RGG-motif proteins regulate mRNA translation upon genotoxic stress

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Abstract

RGG-motif proteins are the second largest group of RNA-binding proteins. These proteins have been implicated in several cellular processes. Here we identify a new role of RGG-motif proteins Sbp1, Scd6 and its human homolog LSM14a in genotoxic stress response. Scd6 and Sbp1 co-localize to cytoplasmic puncta upon hydroxyurea (HU) stress. These puncta are reversible and require RNA for their assembly. RGG-motif is necessary for the localization of both proteins to these granules. Interestingly, Scd6 is required for the efficient localization of Sbp1 to puncta upon HU stress. Strikingly absence of Scd6 increases HU-tolerance under normal conditions but sensitized the cells to HU upon SRS2 overexpression. Human LSM14a like yeast Scd6 and Sbp1 localizes to puncta in response to genotoxic stress such as cisplatin and HU. This localization to puncta also depends on the two RGG-motifs. Proximity ligation assay reveals that LSM14a interacts with EIF4G. Polysome profiling followed by RNA sequencing has identified numerous transcripts that are regulated by LSM14a at the translational level under normal conditions and in response to genotoxic stress. Our work identifies RGG-motif proteins as a key player in orchestrating genotoxic stress response by determining mRNA fate.

Introduction

DNA damage caused by cellular conditions and/or exogenous factors leads to genomic instability and thereby genotoxic stress. Replication errors, defective DNA damage repair machinery and nucleotide misincorporation are some of the cellular events leading to DNA damage [1]. Exogenous factors causing DNA damage include exposure to genotoxins such as hydroxyurea, cisplatin, and MMS. Living systems employ strategies to regulate different gene expression steps to alter the cellular proteome allowing it to mount an effective genotoxic stress response (GSR). The contribution of various gene expression steps to GSR is well documented, however changes in cytoplasmic mRNA fate in response to GSR remain poorly explored [2].

RNA binding proteins (RBPs) play a key role in determining functional states of mRNA. Several classes of RNA binding domains such as RNA Recognition Motif (RRM), KH-domain, Zn-finger motif and PUMILIO have been reported to play a role in post-transcriptional gene control by affecting specific mRNA subsets. RGG-motifs are the second largest class of RBPs which are also implicated in binding to proteins and DNA [3,4]. RGG-motifs, characterized by repeats of RGG-/RGX- which imparts properties of low-complexity sequences [3]. Consistent with that, these sequences contribute towards assembly of mRNP condensates by undergoing liquid-liquid phase separation [5].

Sbp1 and Scd6 are two such RNA-binding proteins with RGG-motif sequences. These proteins target eIF4G to repress translation. The RGG-motif of these proteins is important for localization to granules, interaction with eIF4G and consequently for translation repression activity ([6,7]). Interestingly, RGG-motif of Sbp1 has recently been implicated in P-body disassembly suggesting that RGG-motifs can promote both assembly and disassembly of RNA granules [8]. LSM14, the human homolog of Scd6 is a granule-resident protein which has also been implicated in translation control [9]. Interestingly LSM14 contains two RGG-motifs as compared to a single RGG-motif in Scd6 [10]. LSM14 plays a key role in formation of the mRNA silencing complex via its association with DDX6 [11]. Although xRAP55 (Xenopus ortholog of LSM14/Scd6) has been reported to repress translation [12], the direct role of human LSM14 in repression remains to be demonstrated. Interestingly LSM14 has been reported as a sensor of viral nucleic acid which plays a key role in antiviral response [13]

Scd6 localizes to puncta upon hydroxyurea stress however the functional implication of this observation is unclear [14]. This observation combined with the knowledge that RGG-motif proteins have merged as important regulators of mRNA fate motivated us to hypothesize that RGG-motifs could contribute to genotoxic stress response and that such a role could be conserved. Our results report an exciting new contribution of yeast proteins Sbp1, Scd6 and its human homolog LSM14 towards GSR.

Results

Scd6 and Sbp1 localize to granules in response to HU stress

Scd6 is a known P-body and stress granule resident protein in response to glucose deprivation and oxidative stress. We observed that Scd6 localized to foci upon HU stress (Figure 1A). Within 45 minutes of 0.2 M HU treatment, the number of Scd6 granules per cell increased from 1 to 2.2 (n=3). We wanted to know if localization to foci is specific to Scd6 or general to other RGG-motif proteins. Hence, we shortlisted four more RNA binding, RGG-motif containing proteins (Sbp1, Npl3, Gbp2 and Psp2) to understand their role, if any, in HU induced replication stress. Sbp1 and Npl3 (like Scd6) are known to be involved in translation repression activity mediated by binding to eIF4G1 among other known functions [15]. Psp2 has been implicated in promoting translation of specific mRNA in autophagy response [16]. Gbp2 is mainly involved in mRNA export and processing. Npl3 and Gbp2 are shuttling proteins, mainly localised in the nucleus and have known connections to cellular stress response [17,18]. Out of the four proteins tested, Sbp1 (like Scd6) localized to foci in response to HU stress (Figure 1B) with granules per cell increasing from 0.3 to 0.8 upon 60 minutes of exposure to HU (n=3)

(Figure 1E). For Npl3, Gbp2, and Psp2, no significant localization change was observed even after 120 minutes of HU treatment (Figure 1D). Localization of both Scd6 and Sbp1 to granules is not a function of increased protein levels upon HU exposure, as indicated by western blot analysis (Figure 1C). Due to technical difficulty associated with detecting GFP-Scd6 using anti-GFP antibody, levels of endogenously tagged Scd6-myc were quantified upon HU stress. Based on these observations we conclude that Scd6 and Sbp1 may have a specific role under HU-induced genotoxic stress.

HU-induced Scd6 and Sbp1 puncta colocalize, require RNA and are reversible

Since both Scd6 and Sbp1 localize to foci in response to HU stress, we hypothesized that they could co-localize. To test this hypothesis, live cell imaging was done with GFP-tagged endogenous Sbp1 and plasmid borne Scd6-mCherry. We observed that 91% of Sbp1 foci co-localized with Scd6-mCherry upon 60 minutes of HU stress (Figures 2A and 2B). We further observed that Scd6 puncta form faster than Sbp1 upon HU treatment. Scd6 and Sbp1 puncta become visible after 30 and 45 minutes respectively of HU stress (data not shown). To investigate whether mRNA was present in the Scd6 and Sbp1 granules formed under HU stress, we performed the classical cycloheximide (CHX) experiment. CHX-treated cells show a disappearance of mRNA containing granules as CHX mediated elongation inhibition causes the mRNA to be stuck in the polysome thereby reducing its availability. This prevents formation of new mRNP complexes such as SGs and PBs. When HU-treated cells were subjected to 1 mg/ml of CHX for 5 minutes at 30 ºC, there was a strong decrease in both Scd6 and Sbp1 granules (Figures 2C and 2D). For Scd6, the granule/cell count decreased from 0.95 to 0.15 (Figure 2E, left panel) whereas for Sbp1 no granules were visible upon CHX treatment (Figure 2E, right panel). This suggests that mRNAs are present in Scd6 and Sbp1 granules that form in response to HU stress.

RNA granules disassemble rapidly upon removal of stress [19]. The reversible nature of RNA granules is important as it allows the return of mRNA and various RNA-binding proteins to the cytoplasmic pool. To examine if the HU-dependent formation of Scd6 and Sbp1 granules is reversible and therefore dynamic, we performed the experiments as described earlier but followed by a recovery period. During the recovery period, the cells were resuspended in HU-free media and the status of granules at different time points from 30-90 minutes was observed. Localization to foci decreased for both the proteins gradually with time (Figure 2E and 2F). After 90 minutes of recovery, Scd6 granules decreased from 1 to 0.3/cell. Similarly, Sbp1 granules, showed a decrease in granule count from 0.9 to 0.25 in 90 minutes (Figure 2G). Based

on these observations, we conclude that the formation of Scd6 and Sbp1 granules in response to HU is reversible. Since CHX experiments implicated that these granules contain mRNA, it is likely that Scd6 and Sbp1 localize to these foci to regulate the fate of specific mRNAs in response to HU stress.

Since both Scd6 and Sbp1 localize to foci in response to HU stress, we tested if one protein could affect the localization of the other. We observed a significant decrease (from 0.8 to 0.5 granules/cell) in the localization of Sbp1 to granules in the absence of Scd6 (Figure 3A-D) but not vice versa. This is not due to a decrease in protein levels as GFP intensity quantitation indicate a net increase in Sbp1-GFP levels (Figure 3C, right panel). This suggests that Scd6 is important for localization of Sbp1 to puncta upon HU stress.

