly of SGs, 3) MLN4924 inhibits stress-induced neddylation of SRSF3 in a dose-dependent manner, 5) knockdown of UBE2M dampens stress-induced neddylation of SRSF3, 6) UBE2M and NEDD8 are integral components of SGs, 7) SRSF3 associates with UBE2M in response to arsenite, and 8) SRSF3 neddylation at Lys85 promotes SG assembly likely at the aggregation stage. The latter conclusion is based on the observation that the K85R-expressing cells show similar stress-induced polysome disassembly when compared to WT, whereas blocking neddylation pathway inhibits polysome disassembly (Fig. 22).

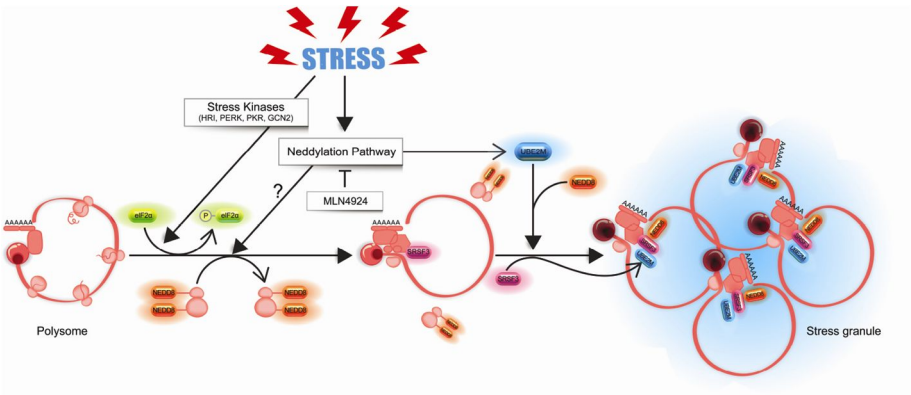

Figure 22.Working model of Neddylation in SG assembly.

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We do understand the concern of possible artificial conjugation of overexpressed Nedd8[47]. Our analyses show that expression of FB-Nedd8 is lower than the endogenous level of Nedd8, and that expressing FB-Nedd8 does not noticeably alter the polysome profile. Also, FB-Nedd8-conjugated proteins are visible in the non-translating monosome fractions by streptavidin-HRP western blot and the pattern is similar to that of anti-Nedd8 Western blot. Most importantly, we showed that Flag-SRSF3 is conjugated with endogenous NEDD8 under arsenite stress and SRSF3 neddylation is effectively inhibited by MLN4924 treatment, arguing that SRSF3 is a bona-fide target[48].

Recently, several studies have identified pathological SGs related to many types of neuro-degenerative diseases such as Alzheimer's Dementia (AD), Multi-system Proteinopathy (MSP), Amyotropic Lateral Sclerosis (ALS), and Frontotemporal Lobar Degeneration (FTLD)[49, 50]. Most of these disease symptoms are likely mediated through mutated RBPs that produce stabilized SG-like aggregates, insoluble inclusion bodies, pathological fibril formation[15] or defects of autophagic system which is required for SG clearance during stress recovery[51]. Hence, unraveling the molecular mechanisms involved in the dynamic assembly and disassembly of SGs may lead to an improved understanding of the pathology of the SG-related neurological disorders. An interesting study has recently identified a pathogenic mutation in hnRNP A2B1 and A1 which potentially causes

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inherited MSP and ALS[15]. Notably, these mutations reside in a prion-like domain, which promote excessive SG aggregation and cytoplasmic inclusions. Our identification of hnRNPs in the proteomic screen for neddylation could add another layer of complexity to the role of hnRNP aggregates in these disease syndromes. It will be important to determine the functional implications of hnRNP neddylation in the assembly of SGs.

Previous study showed that Ube1, a ubiquitin E1 enzyme, mediates conjugation of NEDD8 under diverse stress conditions [33]. Interestingly, treatment of PYR-41, an ubiquitin E1 enzyme inhibitor, strongly inhibited the neddylation of SRSF3 to a similar degree with MLN4924. Knockdown of Ube1 also dampen the arsenite-induced neddylation of Flag-SRSF3 (Fig. 23).

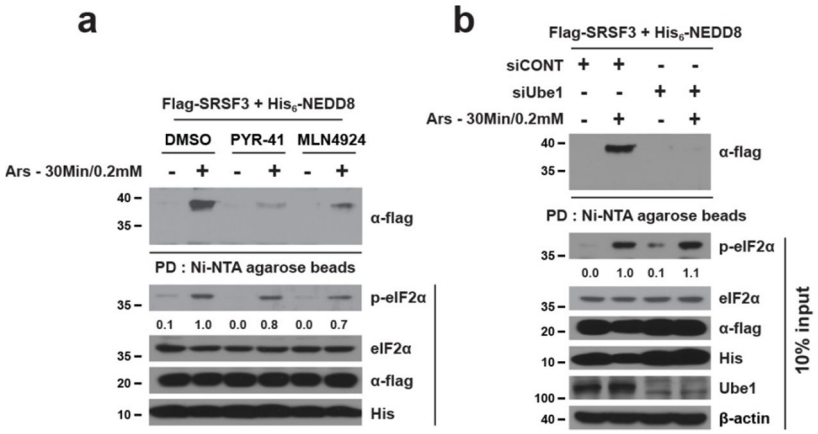

Figure 23. Typical and atypical neddylation pathway involve in SRSF3 neddylation.

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To compare the effects of UBE2M-mediated canonical and Ube1-mediated atypical neddylation pathways on SG formation under arsenite stress, we transfected U2OS cells with siCONT, siUbe1 and siUBE2M and SGs were visualized using eIF3b, Tia-1 and G3BP. Unexpectedly, Ube1-knockdown cells display a slight decrease (~20%) while UBE2M knockdown cells display an outstanding decrease (~60%) in SG formation suggesting that the canonical pathway is more essential for the SG formation (Fig. 24).

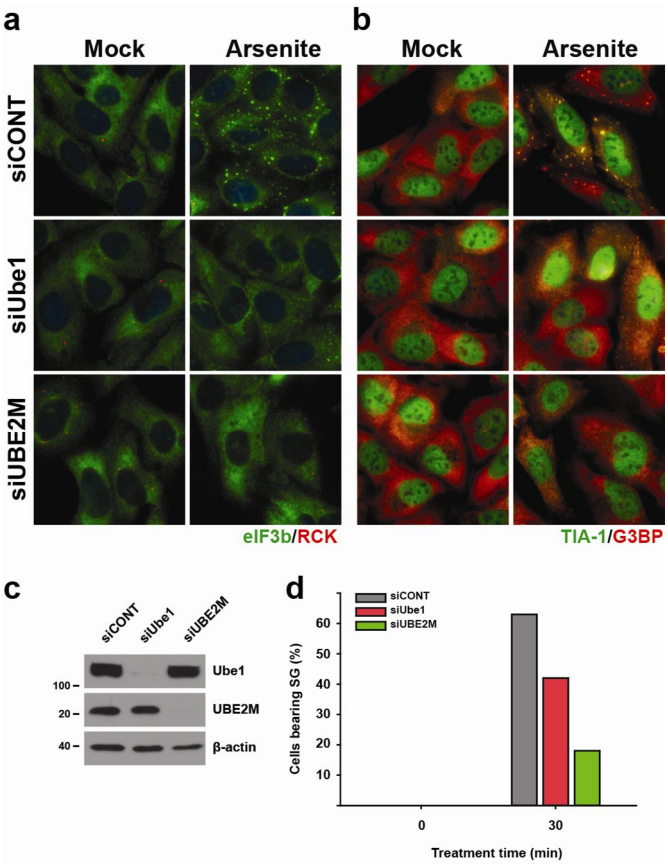

Figure 24. Typical SRSF3 neddylation is major player in SG assembly.

