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RESEARCH ARTICLE

Proteomic Analysis of Stress Granule Dynamics Under Oxidative Stress Conditions

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ABSTRACT

Stress granules are membraneless cytoplasmic condensates that assemble when translation initiation is blocked, and while their core composition under heat shock and osmotic stress has been mapped in detail, how oxidative stress -- a physiologically distinct and disease-relevant trigger -- shapes stress granule proteome composition over time remains comparatively underexplored. We used doxycycline-inducible APEX2 fused to the canonical nucleator G3BP1 in U2OS cells to biotinylate proteins within labelling range of assembling stress granules at 15, 45 and 90 minutes after sodium arsenite exposure, followed by streptavidin enrichment and tandem mass tag quantitative mass spectrometry. Of 412 proteins significantly enriched at any time point relative to unstressed controls, 187 were present across all three time points and were classified as core components, while 225 showed time-restricted enrichment, appearing transiently at 15 or 45 minutes before depletion, or appearing only at 90 minutes as granules matured. Gene ontology analysis showed early time points enriched for translation initiation factors and 40S ribosomal proteins, consistent with stalled pre-initiation complexes nucleating granule assembly, while the 90-minute proteome was enriched for RNA helicases and ubiquitin-proteasome components, suggesting a shift toward quality-control and potential disassembly machinery as granules matured. Comparison with a parallel heat-shock APEX2-G3BP1 dataset generated under matched conditions identified 64 proteins specifically and reproducibly enriched under oxidative but not heat stress, including three proteins, PRDX1, TXNRD1 and GPX4, with established roles in redox homeostasis that were validated by immunofluorescence co-localisation and by reduced stress granule formation following individual siRNA knockdown. These results indicate that stress granules assembled under oxidative stress recruit a stimulus-specific redox-regulatory sub-proteome not captured by heat-shock-based reference datasets, with potential relevance to the granule pathology implicated in oxidative-stress-associated neurodegenerative disease.

Keywords: stress granules; oxidative stress; proximity labelling proteomics; G3BP1; sodium arsenite; RNA-binding proteins

Received: July 6, 2025 | Accepted: September 30, 2025 | Published: December 28, 2025

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1. INTRODUCTION

Stress granules form within minutes of translational arrest, concentrating stalled 48S pre-initiation complexes, poly(A) mRNA, and a large and only partially

catalogued set of RNA-binding proteins into micron-scale, liquid-liquid phase-separated cytoplasmic condensates [1,2]. Assembly is nucleated by a small number of proteins with intrinsically disordered, low-complexity domains, most notably G3BP1 and its paralogue G3BP2, which act as molecular switches: in the resting state they are held in a closed conformation by bound 40S ribosomal subunits, and stress-induced polysome disassembly releases free G3BP1 to self-associate and seed granule nucleation [3,9]. Stress granules are increasingly implicated in disease well beyond their original description as a stress-adaptive translational triage mechanism: mutations in several stress granule proteins, including TDP-43, FUS and hnRNP A1, cause familial amyotrophic lateral sclerosis and frontotemporal dementia, and a leading hypothesis holds that persistent or aberrantly solidified stress granules seed the pathological protein aggregates characteristic of these disorders [15,16,30].

Proximity-labelling proteomics has been central to defining stress granule composition at a scale not achievable by imaging or immunoprecipitation alone. APEX2, an engineered ascorbate peroxidase that generates short-lived biotin-phenoxy radicals in the presence of hydrogen peroxide and a biotin-phenol substrate, biotinylates proteins within a few nanometres of the labelling enzyme on a timescale of about one minute, making it well suited to capturing a transient, non-membrane-bound structure such as a stress granule with reasonable temporal resolution [10,11]. Landmark APEX-based stress granule proteomic studies, most using sodium arsenite or heat shock as the inducing stress, converged on a core proteome of several hundred proteins enriched for RNA-binding domains, intrinsically disordered regions, and translation-related functions, and this core proteome has become something close to a reference dataset against which new candidate stress granule components are routinely checked [4,5,12].