RGG-motif is required for localization of Scd6 and Sbp1 to puncta

Both Scd6 and Sbp1 are multidomain proteins containing a C-terminal and a middle RGG domain respectively. In addition, Scd6 has an N-terminal Lsm domain, and a middle FDF domain (Figure 4A, left schematic). The domain organization is conserved across all the orthologs of Scd6. The RGG domain of Sbp1 is flanked by two RRM domains, RRM1 and RRM2 (Figure 4A, right schematic). RGG-motif rich sequences are low-complexity sequences implicated in the assembly of RNA granules. Also, since previously characterized function of both Scd6 and Sbp1 were mediated through their RGG domain [6,7], we tested the localization of RGG domain deletion mutant of both Scd6 and Sbp1. We observed that localization of Scd6 to granules decreased by almost 50% when RGG domain is deleted (Figures 4B and 4C). The Lsm domain of Scd6 is reported to bind RNA as well as protein. When the Lsm domain was deleted, the localization of Scd6 to granules was almost completely abrogated indicating that Lsm domain is essential for Scd6 localization to granules (Figure4B). Interestingly, deletion of RGG domain caused complete cessation of Sbp1 granule localization and it was similar to the effect observed with the Lsm domain deletion mutant of Scd6. These observations suggest that although the absence of Lsm domain of Scd6 completely compromises its ability to localize to puncta, the RGG-motif of both Scd6 and Sbp1 is important for their localization to puncta.

Scd6 modulates cellular tolerance to HU

To test the physiological role of Scd6 and Sbp1 in HU stress response, we measured growth kinetics of Δ*scd6* and Δ*sbp1* strain upon HU stress. We observed that both Δ*scd6* and Δ*sbp1* strains grew marginally but significantly better than wild type in the presence of HU (Figure 5A and B). Consistent with this observation, we observed an increase in the CFUs of both

Δ*sbp1* and Δ*scd6* strains on HU containing media as compared to wild type (Figure 5C). Taken together these observations indicate that Scd6 and Sbp1 contribute to HU-stress response pathway(s).

We further observed that absence of Scd6 significantly increased the sensitivity of cells to HU upon SRS2 overexpression; however, the absence of SBP1 did not alter the growth defect phenotype. SRS2 is 3' to 5' DNA helicase involved in the regulation of homologous recombination (HR)[20]. Srs2 levels are tightly regulated and overexpression of SRS2 causes growth defect and increases sensitivity to genotoxic stress[21]. Excess SRS2 causes the formation of toxic intermediates upon checkpoint activation and initiation of HR mediated repair [22]. This observation identifies an important player in GSR which is impacted by RGG-motif protein Scd6.

LSM14a localizes to puncta upon Cisplatin and HU stress in RGG-motif dependent manner

To check if the role Scd6 in genotoxic stress was conserved, we decided to focus on LSM14, the human homolog of Scd6. It is notable that Sbp1 has no know human ortholog. Scd6 is conserved across different species and LSM14a is also known to localize to P-bodies [23]. Like Scd6, LSM14a is a modular protein (Figure 6A) but with an additional RGG domain before the FDF domain. LSM14a is also known to localize to P-bodies and to repress translation [23]. We therefore decided to understand the role of LSM14a in genotoxic stress response. We observed that, like Scd6, LSM14a also localizes to puncta upon HU and Cisplatin treatments (Figure 6B & C). This result indicates that LSM14a could be involved in genotoxic stress response. As mentioned earlier, LSM14a contains two RGG-motifs (Figure 6A). Since RGG motif was important for localization of Scd6 and Sbp1 to granules, we next tested the role of RGG-motifs in localization of LSM14a to puncta. We report that the localization to puncta significantly decreased upon deletion of the two RGG-motifs of LSM14 (Figure 6D & E). Based on these results we conclude that the RGG-motifs of LSM14a are important for localization to puncta.

LSM14a modulates translation upon genotoxic stress

To better understand the role of LSM14a in translation control, we used polysome profiling approach. RNAs isolated from translating and non-translating fractions of wild type and LSM14a knockdown cells were sequenced to identify mRNAs whose association with polysomes was perturbed by LSM14a. 112 genes were identified to be differentially regulated

(Figure 7A and B volcano plot). Of these, association to polysomes of 66 mRNAs was decreased whereas the association of 46 mRNAs was increased. To address the role of LSM14 in genotoxic stress response, similar analysis was carried for LSM14A-depleted cells treated with HU. 163 mRNAs were identified to be differentially regulated at the translation level. Of these, 86 mRNAs were downregulated and 77 were upregulated.

We validated changes in association to polysomes of 3 transcripts using RTqPCR-based quantification of specific mRNAs in polysome fractions. These include the mRNAs encoding LIG4 (DNA Ligase IV), SASS6 (SAS-6 centriolar assembly protein) and FAM111B (FAM111 trypsin-like peptidase B). These mRNAs shifted from light to heavy polysome fractions upon HU treatment and LSM14a knockdown (Figure 8A-C). This indicates that HU upregulates the translation of these mRNAs in LSM14A-depleted cells.

Discussion

In this report we have explored and identified a conserved role of RGG-motif proteins in HU stress response. Several observations support this conclusion: (i) Sbp1, Scd6 and its human homolog LSM14 localize to puncta upon HU-induced genotoxic stress, (ii) Localization of Sbp1 to HU-induced puncta is partially dependent on Scd6 localization, (iii) RGG domain of Scd6, Sbp1 and LSM14 is important for their localization to granules, (iv) Absence of Scd6 and Sbp1 decreases HU sensitivity, (vi) Absence of Scd6 increases sensitivity to HU upon SRS2 overexpression (vii) LSM14 regulates translation of multiple transcripts including those important in GSR in response to HU stress. These results identify an exciting new role of RGG-motif proteins in genotoxic stress response.

RNA granules are sites of regulation of mRNA translation and decay. Localization of Sbp1, Scd6 and LSM14 to puncta upon HU stress suggests that such localization could lead to regulation of mRNA fate upon HU stress. Consistent with this, the elongation inhibitor cycloheximide significantly decreases Scd6 and Sbp1 puncta upon HU stress. The reversibility of HU-induced Scd6 and Sbp1 puncta suggests that resident mRNAs may return to translation once the stress is removed.

Our study emphasizes a conserved role of RGG-motifs in HU stress response. RGG-motifs comprise of repeats of arginine and glycine and have been implicated in several cellular processes including translation control. Localization of Sbp1, Scd6 and LSM14 to puncta is dependent on their RGG-motif as the mutants lacking this motif are defective in localizing to puncta [6,7,23]. RGG-motifs are unique as these have been implicated in both assembly and disassembly of RNA granules [24–26]. It must be noted that the Lsm domain of Scd6 has the

strongest impact on localization of Scd6 to HU-induced puncta (Figure 4A). The interactions of RGG-motif with RNA and/or proteins that enable localization of these proteins to puncta remain to be explored.

Direct demonstration of LSM14 affecting the translation status of mRNAs is missing in literature. Through polysome profiling studies we have identified several transcripts regulated by LSM14 upon HU stress (Figure. 8A). This data confirms the role of above RGG-motif proteins in regulating HU stress response by modulating mRNA translation. We have validated change in translation status of three such mRNAs, *LIG4, SASS6* and *FAM111B*. LIG4, (DNA Ligase IV) associates with XRCC4 to promote DNA double strand break repair via the non-homologous end joining (NHEJ) pathway. Our data suggests that under normal conditions, the translation of the *LIG4* mRNA is repressed by LSM14a. In response to genotoxic stress such as HU, it is derepressed to help cell mount a genotoxic stress response. An interesting future direction would be the mechanism that allows repression and de-repression of *LIG4* mRNA translation by LSM14a. We think that repression mechanism of LSM14a is likely similar to Scd6 which targets eIF4G to repress translation [15]. Consistent with this is our observation that LSM14 interacts with EIF4G using proximity ligation assay (Supplementary Figure 2). This interaction depends on arginine methyltransferase PRMT1 (Supplementary Figure 2) as reported for Scd6-eIF4G interaction.

SASS6 (SAS-6 Centriolar assembly protein) is a central component of centrioles which plays an important role in its duplication and function. SASS6 is known to promote the growth of esophageal squamous carcinoma cells [27]. Consistent with this observation knockdown of SASS6 decreases proliferation of triple negative breast cancer cells [28]. HU is known to induce centriole amplification which leads to genome instability. We observe that SASS6 is derepressed in absence of LSM14a upon HU stress suggesting that LSM14a functions to keep SASS6 levels under check.

FAM111B (homolog of FAM111A) belongs to a family of serine protease. This family has been implicated in the clearance of DNA-protein crosslinks (DPC) upon genotoxic stress. DPCs interfere with several cellular processes such as replication, transcription and recombination [29]. Therefore, the DPC proteases like FAM111B play an important role in maintaining genomic stability. Evidence suggest that deregulated levels of FAM111A/B proteases are detrimental to cells [30]. Our study identifies LSM14a as a translation regulator which keeps levels of FAM111B under check suggesting that LSM14a could play a role in maintaining genomic stability by maintaining DPC clearance.

