

1 Loss of OGT activity impairs the dynamic formation of protective stress granules in
2 ischemic cardiomyocytes.

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19 Running title: Targeting O-GlcNAc transferase impairs stress granule formation

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38 **Abstract (250 words max, currently 293)**

39 Stress granules are transient biomolecular condensates of proteins and RNA that form during
40 stress. G3BP1 is an RNA-binding protein, known to be essential in the nucleation process of stress
41 granules. Stress granules are thought to support cell survival by sequestering and protecting
42 components of the translational machinery, suppressing pro-apoptotic signaling, and conserving
43 cellular resources. O-GlcNAcylation, a dynamic modification of cytoplasmic proteins, is
44 recognized as an important regulator of stress responses and is required for efficient stress-
45 granule assembly. Although O-GlcNAcylation is protective in conditions of acute cardiac stress,
46 the downstream mechanisms that couple O-GlcNAcylation to cytoprotection remain elusive.

47 Here, we investigated whether O-GlcNAcylation regulates stress granule formation in
48 cardiomyocytes exposed to sodium arsenite stress, or models of ischemia/reperfusion injury.
49 Using the stress granule reporter G3BP1–EGFP and automated image analysis, we found that
50 OGT activity is necessary for efficient stress-granule formation, whereas OGA appears to have
51 little effect. Although stress granules are highly enriched in O-GlcNAcylated proteins, OGT itself
52 did not localize to granules, suggesting that proteins are modified prior to incorporation.
53 Importantly, we identified T268 of G3BP1 as an O-GlcNAc site and demonstrated its functional
54 importance for stress granule assembly.

55 In primary cardiomyocytes subjected to simulated ischemia/reperfusion, stress granules formed
56 transiently during ischemia, and this response was decreased by OGT-knockdown. Similarly,
57 stress granules were transiently detected at the ischemic phase of ischemia/reperfusion injury,
58 and their numbers were reduced in OGT-deficient hearts. Furthermore, brief pretreatment with
59 sodium arsenite before ischemia/reperfusion conferred cardioprotection. Likewise,
60 pretreatment with sodium arsenite protected cells from staurosporine-induced death and this
61 effect was decreased by OGT-knockdown.

62 Taken together, these findings identify O-GlcNAcylation as an important checkpoint for stress-
63 granule assembly in cardiomyocytes, define G3BP1 as an O-GlcNAc-modified effector, and
64 suggest that stress granules can act as cytoprotective mediators during acute cardiac injury.

65 **Introduction**

66 O-GlcNAcylation is the reversible addition of O-linked N-acetylglucosamine to serine/threonine
67 residues, serving as a signaling modality that regulates thousands of intracellular proteins (1-3).
68 O-GlcNAc transferase, OGT is the enzyme that installs O-GlcNAc on proteins, whereas the O-
69 GlcNAcAse (OGA) removes the GlcNAc moiety (4). O-GlcNAcylation has been shown to coordinate
70 virtually every cellular process including transcriptional control (5, 6), post-transcriptional RNA
71 splicing (7), ribosome function, translational initiation (8, 9), and protein quality control (10-13).
72 Additionally, O-GlcNAcylation is emerging as an essential signaling modification that coordinates
73 cellular stress responses (14-16).

74 Cardiac ischemia/reperfusion (I/R) injury stems from the abrupt deprivation and resupply of
75 nutrients and oxygen, resulting in a burst of reactive oxygen species, ion dysregulation, and
76 mitochondrial Ca^{2+} overload, that strain proteostasis and activate cell-death programs (17-19).
77 Importantly, boosting O-GlcNAcylation is protective in the context of myocardial I/R injury (20-
78 23). Additionally, evidence from knockout mice suggests that OGT is protective against heart
79 failure after acute MI (24). Mechanistically, the protective effect of O-GlcNAcylation in the
80 context of I/R injury has been associated with improved mitochondrial Ca^{2+} and ROS handling
81 and reduced cytosolic Ca^{2+} release (22, 25-28), although further investigations are required to
82 elucidate additional molecular mechanism whereby O-GlcNAc exerts its protective effects.

83 During acute stress, non-translating messenger ribonucleoproteins (stalled mRNA–protein
84 assemblies that include ribosomal proteins and translation initiation factors) coalesce into
85 cytoplasmic, membraneless biomolecular condensates called stress granules (29-31). G3BP1
86 (Ras-GAP SH3 domain-binding protein 1), is a conserved RNA-binding protein, nucleates stress-
87 granule assembly by binding RNA and multimerizing through low-complexity regions (32, 33).
88 Additional RNA-binding proteins, such as TIA1 and Caprin1 act as regulators that tune assembly
89 and dynamics (34-36). Early genetic screening showed that OGT was required for efficient stress-
90 granule assembly, and several stress-granule components, including ribosomal proteins and
91 regulatory RNA-binding proteins undergo O-GlcNAcylation during stress (37, 38). Interestingly,
92 OGT appears to preferentially target low-complexity, intrinsically disordered regions (IDR) of
93 proteins (39, 40). These regions mediate weak, multivalent contacts that drive liquid–liquid phase
94 separation, a defining step in stress-granule formation (41, 42).

95 G3BP1 contains an N-terminal domain, termed NTF2L, that is essential in homodimer formation,
96 an acidic domain, a proline-rich (PxxP) segment that serves as an SH3-binding hub, an RNA-
97 recognition Motif (RRM) crucial for RNA binding, and an arginine-glycine rich region (RGG) (43,
98 44). Within G3BP1, the NTF2L and RRM are predicted to be structured domains, whereas the
99 other regions including the acidic and PxxP domains are predicted to have intrinsically disordered
100 regions (45). During stress, G3BP1 dimers adopt an ‘open’ conformation and the RRM becomes
101 available for multivalent interactions with RNA molecules to initiate stress granule formation (32,
102 46, 33). In cardiomyocytes, G3BP1 suppresses pre-miR-1 processing and promotes hypertrophic
103 growth (47, 48). Furthermore, G3BP1 has been reported to ameliorate inflammatory and
104 mitochondrial readouts in lipopolysaccharide-treated cardiomyocytes (49).

105 Stress granules support survival during apoptotic signaling and oxidative stress, although
106 persistent or abnormal assemblies can be deleterious, particularly in neurodegenerative settings
107 (50-52). Nevertheless, in acute cerebral events, stress granules form transiently during ischemia
108 and are associated with neuroprotection (53, 54). In cardiac cells, stress granules are reported to
109 form transiently in response to acute pacing, and have been associated with mitigation of Ca^{2+}
110 overload, oxidative stress, and doxorubicin toxicity (55, 56). Nevertheless, key signaling
111 modalities and mechanisms regulating stress granule formation in cardiac myocytes remain
112 unclear. Here, we hypothesized that O-GlcNAcylation governs stress-granule assembly in
113 cardiomyocytes. We demonstrate that O-GlcNAcylation regulates assembly and identify G3BP1
114 as a key O-GlcNAc-sensitive effector. Employing cell and mouse models of acute cardiac injury,
115 we show that stress granules form during ischemia and resolve with reperfusion. Finally, we
116 demonstrate that preemptive formation of stress granules confers cytoprotection against cell-
117 death stimulation.

Results

Pharmacologic Inhibition or Genetic Knockdown of OGT Reduces SG formation.

O-GlcNAcylation has been implicated in regulating cellular stress responses, yet its role in modulating stress granule (SG) formation in cardiac myocytes remains unclear. First, we confirmed that O-GlcNAcylation is enriched in stress granules forming in cardiomyocytes. To that end we treated primary neonatal cardiomyocytes (NRVMs) with the stress granule inducer sodium arsenite (SA) and performed co-staining for O-GlcNAc and various stress granule markers (G3BP1, eIF4G1, PABPC1, and TIA1). Indeed, O-GlcNAc showed strong immunoreactivity in subcellular globular formations that were positive for the chosen stress granule markers (Suppl. Fig. 1).

Next, we sought to determine the impact of O-GlcNAcylation on the kinetics of stress granule formation. To address this question, we assessed the rates of SG formation in H9c2 cardiomyocytes. Cells were transduced to express EGFP–G3BP1, enabling real-time visualization and quantification of SG assembly (Fig. 1A). After adenoviral transduction (MOI=50, 24 h), cells were treated with vehicle (DMSO) or the OGT inhibitor OSMI-1 (25 μ M, 6h) that effectively reduces O-GlcNAcylation levels, (Suppl. Fig. 2A-B). The cells were then challenged with a bolus treatment with sodium arsenite (SA, 500 μ M) to induce SG formation (Fig. 1A). SG formation was monitored over a 20-minute period with a confocal microscope (FV300RS, enabled with z-drift compensation system, 1 image/minute). The number of SGs per image was quantified by CellProfiler using the Robust Thresholding feature (Fig. 1B). Using this analytical setup, we found that the sigmoidal curve of SG formation was downwardly shifted in cells treated with OSMI-1, showing significantly lower SG numbers compared with vehicle-treated controls (Figure 1C-D, $P<0.01$). To further corroborate these findings, we employed siRNA-mediated knockdown of OGT (20 nM, 48 h), another approach that is efficient in reducing O-GlcNAcylation levels (Suppl. Fig. 2C-D). Control (NTC) and OGT knockdown cells were subjected to the same real-time SG formation assay as described above. In agreement with the results with OGT inhibition (OSMI-1) we similarly found that OGT knockdown caused a downward shift of the curve, significantly reducing SG formation over the course of imaging (Fig. 1E-F, $P<0.05$).

Next, we examined whether inhibiting OGA, the enzyme removing O-GlcNAc from modified proteins, would shift the SG formation curves upwardly or downwardly. To that end, we treated cells with vehicle, or the OGA inhibitor thiamet-G (TMG, 25 μ M, 6h) that effectively increases O-GlcNAcylation (Suppl. Fig. 3A-B) and performed the assay as above. In these experiments, however, we found that the SG formation was not significantly changed (Fig. 2A-B). Similarly, the SG formation remained unaffected when a second OGA inhibitor, MK8719, was used (25 μ M, 6h, Fig. 2A and C). While MK8719 significantly increased O-GlcNAcylation (Suppl. Fig. 3C-D), the absence of any significant impact on SG rate formation is consistent with the TMG results, suggesting that OGA inhibition does not have a significant impact on SG formation. To further investigate this result, we employed siRNA-mediated knockdown of OGA (20 nM, 48 h), which as expected caused a significant reduction in OGA protein levels and an increase in O-GlcNAcylation

(Suppl. Fig. 3E-G). However, and in agreement with findings from pharmacologic inhibition, we found that OGA knockdown had no significant effect on SG formation (Fig. 2D-E).

Differential recruitment of OGT and OGA enzymes on stress granules upon sodium arsenite treatment.

Building on the functional data above, we next examined the spatial distribution of enzymes OGT and OGA relative to stress granules using immunohistochemistry. To that end, H9c2 cells were treated with SA (500 μ M, 30 min.) and were subsequently fixed and stained for the SG marker G3BP1 with either OGT or OGA. As shown in Fig. 3A, the distribution of OGT was spatially distinct from that of G3BP1, both at baseline and following SA treatment. By contrast, OGA showed a weak colocalization with G3BP1 at baseline, and upon SA treatment, a clear co-localization with G3BP1 within SGs (Fig. 3B). Pearson's correlation analysis within cytoplasmic ROIs yielded values near zero for OGT-G3BP1 under both basal (-0.029) and SA-treated ($+0.074$) conditions, consistent with the absence of visual co-localization (Fig. 3C). In contrast, OGA-G3BP1 correlations increased from 0.084 at baseline to 0.224 after SA treatment ($P < 0.05$), reflecting a shift toward positive correlation. It is important to note that while a strong colocalization is visible between OGA and G3BP1 within SGs, the overall r values remained in the weak range (<0.3), due to the substantial diffuse cytoplasmic OGA signal outside SGs, as it can be assessed visually (Fig. 3B). Taken together, these results show that the spatial distribution of the two O-GlcNAc cycling enzymes changes upon SA treatment and SG formation and OGA can be acutely recruited to SGs. However, given the earlier findings that OGA is dispensable in SG formation, these data suggest that enzyme localization alone does not predict their respective contribution to SG dynamics.

Protein enrichment and mass spectrometry identifies site of O-GlcNAcylation on G3BP1.

To gain a further mechanistic understanding of the impact of O-GlcNAcylation on SG formation we hypothesized that G3BP1, the central mediator of SG formation is O-GlcNAcylated. To that end, cells were transduced with β -gal- or EGFP-G3BP1-expressing adenoviruses, harvested 24 h later, and subjected to immunoprecipitation to enrich for EGFP-G3BP1. Fractions corresponding to input, eluate, and flow-through were collected for each IP group (β -gal, EGFP-G3BP1, HS, Lysate + NAR) (Fig. 4A). Immunoblotting for G3BP1 revealed strong enrichment of EGFP-G3BP1 in the eluate from GFP-Trap IP, with the signal markedly higher than that in the 2 % input and undetectable in the flow-through, indicating near-complete capture. In contrast, incubation of EGFP-G3BP1 lysate with neutral agarose resin (Lysate + NAR) yielded little to no signal in the eluate and detectable protein in the flow-through. Together, these results confirm the high efficiency of the pulldown that enriches EGFP-G3BP1 (Fig. 4A). Following the diagnostic IP immunoblot, the remaining enriched material (~ 90 %) from the three groups (β -gal, EGFP-G3BP1, HS) was resolved on a Bis-Tris 4–12 % gradient gel. After electrophoresis, the gel was stained with Brilliant Blue, revealing a prominent band at the expected molecular weight of EGFP-G3BP1 (~ 79 kDa), that appeared as a doublet (Fig. 4B). A second, higher-molecular-weight doublet (>100 kDa) was also observed. The origin of these doublets is unclear but may reflect post-translationally modified or differently processed forms of the protein. For downstream mass

spectrometric analysis, only the ~79 kDa doublet, corresponding to the predicted size of EGFP–G3BP1, was excised (Fig. 4B).

The excised 79 kDa band was subjected to in-gel tryptic digestion, and peptides were analyzed by LC–MS/MS using a triggered ETHcD strategy. In this approach, HCD scans were first screened for diagnostic oxonium ions indicative of O-GlcNAc, which, when detected above threshold, triggers ETHcD fragmentation of the same precursor. O-GlcNAcylation (S, T), phosphorylation (S, T, Y) oxidation (M), and deamidation (N, Q) were specified as variable modifications. This workflow provided 58% sequence coverage of G3BP1 (Fig. 4C). Upon further examination of the MS/MS spectra, we identified evidence for O-GlcNAcylation on G3BP1. Fig. 4D shows a representative HCD spectrum of peptide (NLPPSGAVPVTGIPPHVVK) with b/y fragment ion series, together with a signal at m/z 204.087, corresponding to the GlcNAc oxonium ion. Closer inspection of the lower m/z range (Fig. 4D, boxed region), shown in greater detail in Fig. 4E, revealed a complete cluster of O-GlcNAc diagnostic fragment ions (m/z 126.055, 138.055, 144.065, 168.066, 186.076), collectively confirming the presence of O-GlcNAc on the identified peptide. To identify the precise residue modified by O-GlcNAc, we examined a collection of ETHcD spectra for that peptide (Suppl. Table S1). This analysis revealed the presence of high confidence peptide-spectral matches (PSMs) for peptide NLPPSGAVPVTGIPPHVVK (Suppl. Table S2). The spectra contained multiply charged precursor ions with neutral loss of GlcNAc, while extensive c- and z-type ion coverage, provided sufficient evidence for the localization of the O-GlcNAc modification to T268 (Fig. 4F).

O-GlcNAc site T268 is required for efficient stress granule formation.

Given the mass spectrometric evidence localizing the O-GlcNAc modification to residue T268, we next sought to determine whether this site contributes functionally to SG assembly. G3BP1 is a modular protein containing five well-characterized domains: an NTF2-like (NTF2L) domain, a central acidic domain, a PxxP motif-rich region, an RNA recognition motif (RRM), and a C-terminal RG-rich domain (Fig. 5A). To examine whether O-GlcNAcylation at this residue influences G3BP1 function, we generated a T268A point mutant, replacing the hydroxyl-containing threonine with alanine to prevent O-GlcNAc attachment (Fig. 5B). Next, wild-type and T268A EGFP-G3BP1 versions were delivered to cells via adenoviral transduction, enabling side-by-side assessment of stress granule assembly in response to sodium arsenite treatment. Real-time, confocal live-cell imaging for 20 minutes after SA addition, revealed that T268A-expressing cells had significantly lower numbers of SGs, compared to WT-expressing cells (Fig. 5C). This was also evident from the aggregate data showing the significant rightward and downward shift of the T268A curve (Fig. 5D, $P < 0.05$). Taken together, these results suggest that O-GlcNAcylation at T268 plays an important role in stress granule assembly, consistent with the slowed kinetics observed upon OGT targeting shown in Fig. 1.

Stress granule formation in ischemic NRVMs and its modulation by OGT depletion.