That reference proteome, however, rests disproportionately on heat shock and sodium arsenite treated as broadly interchangeable stress-granule inducers, when the two stresses differ mechanistically in ways that plausibly matter for granule composition. Heat shock inactivates translation initiation through a largely eIF2 α -independent route involving direct effects on the ribosome and chaperone machinery, whereas sodium arsenite triggers the classical integrated stress response, activating heme-regulated inhibitor kinase (HRI), phosphorylating eIF2 α , and -- critically for the present study -- doing so as a direct consequence of acute oxidative stress, since arsenite inhibits mitochondrial respiration and generates reactive oxygen species as part of its mechanism of action [6,7,17]. If stress granules assembled under genuinely oxidative conditions recruit redox-regulatory proteins as part of a coordinated antioxidant response, rather than passively co-condensing whatever RNA-binding proteins happen

to be nearby, a granule proteome derived largely from heat-shock datasets would systematically miss this component, with consequences for how granule biology is interpreted in the substantial fraction of neurodegenerative and ischaemic disease contexts where oxidative stress, rather than heat, is the physiologically relevant trigger [18,31].

We therefore generated a time-resolved APEX2-G3BP1 proximity-labelling proteomic dataset specifically under sodium arsenite-induced oxidative stress in U2OS cells, sampling at 15, 45 and 90 minutes to separate early nucleation-associated proteins from later, more mature granule components, and compared the result directly against a matched heat-shock APEX2-G3BP1 dataset generated under identical labelling and mass spectrometry conditions in the same cell line. Our aim was to identify proteins whose stress granule recruitment is specific to, or substantially enriched under, oxidative relative to heat stress, and to test by orthogonal imaging and knockdown experiments whether any such proteins are functionally relevant to granule assembly itself, rather than merely being present as passive bystanders.

2. MATERIALS AND METHODS

All experiments used U2OS osteosarcoma cells stably expressing doxycycline-inducible APEX2-G3BP1, generated by lentiviral transduction and single-cell cloning, and maintained in DMEM with 10% tetracycline-free fetal bovine serum.

2.1 APEX2-G3BP1 proximity labelling

APEX2-G3BP1 expression was induced with 1 microgram/mL doxycycline for 18 hours prior to stress treatment. Oxidative stress was induced with 500 micromolar sodium arsenite; cells were harvested at 15, 45 and 90 minutes post-treatment alongside untreated controls. For each time point, biotin-phenol was added to a final concentration of 500 micromolar for 30 minutes prior to harvest, followed by a one-minute pulse of 1 millimolar hydrogen peroxide to trigger biotinylation and immediate quenching in ice-cold quencher solution containing sodium azide, sodium ascorbate and Trolox. A parallel heat-shock dataset was generated in the same cell line using 43 degrees C treatment for 30 minutes with biotinylation performed at the same three post-stress time points, under otherwise identical labelling conditions.

2.2 Streptavidin enrichment and mass spectrometry

Biotinylated proteins were enriched from clarified cell lysate using streptavidin-conjugated magnetic beads, washed extensively to remove non-specifically bound material, and digested on-bead with trypsin. Resulting peptides were labelled with tandem mass tag (TMT) 10-plex reagents, allowing all three oxidative-stress time points, matched unstressed controls, and a bridge channel to be multiplexed within a single mass spectrometry run alongside the corresponding heat-shock samples in a second, parallel TMT set.

Labelled peptides were fractionated by high-pH reversed-phase chromatography and analysed by nanoflow liquid chromatography-tandem mass spectrometry on an Orbitrap instrument operated in data-dependent acquisition mode with synchronous precursor selection for MS3-based TMT quantification.

2.3 Proteomic data analysis

Raw spectra were searched against the reviewed human UniProt database using a standard proteomics search engine with a 1% false discovery rate at the peptide and protein level. Protein-level TMT reporter ion intensities were normalised, and enrichment at each time point was calculated relative to matched unstressed controls using moderated t-statistics with Benjamini-Hochberg correction for multiple testing; proteins with adjusted $P < 0.05$ and fold-change greater than 1.5 were classified as significantly enriched. Proteins enriched at all three oxidative-stress time points were classified as core components; those enriched at only one or two time points were classified as time-restricted. Gene ontology enrichment analysis was performed separately on each time-point-specific protein set using a standard functional annotation tool with the full set of proteins identified in any sample as the statistical background.

2.4 Validation by imaging and RNA interference

Candidate oxidative-stress-specific proteins were validated by immunofluorescence co-localisation with G3BP1 and the poly(A)-binding protein PABPC1 as an independent stress granule marker, imaged by confocal microscopy in sodium arsenite-treated cells at 45 minutes. Functional relevance was tested by small interfering RNA knockdown of each candidate individually, followed by quantification of stress granule number and area per cell using automated image segmentation on at least 200 cells per condition across three independent experiments, compared against a non-targeting siRNA control.