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Consistent with a previous report that the K85 residue of SRSF3 is ubiquitinated [52], we found that SRSF3 indeed is ubiquitinated in both conditions (Appendix 16). These observations suggest that the K85 residue of SRSF3 may be subjected to mixed modification of ubiquitin and NEDD8, or it is possible that distinct pools of ubiquitin and NEDD8 modified SRSF3 may exist under arsenite stress. Further studies are needed to define whether SRSF3 is modified with ubiquitin/nedd8 dipeptide chain and if so, what the physiological significance of this complex modification is.

The responsible E3 ligase for SRSF3 neddylation remains to be determined. So far, the best-characterized NEDD8 E3 ligases are RBX1 and RBX2, which work with UBE2M and UBE2F, respectively[53]. While knockdown of RBX2 had no effect on SG assembly kinetics, RBX1 knockdown produced a slight delay in SG assembly at early time points (Appendix 17), suggesting that RBX1 or RBX2 are not major players in stress-inducible SG assembly. Supporting this conclusion is the fact that; 1) neither these E3 ligases nor Cullin components were identified in our RNAi screen for SG assembly, and 2) RBX1 or RBX2 are not detected in SG (Appendix 18). Hence, we conclude that the inhibitory effect of SG formation upon blocking neddylation is cullin-independent. Based on the observation that Flag-SRSF3 physically interacts with UBE2M, it is possible that UBE2M may act directly on SRSF3 for Nedd8 conjugation. Further studies are needed to validate this result or possibly identify additional E3 ligases.

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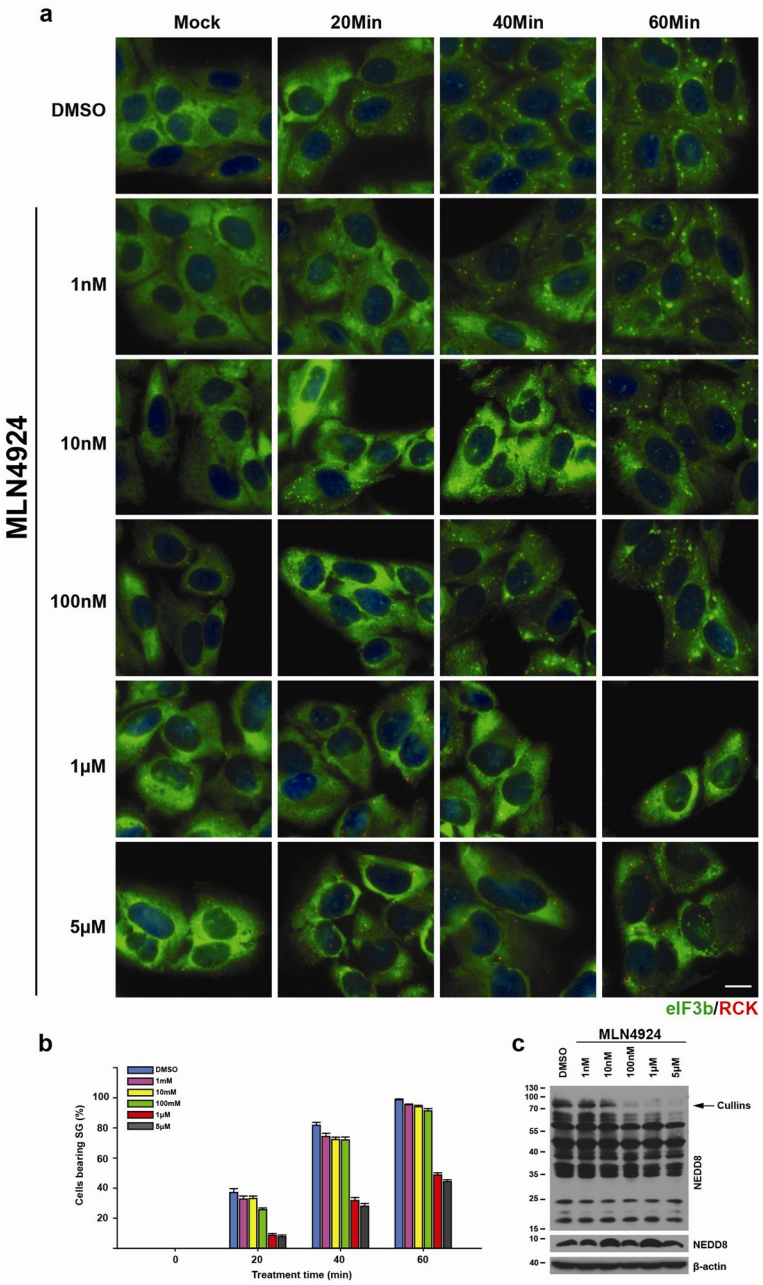

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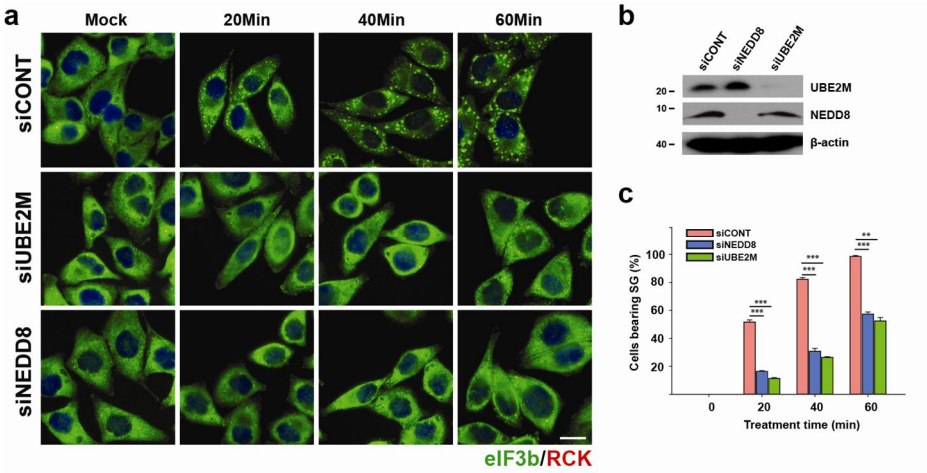

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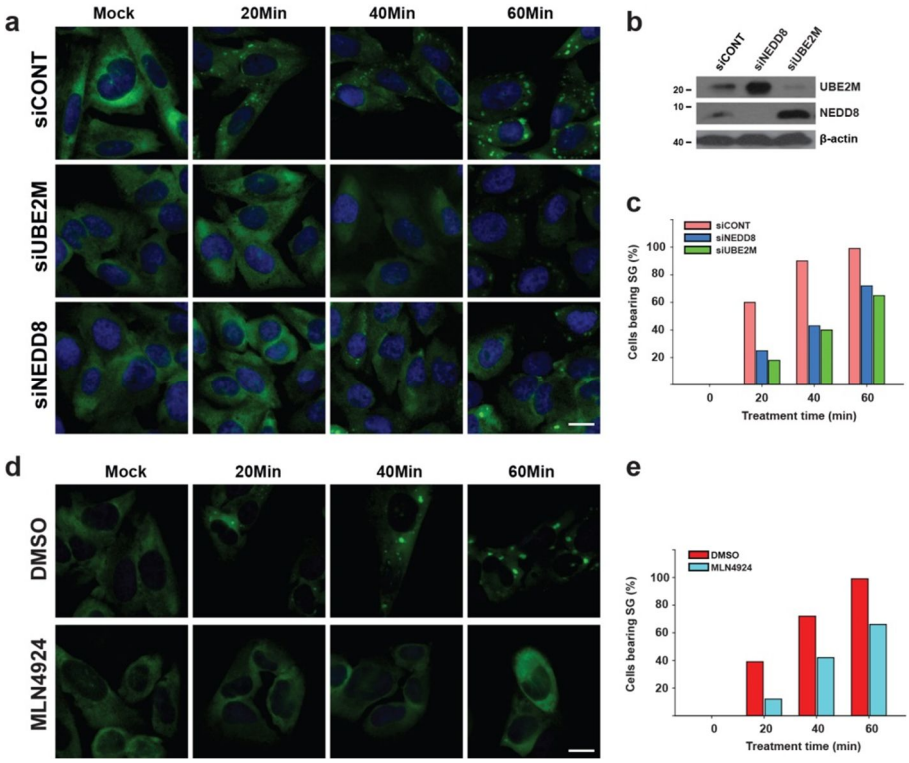