Interestingly absence of Scd6 and Sbp1 affects the ability of cells to deal with HU stress. Both deletion strains increase the tolerance of cells towards HU. This is evident based on the results obtained from the growth curve and CFU experiments (Figure 5A, 5B and 5C). We interpret these results to indicate that Scd6 and Sbp1 are likely involved in repression of the translation of mRNAs encoding proteins important for mounting a stress response to HU. This is consistent with results obtained using LSM14 which represses translation of mRNA encoding proteins such as Ligase 4 and FAM111B. Both these proteins have been implicated in maintaining genomic stability in response to HU stress.

Another exciting new link between RGG-motif protein and GSR presented in this study is the observation that Scd6 regulates Srs2 activity. In the absence of SCD6 and upon Srs2 overexpression, cells are more sensitive to HU (Figure 5D). Under our experimental conditions, Srs2 overexpression or SCD6 deletion alone does not render any sensitivity to HU compared with WT. Srs2 is a tightly regulated protein [21]. It is a key player in homologous recombination and repair [20,31], therefore any alteration in its levels beyond a certain limit are likely to initiate a signalling cascade leading to its feedback inhibition. HU induces SRS2 overexpression[32]. Since strong overexpression (from galactose-inducible promoter) of SRS2 leads to cellular toxicity (Supplementary Figure 1), we believe that Scd6 functions to keep its level under control upon HU stress. Therefore, the absence of Scd6 leads to increased growth defect upon in the presence of HU. The mechanism underlying regulation of Srs2 function by Scd6 will be an exciting future direction.

Overall, this study establishes RGG-motif proteins Sbp1, Scd6 and LSM14 as a regulator of genotoxic stress response. Identifying the regulatory mechanisms that enable genotoxic stress condition-specific repression activity of LSM14a would be a key future direction. It must be noted that both LSM14a and its ortholog Scd6 are known to be methylated at their RGG-motifs [6,33]. Arginine methylation (AM) is known to promote the repression activity of RGG-motif proteins [6,7]. Whether AM of LSM14a modulates its repression activity in response to genotoxic stress will be important to test in the future.

Methods and materials

Yeast transformation

BY4741 cells were grown to 0.6 OD_{600} in complete media and pelleted down. The cells were washed once with water followed by 100 mM Lithium Acetate (LiAc). The cells were resuspended in 100 mM Lithium Acetate and aliquoted into 50 µL fractions. The cell suspension was then layered with 240 µL of 50 % PEG (v/v), 36 µL of 1 M LiAc and 25 µL

of salmon sperm DNA (100 mg/ml) and vortexed. The mixture was then incubated in 30 ºC for 30 minutes followed by 15 minutes of heat shock at 42 ºC. The cells were then pelleted, resuspended in 100 µL water and plated on synthetic defined media lacking Uracil (SD-Ura$^-$) glucose, agar plate. The plates were incubated at 30 ºC for 2 days, before colonies appeared.

HU treatment

Yeast cultures were grown in 10 ml of SD-Ura$^-$with glucose as the sugar, to 0.3-0.4 OD_{600} and split into two equal parts. The fractions were treated with 0.2 M HU or water (control) and incubated for 45 minutes (for Scd6) or 60 minutes (for Sbp1) at 30 ºC with constant shaking.

Cycloheximide treatment

Yeast cultures were grown in 10 ml of SD-Ura$^-$ with glucose as the sugar, to 0.3-0.4 OD_{600} and split into three equal parts of 2.760 ml each. To two fractions, 240µL of 2.5 M HU was added and to one fraction, same volume of water was added (control). They were incubated for 45 or 60 minutes (depending on the strain) at 30 ºC with constant shaking. After incubation, to the HU containing fractions, 3µL of 100 mg/ml Cycloheximide solution (dissolved in methanol) or methanol (control) was added. Cells were kept back in the shaker for 5 minutes followed by pelleting and live cell imaging.

Yeast Live Cell imaging

In all cases for yeast live cell imaging, after the incubation period, the cells were immediately harvested (14000 rpm, 15 seconds), spotted on glass cover slip (No.1) and observed using live cell imaging. Yeast images were acquired using a Deltavision RT microscope system running softWoRx 3.5.1 software (Applied Precision, LLC), using an Olympus 100×, oil immersion 1.4 NA objective. The Green Fluorescent Protein (GFP) channel had 0.2 or 0.25 seconds of exposure (for Scd6GFP and Sbp1GFP respectively) and 32 % transmittance. Minimum 80 - 100 cells were observed for each experiment. Quantification was done as granules per cell. Statistical analysis was done using GraphPad Prism Version 7.0. Statistical significance was calculated using two tailed paired t-test.

Western Blotting

SDS Polyacrylamide gels were transferred onto Immobilon-P Transfer Membrane® (MERCK) using Transfer-Blot® Semi Dry apparatus (BIO-RAD). Transfer was done at 10 V for 1 hour.

Antibodies used in this study	Catalogue No.
Anti-Sbp1	Generated in lab
Anti-Pgk1	Abcam, ab113687
Anti-myc	Sigma, C3956
Anti-Rabbit	Jackson ImmunoResearch Laboratories, Code No. 111-035-003
Anti-Mouse	Jackson ImmunoResearch Laboratories, Code No. 115-035-003
Monoclonal mouse LSM14A antibody	SantaCruz Biotechnology, sc-398552
Rabbit EIF4G antibody	Cell Signalling Technologies, 2498
Mouse EIF4E antibody	Cell Signalling Technologies, 9742
Secondary mouse AlexaFluor 594 antibody	ThermoFisher , A32744

Cell culture

The REP-1, hTERT-immortalized retinal pigment epithelial cell line and A2058 melanoma cell line used in this study were purchased from the ATCC. Cancer cell lines were maintained at 37 °C and 5% CO_2 in a humidified atmosphere and grown in DMEM:F12 or MEM growth media supplemented with 10% FBS, 2 mM glutamine, 50 u ml^{-1} penicillin and 50 mg ml^{-1} streptomycin (Gibco)

Immunofluorescence assay

For immunofluorescence experiments to investigate the localization of endogenous LSM14A upon genotoxic stress. Cells were seeded on coverslips pre-coated with 1 µg/ml fibronectin (Sigma) and 20 µg/ml collagen (Sigma) two day before treatment with genotoxic stress agent. After reaching confluency cells were then treated with either 10 mM hydroxyurea for 30 minutes or 10 µM of cisplatin for 6 hours. After the treatment the coverslips were washed twice in PBS, let the coverslip dry for 2-3 minutes. The cells were then fixed with 4% PFA in PBS for 10 minutes followed by permeabilization for 10 min at room temperature in PBS containing 0.1% Tween-20 (PBST). The cells were blocked for 20 minutes at room temperature with 5% BSA in PBST (PBST-BSA) followed by probing with primary antibody. The primary anti-LSM14A antibody was diluted 1:100 times in PBST-BSA solution, added onto the coverslip and incubated for 1 hour at room temperature. Cells were then washed twice with PBST. Secondary antibody conjugated with Alexa Fluor™ 594 was diluted to 1:1000 in PBST-BSA

solution, added onto the coverslip at room temperature for 1 hour of exposure. Cells were then washed with PBST, followed by one wash with 1X PBS. Cells were let to dry before putting mounting media containing DAPI followed by fixation onto glass slide for confocal microscopy.

For localization experiment with ectopically expressed LSM14A-GFP and LSM14AΔRGG1ΔRGG2-GFP. Cells were seeded on coverslips pre-coated with 1 µg/ml fibronectin (Sigma) and 20 µg/ml collagen (Sigma) two day before treatment with genotoxic stress agent. After reaching confluency cells were then treated with either 10 mM hydroxyurea for 30 minutes or 10 µM of cisplatin for 6 hours. After the treatment the coverslips were washed twice in PBS, let the coverslip dry for 2-3 minutes. The cells were then fixed with 4% PFA in PBS for 10 minutes followed by permeabilization for 10 min at room temperature in PBS containing 0.1% Tween-20 (PBST). Cells were then washed with PBST, followed by one wash with 1X PBS. Cells were dried onto the cover slip before putting mounting media containing DAPI followed by fixation onto glass slide for confocal microscopy (Leica SPE).