To address the broader question of whether cardiomyocyte pathophysiologic injury, such as ischemia/reperfusion (I/R), is associated with SG formation and how the O-GlcNAcylation system

contributes in this context, we employed an established in vitro model of cardiac I/R injury (57). In this approach, a sterile glass coverslip is gently placed over a monolayer of neonatal rat ventricular myocytes (NRVMs), creating a transient barrier that limits oxygen and nutrient exchange with the culture medium. Removal of the coverslip then restores access, mimicking reperfusion injury. (Fig. 6A). This model produces three experimental groups (Control, Ischemia, and Reperfusion). To investigate SG dynamics across these stages, we performed immunostaining for G3BP1. In parallel, we also stained for O-GlcNAc, to monitor how O-GlcNAcylation is spatially redistributed relative to stress granules in the context of cellular ischemia/reperfusion (Fig. 6B). IHC analysis and CellProfiler quantification revealed a statistically significant increase in number of SGs in NRVMs that underwent ischemic injury compared with control-treated NRVMs (incubated in Tyrode's buffer for 120 min, Fig. 6C, $P < 0.05$). Interestingly, NRVMs that underwent reperfusion had significantly lower number of SGs than ischemic NRVMs (Fig. 6C, $P < 0.05$). Taken together, these data suggest that SGs form in NRVMs during ischemia and dissipate during reperfusion. We also observed a marked overlap between O-GlcNAc staining and punctate G3BP1 signal in ischemic NRVMs, suggesting that O-GlcNAcylated proteins readily associate with SGs during ischemic stress.

To investigate the role of OGT in ischemia-induced SG formation, NRVMs were transfected with either non-targeting control (NTC) or OGT-targeting siRNAs, they were subjected to simulated ischemia/reperfusion using the coverslip model and then stained for G3BP1 to monitor SG dynamics under the different conditions (Fig. 7A–B). In line with our earlier observations, NTC-transfected cells showed a significant increase in SG number during ischemia compared with NTC-control (compare blue and green bars) and NTC-reperfusion groups (compare green and orange bars, Fig. 7C). In contrast, OGT-depleted cells did not display a significant increase in SG number under ischemia relative to their own controls (compare red and purple bars, Fig. 7C), while their SG abundance was markedly lower than that of ischemic NTC-transfected cells (compare green and purple bars, Fig. 7C, $P < 0.05$). However, there was no statistically significant difference between NTC-transfected and OGT-depleted NRVMs at reperfusion (compare orange and black bars, Fig. 7C). It is noteworthy that OGT-depleted reperfused cells appeared to contain more SGs than their control counterparts (compare red and black bars, Fig. 7C). Taken together, these data suggest that while OGT-depleted NRVMs subjected to I/R are still capable of forming SGs, their accumulation appears attenuated relative to NTC-transfected cells.

Adaptive stress granules form in ischemic hearts and depend on OGT for efficient assembly.

Building on our findings in cultured NRVMs, we next sought to determine whether ischemia-induced SG formation also occurs in intact cardiac tissue. To this end, we employed an ex vivo Langendorff-perfused mouse heart model of ischemia/reperfusion (I/R), which allowed us to perfuse hearts under three experimental protocols (Control perfusion, ischemia-only, and I/R, Fig. 8A). At the specified time intervals, hearts were collected, fixed and processed for the histological analysis of stress granules, identified as cytoplasmic G3BP1-positive puncta, using confocal microscopy. Consistent with our experiments on simulated ischemic NRVMs, ischemic hearts had higher numbers of G3BP1-positive puncta compared with control-perfused controls

and also compared to I/R hearts (Fig. 8B). Quantification, performed using CellProfiler-based analysis, enabled direct comparison across models and showed that indeed the ischemic hearts had a significantly higher number of stress granules compared to both Control-perfused and I/R-perfused hearts (Fig. 8B–C). There were no statistically significant differences between I/R-injured hearts and hearts that underwent controlled perfusion (Fig. 8C).

To verify the role of OGT in stress granule formation in this context, we employed mice with inducible cardiomyocyte specific knockout of OGT (OGT-icko), a model that we have described recently (58). Since our data from simulated I/R conditions in cells and in hearts had demonstrated that ischemic stress granules form selectively during ischemia and resolve upon reperfusion, we focused on examining ischemia-only wild-type (WT) and OGT-icko hearts. Immunohistochemical staining for G3BP1 and quantitative analysis of confocal images revealed a robust presence of stress granules in ischemic WT hearts that were significantly attenuated in ischemic OGT-icko hearts (Fig. 8D and E). These data support that cardiomyocyte OGT is required for the efficient *in vivo* formation of stress granules in ischemic hearts, consistent with our findings in OGT-depleted cardiac myocytes subjected to SA stress, or simulated coverslip ischemia.

Next, we used sodium arsenite, a robust inducer of stress granule assembly, to assess its potential cardioprotective effect in the context of ischemia/reperfusion injury. To that end mice were injected with a single bolus dose of sodium arsenite (4.5 mg/kg), or an equivalent volume of PBS, 1 h prior to the onset of the ischemia/reperfusion protocol (Fig. 8F). At the end of reperfusion, hearts were collected, sliced and stained with triphenyl-tetrazolium chloride to assess the extent of infarct size in the two groups. Remarkably, this analysis showed that sodium arsenite-pretreated hearts had a significantly smaller infarct size compared to PBS controls (Fig. 8G-H, $P < 0.01$), consistent with a protective role of sodium arsenite against ischemia/reperfusion injury.

Sodium arsenite-induced stress granules protect H9c2 cells from staurosporine-induced cell death.

To further corroborate the cytoprotective potential of stress granules, we used a cell-based model of cell death. In this approach cells were transiently exposed to sodium arsenite (500 μ M, 30 min) before exposed to death inducing Staurosporine (10 μ M, 1h; Fig. 9A). As expected, sodium arsenite treatment readily induced the formation of stress granules, as shown by immunostaining for G3BP1 (Fig. 9B). To monitor cell viability, we assessed cell rounding by phase-contrast microscopy, as a marker of cell death. The number of rounded cells was quantified and normalized to total Hoechst-positive nuclei to provide an index of cell death. As shown with this analysis (Fig. 9C-D), we observed a significant increase in the proportion of dead cells caused by Staurosporine, and this effect was strongly abolished by pre-treatment with sodium arsenite (Fig. 9D, $P < 0.0001$), indicating a protective effect of sodium arsenite-induced stress granule formation against acute cell death.

To further investigate whether the cytoprotective effect of sodium arsenite was mediated by stress granule assembly, we genetically targeted the stress granule orchestrator G3BP1 and its

close homolog G3BP2 (33). First, cells were transfected with siRNAs targeting G3BP1 and G3BP2, or with non-targeting controls (NTC), and exposed to sodium arsenite treatment for 30 min. Next, immunofluorescence analysis was performed to assess the efficiency of stress granule formation in G3BP1/2-targeted cells. Indeed, the knockdown approach significantly impaired the formation of stress granules, as assessed using G3BP1 immunostaining (Fig. 10A). Further confirmation was also provided by staining for the independent stress granule marker, TIA1 which consistently showed a deficit in coalescing into distinct stress granules upon sodium arsenite treatment (Fig. 10A), confirming the deficit in stress granule formation in G3BP1/2-knockdown cells. Next, we examined the impact of G3BP1/2 depletion on sodium arsenite-induced cytoprotection. While G3BP1/2 depletion did not affect basal or staurosporine-only-induced cell death, it caused a significant increase in cell death in cells pretreated with sodium arsenite and exposed to staurosporine (compare brown and lavender blue, Fig. 10B-C, $P < 0.05$). Collectively, these findings support the conclusion that stress granule assembly downstream of sodium arsenite treatment contributes significantly to the cytoprotective effect against staurosporine treatment.

Finally, to address the importance of OGT and O-GlcNAcylation in the context of stress granule cytoprotection, we next targeted OGT using siRNA. Immunofluorescence analysis demonstrated a marked reduction in OGT signal, both in the cytoplasm and the nucleus of cells, consistent with effective knockdown (Fig. 11A). Consistently, western blot analysis showed substantially lower OGT levels in OGT siRNA-transfected cells, vs. NTC-transfected cells, and the O-GlcNAcylation levels were visibly decreased in OGT-depleted cells (Fig. 11B). Next, we assessed the propensity of OGT-depleted cells to undergo cell death at baseline, and in staurosporine-treated conditions with or without prior treatment to sodium arsenite (Fig. 11C). OGT depletion did not affect cell death at baseline or in response to Staurosporine, or sodium arsenite alone. By contrast, sodium arsenite pretreatment failed to fully protect OGT-depleted cells, which showed significantly greater cell death upon staurosporine challenge than NTC controls (compare brown and lavender blue, Fig. 11D, $P < 0.001$). These results indicate that OGT is required for the full protective effect of sodium arsenite pretreatment and stress granule formation, indicating a novel link between OGT and granule-mediated cytoprotection.

Discussion

Stress granules are increasingly recognized as central regulators of cellular stress responses and are implicated in diverse diseases including neurodegeneration and cancer (59-62), yet their regulation and role in the context of cardiac injury remains largely unexplored. Here, we demonstrate that O-GlcNAcylation is an important determinant of stress granule formation in cardiac cells. Using complementary time-lapse imaging approaches, stress-specific models, and genetic approaches, we establish that OGT activity is necessary for efficient stress granule assembly in cardiac myocytes. Using protein enrichment and O-GlcNAc site-mapping on the stress granule orchestrator G3BP1, we identified T268 as O-GlcNAcylated residue with functional significance. Importantly, we show that stress granules form in cardiomyocytes under ischemic conditions and dissipate upon reperfusion, and that OGT plays an important role in stress granule formation in the ischemic heart. Finally, we found that pharmacologic induction of stress granules in cultured cardiomyocytes confers resistance to acute cell death, and that pre-emptive induction of stress granules in vivo by sodium arsenite protects hearts against ischemia/reperfusion injury. Collectively, our findings identify OGT and O-GlcNAcylation as a central regulatory modality coordinating stress granule assembly and promoting cardiomyocyte adaptation and survival during ischemic stress.

Our data establish OGT activity as a critical mediator of stress granule formation in cardiac cells, consistent with previous work showing that OGT depletion delays arsenite-induced stress granule formation (37, 63). In contrast, pharmacological or genetic targeting of OGA did accelerate stress granule formation. By contrast, pharmacological or genetic inhibition of OGA did not accelerate stress granule formation. These findings are in line with earlier experiments showing that shRNA-mediated depletion of OGA/MGEA5 did not affect arsenite-induced stress granule assembly in cells (37). One possible explanation is that in our experimental conditions arsenite elicits a near-maximal stress granule response on its own, leaving little room for additional potentiation to be observed upon OGA inhibition. Alternatively, OGA may exert a greater influence on stress granule disassembly or clearance than on their initiation, a role that would not be captured within the time frame of the stress granule formation assays used here. Intriguingly, our data show that OGA, but not OGT, is recruited to stress granules, suggesting that OGA may be more geared toward later remodeling steps, rather than in the initial granule nucleation events. Moreover, the absence of OGT from stress granules aligns with a model where proteins already O-GlcNAcylated are preferentially sequestered, rather than being modified after being recruited into the stress granules.

Following the initial observation linking the stress granule inducer sodium arsenite with O-GlcNAcylation (14), multiple stress granule constituents across diverse functional classes have been reported as O-GlcNAc targets. These include ribosomal subunits (37), translation-initiation factors such as eIF4G1 (64), and the core scaffold Caprin1 (38). In addition, DEAD-box (DDX) RNA helicases feature frequently both as stress granule components (65, 66), and as O-GlcNAc targets (e.g. DDX3X) (67, 68). Similarly, heterogeneous nuclear ribonucleoproteins (hnRNPs) are

recurrent stress-granule constituents (69, 70) and carry O-GlcNAc modifications (e.g., hnRNPA1) (71). However, in most of these studies, O-GlcNAcylation is functionally linked to the regulation of stress granule formation, or dynamics. In this regard, our demonstration that the core nucleator G3BP1 is O-GlcNAcylated, provides key evidence supporting O-GlcNAcylation as an active regulator of stress granule formation.

G3BP1 is a modular RNA-binding protein comprising an NTF2L domain, an acidic middle region that contains a proline-rich domain (PxxP, amino acids 221-339), and a C-terminal region that contains the RRM/RG-rich domains (32, 33, 44, 72). Our work identified at least one functional site of O-GlcNAcylation, at position T268, that structurally belongs to the disordered acidic region and within the boundaries of the PxxP domain. The PxxP domain of G3BP1 constitutes a canonical SH3-binding interface (43) and has been suggested to have important roles in the antiviral activity of G3BP1, but also in the initial nucleation events of stress granules (73, 74). Given PxxP's role in protein-protein interactions, it is possible that the presence of O-GlcNAc in that region could sterically weaken the binding of putative SH3-containing proteins with perhaps favorable effects on condensation and stress granule nucleation. Moreover, T268 lies within G3BP1's acidic region, which forms intramolecular contacts with the C-terminal RGG/RG-rich tail that mask the RRM and limit RNA engagement during stress-granule assembly (32, 33). In this regard, it is possible that the presence of O-GlcNAc at T268 weakens these intermolecular contacts, effectively relieving autoinhibition, favoring G3BP1:RNA interactions and promoting stress granule nucleation.

Stress granule formation is a multi-step process that frequently involves liquid-liquid phase separation, as untranslating mRNPs and associated proteins coalesce into droplet-like clusters (75, 29, 76). Evidence from different experimental systems indicates that O-GlcNAcylation on individual proteins can either promote or dampen their phase separation. For example, O-GlcNAcylation on the RNA-binding protein EWS reduces its phase separation in vitro and enhances the liquid-like nature of EWS condensates (77). Consistently, independent work correlated EWS O-GlcNAcylation with reduced partitioning into cytoplasmic granules, during hyperosmotic stress (78). Furthermore, NMR work on CAPRIN1 indicates that O-GlcNAcylation on S644/S649 lowers its phase-separation propensity by weakening key intermolecular interactions (79). In another experimental system, more relevant to synaptic transmission, O-GlcNAcylation of SynGAP at T1306 blocks its interaction with PSD-95 and suppresses phase separation of the SynGAP/PSD-95 assembly (80). Finally, a study investigating the biological functions of N⁶-methyladenosine (m⁶A)-RNA reader proteins showed that YTHDF1/3, undergo O-GlcNAcylation and this dampens their condensation into stress granules (81). Additionally, O-GlcNAcylation of YTHDF1/3 is suggested to be important for their dynamic movement in and out of stress granules and favoring a more efficient disassembly of the stress granules (81). In an independent study however, O-GlcNAcylation on YTHDF3 appears to promote the formation of YTHDF-containing stress granules (82). The same was also observed for another protein, NUFIP2. Moreover, OGT inhibition with OSMI in that setting was inhibitory for condensate formation (82). Taken together, these findings suggest a more nuanced role of O-GlcNAcylation's effects that

appear to be protein- or even context-dependent. In our experimental systems (arsenite-induced stress granule formation, simulated coverslip ischemia/reperfusion injury, and whole heart ischemia/reperfusion injury) global O-GlcNAcylation suppression delays stress granule assembly, consistent with the initial work placing O-GlcNAcylation upstream of stress granule regulation (37). Moreover, our mutational analysis further implicates O-GlcNAcylation on G3BP1 as a positive determinant of stress granule formation.

In primary rat cortical neurons or PC12 cells subjected to oxygen-glucose deprivation/reperfusion (OGD/R), TIA1/G3BP1-positive stress granules were shown to form during the 6 hours of deprivation and then progressively decreased upon reperfusion (83, 84). TIA1/G3BP1-containing stress granules are also detected in cerebral the cortex in a model of focal cerebral ischemia/reperfusion model, and their formation is associated with ameliorated injury (84). These findings are consistent with earlier studies of global brain ischemia documenting translational arrest and formation of TIA1-containing stress granules rise early in reperfusion (~10 min) and decline by ~90 min (85). Furthermore, brain regions that assemble stress granules promptly at reperfusion and resolve them rapidly (i.e., Cornu Ammonis, CA3) are relatively injury-resistant, whereas regions with delayed SG dynamics (e.g., CA1) are more vulnerable and later show convergence with ubiquitin-positive protein aggregates (85, 86). The increased vulnerability in CA1 was also associated with a non-canonical pathway for stress granule formation (53). Stress granules were also detected in the context of renal I/R (87). Complementing these I/R observations, metabolic inhibition (combining glycolytic blockade with ATP depletion in neuronal cells) stimulates the formation of stress granules (88). Moreover, efficient recovery of ATP synthesis is necessary for the effective clearance of stress granules (88). In the heart, within 1 hr of coronary occlusion, eIF4G redistributes into TIAR-positive RNP granules (89), consistent with the formation of stress granule-like structures in the ischemic myocardium. Taken together, these findings are consistent with our data both in cardiomyocytes exposed to simulated ischemia/reperfusion injury and in I/R-injured hearts, demonstrating that stress granules assemble transiently over the course of ischemia and then resolve upon reperfusion.