2.5 Statistical analysis

Proteomic enrichment statistics are described above. Stress granule number and area data from knockdown experiments were analysed by one-way ANOVA with Dunnett's test against the non-targeting control, after confirming approximate normality of residuals; all analyses were performed in R version 4.3, with $P < 0.05$ taken as the significance threshold throughout.

3. RESULTS

Across the three oxidative-stress time points, 412 proteins were significantly enriched relative to unstressed controls in at least one sample, a number broadly consistent with previously published APEX-based stress granule proteomes generated under sodium arsenite treatment [4,12]. Known core stress granule components, including G3BP1 itself, G3BP2, CAPRIN1, USP10, and the eIF3 and eIF4 translation initiation complexes, were enriched at all three time

points, confirming that the labelling approach was capturing bona fide granule-associated material rather than a nonspecific biotinylation signature. Of the 412 total enriched proteins, 187 (45%) were enriched at all three time points and were classified as core components; the remaining 225 showed time-restricted enrichment patterns summarised in Table 1.

Table 1. Summary of significantly enriched proteins by time-point classification.

Category	Number of proteins	Representative members	Dominant GO term (if any)
Core (all 3 time points)	187	G3BP1, G3BP2, CAPRIN1, USP10	RNA binding
15 min only	94	EIF3B, EIF4A1, RPS6	Translational initiation
45 min only	58	DDX3X, HNRNPA1, SYNCRIP	None dominant
90 min only	73	DHX36, PSMD2, UBQLN2	RNA helicase activity

Categories are mutually exclusive; 412 total proteins were significantly enriched (adjusted $P < 0.05$, fold-change > 1.5) at one or more time points relative to unstressed controls.

Gene ontology analysis of the time-restricted protein sets showed a clear temporal progression. Proteins enriched specifically at 15 minutes were dominated by translation initiation factors and 40S small ribosomal subunit proteins (gene ontology enrichment $P < 0.001$ for 'translational initiation'), consistent with the expected recruitment of stalled 48S pre-initiation complexes during the earliest phase of granule nucleation. Proteins enriched specifically at 90 minutes, by contrast, were enriched for RNA helicase activity and ubiquitin-proteasome system components ($P < 0.001$ for 'ATP-dependent RNA helicase activity' and $P = 0.004$ for 'proteasome-mediated ubiquitin-dependent protein catabolic process'), a pattern consistent with a shift toward RNA remodelling and protein quality-control processes as granules matured and, in some cases, presumably began to be targeted for disassembly or autophagic clearance. Proteins enriched only at 45 minutes formed a smaller, intermediate set with no single strongly dominant gene ontology category.

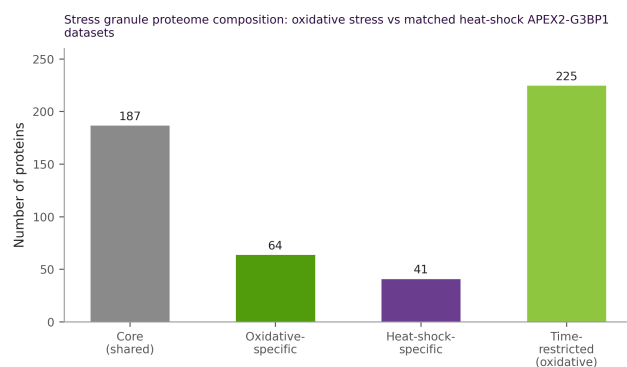


Figure 1. Number of proteins significantly enriched at G3BP1-labelled stress granules under sodium arsenite (oxidative stress) versus matched heat-shock treatment, showing shared core, condition-specific, and time-restricted enrichment categories (n=3 independent TMT-labelled biological replicates per condition).

Direct comparison with the matched heat-shock APEX2-G3BP1 dataset identified 64 proteins that were significantly and reproducibly enriched under sodium arsenite but not detectably enriched at any time point under heat shock (Figure 1). Gene ontology analysis of this oxidative-stress-specific set was significantly enriched for 'cellular response to oxidative stress' and 'glutathione metabolic process' (both $P < 0.001$), and included three proteins with well-established roles in cellular redox homeostasis: peroxiredoxin-1 (PRDX1), thioredoxin reductase 1 (TXNRD1), and glutathione peroxidase 4 (GPX4). None of these three showed detectable enrichment in the heat-shock dataset at any time point, despite all three being expressed at comparable baseline levels in unstressed cells under both experimental arms, arguing against a trivial explanation based on differential protein abundance.