Proximity ligation assay

Interactions between EIF4E and EIF4G (EIF4E–EIF4G) or LSM14A and EIF4G (LSM14A-EIF4G) were detected by in situ proximity ligation assay (PLA) in A2058 melanoma cell lines untreated or treated with hydroxyurea. PLAs were performed on both fixed and permeabilized melanoma cells. The fixed, permeabilized cells were treated identically, and the PLA protocol was followed according to the manufacturers' instructions (Olink Bioscience), with incubation of the primary antibodies at 4 °C overnight. After blocking, the antibodies were used at the following concentrations: for LSM14A (mouse, sc-398552, Santa Cruz Biotechnology, 1:200); for eIF4G (rabbit, 2498; Cell Signaling Technology, 1:200); for EIF4E (mouse, 9742, Cell Signaling Technology, 1:200). PLA minus and PLA plus probes (containing the secondary antibodies conjugated to oligonucleotides) were added and incubated for 1 h at 37 °C. More oligonucleotides were then added and allowed to hybridize to the PLA probes. Ligase was used to join the two hybridized oligonucleotides into a closed circle. The DNA was then amplified (with rolling circle amplification), and detection of the amplicons was carried out using the Brightfield detection kit for chromogenic development or using the Far-Red detection kit for fluorescence. Cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). The sections were mounted with Olink Mounting Medium. The first results were visualized by confocal microscopy (Leica SPE), and the analysis was supported by Volocity software. To

improve the sensitivity of the fluorescence detection, we next used a scanner (Olympus VS120) (magnification 20×; 2-ms exposure for the DAPI channel and 300-ms exposure for the Cy5 channel; 1 pixel = 0.32 μm), and the number of PLA signals per cell was counted (more than three fields) by semi-automated image analysis (ImageJ and OlyVIA).

Polysomal fractionation and microarray experiment.

To understand the translation control effect of LSM14A, we either transfected A2058 cells with scrambled siRNA or siRNA specific to LSM14A cells. We stress the cells with or without 10 mM hydroxyurea for 2 hours, lysed the cells, and carried out polysome profiling. Sucrose density gradient centrifugation was used to separate the sub-polysomal and the polysomal ribosome fractions. Fifteen minutes before collection, cells were incubated at 37 ºC with 100 mg/ml cycloheximide added to the culture medium. Next, cells were washed, scraped into ice-cold PBS supplemented with 100 mg/ml cycloheximide, centrifuged at 3,000 r.p.m. for 5 min and then collected into 400 ml of LSB buffer (20 mM Tris, pH 7.4, 100 mM NaCl, 3 mM MgCl2, 0.5 M sucrose, 2.4% Triton X-100, 1 mM DTT, 100 U ml21 RNasin and 100 mg ml21 cycloheximide). After homogenization, 400 ml LSB buffer supplemented with 0.2% Triton X-100 and 0.25 M sucrose was added. Samples were centrifuged at 12,000g for 10 min at 4 ºC. The resultant supernatant was adjusted to 5 M NaCl and 1 M MgCl2. The lysates were loaded onto a 15–50% sucrose density gradient and centrifuged in an SW41 rotor at 38,000 r.p.m. for 2 h at 4 ºC. Polysomal fractions were monitored and collected using a gradient fractionation system (Isco). We extracted all the RNAs from each polysome fractions. Total RNA was extracted from the four heaviest fractions and the input samples using the TRIzol–chloroform method.

Real-time PCR

RNA from each of the polysome fractions (16 fractions) was isolated using TRIzol LS reagent (TRIzol™ LS Reagent, Invitrogen, Cat ID 10296010) using the manufacturer's protocol. The isolated total RNA was quantified and a total of 1 ug of RNA was used for complementary DNA synthesis. The cDNAs were diluted to 1:10 times from which equal volume (2 ul) cDNA were used along with SYBR green (TB Green Premix Ex Taq II (Tli RNase H Plus); TaKaRa, Cat ID RR820B) and primers (Bioserve, India) for validation of mRNA levels using quantitative real-time PCR (BioRad CFX96 Touch Real-Time PCR Detection System).

RNA interference

Cells were transfected with 20 nM of each siRNA against LSM14A (Dharmacon) and PRMT1 (Dharmacon) using Lipofectamine RNAiMAX Reagent (Life Technologies) following the supplier's instructions.

References

1. Marnett, L. Endogenous DNA damage and mutation. *Trends in Genetics* **17**, 214–221 (2001).
2. Mohanan, G., Das, A. & Rajyaguru, P. I. Genotoxic stress response: What is the role of cytoplasmic mRNA fate? *BioEssays* **43**, 2000311 (2021).
3. Thandapani, P., O'Connor, T. R., Bailey, T. L. & Richard, S. Defining the RGG/RG Motif. *Molecular Cell* **50**, 613–623 (2013).
4. Chowdhury, M. N. & Jin, H. The RGG motif proteins: Interactions, functions, and regulations. *WIREs RNA* (2022) doi:10.1002/wrna.1748.
5. Chong, P. A., Vernon, R. M. & Forman-Kay, J. D. RGG/RG Motif Regions in RNA Binding and Phase Separation. *Journal of Molecular Biology* **430**, 4650–4665 (2018).
6. Poornima, G., Shah, S., Vignesh, V., Parker, R. & Rajyaguru, P. I. Arginine methylation promotes translation repression activity of eIF4G-binding protein, Scd6. *Nucleic Acids Research* **44**, 9358–9368 (2016).
7. Bhatter, N. *et al.* Arginine methylation augments Sbp1 function in translation repression and decapping. *FEBS Journal* **286**, 4693–4708 (2019).
8. Roy, R., Das, G., Kuttanda, I. A., Bhatter, N. & Rajyaguru, P. I. Low complexity RGG-motif sequence is required for Processing body (P-body) disassembly. *Nature Communications* **13**, 2077 (2022).
9. Yang, W. H., Jiang, H. Y., Gulick, T., Bloch, K. D. & Bloch, D. B. RNA-associated protein 55 (RAP55) localizes to mRNA processing bodies and stress granules. *RNA* (2006) doi:10.1261/rna.2302706.
10. Roy, D. & Rajyaguru, P. I. Suppressor of clathrin deficiency (Scd6)—An emerging RGG-motif translation repressor. *Wiley Interdisciplinary Reviews: RNA* **9**, e1479 (2018).
11. Brandmann, T. *et al.* Molecular architecture of LSM14 interactions involved in the assembly of mRNA silencing complexes. *The EMBO Journal* **37**, (2018).
12. Tanaka, K. J. *et al.* RAP55, a Cytoplasmic mRNP Component, Represses Translation in Xenopus Oocytes. *Journal of Biological Chemistry* **281**, 40096–40106 (2006).

13. Li, Y. *et al.* LSm14A is a processing body-associated sensor of viral nucleic acids that initiates cellular antiviral response in the early phase of viral infection. *Proceedings of the National Academy of Sciences* **109**, 11770–11775 (2012).
14. Tkach, J. M. *et al.* Dissecting DNA damage response pathways by analysing protein localization and abundance changes during DNA replication stress. *Nat Cell Biol* **14**, 966–976 (2012).
15. Rajyaguru, P., She, M. & Parker, R. Scd6 Targets eIF4G to Repress Translation: RGG Motif Proteins as a Class of eIF4G-Binding Proteins. *Molecular Cell* **45**, 244–254 (2012).
16. Yin, Z. *et al.* Psp2, a novel regulator of autophagy that promotes autophagy-related protein translation. *Cell Research* (2019) doi:10.1038/s41422-019-0246-4.
17. Santos-Pereira, J. M., Herrero, A. B., Moreno, S. & Aguilera, A. Npl3, a new link between RNA-binding proteins and the maintenance of genome integrity. *Cell Cycle* (2014) doi:10.4161/cc.28708.
18. Poornima, G. *et al.* RGG-motif containing mRNA export factor Gbp2 acts as a translation repressor. *RNA Biology* (2021) doi:10.1080/15476286.2021.1910403.
19. Decker, C. J. & Parker, R. P-bodies and stress granules: possible roles in the control of translation and mRNA degradation. *Cold Spring Harb Perspect Biol* **4**, a012286–a012286.
20. Marini, V. & Krejci, L. Srs2: The "Odd-Job Man" in DNA repair. *DNA Repair* **9**, 268–275 (2010).
21. Bronstein, A., Bramson, S., Shemesh, K., Liefshitz, B. & Kupiec, M. Tight Regulation of Srs2 Helicase Activity Is Crucial for Proper Functioning of DNA Repair Mechanisms. *G3 Genes|Genomes|Genetics* **8**, 1615–1626 (2018).
22. Bronstein, A., Gershon, L., Grinberg, G., Alonso-Perez, E. & Kupiec, M. The main role of Srs2 in DNA repair depends on its helicase activity, rather than on its interactions with PCNA or Rad51. *mBio* (2018) doi:10.1128/mBio.01192-18.
23. Yang, W. H., Jiang, H. Y., Gulick, T., Bloch, K. D. & Bloch, D. B. RNA-associated protein 55 (RAP55) localizes to mRNA processing bodies and stress granules. *RNA* (2006) doi:10.1261/rna.2302706.
24. Schuster, B. S. *et al.* Controllable protein phase separation and modular recruitment to form responsive membraneless organelles. *Nature Communications* (2018) doi:10.1038/s41467-018-05403-1.