Across different experimental systems, the rapid assembly of stress granules is cytoprotective. For example, blocking stress granule assembly by oxidizing TIA1 increases apoptosis, and depleting the core nucleators G3BP1/2, heightens stress-induced cell death (90, 91). Mechanistically, assembled stress granules are suggested to be cytoprotective by sequestering executioner caspases and other death-inducing factors (92, 50, 93, 94, 91). Accordingly, stress granules have been shown to be cytoprotective in the context of viral infection, proteotoxic stress, and lysosomal damage (95-97). In this work, we developed an on/off strategy that first exposes cells to sodium arsenite to induce stress granules, followed by arsenite wash off and challenge by staurosporine, that robustly activates intrinsic apoptotic pathways (98, 99), yet it does not elicit stress granules (100, 91). In this setup, arsenite priming protected against staurosporine-induced killing, and critically, this benefit was significantly attenuated by G3BP1/2 depletion. While arsenite can trigger broader stress responses, our data indicate that stress

granule assembly is required for the full protective effect observed in this setting and align with the notion that acute, transient stress granule formation is an important survival mechanism.

In addition to a cytoprotective effect in transiently exposed cells, our work showed that acutely injecting sodium arsenite to mice 1h prior to I/R injury has a significant cardioprotective effect, evident very early at reperfusion. These results are consistent with multiple preclinical studies showing that acute, single-dose arsenite can act as a chemical preconditioning stimulus. For example, one study reported that arsenite bolus (4.5 mg/kg) given 30 minutes before coronary occlusion reduced infarct size, although this effect was associated primarily with effects on mitochondrial respiration, and inflammation, and was observed late in reperfusion (24h) (101). Beyond the heart, studies on kidney and liver demonstrated that arsenite pretreatment (6 or 8 mg/kg) limits apoptosis and tissue injury markers after I/R, and in these studies the effects were associated with the induction of Hsp70 and were again observed mid-to-late in reperfusion (i.e. 6, or 24h) (102, 103). However, it should be noted that outside a narrow dose and time window, prolonged arsenite administration at the micromolar level can induce cardiac, smooth muscle, or endothelial cell death (104-106). Moreover, low dose exposure to arsenite (at sub micromolar levels) over the period of weeks (typically from 4-22) is known to induce, or exacerbate adverse remodeling in preclinical cardiac models (107-110) and chronic exposure to arsenite has been viewed as an environmental pollutant contributing to cardiovascular disease (111). Taken together, arsenite's effects on the heart are highly dependent on dose and exposure duration. Our data have identified an acute, pre-ischemic window in which transient arsenite administration confers cardioprotection, and we attribute this response to a stress-granule-linked adaptation in the acutely ischemic heart.

Prior work shows that targeting OGT in cardiomyocytes sensitizes the heart to injury and adverse remodeling (24), while acute augmentation of O-GlcNAc is protective in stressed cardiac myocytes (112, 26) and in I/R-injured hearts (21-23). Similar directionality holds in the brain, where moderate O-GlcNAc elevation lessens stroke injury and neuron-specific OGT deletion aggravates regional cerebral ischemia/reperfusion injury outcomes (113, 114). While the cardio- and neuro-protective effects of OGT in these contexts are likely to be multifaceted, our data identify a specific requirement for OGT in efficient assembly of G3BP1-positive stress granules under ischemic stress. These findings in isolated cardiomyocytes were also recapitulated in perfused OGT-deficient hearts exposed to ischemic stress, providing genetic *in vivo* evidence that OGT coordinates stress granule assembly in the ischemic myocardium. Moreover, in our arsenite/staurosporine on/off experiment, arsenite priming reduced staurosporine cytotoxicity, and this benefit was blunted by OGT depletion, supporting a contributory role for OGT in stress granule-linked cytoprotection.

Together, these data show that O-GlcNAcylation promotes timely assembly of G3BP1-defined stress granules in cardiomyocytes exposed to arsenite, or ischemic stress. We map a functionally important O-GlcNAc site on G3BP1 and propose that pre-existing O-GlcNAcylation primes stress granule proteins to coalesce in a timely manner. Pre-emptive formation of stress granules in

500 cardiac myocytes is cytoprotective as it preserves proteostasis and suppresses pro-death
501 signaling particularly during the vulnerable early reperfusion window. These findings define a
502 novel mechanistic framework connecting O-GlcNAcylation, G3BP1, and stress granules in
503 stressed cardiac myocytes and ischemic hearts, opening opportunities for new therapeutic
504 approaches in ischemic and related cardiac conditions.

Experimental Procedures

Materials

A detailed list of key reagents including their identifiers, vendors, and catalog numbers is provided in [Supporting Table S3](#). Additional information on other reagents can also be found in the description of methods in the text below. Procedures involving the use of animals were approved by the Institutional Animal Care and Use Committee (IACUC) at Johns Hopkins University School of Medicine. All mice were maintained on a C57BL/6J background and housed under standard laboratory conditions with free access to food and water. The tamoxifen inducible cardiomyocyte specific OGT knockout mice were previously described (58).

Plasmids and adenoviral constructs

A recombinant adenoviral vector expressing enhanced green fluorescent protein (EGFP, codon optimized) fused to the N-terminus of human G3BP1 (RefSeq: NM_005754.3) was custom-designed by VectorBuilder (Chicago, IL, USA). The construct included a Kozak consensus sequence (GCCACCATGG) immediately upstream of the EGFP start codon to enhance translation initiation. The coding sequence was inserted into a standard deleted adenovirus serotype 5 (Δ Ad5) viral backbone under the control of the CBH promoter (hybrid promoter composed of the cytomegalovirus (CMV) enhancer and chicken β -actin promoter with a hybrid intron). A second adenovirus carrying a threonine-to-alanine point mutation at residue 268 (T268A) in G3BP1 was generated using the same construct design and backbone. Adenoviruses were packaged in HEK293 producer cells, amplified to high titer ($\sim 1 \times 10^{12}$ viral particles/ml), purified by two sequential cesium chloride gradients, dialyzed, and titrated by UV spectrophotometry and confirmed by plaque formation assay. Cells were transduced with each virus at a multiplicity of infection (MOI) of 50, 24 h prior to experiments.

Cell culture of H9c2 cells, siRNA transfection and adenoviral transduction

H9c2 rat cardiomyoblasts (ATCC) were cultured in DMEM (4.5 g/l glucose) supplemented with 10% (v/v) fetal bovine serum and 100 U/ml penicillin–streptomycin (both from Gibco), along with 2 mM GlutaMAX, MEM non-essential amino acids, and 10 mM HEPES (all from Invitrogen). For gene knockdown, cells were transfected with Dicer-substrate siRNA (DsiRNA) using Lipofectamine RNAiMAX (Thermo Fisher Scientific) 48 h prior to experimentation. Transfection complexes were formed at room temperature for 15 min according to the manufacturer's protocol and added at a final ratio of 40 pmol per 1×10^6 cells. A non-targeting DsiRNA (IDT) was used as control. The sequences of all DsiRNAs used are provided in [Supporting Table S3](#).

To monitor rates of stress granule formation, cells were transduced with adenovirus encoding EGFP–G3BP1 (multiplicity of infection [MOI] = 50) 24 h prior to confocal imaging. Cells were treated with the indicated pharmacological inhibitors for durations specified in the figure legends and main text.

Live cell imaging, data acquisition and analysis

For stress granule induction, 500 μ M sodium arsenite was added directly to the culture medium, and cells were imaged over the subsequent 20 min at 1 min intervals. Imaging was performed on an Olympus FV3000 scanning confocal microscope equipped with a 100 $\times$ /1.45 NA oil-immersion objective at 37 °C and 5% CO₂. Nuclei and G3BP1 signals were visualized using 405 nm and 488 nm lasers, respectively, in the presence of NucBlue.

For data analysis, .oir images were uploaded into a Fiji-run custom-made macro to convert composite images into individual .tiff single-color channels (blue, green, and red). The images were then uploaded into CellProfiler (version 4.2.1., <https://cellprofiler.org/>). The CellProfiler pipeline extracts nuclei from the blue channel and uses those as primary objects. Next, using nuclei as primary objects, the pipeline identifies the cells from the G3BP1 signal using the 'Propagation' method. Next, stress granules are identified within the cellular space using the 'RobustBackground' thresholding feature. The main output parameter of the pipeline used for quantification of stress granules formation was 'Count Stress Granules.'

Cell culture of NRVM cardiomyocytes, siRNA transfection

Neonatal rat ventricular myocytes (NRVMs) were isolated from P0-P1 rats of either sex using enzymatic dissociation, according to a standard methodology as previously described (115, 116). Following a pre-plating step, to remove fibroblasts and other non-myocytes, the cardiomyocytes (suspensions at 0.5 $\times 10^6$ cells/ml) were seeded for experiments on culture plates pre-coated with 10 μ g/mL fibronectin (Sigma Aldrich) and incubated at 37 °C, 5% CO₂ for 24 hrs. Typical seeding densities were 1.0 $\times 10^6$ cells per well in a 6-well plate for western blot experiments, or 0.8 $\times 10^6$ cells per chamber in glass bottom cell culture dishes (NEST) for confocal microscopy. The following day, cells were switched to serum-free DMEM (Corning) supplemented with insulin–transferrin–selenium (ITS; Gibco) and 10 mM HEPES (Invitrogen). For knockdown experiments, cells were transfected with a Lipofectamine/siRNA mix 48 h prior to treatment.

NRVM model of simulated ischemia/reperfusion injury by glass coverslip application

Prior to the experiment, the culture medium was replaced with fresh Tyrode's buffer (130 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 10 mM NaHEPES, 1 mM CaCl₂, and 5 mM glucose). Simulated ischemic injury was induced by placing a glass coverslip over the cells for 60 min to restrict oxygen and nutrient exchange, as previously described (117, 57). Where indicated, simulated reperfusion was achieved by removing the coverslip and incubating the cells for an additional 60 min in Tyrode's buffer. Control cells were incubated in Tyrode's buffer for 120 min without coverslip placement.

Immunocytochemistry staining and confocal imaging of NRVMs subject to simulated ischemia/reperfusion injury

Following coverslip applications, NRVMs from the different treatment groups (Control, Ischemia, Reperfusion) were washed with 37 °C prewarmed PBS and then fixed with prewarmed 4% paraformaldehyde for 15 min. The fixative was then washed off with PBS and cells were

permeabilized with 0.1% Triton X-100 for 5 min at room temperature. This was followed by PBS washes and blocking overnight at 4 °C with 2.5% BSA and 2.5% normal goat serum (NGS, Cell Signaling Technology, catalog number: 5425) in 0.1% tween-20 PBS (PBS-T). Primary antibodies against O-GlcNAc (200:1, RL2, host mouse), or G3BP1 (1000:1, Cat. No. 13057-2-AP, host rabbit) were diluted in blocking solution (2.5% BSA, 2.5% normal goat serum, 0.1% Tween-20 in PBS) and incubated with samples at 4 °C with gentle shaking overnight. After washes (PBS-T, 3×) secondary antibodies were applied for 1 h at room temperature in blocking buffer as follows: Alexa Fluor 546 anti-rabbit IgG (Thermo Fisher, A11071, 1:1000) and Alexa Fluor 488 anti-mouse IgG (Thermo Fisher, A21121, 1:1000). Nuclei were counterstained with Hoechst 33342 (1:5000 in PBS, 5 min). Imaging was carried out with the Olympus FV3000RS confocal microscope using the 100× objective and 405 nm, 488 nm, and 561 nm lasers to excite Hoechst, Alexa 488- and Alexa 546-conjugated immunocomplexes respectively. Laser power and gain were kept constant across imaging sessions, and four fields of view were captured per plate.

Immunocytochemistry and Colocalization analysis of OGT and OGA in stress granules

Cells plated on ibidi chamber slides (4-well or 8-well, [Supporting Table S3](#)) were treated with or without sodium arsenite (500 μM, 30 min), washed with 37 °C prewarmed PBS (3×) and then fixed with prewarmed 4% paraformaldehyde in PBS for 15 min. The fixative was washed off with PBS (3×) and cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min at room temperature. Primary antibodies against OGT (200:1, AL24, host rabbit), or OGA (200:1, Cat. No. 14711-1, host rabbit) were added to samples and co-incubated with anti-G3BP1 (1000:1, Cat. No. 66486-1-Ig, host mouse) at 4 °C with gentle shaking overnight. After washes (PBS-T, 3×) secondary antibodies were applied for 1 h at room temperature in blocking buffer (2.5% BSA, 2.5% normal goat serum, 0.1% Tween-20 in PBS): Alexa Fluor 546 anti-rabbit IgG (Thermo Fisher, A11071, 1:1000) and Alexa Fluor 488 anti-mouse IgG (Thermo Fisher, A21121, 1:1000). Nuclei were counterstained with Hoechst 33342 (1:5000 in PBS, 5 min). Images were acquired on an Olympus FV3000RS confocal microscope using a 100×/1.35 NA silicone oil immersion objective (UPLSAPO100XS).

Co-localization between G3BP1 and OGT, or G3BP1 and OGA within granules was quantified in CellProfiler using the MeasureColocalization module. Stress granule masks were generated from the G3BP1 channel and segmented with IdentifyPrimaryObjects using two-class Global Otsu thresholding (threshold smoothing scale = 1; correction factor = 3; bounds = 0.005–1.0), typical diameter 5–100 pixels, and shape-based declumping (smoothing filter = 10 pixels; minimum peak distance = 7 pixels; border objects discarded). Pearson's correlation coefficients were computed using MeasureColocalization for G3BP1↔OGT and G3BP1↔OGA pixel intensities restricted to the stress granule masks.

Langendorff ischemia/reperfusion injury

The procedure to perfuse isolated mouse hearts and subject them to ischemia/reperfusion injury was performed as previously described (57). Briefly, a controlled digital peristaltic pump (Reglo, Ismatec) was used to deliver the buffer at a constant flow through Tygon tubing (Cole Parmer).

The freshly-made perfusion buffer (Krebs-Henseleit, consisting 118 mM NaCl, 4.7 mM KCl, 25 mM NaHCO₃, 1.2 mM KH₂PO₄, 10 mM Glucose, 1.2 mM MgSO₄ and 2 mM CaCl₂, pH 7.4) was vacuum-filtered and loaded onto the perfusion apparatus where it was continuously bubbled with a mixture of 95% CO₂ and 5% O₂ and warmed at 37 °C. The temperature in the perfusate right above the heart was monitored (NBD TC2 temperature controller). 1 hr prior to heart excision, mice were injected intraperitoneally with a single bolus dose of sodium arsenite (4.5 mg/kg) or the equivalent volume of PBS. After 45 min, the mice were injected intraperitoneally with heparin (200 U/g body weight, Sagent) and 15 minutes later they were anesthetized with 2,2,2, tribromoethanol (0.2 mg/g body weight). After deep anesthesia the hearts were quickly excised, weighed, cannulated through the aorta and mounted onto the perfusion rig. Perfusions were performed at the constant pressure mode corresponding to 70 mmHg. The heart was immersed in 37 °C perfusion buffer warmed in water-jacketed tissue bath (Radnoti) and allowed to beat with its intrinsic rate without pacing. After an initial phase of normoxic perfusion for 20 minutes the hearts underwent global no-flow (ischemia) for 30 min. followed by 20 min. of reperfusion.

At the end of reperfusion, the hearts were arrested in diastole by switching to KH supplemented with KCl (1% w/v) and then perfused with TTC (1% w/v in KH, 37 °C) for 5 min until viable tissue developed a uniform red coloration. Following TTC staining, the hearts were removed from the rig, rinsed in PBS and sliced into short axis slices (1–2 mm thickness) from apex to base. Slices were placed between two coverslips and photographed at both sides using a stereoscope using identical illumination and magnification. Digital planimetry was performed using ImageJ with fixed threshold settings to segment the pale TTC-negative area which was expressed as a percentage of the total slice area. The results were averaged for each slice, corrected for slice mass and aggregated to yield % infarct size for the total ventricular mass. Image analysis was performed by an investigator blinded to group allocation.

In other experiments, the perfusion of the hearts was stopped at the indicated stages (control perfusion, ischemia-only, ischemia/reperfusion) and the hearts were immediately immersed in 4% PFA in KH buffer containing 1% KCl to maintain diastolic arrest, fixed overnight at room temperature and were subsequently processed for immunohistochemistry.

Immunohistochemistry staining, confocal imaging and analysis

Hearts were carefully extracted and post-fixed by immersion in 4% PFA overnight at room temperature. Following post-fixation, hearts were transferred to a 30% sucrose solution in PBS for cryoprotection and stored at 4°C until they sank to the bottom of the container, typically 24-48 hours later. The heart was then embedded in Optimal Cutting Temperature (OCT) compound, frozen on dry ice, and stored at -80°C until sectioning. Coronal sections (30 µm thick) were obtained using a Tanner TN60 cryostat maintained at -20°C. Sections were serially collected and stored in PBS supplemented with 0.1% sodium azide.