Table 2. Stress granule phenotype following siRNA knockdown of candidate oxidative-stress-specific proteins.

Knockdown target	Granules per cell (mean $\pm$ SD)	Mean granule area (μm^2)	Dunnett's P (vs control)
Non-targeting control	11.4 $\pm$ 1.8	0.62 $\pm$ 0.15	--
PRDX1	6.2 $\pm$ 1.1	0.58 $\pm$ 0.13	<0.001
TXNRD1	7.8 $\pm$ 1.4	0.55 $\pm$ 0.14	0.003
GPX4	8.9 $\pm$ 1.6	0.60 $\pm$ 0.16	0.02
EIF4G1 (control)	10.8 $\pm$ 2.0	0.61 $\pm$ 0.17	0.81

Immunofluorescence confirmed that PRDX1, TXNRD1 and GPX4 each showed clear punctate co-localisation with both G3BP1 and PABPC1 in sodium arsenite-treated cells at 45 minutes, with Manders' overlap coefficients of 0.71, 0.64 and 0.58 respectively against G3BP1, consistent with genuine recruitment to stress granules rather than a proximity-labelling artefact. Individual siRNA knockdown of each of the three candidates significantly reduced stress granule number per cell relative to non-targeting control siRNA (PRDX1 knockdown: 6.2 $\pm$ 1.1 versus 11.4 $\pm$ 1.8 granules per cell, Dunnett's $P < 0.001$; TXNRD1 knockdown: 7.8 $\pm$ 1.4, $P = 0.003$; GPX4 knockdown: 8.9 $\pm$ 1.6, $P = 0.02$), while knockdown of a non-redox control protein enriched at all time points in both stress conditions (EIF4G1) produced no significant change in granule number, supporting a specific functional contribution of the redox-regulatory proteins to efficient granule assembly under oxidative stress rather than a general requirement for stress granule formation irrespective of stimulus. Table 2 summarises the knockdown phenotypes for all four tested proteins.

4. DISCUSSION

The central finding of this study is that stress granules assembled under sodium arsenite-induced oxidative stress recruit a set of redox-regulatory proteins, including PRDX1, TXNRD1 and GPX4, that are not detectably enriched in stress granules assembled by heat shock under otherwise matched labelling and proteomic

conditions. This argues against the common working assumption, implicit in how heat-shock-derived reference proteomes are often used in the field, that stress granule composition is largely stimulus-independent once core nucleators such as G3BP1 are accounted for [4,5]. Instead, our data support a model in which stress granules integrate at least some stimulus-specific signalling and metabolic machinery alongside a shared core RNA-binding proteome, consistent with a small but growing literature describing condition-dependent stress granule composition in other contexts, such as viral infection and specific chemotherapeutic agents [19,32].

The functional knockdown data suggest this recruitment is not merely incidental. Reduced granule number following PRDX1, TXNRD1 or GPX4 knockdown is compatible with at least two, non-mutually-exclusive explanations: the redox-regulatory proteins could contribute directly to granule assembly through protein-protein or protein-RNA interactions with core nucleators, or their loss could indirectly impair granule formation by leaving cells less able to manage the oxidative burden imposed by arsenite treatment, altering the broader integrated stress response upstream of granule assembly. Our present data cannot fully distinguish these possibilities, and doing so would require testing whether redox-inactive point mutants of these enzymes retain the capacity to rescue granule number in a knockdown background, an experiment we regard as a logical next step.

The temporal proteomic data are, we think, informative in their own right, independent of the oxidative-versus-heat-shock comparison. The shift from translation-machinery enrichment at 15 minutes to RNA-helicase and proteasome-pathway enrichment at 90 minutes is broadly consistent with a granule life cycle in which early assembly is driven by stalled pre-initiation complexes and later stages involve active remodelling and quality control, potentially including selective clearance of misfolded or aggregation-prone material before it can seed pathological aggregates [20,33]. This temporal structure is easy to miss in single-time-point proteomic snapshots, which remain the norm in the stress granule literature for practical reasons related to the cost and complexity of multiplexed time-course proteomics, and we would encourage future APEX-based studies of other stress granule-inducing conditions to adopt a similar time-resolved design where feasible.