25. Godin, K. S. & Varani, G. How arginine-rich domains coordinate mRNA maturation events. *RNA Biology* (2007) doi:10.4161/rna.4.2.4869.
26. Roy, R., Das, G., Kuttanda, I. A., Bhatter, N. & Rajyaguru, P. I. Low complexity RGG-motif sequence is required for Processing body (P-body) disassembly. *Nature Communications* **13**, 2077 (2022).
27. Xu, Y. *et al.* SASS6 promotes proliferation of esophageal squamous carcinoma cells by inhibiting the p53 signaling pathway. *Carcinogenesis* (2021) doi:10.1093/carcin/bgaa067.
28. Lili, D. U. *et al.* Knockdown of SASS6 reduces growth of MDA-MB-231 triple-negative breast cancer cells through arrest of the cell cycle at the G2/M phase. *Oncology Reports* (2021) doi:10.3892/or.2021.8052.
29. Ruggiano, A. & Ramadan, K. DNA–protein crosslink proteases in genome stability. *Communications Biology* (2021) doi:10.1038/s42003-020-01539-3.
30. Hoffmann, S. *et al.* FAM111 protease activity undermines cellular fitness and is amplified by gain-of-function mutations in human disease. *EMBO Rep* (2020) doi:10.15252/embr.202050662.
31. Niu, H. & Klein, H. L. Multifunctional Roles of Saccharomyces cerevisiae Srs2 protein in Replication, Recombination and Repair. *FEMS Yeast Research* fow111 (2016) doi:10.1093/femsyr/fow111.
32. Liberi, G. Srs2 DNA helicase is involved in checkpoint response and its regulation requires a functional Mec1-dependent pathway and Cdk1 activity. *The EMBO Journal* **19**, 5027–5038 (2000).
33. Matsumoto, K. *et al.* PRMT1 is required for RAP55 to localize to processing bodies. *RNA Biology* **9**, 610–623 (2012).

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Competing interests

The authors declare no competing interests.

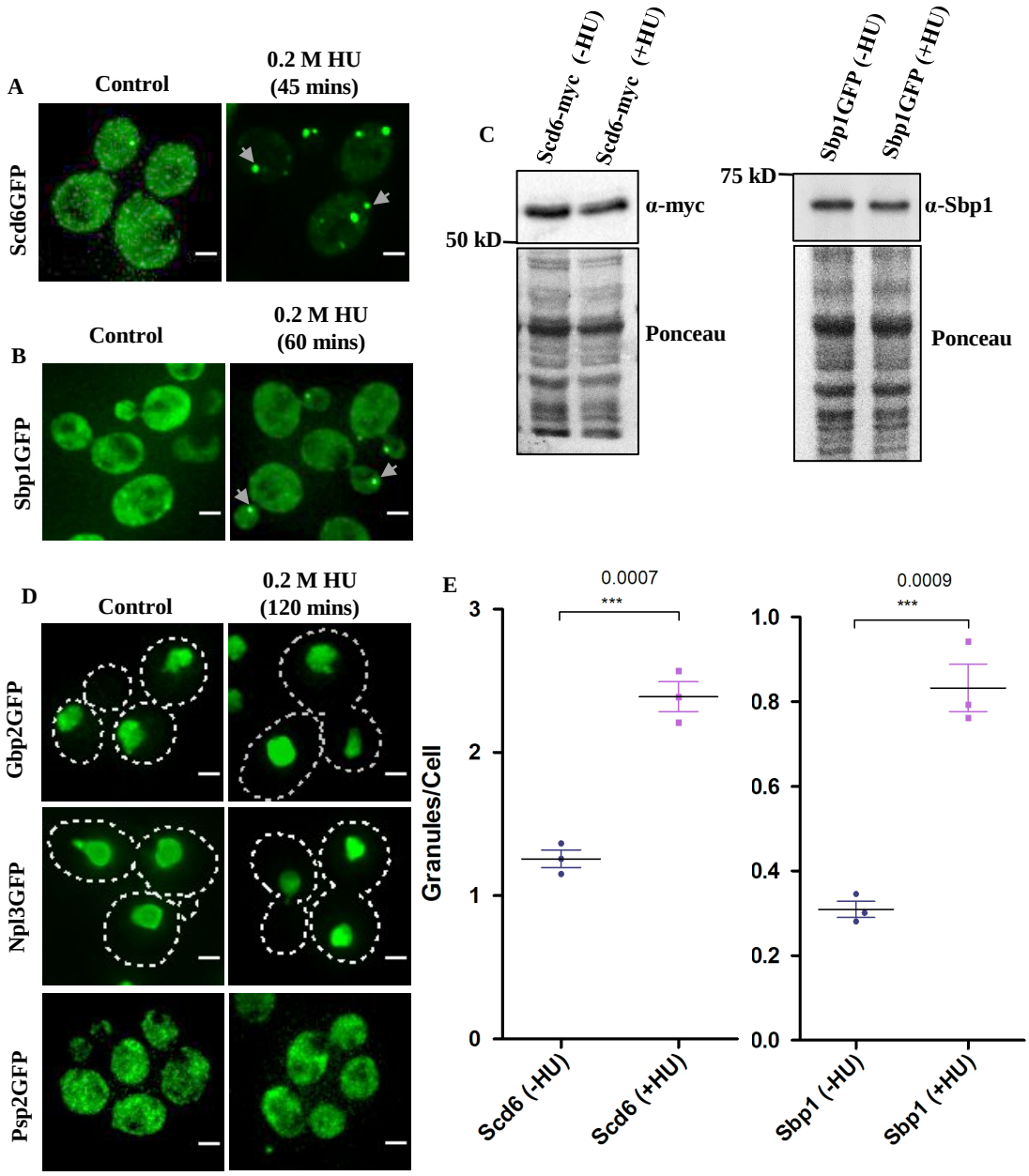

Figure 1. **Scd6 and Sbp1 localize to granules in response to HU stress.** (A) and (B) Live cell imaging showing localization of Scd6 and Sbp1 to granules upon HU stress. (C) Western blot showing protein levels of Scd6 and Sbp1 upon HU stress. Ponceau stained gel was used as loading control. (D) Gbp2, Npl3, and Psp2, another set of RNA binding RGG motif proteins, do not show change in localization to granule even after 120 mins of HU treatment. (E) Quantification of granules as granules per cell (n=3,~300 cells were counted for each protein). P-values are indicated on the graph and were calculated using unpaired t-test. Scale bar denotes 2 μm.