For antibody staining, sections were first blocked and permeabilized for 1 hour at room temperature in a solution containing 10% NGS and 0.05% Triton X-100 in PBS. Following this step,

sections were incubated with anti-G3BP1 (1:1000, Proteintech, Cat. No. 13057-2-AP, Host: Rabbit), diluted in 5% NGS in PBS-T overnight at 4°C with gentle orbital shaking. After primary antibody incubation, sections were washed four times with PBS-T to remove unbound antibodies. Following the washes, sections were incubated with Alexa Fluor 546 anti-rabbit IgG (Thermo Fisher, A11071, 1:1000). Incubations with secondary antibodies were performed at room temperature with gentle orbital shaking for 1 hour, protected from light. This was followed by staining with Hoechst 33342 (1:5000 dilution from a 10 mg/mL stock solution, Cat. No. H3570) for 15 minutes in the dark. Sections were then thoroughly washed in PBS-T and mounted on glass slides using Vibrance anti-fading medium (Vector Laboratories). Images were acquired using the 100×/1.45 NA oil-immersion objective of the Olympus FV3000 confocal microscope equipped with 405 nm, 488 nm, and 561 nm laser lines. Multiple non-overlapping FOVs were collected from different anatomical positions of the heart, and at least three sections per heart were collected and quantified and data averaged.

Immunoprecipitation of EGFP-G3BP1 and O-GlcNAc site discovery

Cells were transduced with either a control β -galactosidase-expressing adenovirus or an adenovirus encoding EGFP-G3BP1 (MOI 50), and harvested 24 h post-transduction. All samples were lysed in IP buffer (150 mM NaCl, 1% (v/v) NP-40, 0.5 mM EDTA, protease and phosphatase inhibitors, 1 mM freshly added PMSF and 2 μ M Thiamet-G in 10 mM Tris-HCl, pH 7.6), followed by clarification at 21,300 $\times$ g for 15 min at 4°C. Lysates were then pre-cleared by end-over-end rotation for 1 h with neutral agarose resin to remove proteins binding non-specifically to the matrix, prior to immunoprecipitation with GFP-Trap resin (alpaca nanobody conjugated to agarose resin; Cat. No. gta-10, Proteintech).

To assess non-specific binding, control IP reactions were set up using harvesting buffer alone incubated with GFP-Trap resin, as well as pooled lysate incubated with neutral agarose resin. Five hundred micrograms of total protein were incubated with 25 μ l of GFP-Trap agarose slurry (1:1 resin suspension; Cat. No. gta-10, Proteintech) overnight at 4°C with end-over-end rotation. During the IP procedure, 2% of the input, flow-through, and final wash fractions were collected in parallel to evaluate pulldown efficiency and specificity by western blot. Beads were then washed five times with 450 μ l wash buffer (150 mM NaCl, 1% (v/v) NP-40, 0.5 mM EDTA in 10 mM Tris-HCl, pH 7.6).

Bound proteins were eluted by boiling the resin in 1 $\times$ LDS sample buffer (Invitrogen) supplemented with 50 mM DTT, RIPA buffer, and protease/phosphatase inhibitors at 95°C for 10 min. The resin was pelleted by centrifugation at 1,500 $\times$ g for 3 min, and the supernatant was collected for SDS-PAGE. Elution fractions, together with the previously collected input, flow-through, and wash samples, were analyzed by western blot using an anti-G3BP1 antibody (Cat. No. 13057-2-AP, Proteintech). Following quality assessment, the eluate was resolved on a 4–12% Bis-Tris gradient gel and stained with SimplyBlue SafeStain (Invitrogen). The band corresponding

to EGFP–G3BP1 (molecular mass: 79.1 kDa) was excised and processed for O-GlcNAc site mapping by mass spectrometry.

The excised gel band corresponding to EGFP–G3BP1 was subjected to in-gel trypsin digestion. Peptides were extracted and desalted using C18 reverse-phase and then separated by nano-flow reversed-phase liquid chromatography and analyzed on an Orbitrap Fusion Lumos mass spectrometer (Thermo Scientific) operating in data-dependent acquisition mode. An HCD-triggered EThcD acquisition strategy was employed: high-resolution HCD MS2 scans were first acquired in the Orbitrap and screened for the presence of diagnostic oxonium ions characteristic of O-GlcNAc modifications (m/z 204.0867, 186.0761, 168.0655, 144.0652, 138.0545, and 126.0550). Upon detection, EThcD fragmentation was triggered to enable confident site localization (118-120). Raw data were analyzed using Proteome Discoverer (Thermo Scientific) with Mascot (Matrix Science) as the search engine and searched against the UniProt human proteome database (G3BP1 in the EGFP–G3BP1 construct was of human origin). Variable modifications included O-GlcNAc (HexNAc, +203.079 Da) on serine and threonine, oxidation (M), deamidation (NQ), and phosphorylation (S/T). A maximum of one missed cleavage was allowed. Precursor and fragment mass tolerances were set at 5 ppm and 10 ppm, respectively. O-GlcNAc site assignments were manually validated in Scaffold, based on the presence of diagnostic oxonium ions (e.g., m/z 204.0867, 186.0761, 168.0655) and supportive fragment ion patterns when available, to ensure high-confidence localization.

Western blot analysis

Cells were washed with ice-cold PBS (3×) and lysed in RIPA buffer (150 mM NaCl, 1.0% IGEPAL CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0, Cat. No. R0278, Sigma) supplemented with 2 μ M TMG, 1 mM PMSF, 1× protease and phosphatase inhibitors (cOMplete, mini, EDTA-free, and PhosSTOP respectively, Millipore, Sigma), 50 mM DTT, and 1× LDS sample buffer (Thermo Fisher Scientific, NP0008). For the subsequent Western Blot analysis, each lane was loaded with 10 μ g of protein and run on NuPAGE Bis-Tris 4% to 12% gradient gels (Thermo Fisher Scientific, Cat. No. WG1403). Following electrophoresis, proteins were transferred onto nitrocellulose membranes (Thermo Fisher Scientific, iBlot2 transfer stacks, Cat. No. IB23001). The membranes were stained using REVERT 700 Total Protein Stain (LI-COR, Cat. No. 926-11010) to visualize the total protein loading and blocked in 5% bovine serum albumin (BSA, Cat. No. 9998, Cell Signaling) in TBS for one hour at room temperature. Primary antibodies (diluted 1000:1 in 5% BSA and 0.1% Tween 20 in TBS) were incubated with membranes overnight at 4 °C. Post-incubation, the membranes were thoroughly washed with TBS-T. Subsequently, IR Dye-conjugated host-specific secondary antibodies were applied (dilution of 5000:1 in 5% BSA and 0.1% Tween20-TBS). The specific antibodies used in this study are detailed in [Supplemental Table 3](#). Detection and quantification of band intensities were accomplished using the Odyssey system and Image Studio Lite software (LI-COR). Normalization for protein loading was achieved by comparing the band intensities to the total protein loading.

Cell Viability Assays

To validate the protective effect of stress granules, four-chamber ibidi slides were utilized. After seeding, cells were allowed to adhere and proliferate in CGM for 24 hours. At this point, the medium was replaced with fresh CGM to maintain nutrient levels and optimal conditions for cell

growth. At 48 hours post-passaging, two wells of the slide were treated with 500 μ M sodium arsenite for 30 minutes to induce cellular stress, while the remaining two wells served as controls and were treated with CGM alone. Following this initial treatment, all medium was carefully removed. One well from the sodium arsenite treated group and one well from the control group were exposed to 10 nM staurosporine for 1 hour to induce apoptosis. The other two wells were treated with a 1:1000 dilution of dimethyl sulfoxide (DMSO) in CGM as a vehicle control. During the final 15 minutes of the incubation period, cells were counterstained with NucBlue to visualize nuclei. The staining allowed accurate identification of apoptotic and viable cells during imaging. Following the incubation and staining processes, cells were imaged to capture treatment-specific cellular responses.

Gene knockdown experiments were conducted using eight-chamber ibidi μ -slides to evaluate the functional impact of specific gene silencing on cell viability under stress conditions. To facilitate comparative analysis, the top row of the eight-chamber slide was designated as the non-targeting control (NTC) group, while the bottom row contained cells transfected with siRNAs targeting OGT or G3BP1/2. Once transfection was complete, the experimental procedures performed in the four-chamber ibidi slides, including sodium arsenite and staurosporine treatments, were replicated for the NTC and siRNA-transfected groups, allowing direct comparisons of treatment effects between groups.

Cellular imaging was performed using the Invitrogen™ EVOS™ XL Core Imaging System, equipped with 20 $\times$ phase contrast and fluorescence microscopy capabilities. For each chamber, three distinct fields of view were selected to ensure representative sampling of both phase contrast and fluorescent micrographs. Images captured included a diversity of cell populations and morphological features to account for spatial heterogeneity. Fluorescent micrographs were utilized to identify nuclei stained with NucBlue, facilitating differentiation between apoptotic and viable cells. Image analysis was conducted using ImageJ software, which allowed precise quantification of condensed nuclei, a hallmark of apoptosis. The total number of apoptotic cells identified was divided by the total number of nuclei to calculate the proportion of viable cells following each treatment. This metric provided a quantitative measure of cell survival and enabled robust comparisons across experimental conditions.

Statistical analysis

Data was handled and plotted using Microsoft Excel and GraphPad Prism. Values in the graphs are reported as means $\pm$ standard error. The numbers of biological replicates are shown inscribed in their respective bars or on the side of curves. Data were examined for normality of distribution using the Shapiro-Wilk test. To identify statistically significant differences between two groups we performed unpaired student's t-test. When comparing three or more groups, we used one-way or two-way ANOVA depending on the number of factors (e.g. for comparing the effect across 3 or more drugs we used one-way whereas for comparing groups resulting from a combination of drugs across several time points we used two-way). To identify statistically significant differences, the data were assessed for normality of distribution. If the majority of groups within

774 an experiment had normally distributed data, we used the Tukey *post-hoc* test. If the distribution
775 of the data in the majority of groups was not normal, we used the Dunnett *post-hoc* test. In all
776 cases, the α level for statistical significance was 0.05. *P* values less than 0.05, 0.01, 0.001, and
777 0.0001 are symbolized as *, **, ***, and **** respectively.

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The authors declare no competing interests.

Statement for the use of animals

Procedures involving animals were approved by the local Institutional Animal Care and Use Committees (IACUC) at Johns Hopkins School of Medicine. All experiments conformed to the principles outlined in the *NIH Guide for the Care and Use of Laboratory Animals* (8th edition, National Academies Press, 2011).

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Figure Legends

Figure 1. Inhibition and knockdown of OGT reduces SG formation.

A. Schematic showing formation of GFP-G3BP1-positive granules in H9c2 cells treated with SA. **B.** Representative images of GFP-G3BP1 signal in H9c2 cells 20 minutes following treatment with 500 μ M sodium arsenite (left). Images were acquired using an FV3000 Olympus confocal microscope using the 100x objective. Scale bars: 20 μ m. Regions of interest (ROIs) identified as SGs by CellProfiler using Robust Thresholding (right). **C.** Representative images of GFP-G3BP1 signal in vehicle-treated and 25 μ M OSMI-1-treated H9c2 cells in minutes following treatment with 500 μ M sodium arsenite. Scale bars: 20 μ m. **D.** Number of SGs identified in images of vehicle-treated H9c2 cells compared with 25 μ M OSMI-1-treated H9c2 cells in regions of comparable confluence (n=10 replicates per group). Statistical comparisons were performed using two-way repeated measures ANOVA (** p=0.0014). **E.** Number of SGs identified in images of NTC H9c2 cells (blue) compared with OGT-KD H9c2 cells (green) in regions of comparable confluence (n=7 replicates per group). Statistical comparisons were performed using two-way repeated measures ANOVA (***) p=0.0002). **F.** Representative images of GFP-G3BP1 signal in NTC and OGT knockdown H9c2 cells in minutes following treatment with 500 μ M sodium arsenite. Scale bars: 20 μ m.

Figure 2. Inhibition and knockdown of OGA does not impact SG formation.

A. Representative images of GFP-G3BP1 signal in vehicle-treated, TMG-treated (25 μ M), and MK-8719-treated (25 μ M) H9c2 cells in minutes following treatment with 500 μ M sodium arsenite. Scale bars: 20 μ m. **B.** Number of SGs identified in images of vehicle-treated H9c2 cells compared with 25 μ M TMG-treated H9c2 cells in regions of comparable confluence (n=10 replicates per group). Statistical comparisons were performed using two-way repeated measures ANOVA (p=0.9641). **C.** Number of SGs identified in images of vehicle-treated H9c2 cells compared with 25 μ M MK-8719-treated H9c2 cells in regions of comparable confluence (n=6 replicates per group). Statistical comparisons were performed using two-way repeated measures ANOVA (p=0.9997). **D.** Number of SGs identified in images of NTC H9c2 cells compared with OGA-KD H9c2 cells in regions of comparable confluence (n=7 replicates per group). Statistical comparisons were performed using two-way repeated measures ANOVA (p=0.2075). **E.** Representative images of eGFP-G3BP1 signal in NTC (top) and OGA-knockdown (bottom) H9c2 cells in minutes following treatment with 500 μ M sodium arsenite. Scale bars: 20 μ m.

Figure 3. OGA colocalizes with G3BP1 in SGs, but OGT does not.

A. Fluorescent immunostaining for OGT and G3BP1 with nuclear Hoechst staining of H9c2 in the presence and absence of 500 μ M sodium arsenite. Scale bars: 20 μ m. **B.** Fluorescent immunostaining for OGA and G3BP1 with nuclear Hoechst staining of H9c2 in the presence and absence of 500 μ M sodium arsenite. Scale bars: 20 μ m. Arrows indicate colocalization of OGA and G3BP1 in SGs. **C.** Quantification of colocalization between G3BP1 and OGT in H9c2 cells in the presence (n=6) and absence (n=6) of 500 μ M sodium arsenite reported as Pearson's

correlation coefficient in cytoplasmic ROIs identified by Cell Profiler. Statistical comparisons were performed using a two-tailed t-test (** $p=0.0091$). **D.** Quantification of colocalization between G3BP1 and OGA in H9c2 cells in the presence ($n=6$) and absence ($n=6$) of 500 μM sodium arsenite reported as Pearson's correlation coefficient in cytoplasmic ROIs identified by CellProfiler. Statistical comparisons were performed using a two-tailed t-test (* $p=0.0259$).

Figure 4. Immunoprecipitation and mass spectrometric analysis of O-GlcNAcylation on G3BP1.

A. Representative immunoprecipitation (IP) of EGFP-G3BP1 from H9c2 cell lysates using GFP-Trap resin. NAR = neutral agarose resin control (unconjugated agarose beads used for pre-clearing and as IP negative control). **B.** Coomassie Brilliant Blue-stained Bis-Tris gradient gel of the GFP-Trap eluates from the three experimental groups (β -gal, EGFP-G3BP1, HS). The EGFP-G3BP1 signal appeared as a distinct doublet at ~ 79 kDa, which was excised for LC-MS/MS analysis (red box). **C.** Amino acid sequence of human G3BP1 showing tryptic peptides unequivocally identified by LC-MS/MS (yellow). Amino acids found to have fixed or variable modifications are shown in green. The identified O-GlcNAcylation site is boxed in blue. Sequence coverage was calculated relative to the UniProt reference sequence Q13283 (RefSeq: NM_005754.3). **D.** Representative HCD MS/MS spectrum of a precursor ion corresponding to the O-GlcNAc-modified G3BP1 peptide containing T268. Prominent b and y fragment ions are annotated, along with a boxed low- m/z region highlighting the diagnostic oxonium 204.09 for GlcNAc. **E.** Expanded view of the low- m/z region from (**D**) showing additional O-GlcNAc-specific diagnostic fragment ions, including m/z 138.055, 144.065, 168.066, 186.076, and 204.087. **F.** Representative EThcD spectrum of a G3BP1 peptide containing T268. Extensive c- and z-type fragment ions spanning the modified residue support confident localization of the O-GlcNAc to T268. The spectrum also contains ions consistent with neutral loss of GlcNAc (HexNAc) from multiply charged precursors.

Figure 5. Design of T268A G3BP1 mutant and assessment of its impact in SG formation.

A. Schematic representation of G3BP1 domain organization, showing the NTF2-like (NTF2L) domain, acidic domain, PxxP motif-rich region, RNA recognition motif (RRM), and RG-rich domain. The O-GlcNAc-modified threonine residue identified by mass spectrometry (T268) is indicated as the blue square within the PxxP domain. **B.** Diagrams depicting the EGFP-tagged wild-type (WT) and T268A mutant G3BP1 constructs used in these experiments. Proteins were expressed in cells via adenoviral transduction for functional characterization. **C.** Representative images of GFP signal in H9c2 cells transduced with viruses expressing WT or T68A mutant G3BP1. Time after addition of 500 μM is indicated as 0, 5, 10, 15, and 20 min. Scale bars: 20 μm . **D.** Number of SGs identified in images of cells expressing WT or T268A mutant EGFP-G3BP1 ($n=9$ replicates per group). The solid lines represent the mean SG number in WT or T268A groups and the shaded areas represent $\pm$ standard deviation. Statistical comparisons were performed using two-way repeated measures ANOVA (* $p=0.0429$).

Figure 6. Ischemia induces O-GlcNAc-containing SGs in NRVMs, which dissipate during reperfusion.