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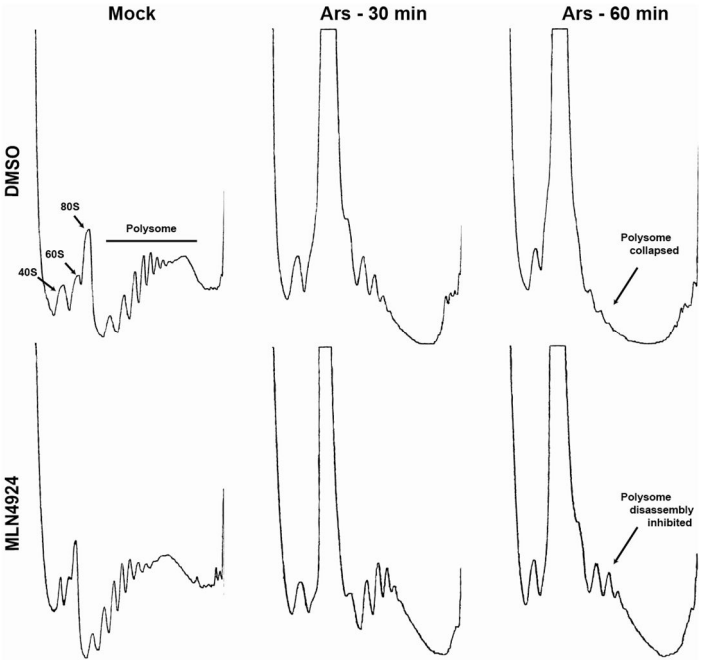

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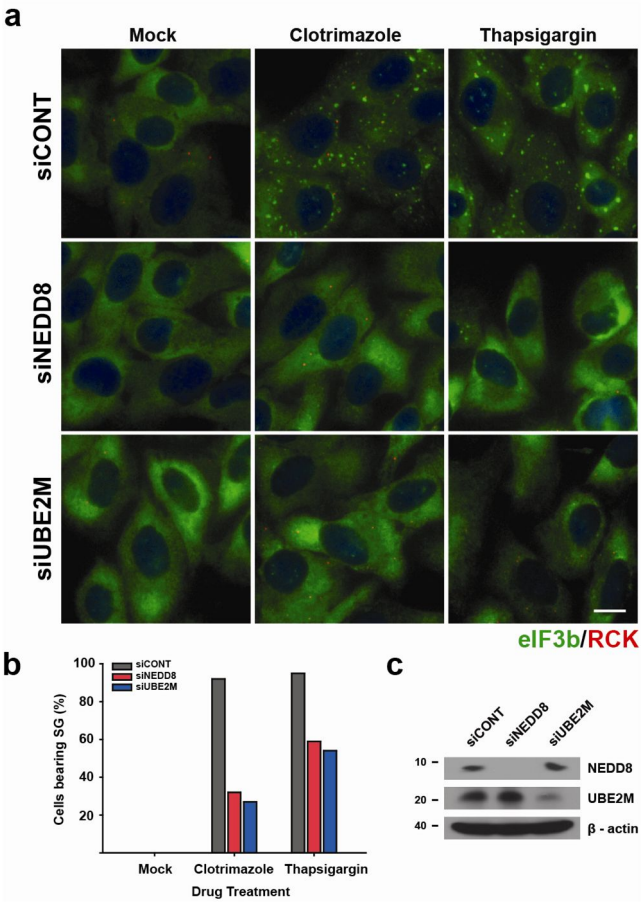

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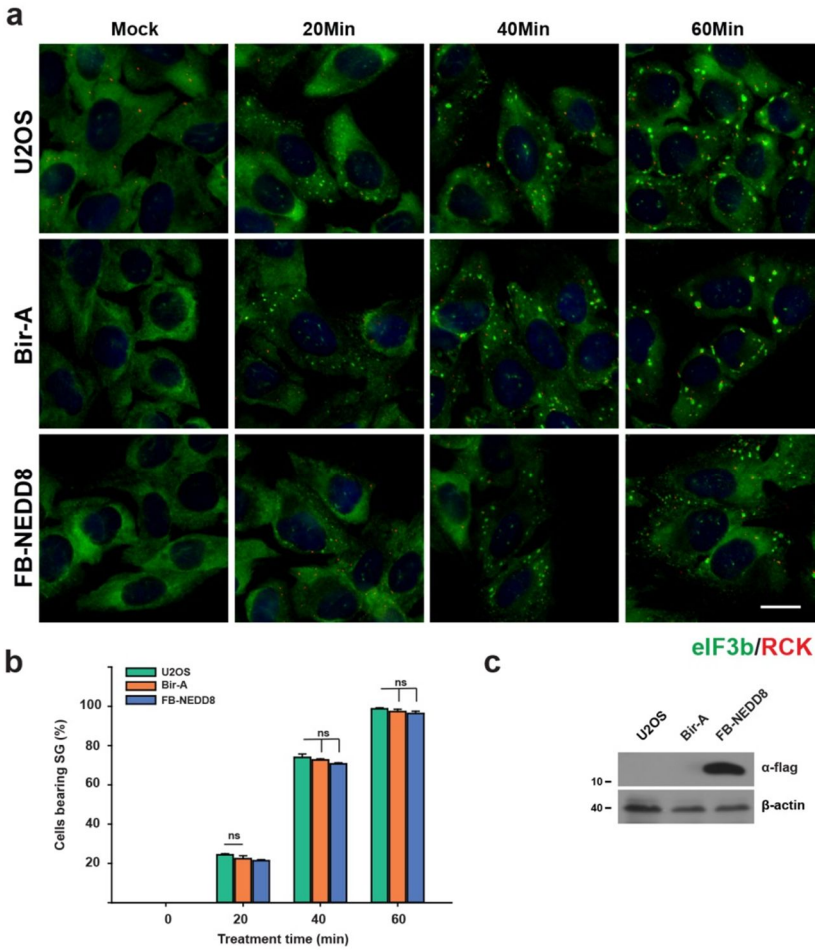

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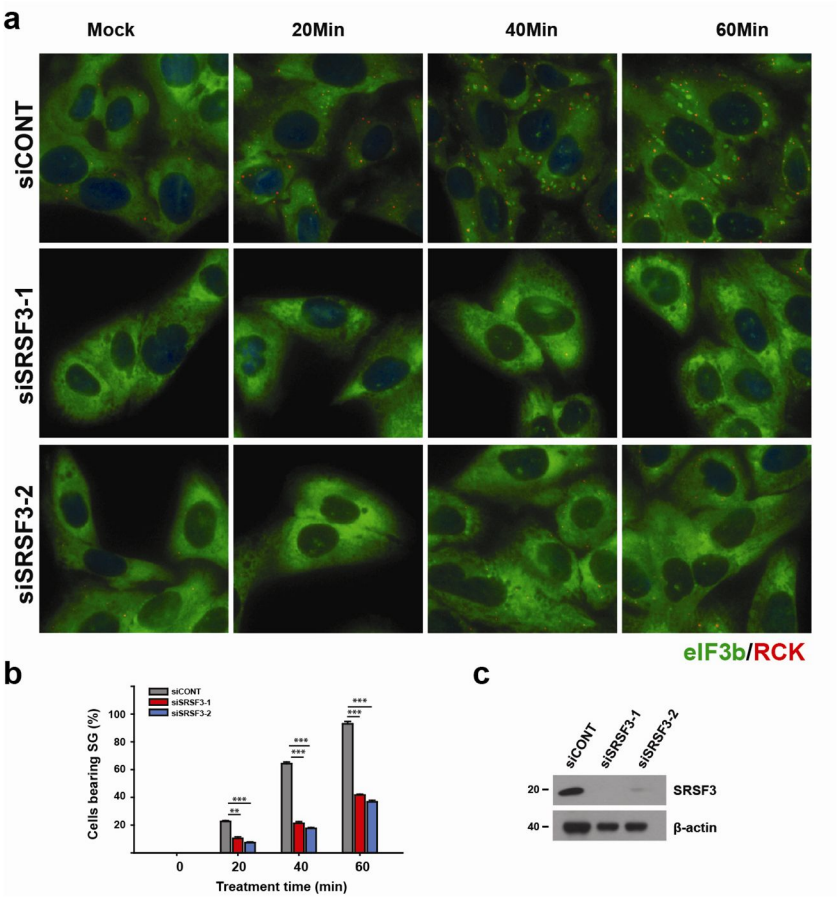