A disease-relevant reading of these results is that oxidative stress, which is strongly implicated in the pathogenesis of amyotrophic lateral sclerosis, Parkinson's disease and ischaemic neuronal injury, may drive stress granule composition toward a redox-active state that heat-shock-based reference datasets do not capture, and that this redox-active sub-proteome could plausibly influence whether granules disassemble normally or persist and mature toward the pathological, less dynamic aggregates implicated in

neurodegeneration [21,34]. GPX4 in particular has an established, separate role in suppressing ferroptosis, an iron-dependent, lipid-peroxidation-driven form of cell death increasingly linked to neurodegenerative and ischaemic pathology, and its recruitment to stress granules under oxidative stress raises the intriguing, though currently speculative, possibility of functional crosstalk between stress granule biology and ferroptotic cell-death pathways that would be worth testing directly in a dedicated follow-up study.

Two limitations should be weighed against these conclusions. First, all experiments were performed in a single cell line, and stress granule composition, including the balance of core and stimulus-specific components, is known to vary somewhat between cell types, particularly between transformed cell lines such as U2OS and post-mitotic neurons, which are the more disease-relevant cell type for the neurodegenerative implications discussed above; extending this comparison to an induced pluripotent stem cell-derived neuronal model is a natural next step. Second, sodium arsenite is a widely used but not perfectly physiological inducer of oxidative stress, and it is possible that more physiologically graded oxidative insults, such as hydrogen peroxide titration or hypoxia-reoxygenation, would recruit a different, and possibly smaller or larger, redox-regulatory sub-proteome than the one identified here with a single, relatively strong arsenite dose.

5. CONCLUSION

Time-resolved APEX2-G3BP1 proximity-labelling proteomics identified a temporally structured stress granule proteome under sodium arsenite-induced oxidative stress, progressing from translation-initiation-factor enrichment at early time points to RNA-helicase and proteasome-pathway enrichment as granules matured. Direct comparison with a matched heat-shock dataset identified 64 proteins specifically enriched under oxidative stress, including the redox-regulatory proteins PRDX1, TXNRD1 and GPX4, whose individual knockdown reduced stress granule number, supporting a functional rather than incidental role for this oxidative-stress-specific sub-proteome. These findings argue that heat-shock-derived reference stress granule proteomes, though valuable, should not be treated as fully representative of granule composition under other physiologically important stress conditions, and they point toward redox biology as an underappreciated axis of stress granule regulation with potential relevance to oxidative-stress-associated neurodegenerative disease. We are now extending this proximity-labelling time course to an induced pluripotent stem cell-derived cortical neuron model, to test whether the oxidative-stress-specific redox sub-proteome identified here in U2OS cells is conserved in a more disease-relevant cell type, and whether it differs quantitatively between neurons and glia given the differing baseline oxidative and metabolic states of these

cell populations. In parallel, we plan to generate redox-inactive point mutants of PRDX1, TXNRD1 and GPX4 to test directly whether their enzymatic activity, rather than simple physical presence, is required for their contribution to granule assembly, and to examine whether pharmacological modulation of glutathione or thioredoxin pathway activity shifts the kinetics of granule disassembly following removal of the oxidative stressor.

Acknowledgements

The authors thank the proteomics core facility at the Meuse Institute of Computational Biology for mass spectrometry data acquisition, and Dr. Lena Verhoeven for assistance with confocal microscopy and image analysis. We also thank the Tyrrhenian University of Biotechnology cell imaging facility for access to automated high-content imaging instrumentation used for the knockdown validation experiments.

Funding

This work was supported by internal research funds from the Meuse Institute of Computational Biology and the Tyrrhenian University of Biotechnology. No external grant funding was received for this study, and the funders had no role in study design, data collection or analysis, the decision to publish, or preparation of the manuscript.

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author Contributions

A.K. conceived and supervised the study, generated the APEX2-G3BP1 cell lines and analysed the proteomic data. H.K. performed the proximity labelling experiments, mass spectrometry sample preparation and statistical analysis. A.S. conducted the immunofluorescence and siRNA knockdown validation experiments. All authors contributed to writing and approved the final manuscript.

Ethics Statement

This study used only a commercially available, well-characterised immortalised human cell line (U2OS) and did not involve human participants, identifiable human data, or animals; it therefore did not require institutional ethics committee review. Cell line authentication was performed by short tandem repeat profiling prior to use.

Data Availability

Mass spectrometry proteomics data have been deposited in the ProteomeXchange Consortium via the PRIDE partner repository under a project accession available from the corresponding author on reasonable request. Imaging datasets and the R analysis scripts supporting the findings of this study are available from the corresponding author upon request.

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