A. Schematic illustration of the coverslip-based procedure used to simulate ischemia and reperfusion injury in neonatal rat ventricular myocytes (NRVMs). Each protocol lasted 120 min and included three experimental groups: *Control*, cells maintained in Tyrode's solution for 120 min; *Ischemia*, cells subjected to 60 min of glass coverslip application to restrict oxygen and nutrient exchange; and *Reperfusion*, cells subjected to 60 min ischemia followed by 60 min recovery after coverslip removal. At the conclusion of each condition, cells were fixed and processed for immunohistochemistry. **B.** Representative immunofluorescence images showing the three experimental conditions (Control, Ischemia, Reperfusion). Hoechst staining (blue) marks nuclei, anti-O-GlcNAc staining (green) highlights O-GlcNAcylated proteins, and anti-G3BP1 punctate staining (red) visualizes stress granules. The merged panels demonstrate punctate G3BP1-positive granules, with O-GlcNAc signal frequently overlapping these foci during ischemia. A lower-magnification field of view is included to illustrate the overall monolayer morphology. Images were acquired using an Olympus FV3000RS confocal microscope with a 100× oil-immersion objective. Scale bars: 20 μm (zoomed-in panels); 50 μm (overview images). **C.** SGs were quantified across three experimental groups (Control, Ischemia, Reperfusion) using CellProfiler with robust thresholding. For each condition, 12 independent image fields were analyzed. Statistical comparisons were performed using one-way ANOVA followed by Tukey's post-hoc test (*P < 0.05; ns, not significant).

Figure 7. OGT depletion delays stress granule formation in ischemic NRVMs.

A–B. Representative immunofluorescent images of Hoechst (blue) and G3BP1 (red) staining in NRVMs subjected to simulated ischemia/reperfusion (Control, Ischemia, Reperfusion) following 48 h of siRNA transfection (NTC or OGT-targeting siRNAs, 20 nM; 8×10^5 cells). Images were acquired using an FV3000RS confocal microscope (100× objective). Scale bars: 20 μm. **C.** Quantification of G3BP1 puncta per cell using CellProfiler robust thresholding. n = 22 independent image fields from three biological replicates. Data were analyzed using two-way ANOVA followed by Tukey's post hoc test (****P < 0.0001, ***P < 0.001, **P < 0.01, *P < 0.05; ns not significant).

Figure 8. SG formation occurs in mouse heart tissue during ischemia and is reduced by OGT KO.

A. Diagrams showing the different heart perfusion protocols. Hearts were assigned to three groups: control perfusion (70 min continuous perfusion), ischemia-only (20 min baseline perfusion followed by 30 min index ischemia) and or ischemia/reperfusion (20 min baseline/30 min index ischemia/20 min reperfusion). **B.** Mouse hearts from the different experimental protocols were fixed in 4% paraformaldehyde, sectioned and processed for immunofluorescent staining for G3BP1 to indicate stress granules. Confocal images were acquired on the FV3000RS confocal microscope with a 100× objective. The scale bars are 20 μm. **C.** Quantification of numbers of stress granules, identified G3BP1-positive puncta, using CellProfiler with robust thresholding. Data are presented as mean ± SEM from n = 3-4 hearts per group. Statistical comparisons were performed by one-way ANOVA with Tukey's post hoc test (*P < 0.05; ns = not significant). **D–E.** Experiments performed in inducible, cardiomyocyte-specific OGT knockout

(OGT-icko) mice and littermate controls (WT), subjected to the same ischemia-only protocol. **D.** Representative confocal images showing G3BP1 staining in heart sections. Scale bars are 20 μ m. **E.** Quantification of stress granules as in **C**. Data are presented as mean $\pm$ SEM from $n = 3$ hearts per group. Statistical comparisons were performed using unpaired two-tailed t -test ($*P < 0.05$). **F.** Schematic showing the protocol followed to assess the protective effect of sodium arsenite in ischemia/reperfusion injury. Mice were injected intraperitoneally with a single bolus dose of sodium arsenite (4.5 mg/kg) or the equivalent volume of PBS, 1 h prior to ex vivo perfusion and ischemia/reperfusion. **G.** Representative triphenyl-tetrazolium chloride (TTC)-stained heart slices collected at the end of reperfusion. Viable myocardium is stained red, while infarcted tissue appears pale. Infarct area was quantified using planimetric analysis in Fiji/ImageJ as described in the methods. **H.** Quantification of infarct size expressed as a percentage of total left ventricular volume, accounting for both the area and the weight of each slice. Infarct size was determined using planimetric analysis in Fiji/ImageJ. Data represent mean $\pm$ SEM for PBS ($n = 8$) and sodium arsenite ($n = 7$) groups. Statistical comparisons were performed using an unpaired two-tailed Student's t -test ($P < 0.01$).

Figure 9. Sodium arsenite-induced stress granule formation protects against Staurosporine-mediated cell death in cardiac myocytes.

(A) Schematic illustrating the treatment protocol established to assess the impact of sodium arsenite on cell viability. **(B)** Fluorescent micrographs depicting staining for the stress granule marker, G3BP1, in cells treated or untreated with 500 μ M sodium arsenite (SA). Scale bars are 20 μ M. **(C)** Representative phase-contrast images of H9c2 cells at the end of the treatment protocol. Cells were pretreated with or without sodium arsenite (500 μ M, 30 min) and then challenged with staurosporine (10 μ M) or vehicle (0.1% DMSO) for 1 h. Nuclei were counterstained with Hoechst and images were acquired on an EVOS imaging system equipped with a 20 $\times$ phase-contrast objective. Cell rounding, assessed by phase-contrast microscopy, was quantified as a morphological marker of acute cell death and normalized to total Hoechst-positive nuclei to provide an index of viability. **(D)** Quantification of cell death expressed as the fraction of rounded up cells normalized to the total number of cells (detected by Hoechst-positive nuclear staining). Data are presented as mean $\pm$ SEM. $n = 9$ for all treatment groups. Comparisons across multiple groups resulting from different drug treatments were performed using two-way ANOVA followed by Tukey's post-hoc test. **** $P < 0.0001$.

Figure 10. The cytoprotective effect of sodium arsenite is attenuated by genetically depleting the stress granule forming genes G3BP1/2.

(A) Representative immunofluorescence showing reduced formation of stress granules following siRNA-mediated knockdown of G3BP1 and G3BP2, compared with non-targeting control (NTC). Cells were transfected with siRNA (20 nM, Lipofectamine RNAiMAX) and 48 h later were exposed to sodium arsenite (500 μ M, 30 min) to induce the formation of stress granules. After stress exposure, cells were washed and fixed in 4% paraformaldehyde and subsequently processed for

immunocytochemistry for stress granule markers G3BP1 and TIA1. Nuclei were counterstained with Hoechst. Scale bar: 20 μ m. **(B)** Representative phase-contrast images of cells subjected to sodium arsenite pretreatment (500 μ M, 30 min) followed by either staurosporine challenge (10 μ M, 1 h) or vehicle control (0.1% DMSO). Cell death was assessed as morphological rounding, normalized to Hoechst-positive nuclei. Scale bar: 20 μ m. **(C)** Quantification of cells with rounded morphology in NTC or G3BP1/2-KD cells and across the different treatment conditions. Cells were seeded in 8-chamber slides and transfected with NTC or G3BP1/2 siRNAs (20 nM for each). 48 hours later cells were exposed to staurosporine challenge with or without prior sodium arsenite pretreatment. Comparisons across multiple groups resulting from genetic knockdown and drug treatments were performed using two-way ANOVA with Tukey's post-hoc test. * $P < 0.05$, ns: not significant, n = 9 replicates.

Figure 11. OGT depletion attenuates sodium arsenite-mediated protection against staurosporine-induced cell death.

(A) Representative immunofluorescence showing reduced O-GlcNAc (green) and OGT (red) signal following siRNA-mediated knockdown of OGT, compared with non-targeting control (NTC). Cells were transfected with siRNA (20 nM) using Lipofectamine RNAiMAX, and fixed 48 h after transfection for immunocytochemistry. Nuclei were counterstained with Hoechst. **(B)** Western blot analysis in NTC siRNA-, or OGT siRNA-transfected cells to assess for OGT protein levels and overall changes in O-GlcNAcylation. Similar to A, cells were transfected with 20 nM siRNAs and processed for analysis 48 hours later. **(C)** Representative phase-contrast images of cells subjected to sodium arsenite pretreatment (500 μ M, 30 min) and then exposed to staurosporine challenge (10 μ M, 1 h) followed by imaging on the EVOS system. Cell death was assessed as morphological rounding normalized to Hoechst-positive nuclei. Scale bar: 20 μ m. **(D)** Quantification of cell death fraction in NTC or OGT-KD cells and across the different treatment conditions. Cells seeded in 8-chamber slides were transfected with NTC or OGT siRNAs and 48 hours later were exposed to staurosporine challenge with or without prior sodium arsenite pretreatment. Statistical analysis was performed using two-way ANOVA with Tukey's post hoc test *** $P < 0.001$, ns: not significant, n = 9 replicates.

Loss of OGT activity impairs the dynamic formation of protective stress granules in ischemic cardiomyocytes

Hannah Swimm, Wenxi Zhang, Yashas Mallikarjun, Rohit Satish, Hisham Qureshi, Aidan Dunphy, Deepthi Ashok, D. Brian Foster, Brian O'Rourke, Natasha E. Zachara & Kyriakos N. Papanicolaou