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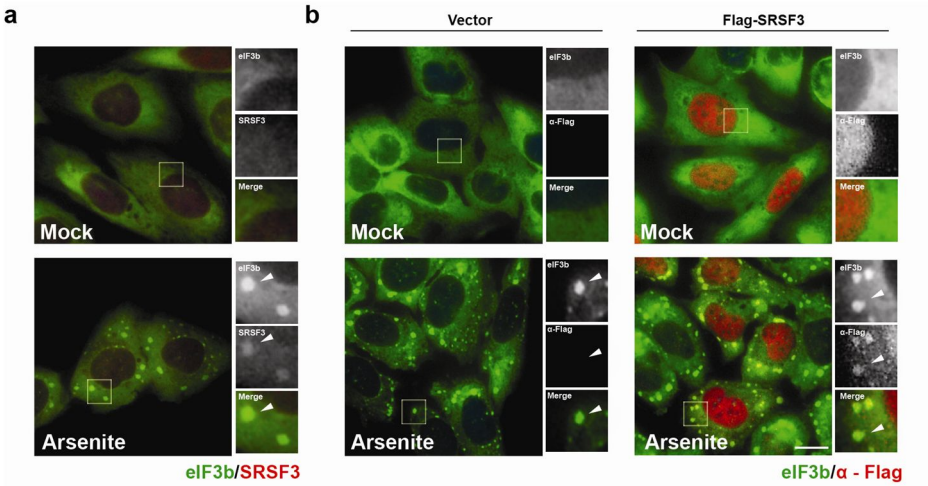

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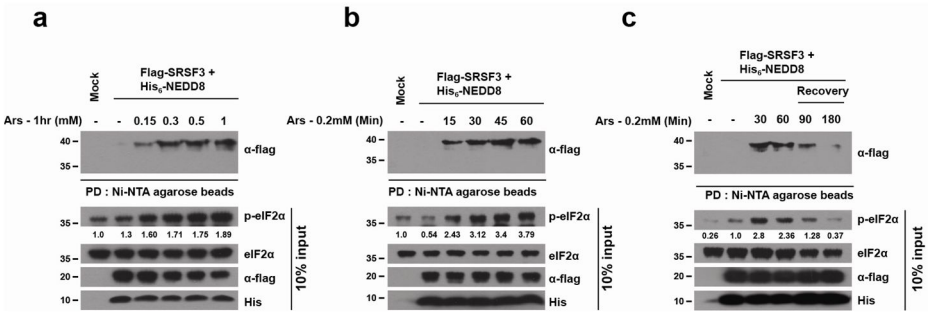

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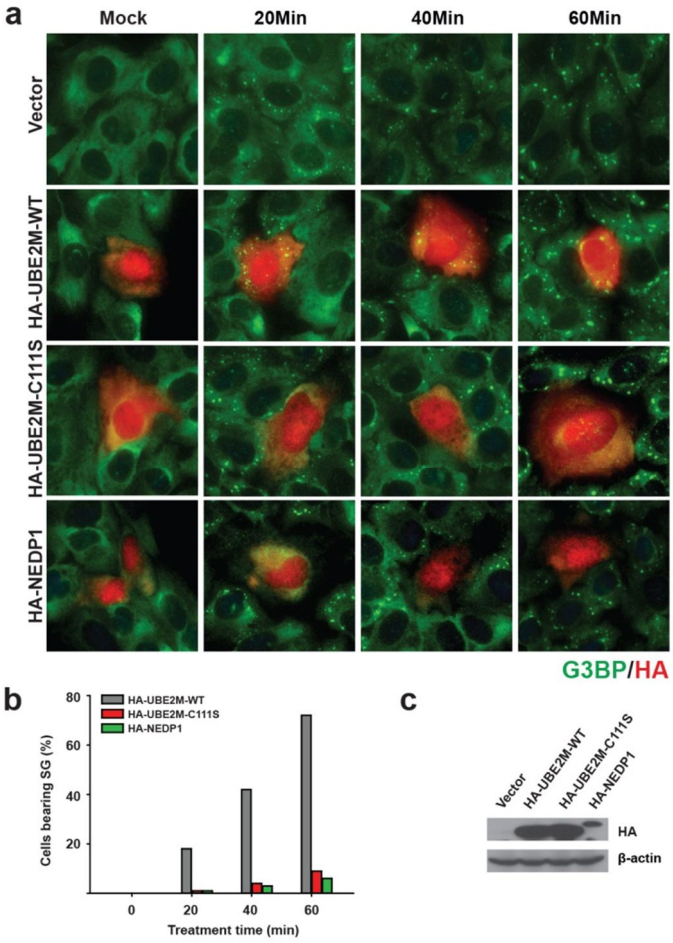

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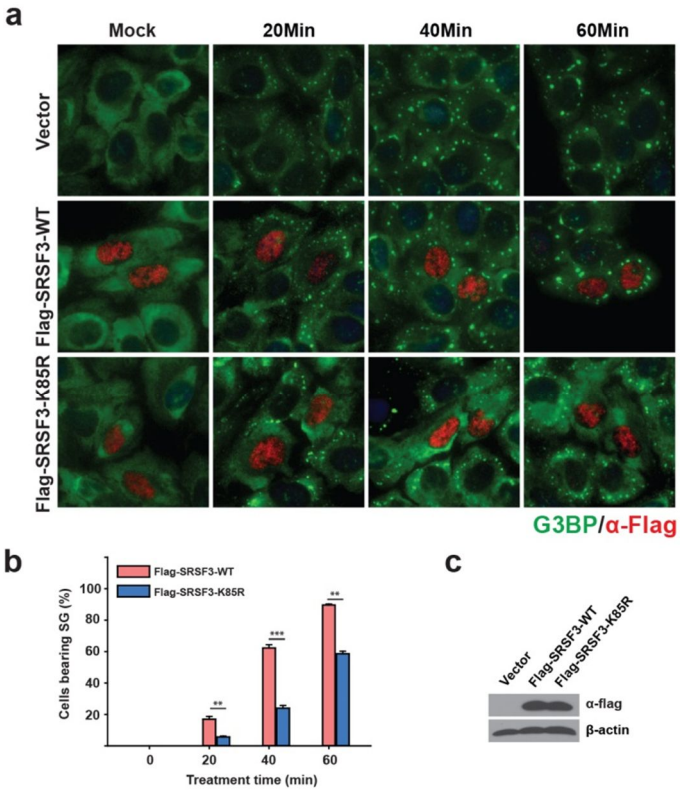