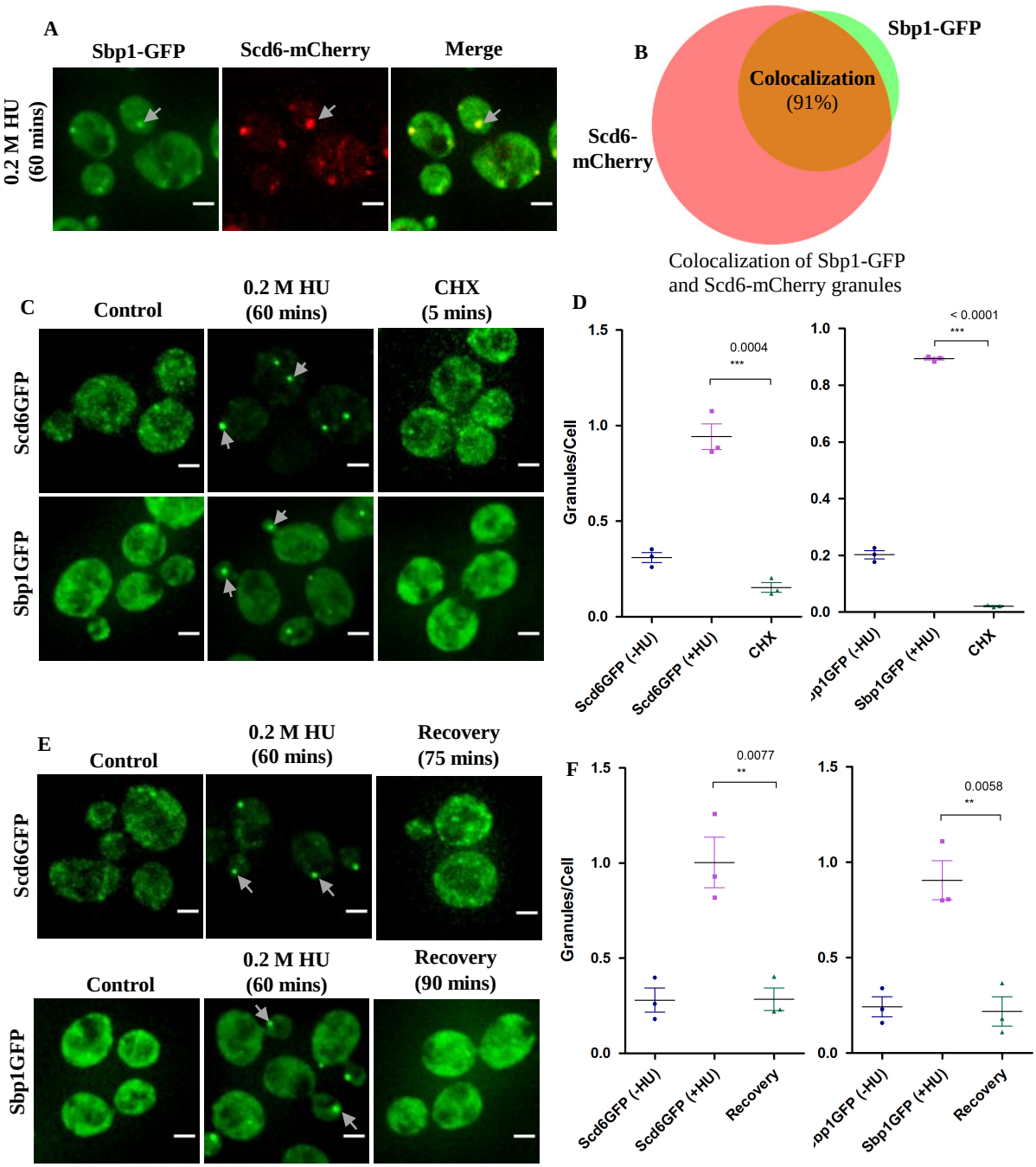

Figure 2. Scd6 and Sbp1 form reversible, RNA containing granules upon HU stress. (A) Live cell imaging showing colocalization of Sbp1 and Scd6 granules in HU stress. (B) Venn diagram showing colocalization of Scd6mCherry and Sbp1GFP granules (Red denotes Scd56mCherry granules and green indicates Sbp1GFP granules. Brown colour indicates colocalizing granules. ~90% of Sbp1 granules colocalize with Scd6 granules upon 60 mins of HU treatment(n3~300 cells were counted). . Percentage colocalization was calculated by dividing total colocalizing foci by total number of Sbp1 foci. (C) Live cell image showing change in localization in Scd6 and Sbp1 granules post 5-minute CHX treatment. (D) Quantification of granules as granules per cell (Left panel shows granule count for Scd6 and right panel for Sbp1). (E) Live cell imaging showing Scd6 and Sbp1 localization after incubating in non-HU containing media (Recovery period is 75 min for Scd6 and 90 minutes for Sbp1). (F) Quantification of granules in terms granules per cell (Left panel shows change in granules for Scd6 and the left panel shows the same for Sbp1. n=3,~300 cells were counted for analysis for all experiments. Statistical significance was calculated using unpaired t-test and p- values are indicated on the graphs. Scale bar denotes 2 µm.

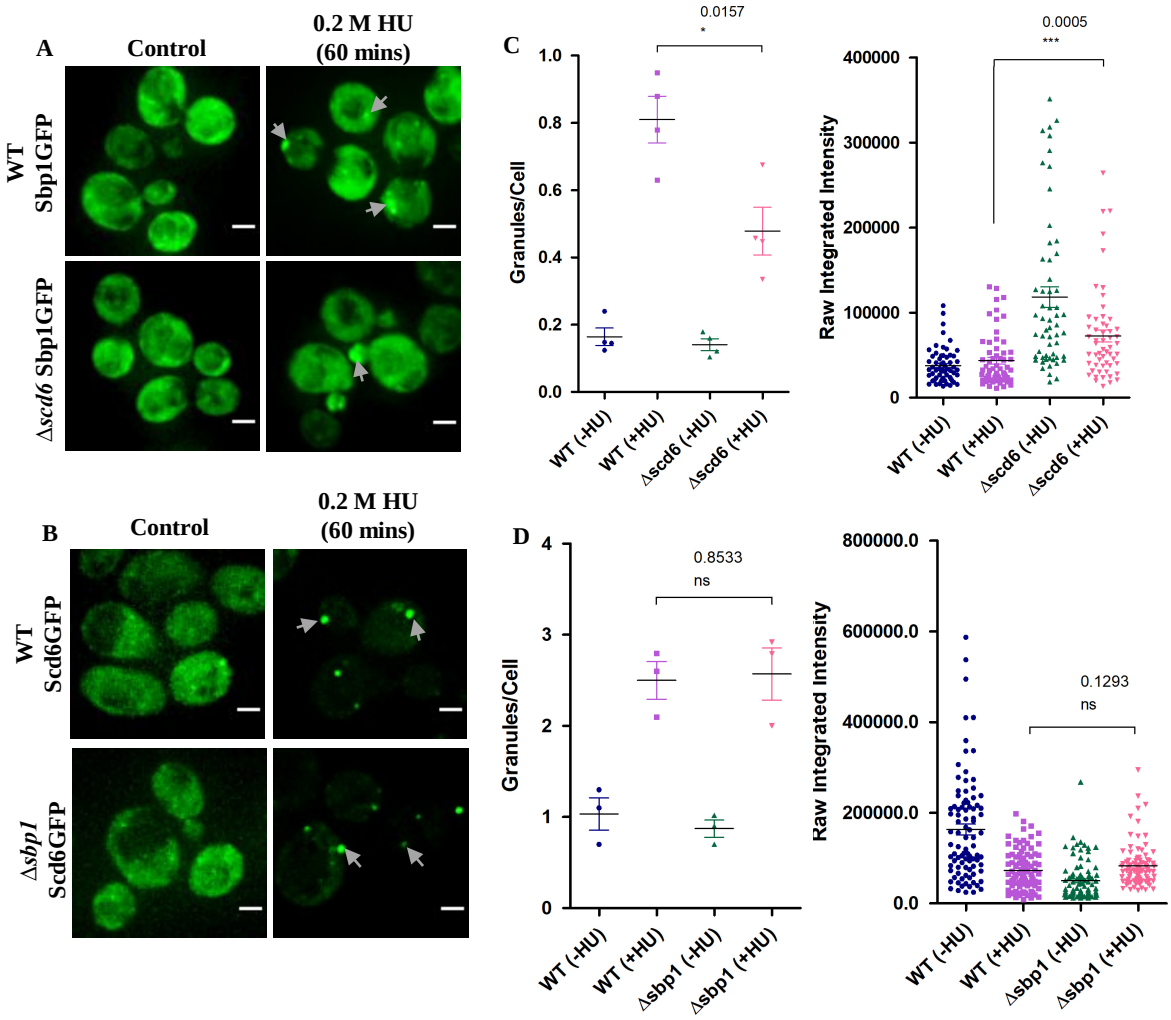

Figure 3. Sbp1 localizes to granules in Scd6-dependant manner. (A) and (B) Live cell imaging showing localization of Sbp1GFP and Scd66GFP in wild type and *Δscd6* or *Δsbp1* background, respectively. (B) and (C) Quantification (of A and B) as granules/cell and raw integrated GFP intensity (n=3;~400 cells were counted; 100 cells were used for intensity calculations;).. Statistical significance was calculated using unpaired t-test and p-values are indicated on the graphs (ns: non-significant change). Scale bar denotes 2 µm.