Main figures

Figures 1-11

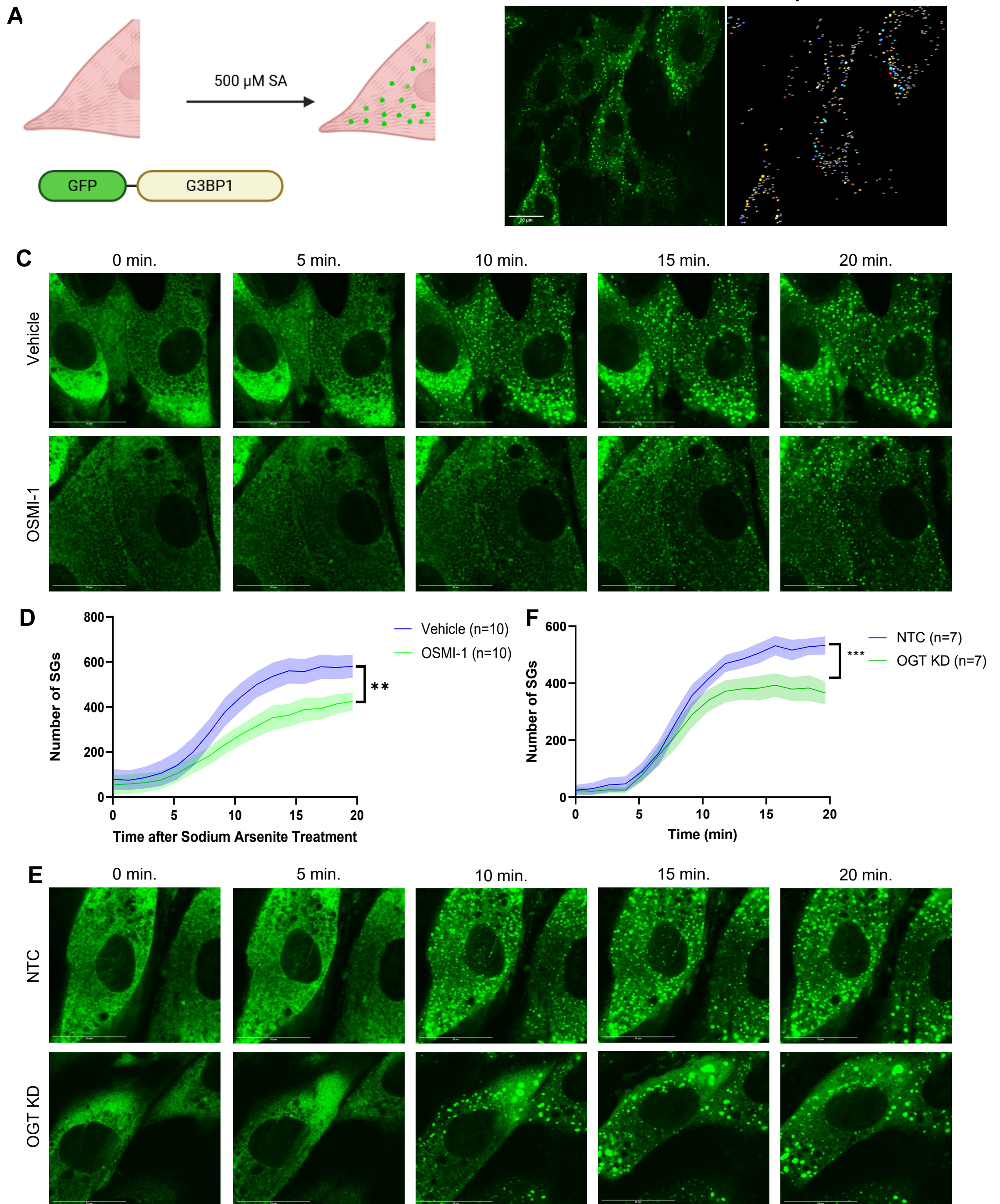
Figure 1

Figure 2

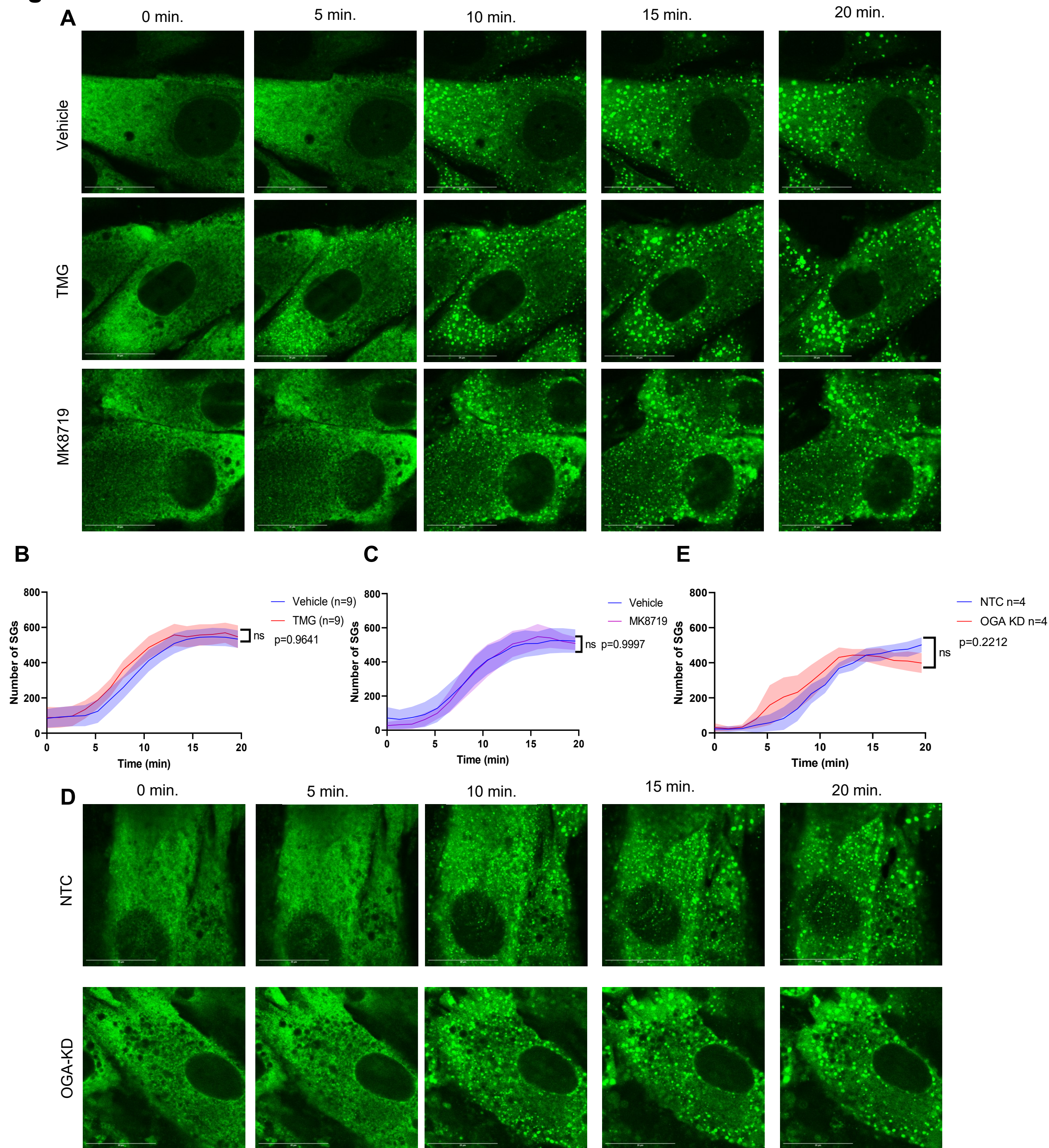


Figure 3

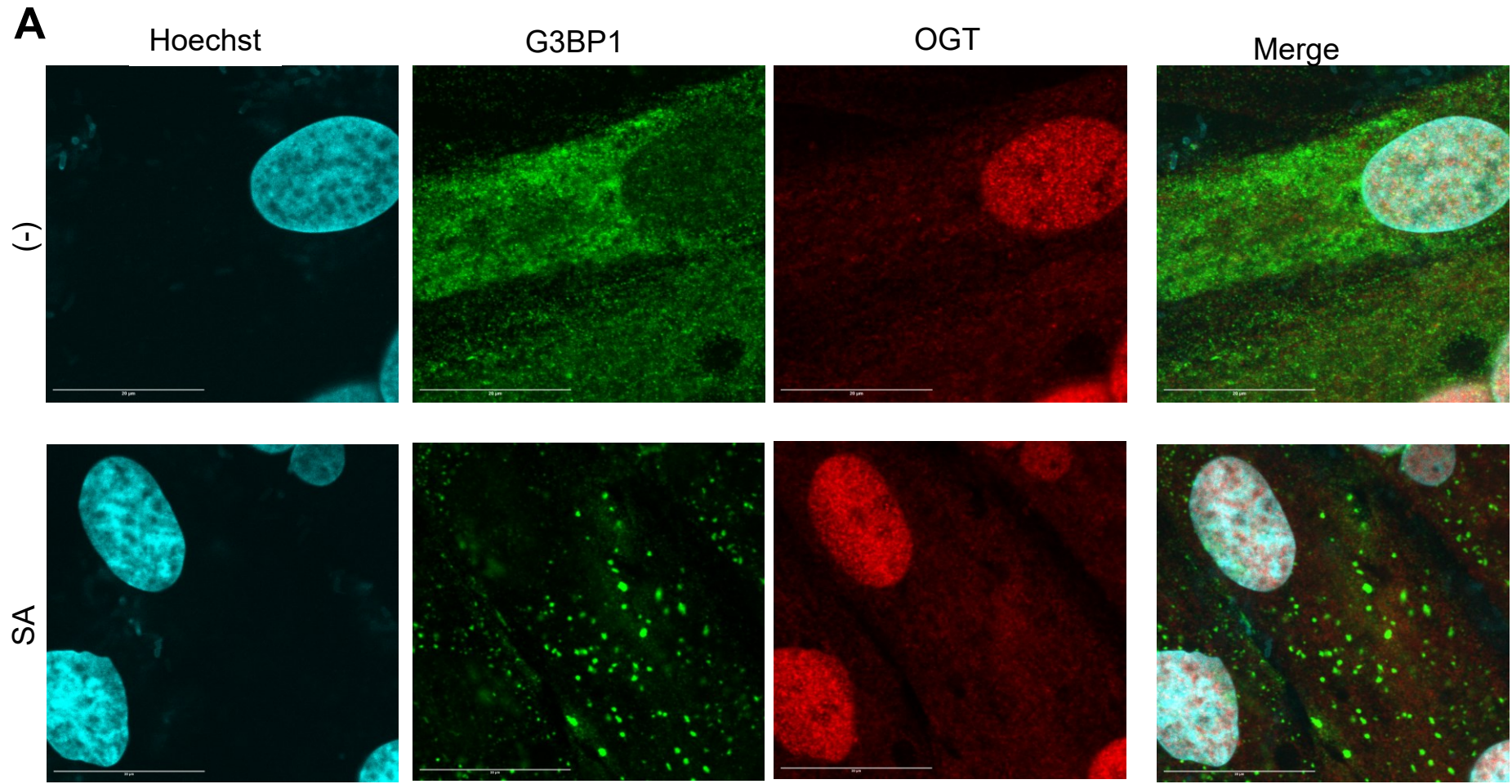
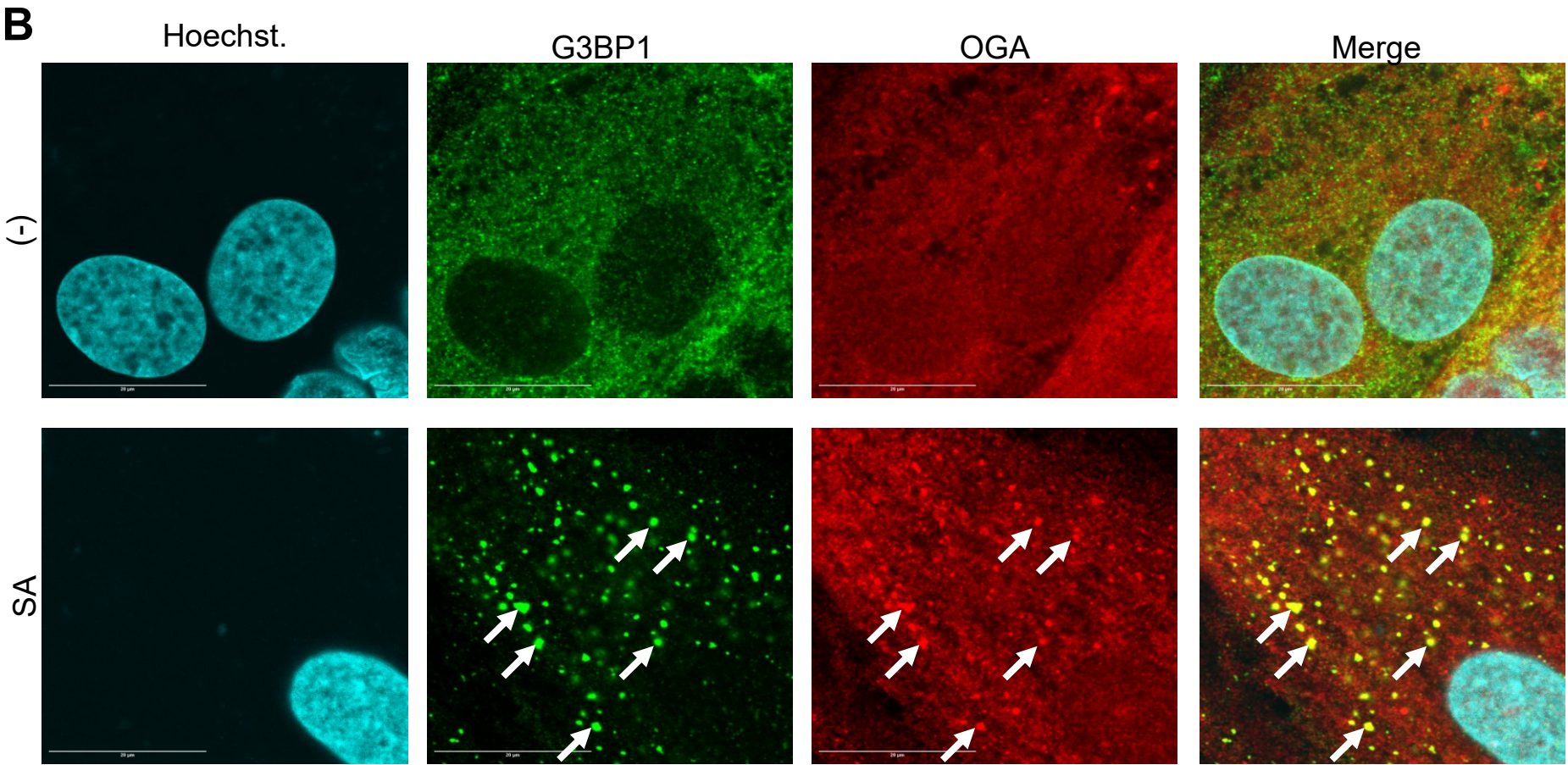
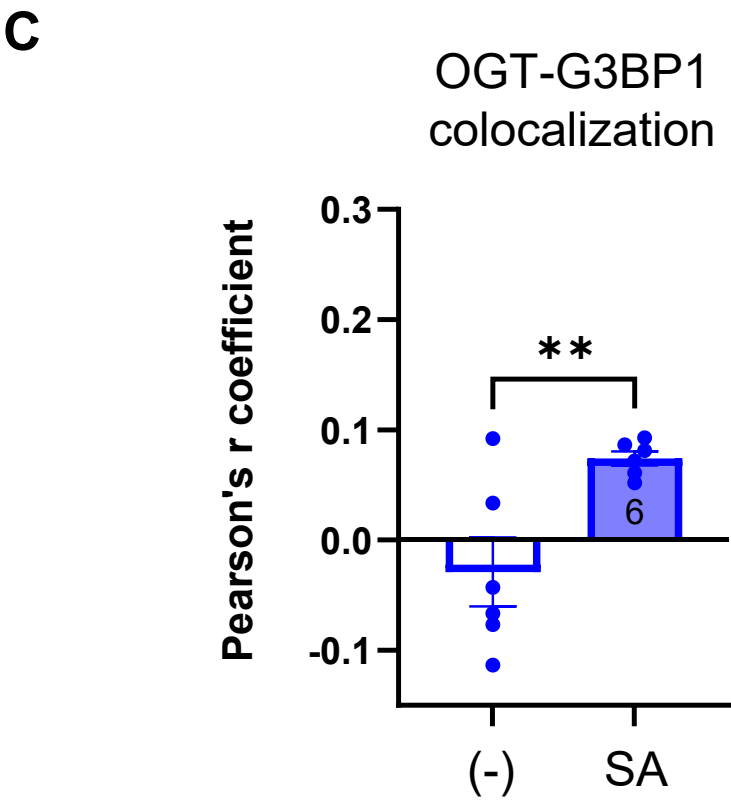
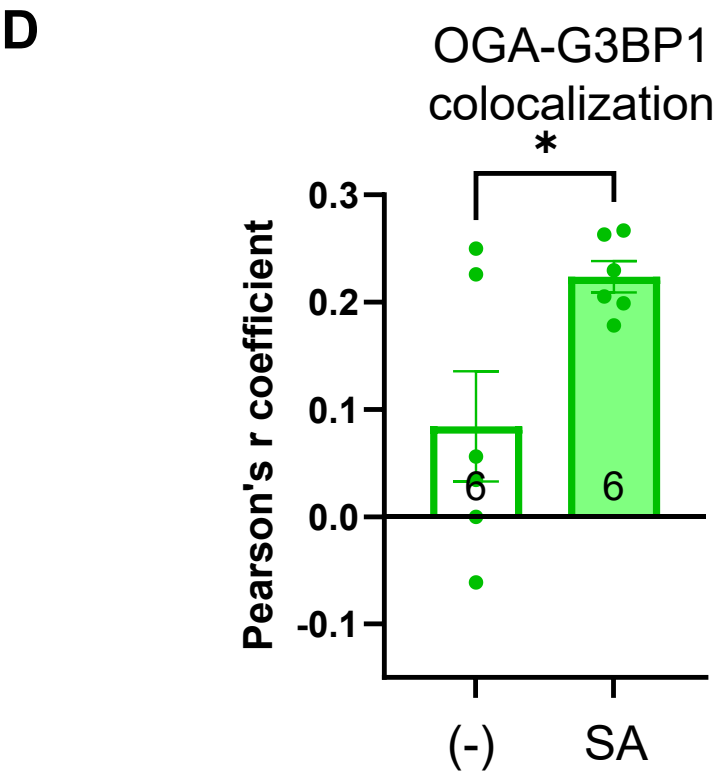


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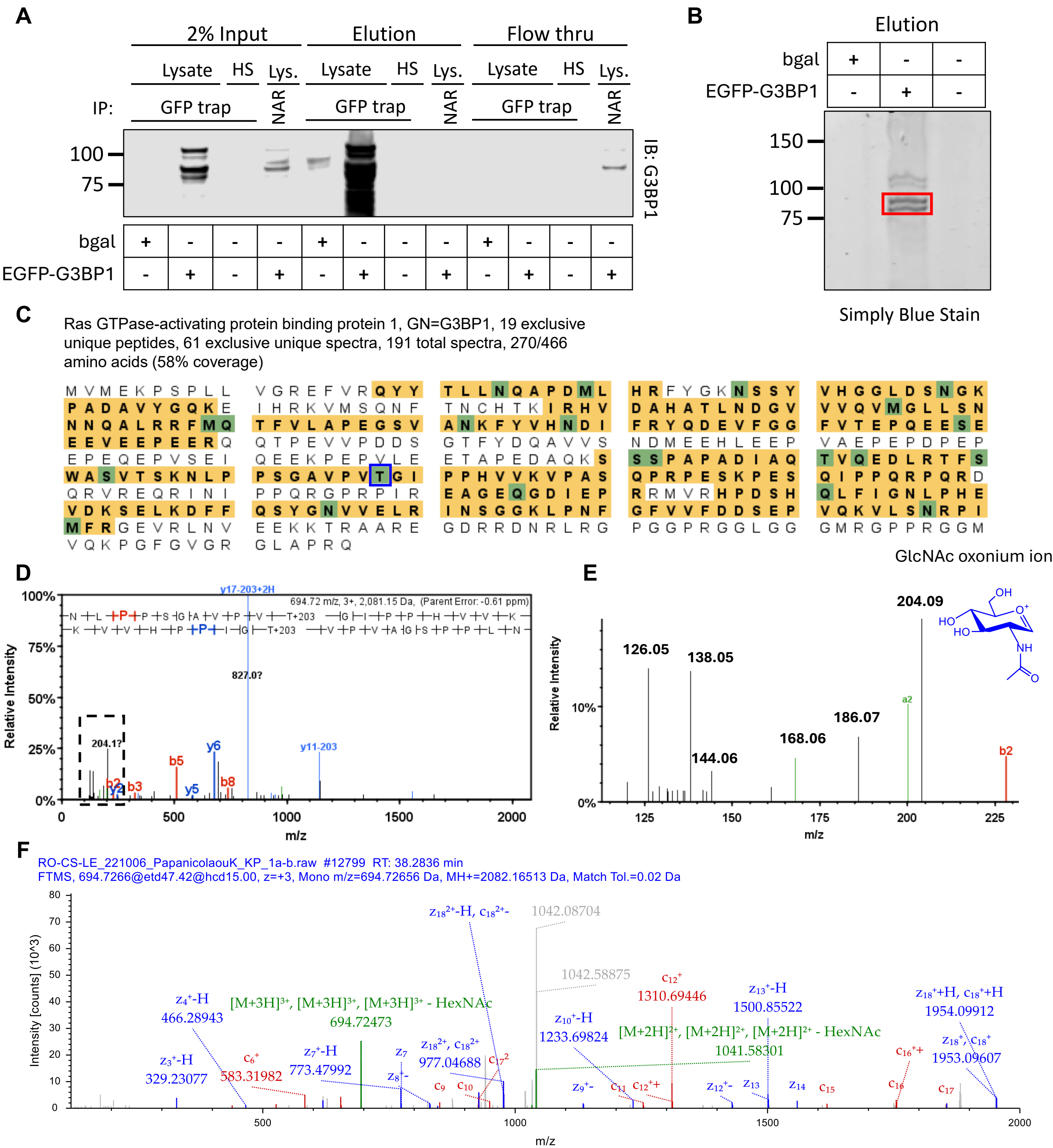