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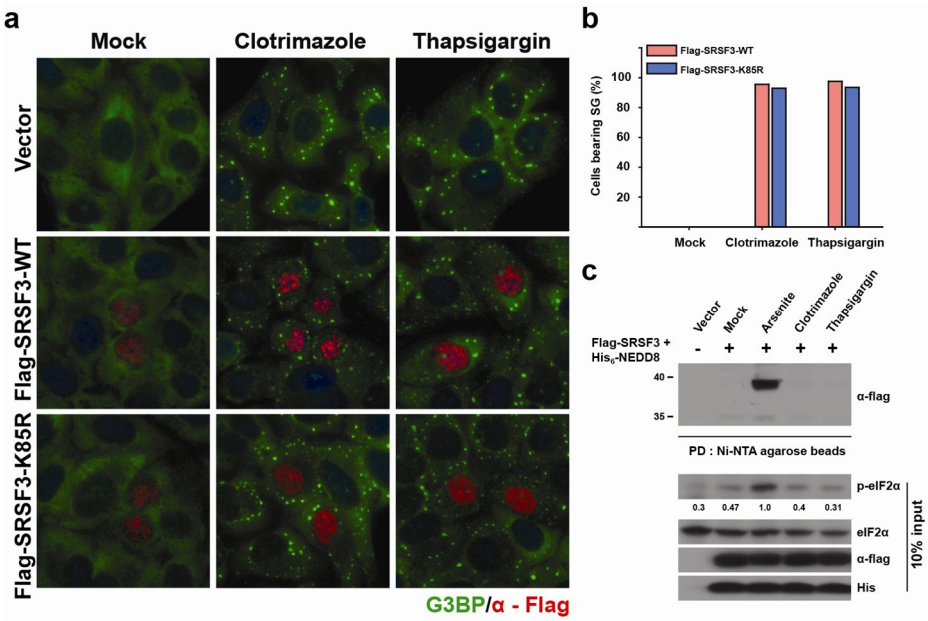

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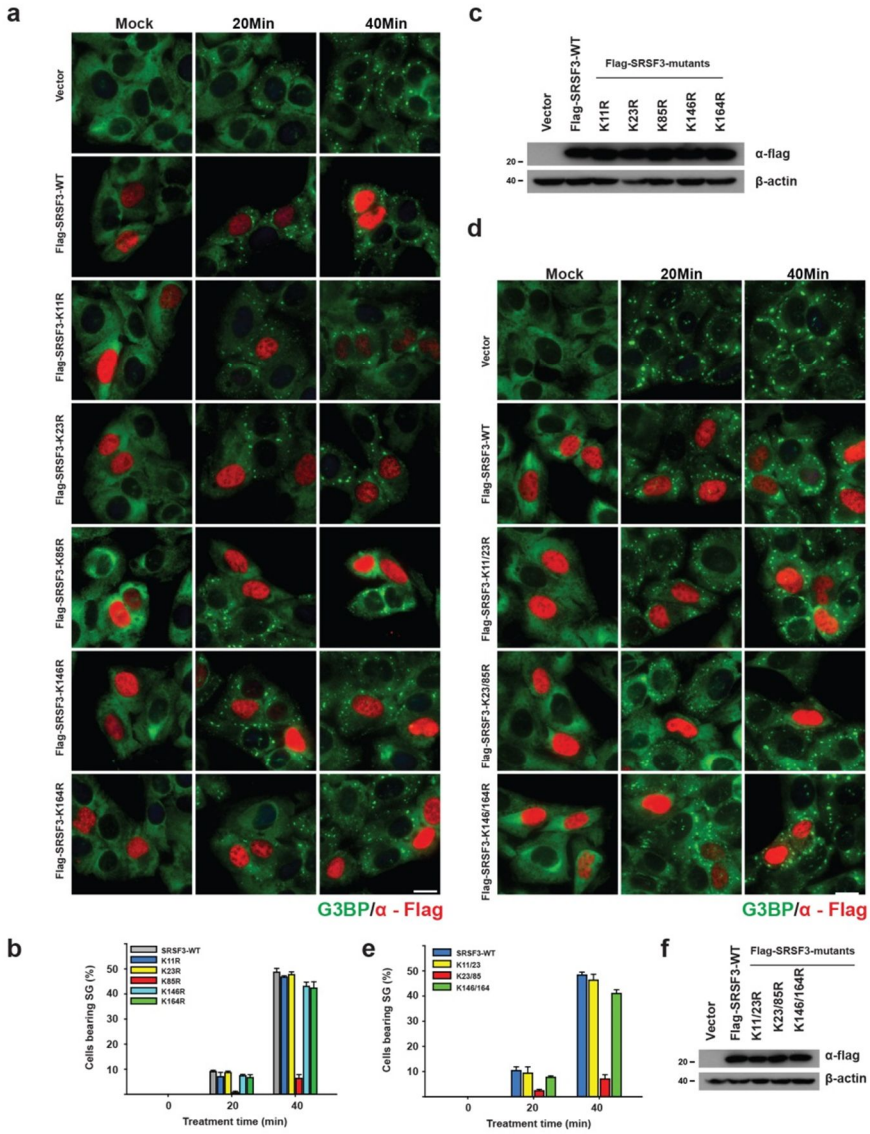

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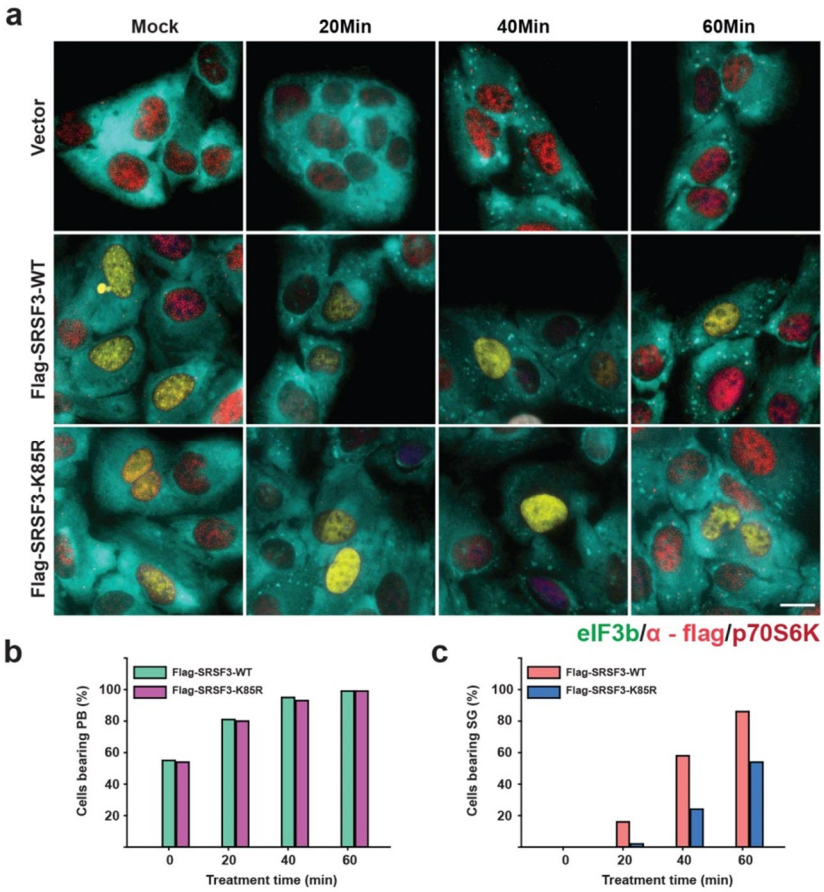