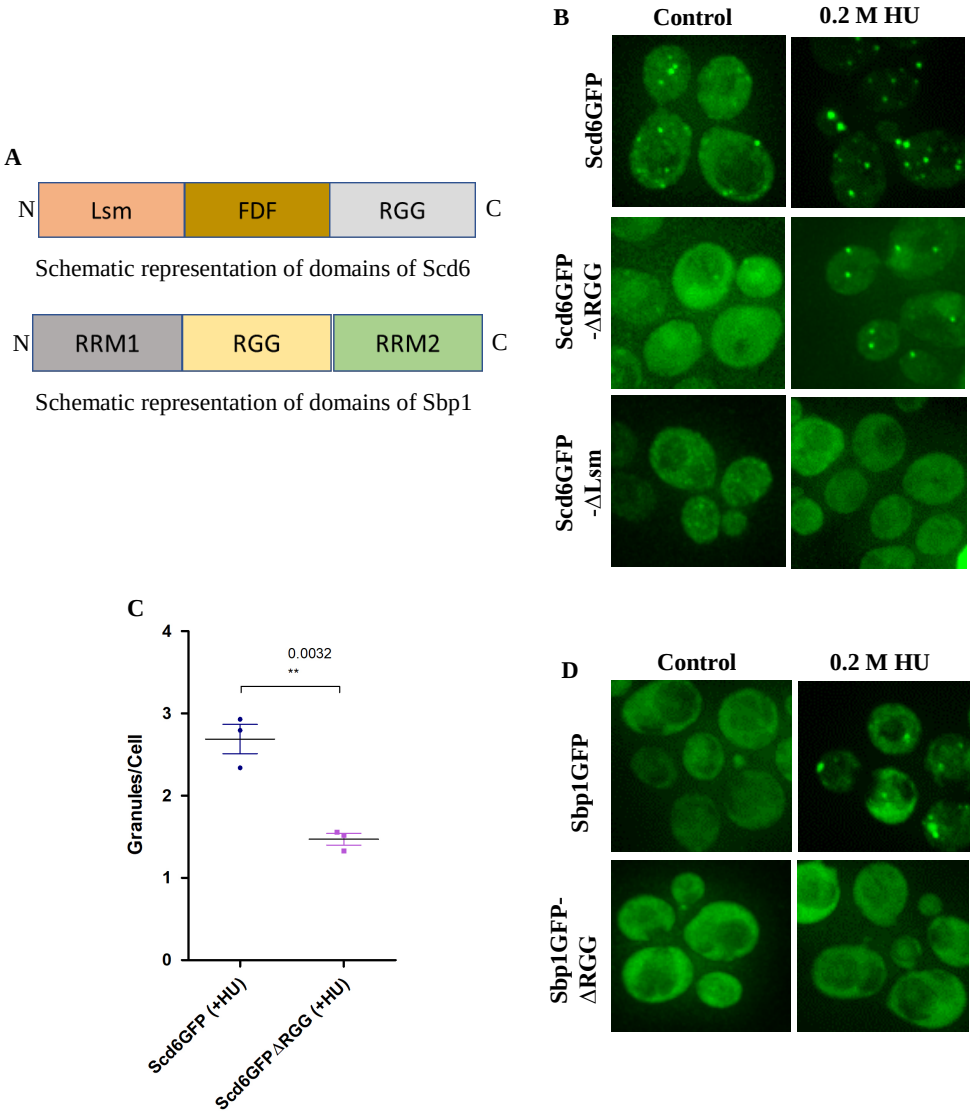

Figure 4. Subcellular localization of Scd6 and Sbp1 domain deletion mutants. (A) Schematic representation shows domain organization of Scd6 and Sbp1. (B) and (D) Live cell imaging showing localization of Scd6 and Sbp1 and their domain deletion mutants upon HU stress. (C) Quantification of Scd6GFP WT and ΔRGG mutant granules as granules per cell (n=3,~300 cells were counted for each protein). Statistical significance was calculated using unpaired t-test and p- values are indicated on the graphs.

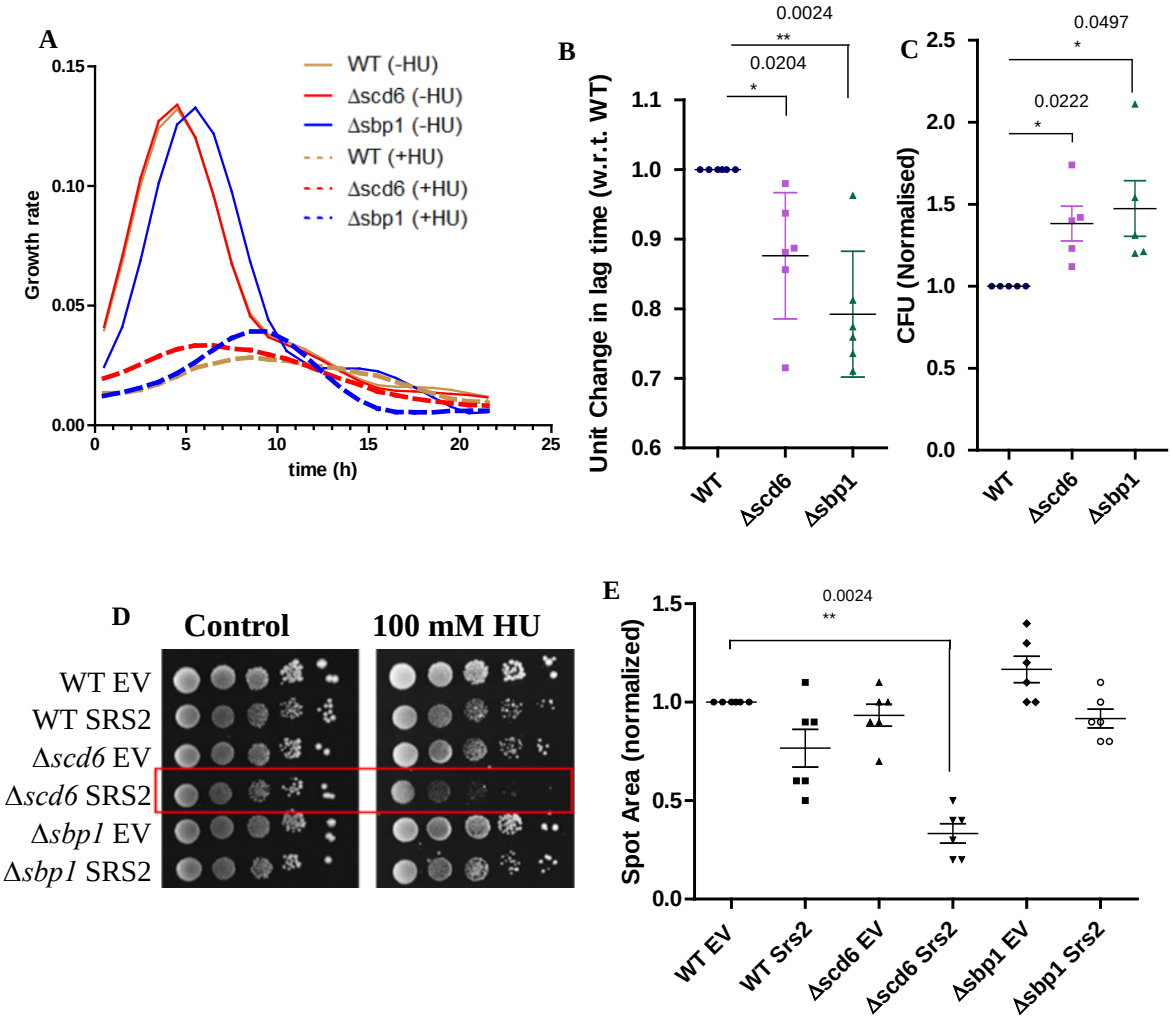

Figure 5. Scd6 modulates cellular tolerance to HU. (A) Representative growth rate curve comparing WT and deletion strains in untreated, and 200 mM HU treated media. (B) Quantification of growth rate in terms of lag time, plotted as change w.r.t. WT (n=6). (C) Quantification of plating assay, plotted as CFUs normalized against WT(n=5). 100 ul of 10^{-4} dilution cell culture were plated on control or 100 mM HU containing YEPD plates. (D) Spot assay of WT and deletion strains either carrying the empty vector (EV) or SRS2 on CEN plasmid, spotted on Uracil dropout agar plate and 2% glucose, in the presence or absence of 100 mM HU. The plates were incubated in 30ºC. (E) Quantification of growth assay by measuring the intensity of the second spot and normalizing the values of spots on HU plate to the respective control values for each strain (n=5).