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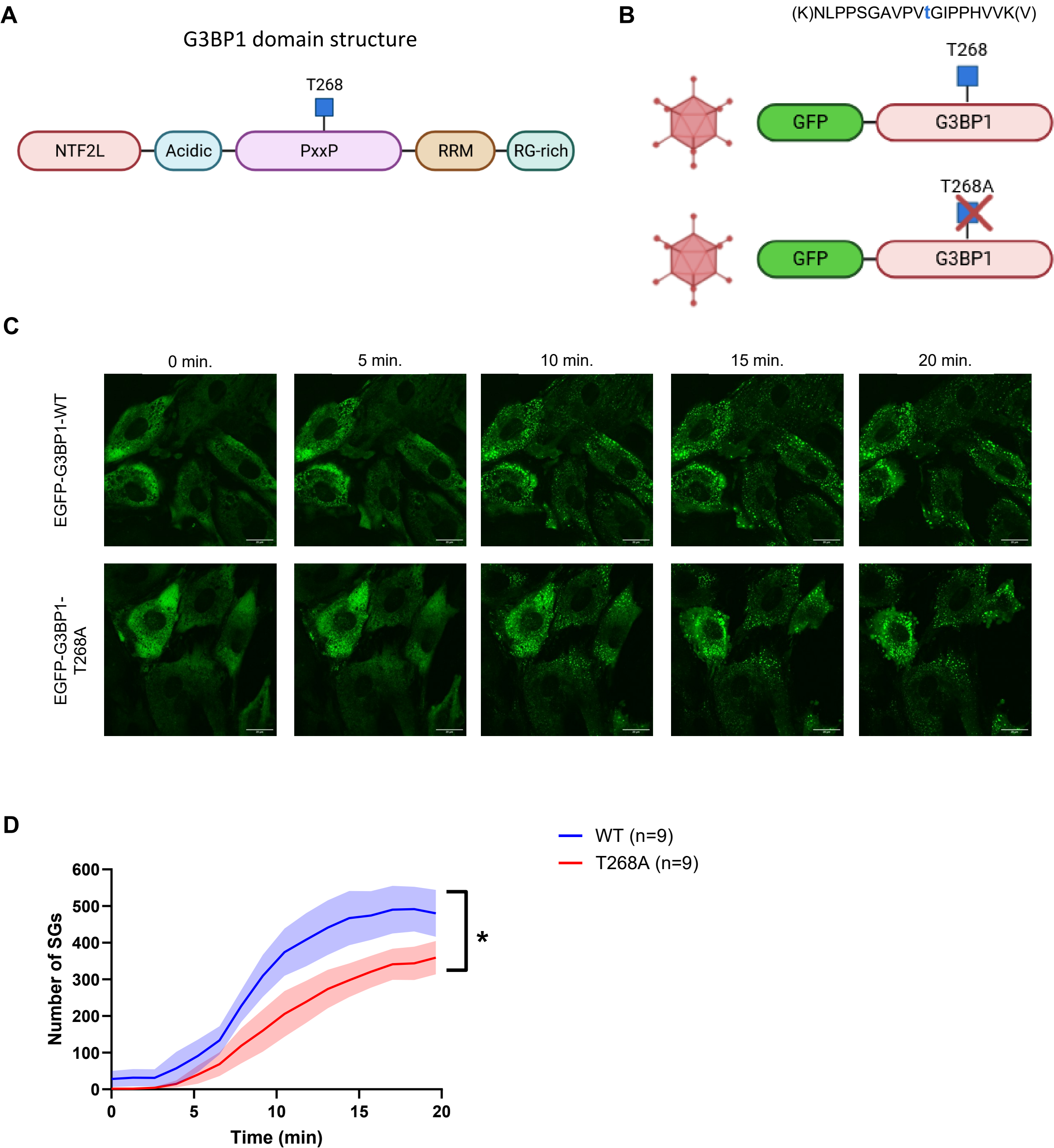
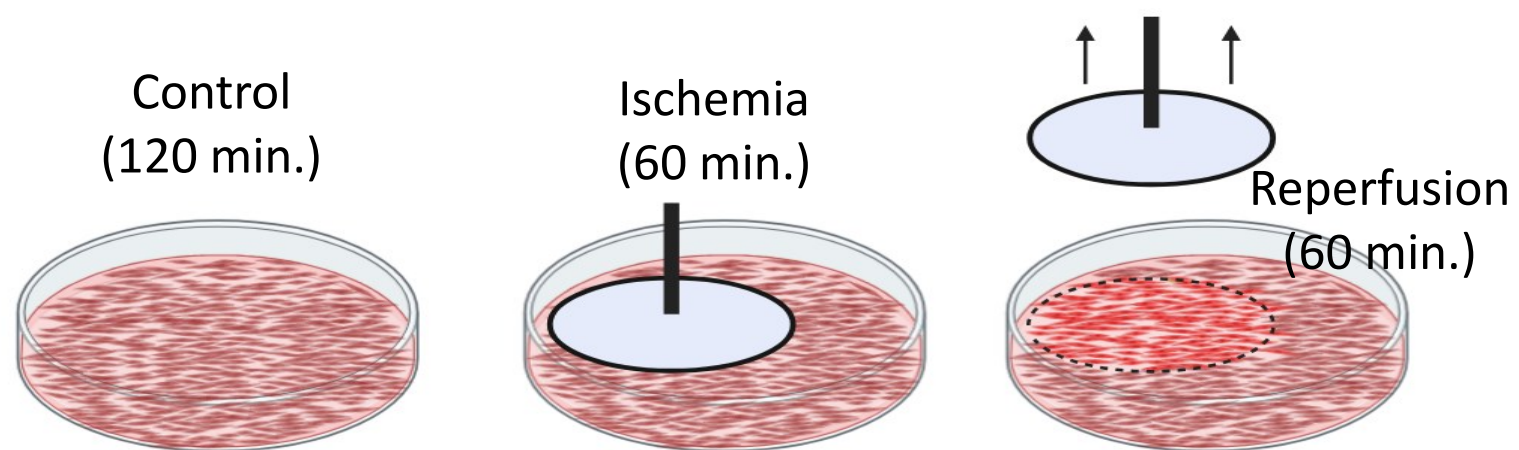
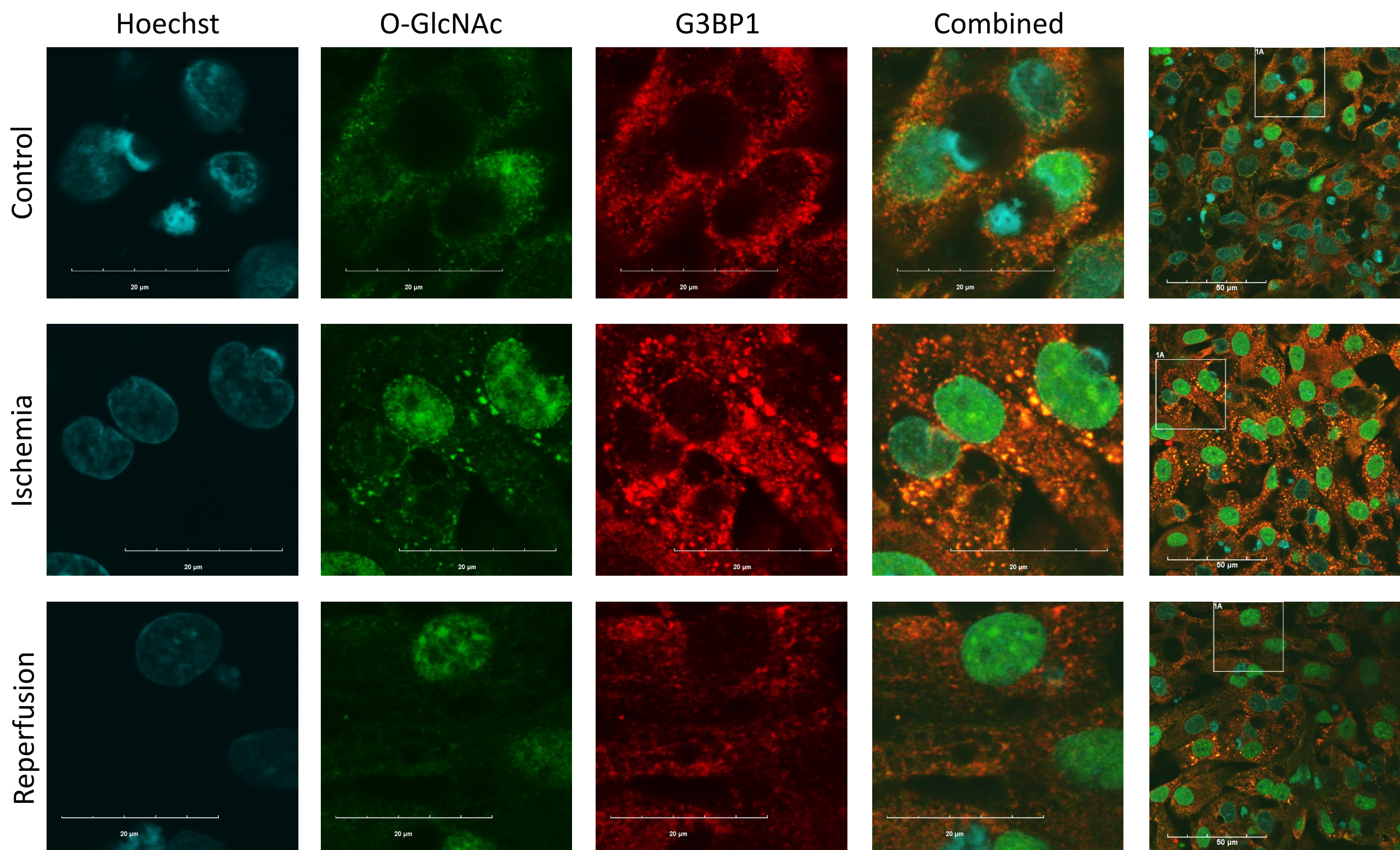


Figure 6

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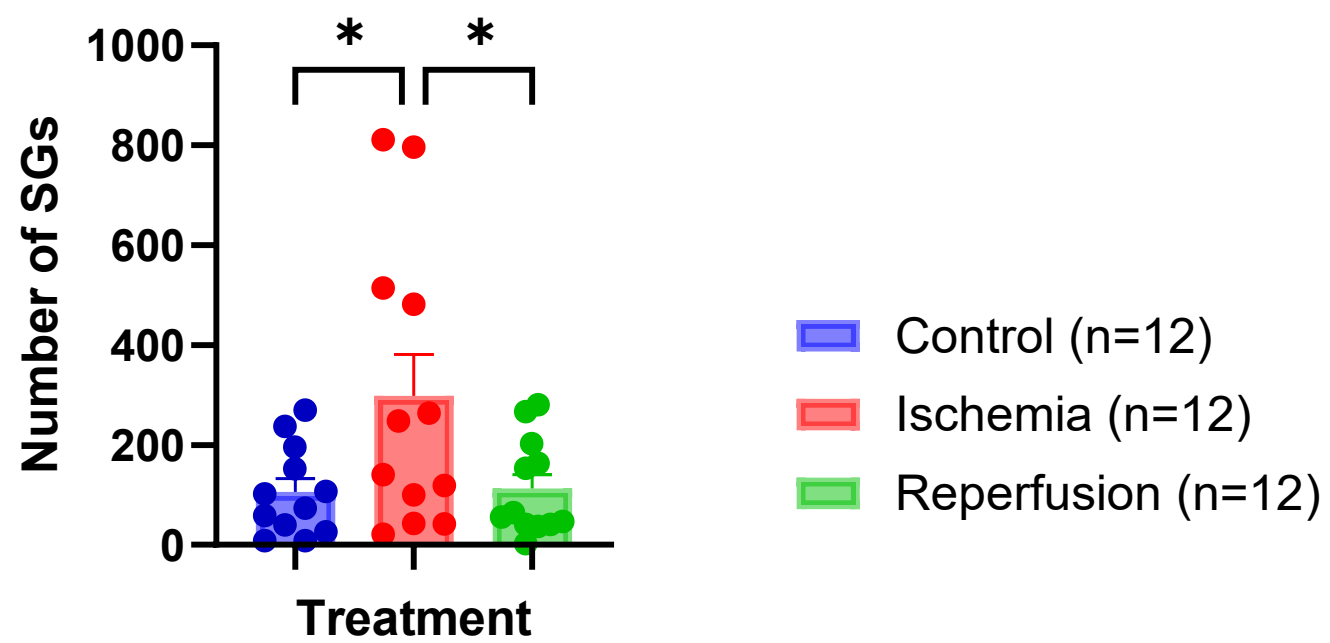
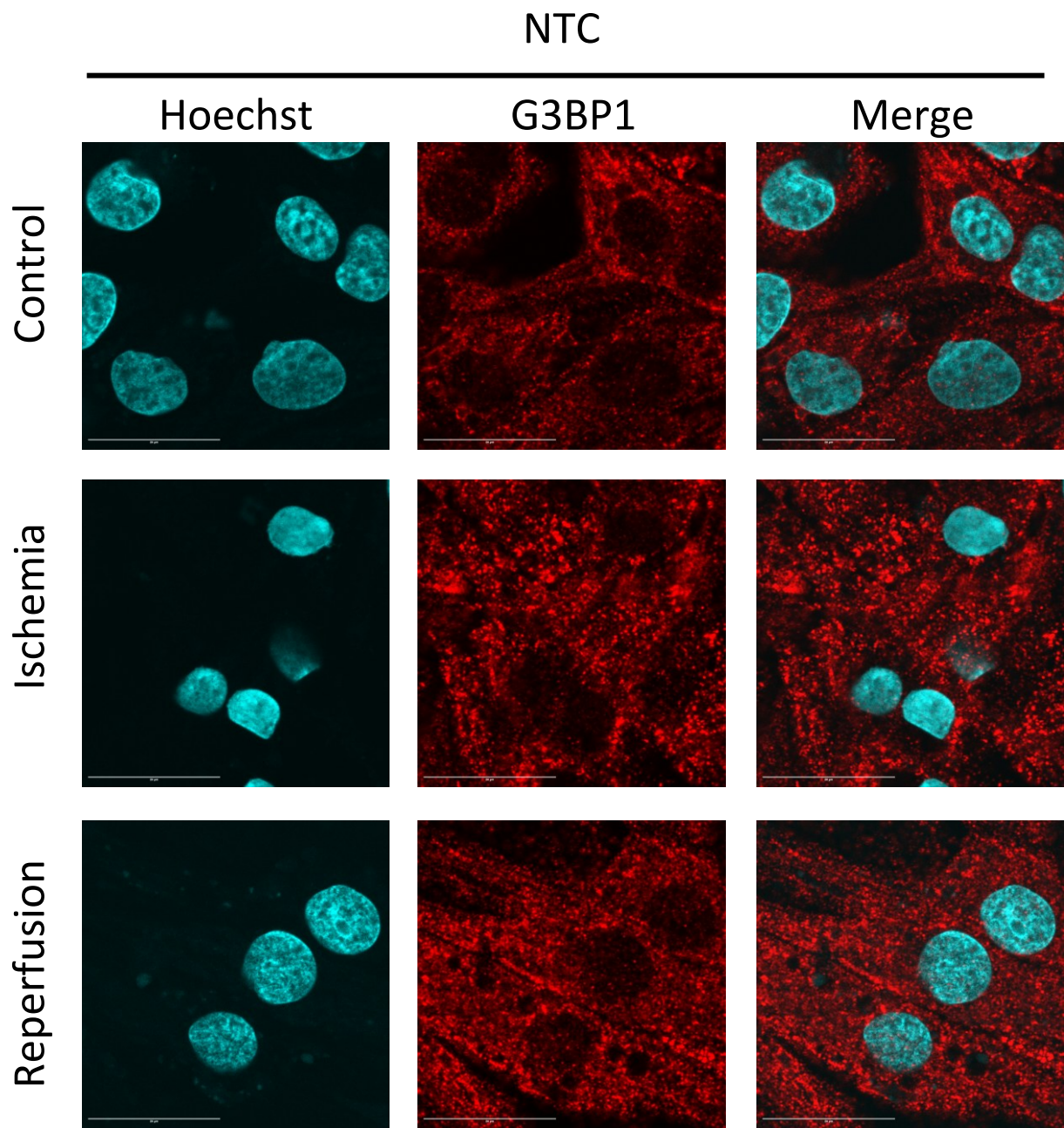
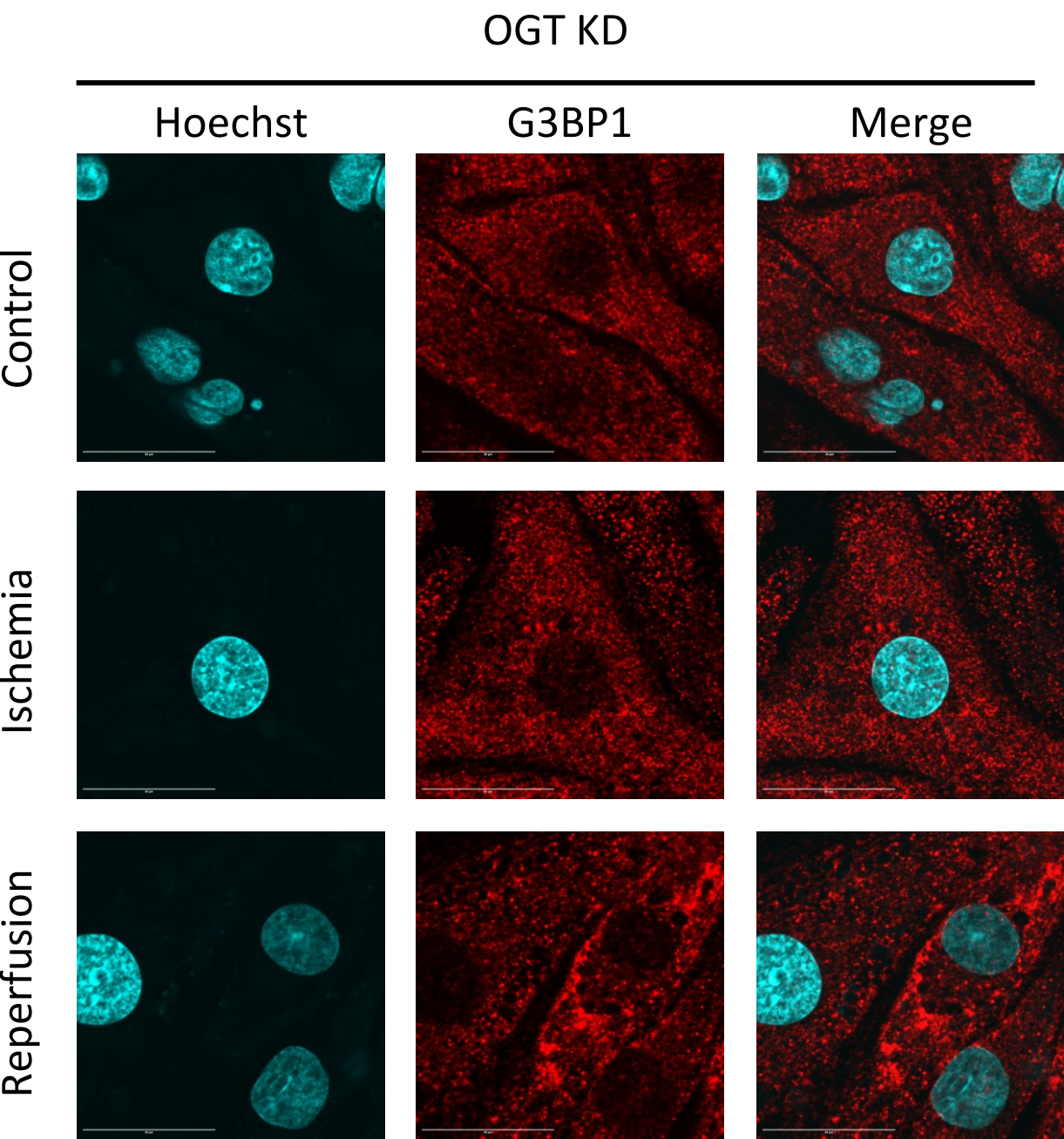