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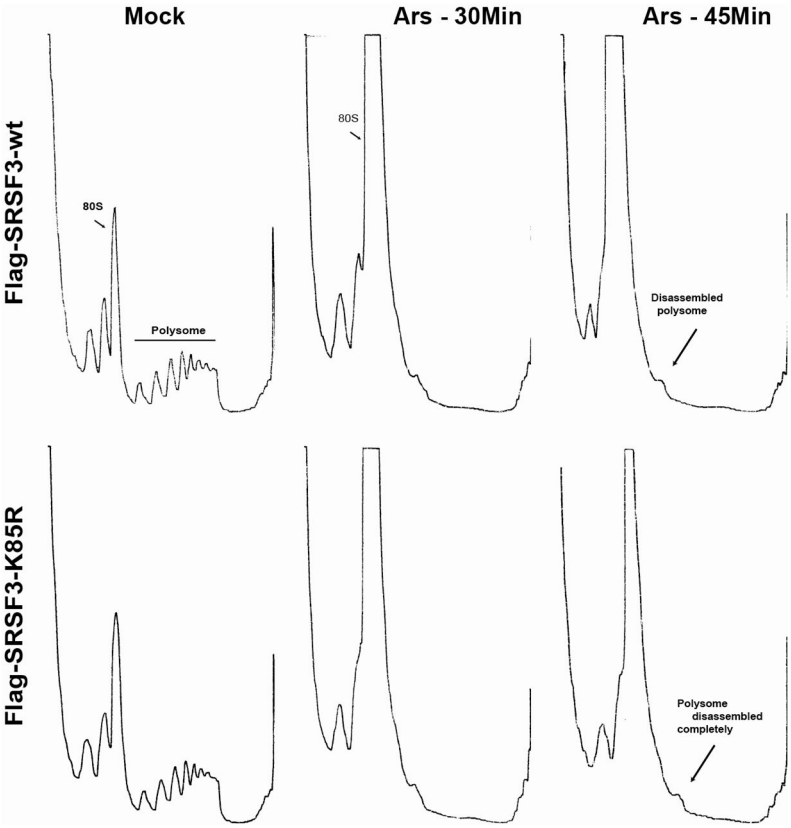

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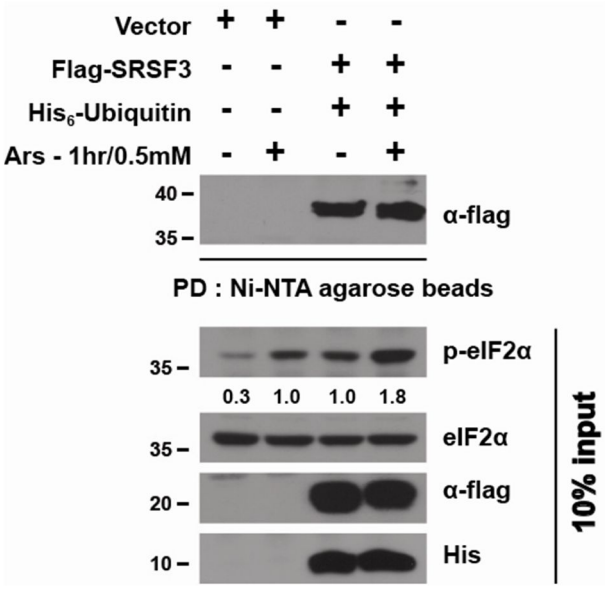

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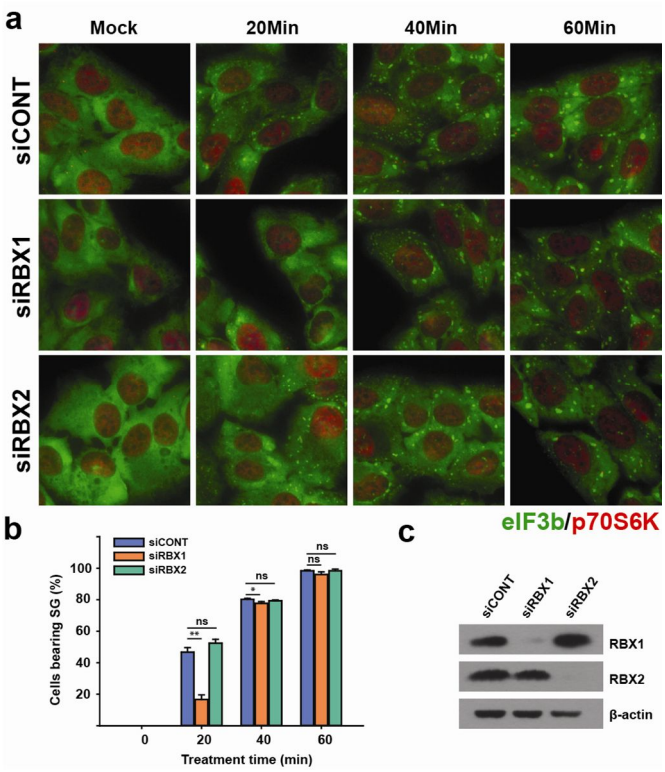

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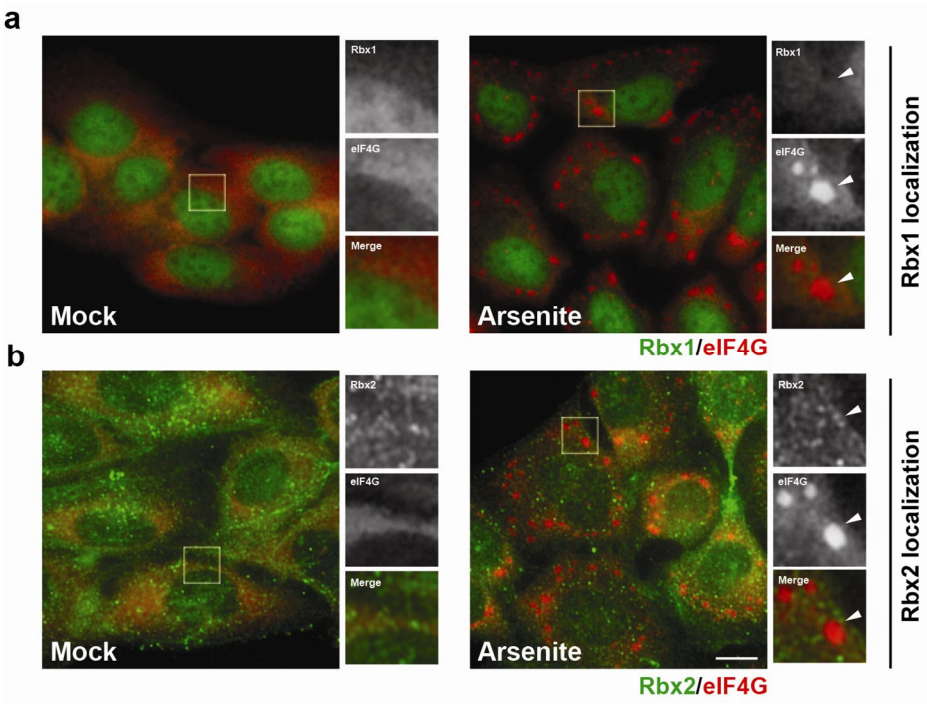

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VI. LEGENDS

Figure 1. Images from siRNA screen plates with RDG3 stable cells showing knockdown effect of UBE2M on SG assembly.

Figure 2. (**a**) U2OS cells transfected with siCONT, siNEDD8 or siUBE2M were treated with 0.2 mM arsenite for indicated time points prior to processing for immunofluorescence microscopy using anti-eIF3b and anti-RCK antibodies. (**b**) Statistical graph showing percentage of cells bearing SGs. Error bars indicate s.e.m. (n=4). $^{**}p < 0.01$, $^{***}p < 0.001$, Student's t-test. (**c**) Western analysis for knockdown efficiency of NEDD8 and UBE2M.

Figure 3. (**a**) U2OS cells pretreated with DMSO or 1 μM ML4924 for 18 h were treated with 0.2 mM arsenite for indicated time points and stained against eIF3b and RCK. (**b**) Statistical data showing percentage of cells bearing SGs. Error bars indicate s.e.m. (n=3). $^{**}p < 0.01$, $^{***}p < 0.001$, Student's t-test. Scale bar, 10 μm.

Figure 4. (**a**) U2OS cells transfected with siCONT or mixture of siUBE2M and siNEDD8 were untreated (mock) or treated with 0.2 mM arsenite for 45 mins and subjected to polysome profiling. (**b**) Areas under monosome and polysome curve were quantified and the ratio of 80S to polysome is shown. (**c**) Western blot analysis for knockdown efficiency of NEDD8 and UBE2M.