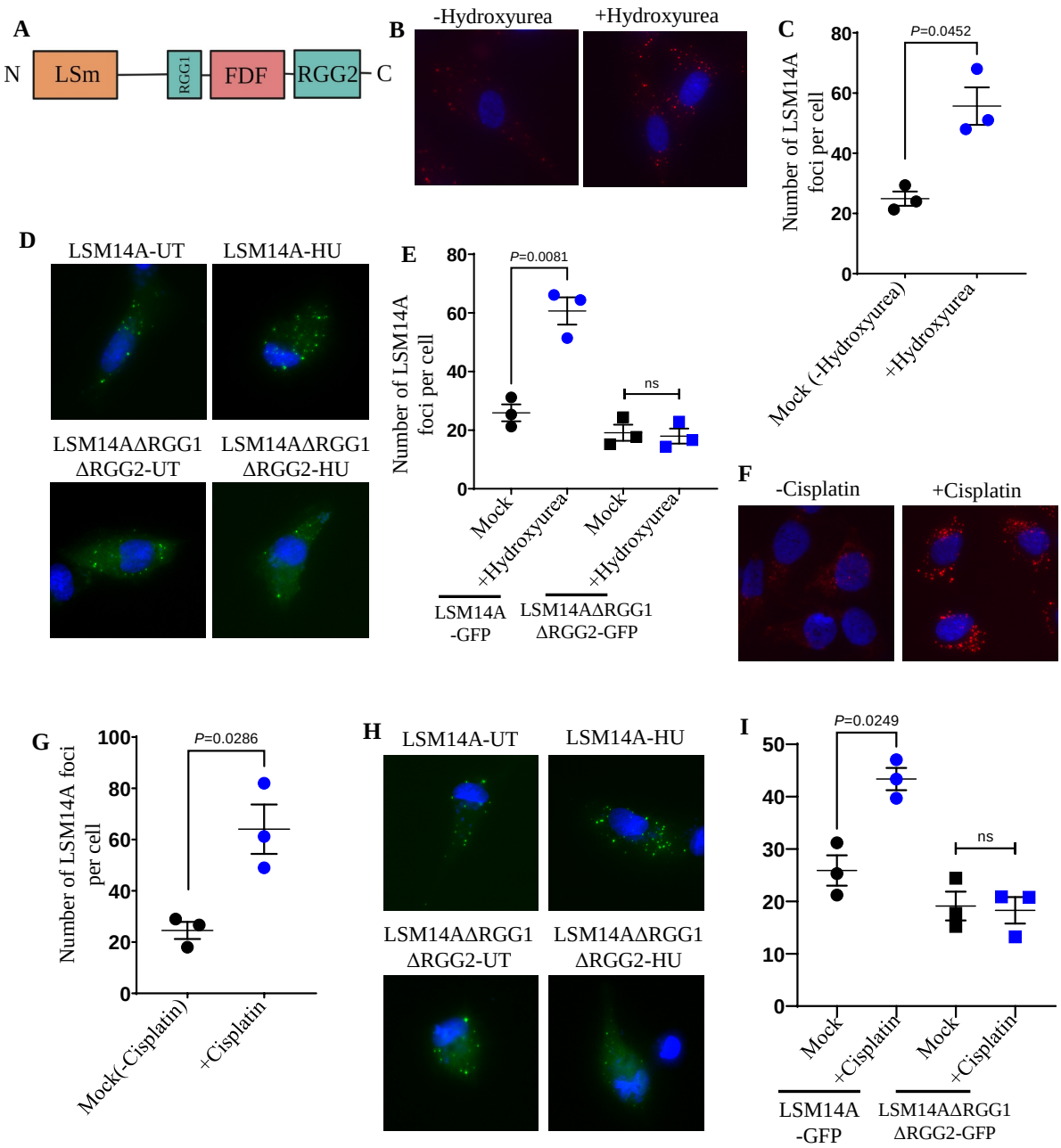

Figure 6: LSM14a localizes to puncta upon genotoxic stress in RGG-motif dependent manner. (A) Domain organization of LSM14A. LSM14A contains a N-terminal LSm domain, a FDF motif and two RGG motifs flanking the FDF motif. (B) Localization of LSM14A in response to hydroxyurea treatment (considered as genotoxic to cells) in the cytoplasm of RPE cells. (C) Quantitation of LSM14A localization as depicted in B. (D) Localization of LSM14A RGG-deletion mutants upon hydroxyurea treatment. (E) Quantitation of localization of LSM14A RGG-deletion mutants as depicted in D. (F) Localization of LSM14A in response to cisplatin treatment in the cytoplasm of RPE cells. (G) Quantitation of LSM14A localization as depicted in B. (H) Localization of LSM14A RGG-deletion mutants upon cisplatin treatment. (I) Quantitation of localization of LSM14A RGG-deletion mutants as depicted in D.

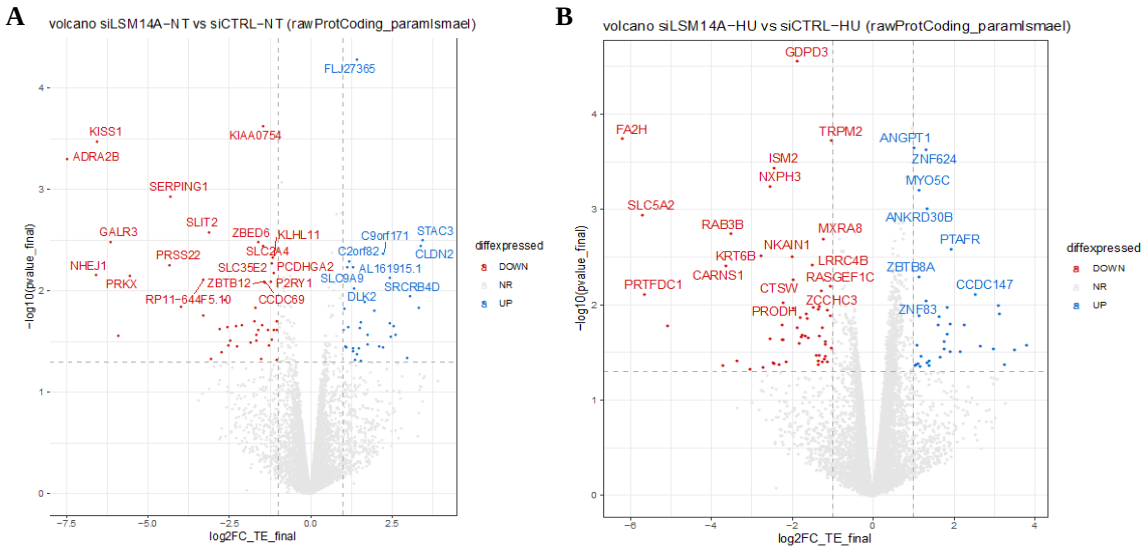

Figure 7: Polysome profiling reveals several mRNA targets of LSM14 under normal (NT) and HU-treated conditions. (A) Volcano plot showing showing changes in transcript levels in siLSM14a knock down cells as compared to wild type cells. (B) Volcano plot showing showing changes in transcript levels in siLSM14a knock down cells as compared to wild type cells upon HU treatment.

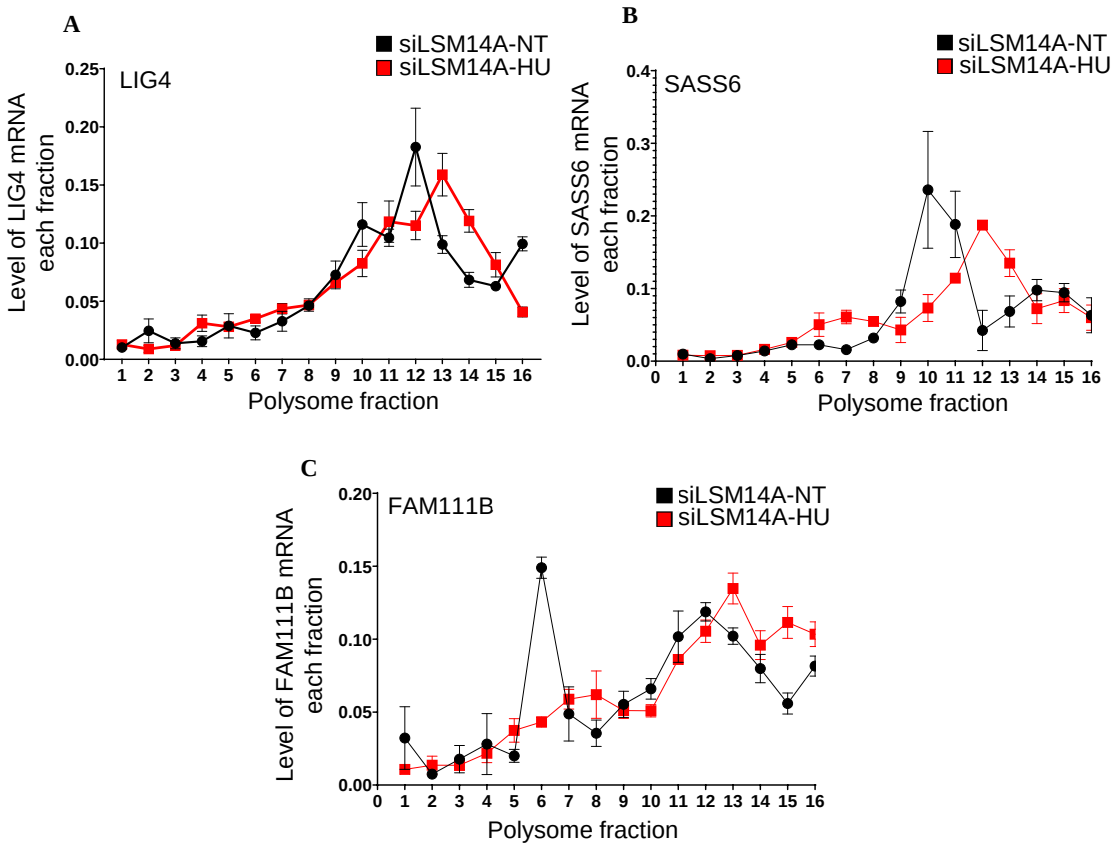

Figure 8: Validation of targets mRNAs identified through polysome RNA sequencing from A2058 cells. (A) Validation of *LIG4* mRNA in each of the polysome fraction comparing siLSM14A untreated (NT) condition with siLSM14A hydroxyurea (HU) treated condition. (B) Validation of *SASS6* mRNA in each of the polysome fraction comparing siLSM14A untreated (NT) condition with siLSM14A hydroxyurea (HU) treated condition. (C) Validation of *FAM111B* mRNA in each of the polysome fraction comparing siLSM14A untreated (NT) condition with siLSM14A hydroxyurea (HU) treated condition.