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Figure 5. (**a**) U2OS cells grown on coverslips were either (**a**) untreated (mock) or treated with (**b**) 0.5 mM arsenite (**c**) 20 μM clotrimazole (**d**) 1 μM thapsigargin for 1 h. Samples were processed and immunostained against eIF3b (green) and UBE2M (red). Scale bar, 10 μm.

Figure 6. U2OS cells either (**a**) untreated or treated with (**b**) 0.5 mM arsenite (**c**) 20 μM clotrimazole (**d**) 1 μM thapsigargin for 1 h were processed and immunostained against G3BP (green) and NEDD8 (red). Scale bar, 10 μm.

Figure 7. (**a**) U2OS cells transfected with siCONT, siNEDD8 or siUBE2M were treated with 0.2 mM arsenite and immunostained against SG marker TIA-1 (green), PB markers RCK (red) and p70S6K (far red). (**b**) Bar graph showing percentage of cells bearing PBs. Error bars indicate s.e.m. (n=3). ns, non-significant, Student's t-test. (**c**) Western blot analysis showing knockdown efficiency of NEDD8 and UBE2M. Scale bar, 10 μm.

Figure 8. Mock or arsenite (0.5 mM for 1 h) treated U2OS cells were subjected to polysome profiling analysis as described in methods. A total of 15 fractions collected from sucrose gradient were acetone precipitated, centrifuged and the air-dried pellets were dissolved in SDS sample buffer. The samples were resolved in SDS-PAGE and blotted with anti-NEDD8 antibody.

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Figure 9. Schematic representation for generation of Flag-biopeptide (FB) tagged NEDD8 stable cell line (see methods

Figure 10. Stably expressing BirA and FB-NEDD8 cells treated with 0.5 mM arsenite for 1 h were subjected to sucrose gradient fractionation, acetone precipitated, and FB-NEDD8 conjugated proteins were affinity-purified using streptavidin beads. The precipitates were resolved in 12% SDS-PAGE and stained with coomassie blue.

Figure 11. (**a**) HEK293T cells were transiently co-transfected with indicated plasmids. After 36 h, cells were treated with either mock or arsenite, lysed under denaturing condition and affinity-purified using Ni^{2+}-NTA agarose beads (see methods). The precipitates were then blotted against anti-Flag antibody. (**b**) HEK293T cells transfected with empty vector or Flag-SRSF3 were treated with arsenite, lysed under denaturing condition (1% SDS) and immunoprecipitated using Flag beads (see methods).

Figure 12. NAE inhibitor MLN4924 significantly attenuates SRSF3 neddylation. HEK293T cells pretreated with DMSO, MLN4924 (1 μM) for 18 h or MLN4924 (3 μM) for 1 h prior to arsenite treatment were subjected to neddylation assay.

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Figure 13. (**a**) *in vivo* neddylation assay was performed for HEK293T cells knocked down with siCONT or siUBE2M under arsenite stress. (**b**) HEK293T cells co-transfected with indicated plasmids were treated with arsenite and subjected to neddylation assay. (**c**) Empty vector or flag-SRSF3 transfected cells were treated with arsenite, lysed in IP buffer and immunoprecipitated using Flag agarose beads. The precipitates were subjected to Western analysis using anti-UBE2M antibody.

Figure 14. NEDP1 overexpression inhibits SRSF3 neddylation. HEK293T cells transfected with indicated plasmids were treated with arsenite and affinity purified using Ni^{2+}-NTA agarose beads.

Figure 15. Schematic representation of SRSF3 protein domains and positions of lysine residue.

Figure 16. His-NEDD8 was co-transfected with empty vector or Flag-SRSF3-WT and series of single lysine mutant (11, 23, 85, 146, 164), double lysine mutant (11/23, 23/85, 146/164) or null mutant (all lysine mutant) as indicated and treated with 0.5 mM arsenite for 30 mins before subjected to neddylation assay.

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Figure 17. (**a**) Stably expressing empty vector, Flag-SRSF3-WT or K85R mutant cells transfected with siCONT or siSRSF3-3'UTR were treated with 0.2 mM arsenite before processing for immunofluorescence microscopy using anti-G3BP and anti-Flag antibody. Percentage of cells bearing SGs in (**b**) siCONT transfected cells and (**c**) siSRSF3-3'UTR transfected cells is shown as bar graph. Error bars indicate s.e.m. (n=4). $^{**}p < 0.01$, Student's t-test. Scale bar, 10 μm. (**d**) Knockdown efficiency of endogenous SRSF3 and the expression Flag constructs were assessed by Western blot analysis.

Figure 18. Empty vector, Flag-SRSF3-WT or K85R mutant transfected cells were treated with arsenite for 30 mins, lysed in IP buffer and immunoprecipitated using Flag agarose beads. The precipitates were subjected to Western analysis using antibodies against SG proteins.

Figure 19. Immunoprecipitation was carried out as described in fig. 18 and the precipitates were blotted against TIA-1 and G3BP.

Figure 20. U2OS cells transiently transfected with Flag-SRSF3-WT or K85R mutant were treated with either mock or 0.2 mM arsenite and subjected to immunofluorescence microscopy. Recruitment of TIA-1 into SGs was assessed by co-staining with anti-TIA-1 and anti-Flag antibodies. White arrow indicates SG.

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Figure 21. siCONT or siHRI transfected cells were subsequently transfected with Flag-SRSF3 and His_6-NEDD8 for 36 hours. Cells were then treated with 0.2 mM arsenite for 30 mins and subjected to *in vivo* neddylation assay.

Figure 22. Working model for SG assembly by neddylation pathway.

Figure 23. (**a**) Flag-SRSF3 and His-NEDD8 co-transfected cells were pre-treated with DMSO, 50 nM PYR-41 for 2 h or 1 μM MLN4924 for 18 h. Cells were then treated with 0.2 mM arsenite for 30 mins and subjected to neddylation assay. (**b**) HEK293T cells knocked down with siCONT or siUbe1 were co-transfected with Flag-SRSF3 and His_6-NEDD8. Cells were then treated with 0.2 mM arsenite for 30 mins and *in vivo* neddylation assay was carried out.

Figure 24. U2OS cells transfected with siCONT, siUbe1 or siUBE2M were treated with 0.2 mM arsenite for 30 mins and stained against (**a**) eIF3b and RCK, (**b**) TIA-1 and G3BP. (**c**) Western blot analysis for Knockdown efficiency of Ube1 and UBE2M. (**d**) Percentage of cells bearing SGs.

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APPENDIX 1. (**a**) U2OS cells grown on coverslips were pre-treated with DMSO or MLN4924 (concentration ranging from 1 nM to 5 μM) for 18 h prior to 0.2 mM arsenite treatment at different time points and then immunostained against eIF3b and RCK. (**b**) Percentage of cells bearing SGs. Error bars indicate s.e.m. (n=3). (**c**) Western blot analysis showing the effect of MLN4924 treatment on total neddylation. Scale bar, 10μm.

APPENDIX 2. (**a**) HeLa cells transfected with indicated siRNAs were treated with 0.2 mM arsenite and immunostained against eIF3b and RCK. (**b**) Western blot analysis for knockdown efficiency of NEDD8 and UBE2M. (**c**) Percentage of cells bearing SG is shown as bar graph. Error bars indicate s.e.m. (n=3). $^{**}p < 0.01$; $^{***}p < 0.001$, Student's t-test.

APPENDIX 3. (**a**) Experiment in (APPENDIX 2) was repeated in stable cell lines expressing EGFP-G3BP. Presence of SG was visualized using GFP immunoflouresence. (**b**) Western blot analysis showing knockdown efficiency of NEDD8 and UBE2M. (**c**) Percentage of cells bearing SGs. (**g**) EGFP-G3BP stable cells were pre-treated with DMSO or MLN4924 (1 μM) for 18 h were treated with 0.2 mM arsenite for indicated time points. (**h**) Statistical data showing percentage of SG inhibition. Scale bar, 10 μm.

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APPENDIX 4. U2OS cells pretreated with DMSO or MLN4924 (1 μM) for 18 h were cultured in presence or absence of 0.2 mM arsenite for 30 and 60 mins. Cells were then lysed and subjected to polysome profiling.

APPENDIX 5. (**a**) U2OS cells transfected with siCONT, siUBE2M or siNEDD8 were either untreated, treated with clotrimazole (20 μM) or thapsigargin (1 μM) for 45 mins. Cells were then immunostained against eIF3b and RCK antibodies. (**b**) Statistical data showing percentage of cells bearing SG. (**c**) Western data for knockdown efficiency. Scale bar, 10μm.

APPENDIX 6. (**a**) Indicated cell lines grown on coverslips were treated with 0.2 mM arsenite at different time points and immunostained against SG marker eIF3b and PB marker RCK. (**b**) Statistical graph for percentage of cells bearing SGs. Error bars indicate s.e.m. ns, non-significant, Student's t-test. (**c**) Western blot analysis for FB-NEDD8 expression. Scale bar, 10 μm.

APPENDIX 7. (**a**) U2OS cells transfected with siCONT or two siRNAs targeting different sites of SRSF3 were treated with 0.2 mM arsenite and stained against eIF3b and RCK. (**b**) Percentage of cells bearing SGs. Error bars indicate s.e.m. (n=3). $^{**}p < 0.01$; $^{***}p < 0.001$, Student's t-test. (**c**) Immunoblot for SRSF3 knockdown efficiency.

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APPENDIX 8. U2OS cells either non-transfected or transiently transfected with empty vector or Flag-SRSF3 were treated with 0.5 mM arsenite for 1 h and immunostained against (**a**) eIF3b and SRSF3 or (**b**) eIF3b and Flag antibody. White arrow indicates SG. Scale bar, 10 µm.

APPENDIX 9. *in vivo* neddylation assay was carried out for cells treated with arsenite in (**a**) dose dependent manner (0.15 mM to 1 mM) for 1 h (**b**) 0.2 mM arsenite at different time points (0-60 mins) as indicated. (**c**) HEK293T cells transfected with indicated plasmids were treated with 0.2 mM arsenite in lane 3 and 4 for 30 and 60 mins respectively. Cells in lane 5 and 6 were treated with 0.2 mM arsenite for 60 mins and then allowed to recover from stress by changing with fresh medium for 90 mins (lane 5) and 180 mins (lane 6) and subjected to neddylation assay.

APPENDIX 10. (**a**) U2OS cells transiently transfected with empty vector, HA-tagged UBE2M-WT, UBE2M-C111S or NEDP1 were treated with 0.2 mM arsenite. Cells were then immunostained against G3BP and HA antibody. (**b**) Bar graph depicting the percentage of cells bearing SG. (**c**) Western blot analysis showing the expression of transfected constructs. Scale bar, 10 µm.

APPENDIX 11. (**a**) U2OS cells stably expressing empty vector, Flag-SRSF3-WT and Flag-SRSF3-K85R were treated with 0.2 mM arsenite and

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immunostained against G3BP and Flag antibody. (**b**) Bar graph depicting the percentage of cells bearing SGs in transfected cells. Error bars indicate s.e.m. (n=3). $^{**}p < 0.01$; $^{***}p < 0.001$, Student's t-test. (**c**) Western blot anaysis showing the expression of Flag-tagged SRSF3 constructs. Scale bar, 10 μm.

APPENDIX 12. (**a**) U2OS cells transfected with Flag-SRSF3-WT/K85R were treated with 20 μM clotrimazole and 1 μM thapsigargin for 45 mins and stained against G3BP and Flag antibody. (**b**) Percentage of cells bearing SGs in transfected cells. (**c**) HEK293T cells co-transfected with Flag-SRSF3 and His_6-NEDD8 were treated with 0.5 mM arsenite, 20 μM clotrimazole and 1 μM thapsigargin for 1 h before subjected to neddylation assay.

APPENDIX 13. U2OS cells transiently transfected with empty vector, Flag-SRSF3-WT or (**a**) single lysine mutant (11, 23, 85, 146, 164) (**d**) double lysine mutant (11/23, 23/85, 146/164) were treated with 0.2 mM arsenite and stained against G3BP and Flag antibody. (**b,e**) Percentage of cells bearing SGs in Flag transfected cells. Error bars indicate s.e.m. (n=3). (**c,f**) Western blot analysis for the expression of Flag-SRSF3 constructs. Scale bar, 10μm.

APPENDIX 14. (**a**) Stably expressing empty vector, Flag-SRSF3-WT or Flag-SRSF3-K85R cells were treated with 0.2 mM arsenite and immunostained against eIF3b (green) and p70S6K (red) and Flag antibody

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(far red). Percentage of cells bearing (**b**) PBs and (**c**) SGs in flag expressed cells were quantified and represented as bar graph. Scale bar, 10 µm.

APPENDIX 15. U2OS cells stably expressing Flag-tagged SRSF3-WT and SRSF3-K85R were untreated or treated with 0.2 mM arsenite at two time points (30 and 45mins) and subjected to polysome profiling analysis.

APPENDIX 16. HEK293T cells transiently transfected with vector or Flag-SRSF3 and His_6-Ubiquitin were mock treated or treated with 0.5 mM arsenite for 1 h and subjected to *in vivo* ubiquitination assay. Scale bar, 10 µm.

APPENDIX 17. (**a**) U2OS cells transfected with siCONT, siRBX1 or siRBX2 were treated with 0.2 mM arsenite for indicated time points and immunostained against eIF3b and p70S6K. (**b**) Statistical graph showing the percentage of cells bearing SGs. Error bars indicate s.e.m. (n=3). $^{*}p < 0.05$; $^{**}p < 0.01$; ns, non-significant, Student's t-test. (**c**) RBX1 and RBX2 knockdown efficiency was confirmed with Western blot analysis.

APPENDIX 18. U2OS cells untreated (mock) or treated with 0.5 mM arsenite for 1 hour were immunostained against SG marker eIF4G (green) and (**a**) RBX1 or (**b**) RBX2 (red channel) as indicated. Scale bar, 10 µm.

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ABSTRACT

Functional Analysis of NEDDylation on the Assembly of Stress Granule

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Stress granules (SGs) are cytoplasmic RNA – protein (RNP) aggregates formed in response to stress exposure. SGs usually harbor translationally stalled mRNPs, RNA binding proteins, signaling molecules and play important roles in regulating gene expression and cell fate. Formation of these cytoplasmic compartments has been linked to several pathological conditions including frontotemporal dementia (FTD), amyotrophic lateral sclerosis (ALS) and cancer, whereas the cellular signaling pathways that lead to SG assembly are incompletely understood. Here we show that neddylation promotes SG assembly in response to arsenite-induced oxidative stress. Inhibition or depletion of key components of the neddylation machinery concomitantly inhibits stress-induced polysome disassembly and SG assembly. Affinity purification and subsequent mass-spectrometric analysis of Nedd8-conjugated proteins from translationally stalled ribosomal fractions

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identifies ribosomal proteins, translation factors and RNA-binding proteins (RBPs), including SRSF3, a previously identified SG regulator. We show that SRSF3 is selectively neddylated at Lys 85 in response to arsenite. Cells expressing a non-neddylatable SRSF3 (K85R) mutant inhibit SG assembly, but do not affect arsenite-induced polysome disassembly. Our observations suggest that the neddylation pathway plays an important role in the SG assembly and that the neddylation of SRSF3 is at least one critical event for mRNP remodeling.

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"I dedicated this PhD thesis to my Parents, Brother, Sister and Prof. Takbum Ohn"

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