Figure 7

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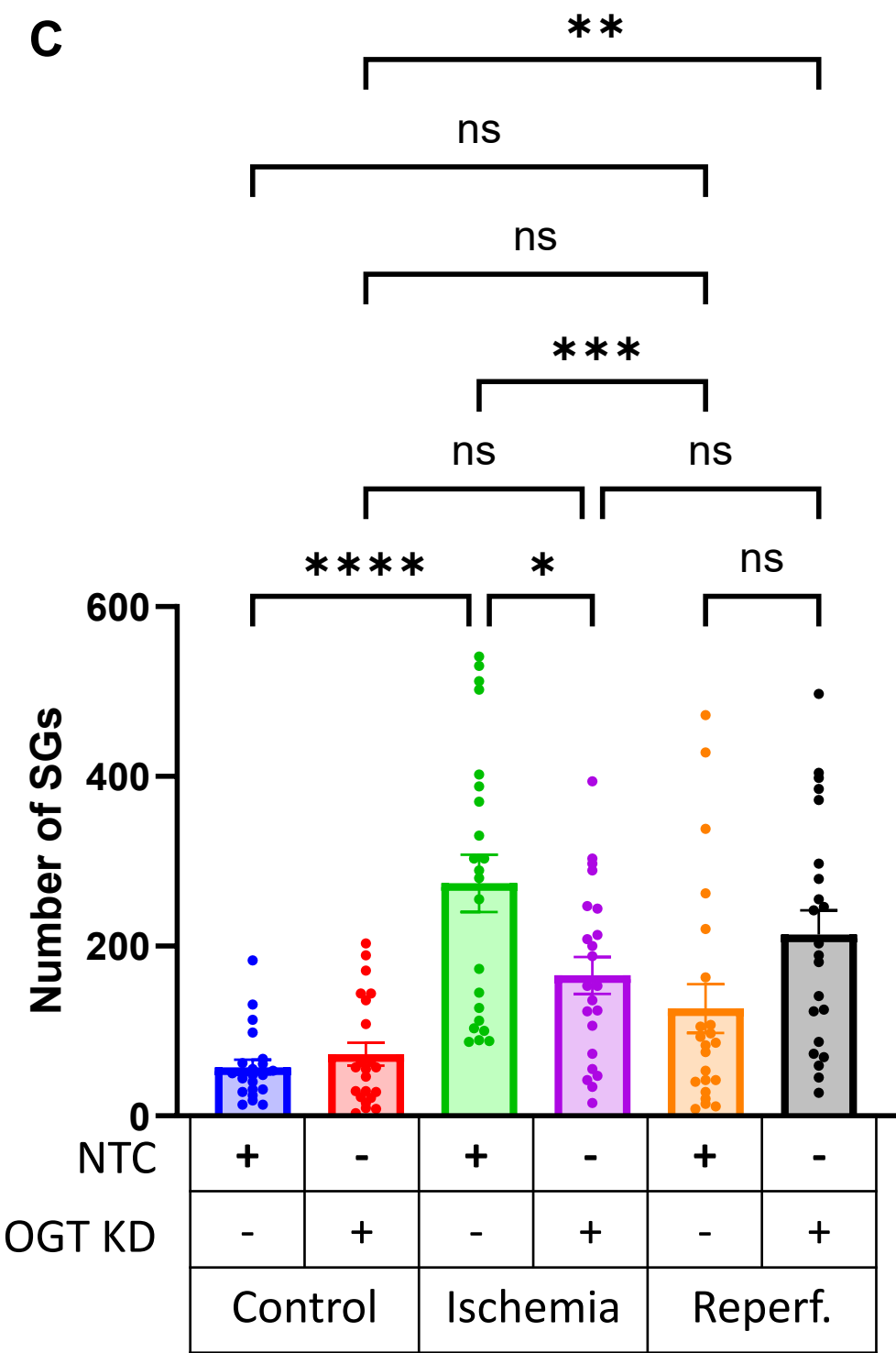
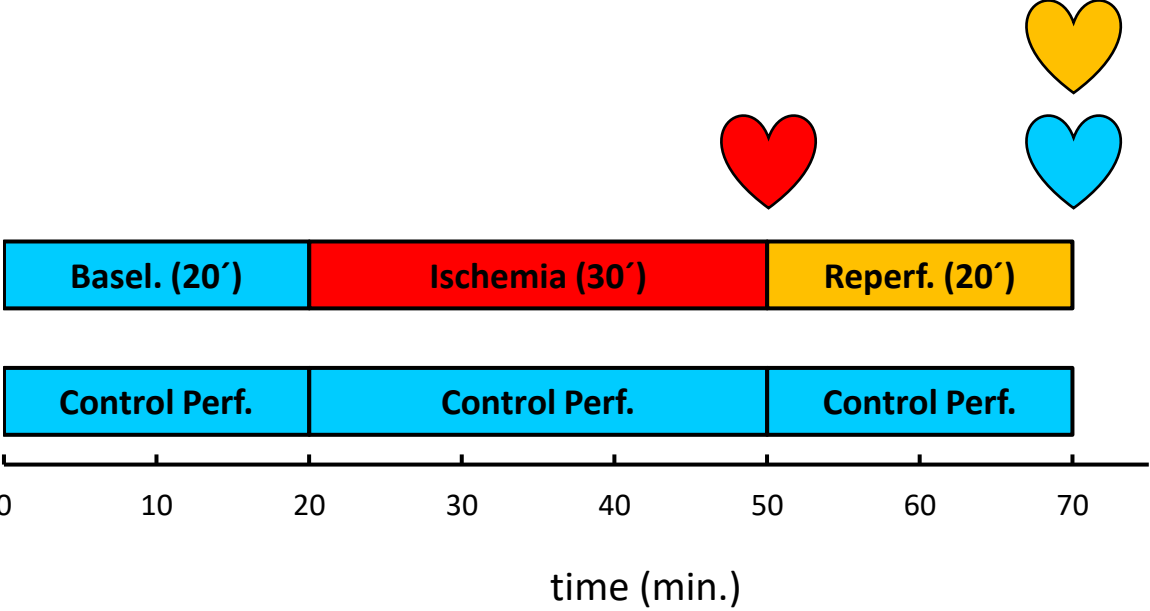
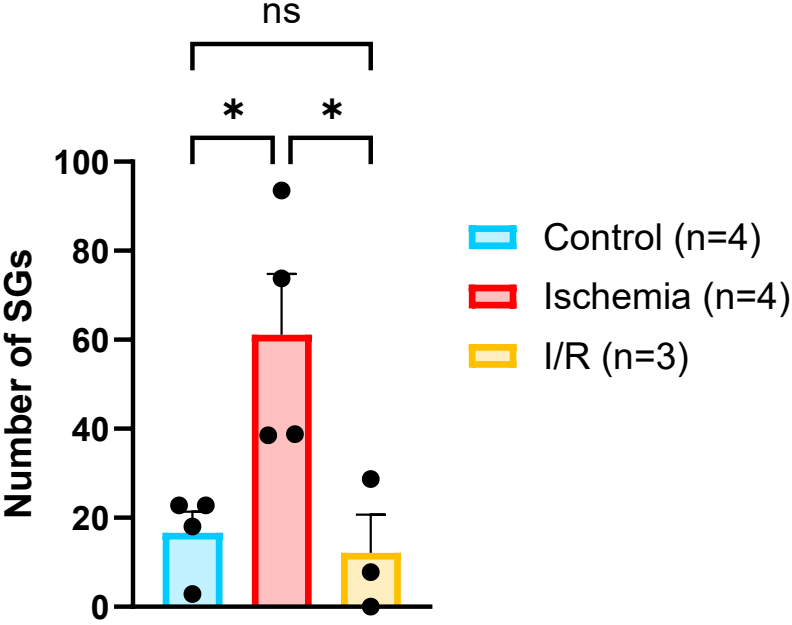


Figure 8

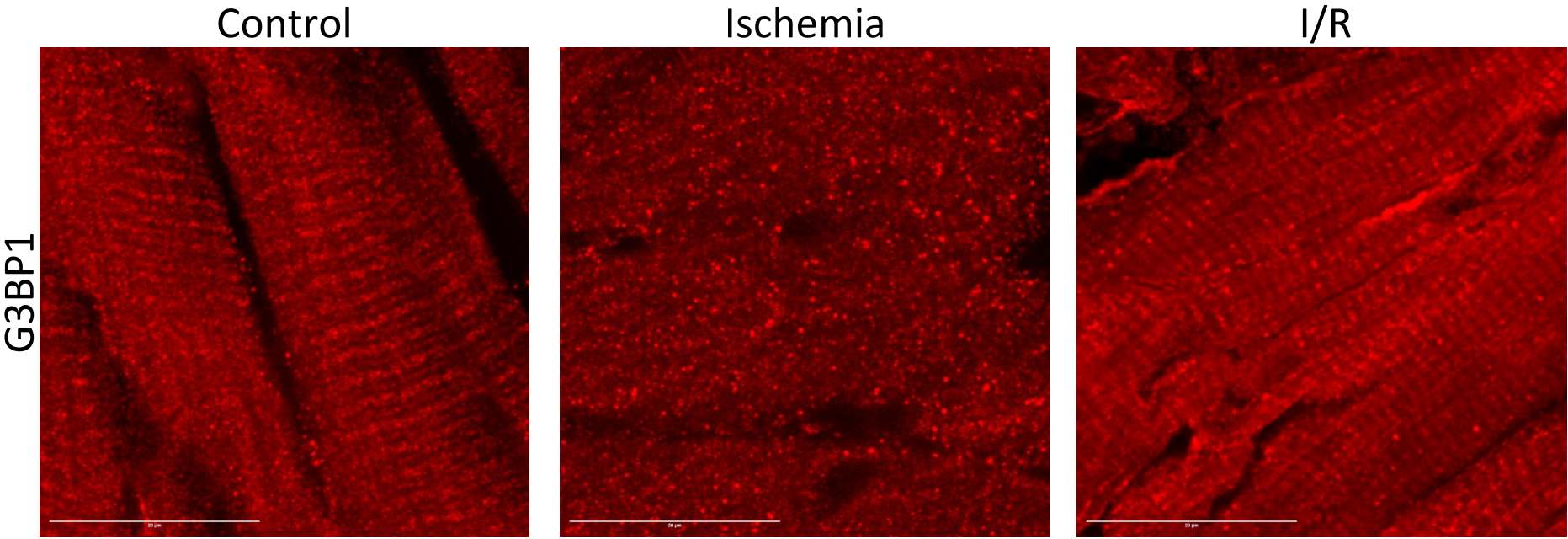
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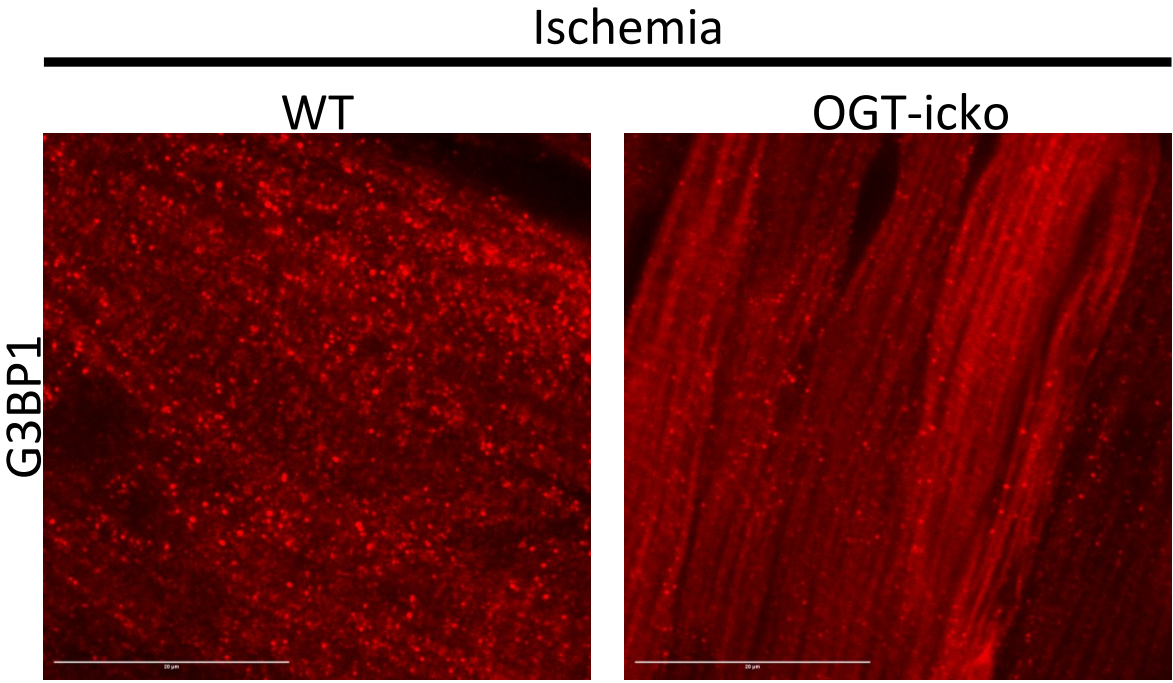
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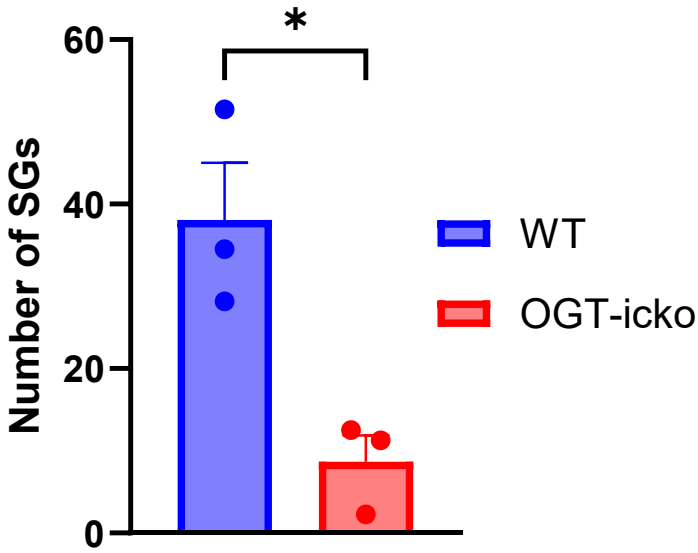
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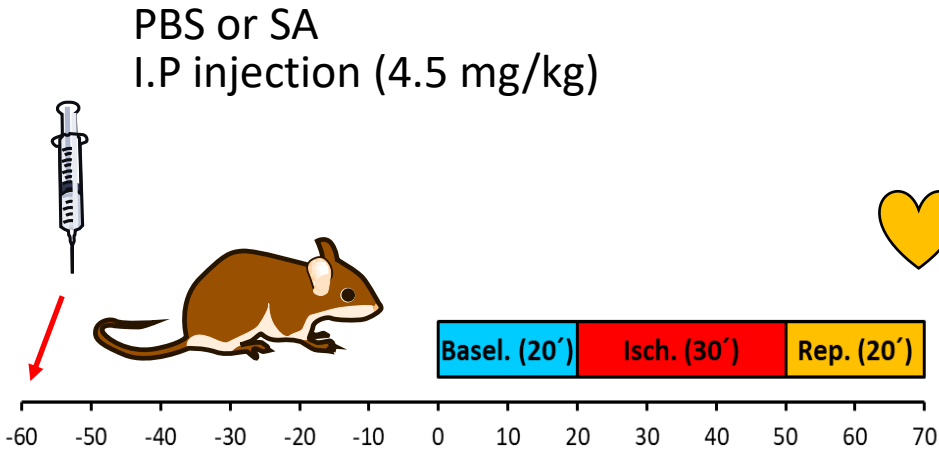
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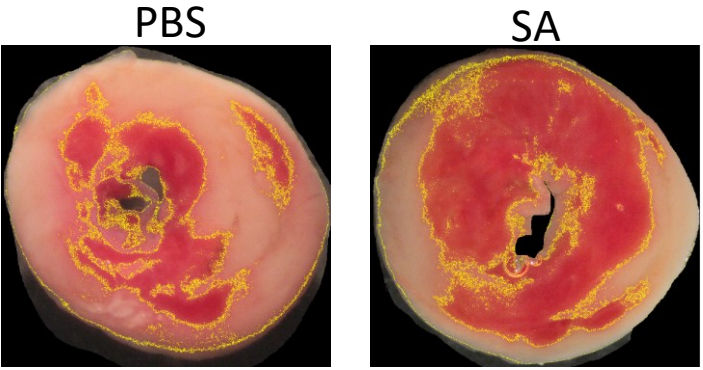
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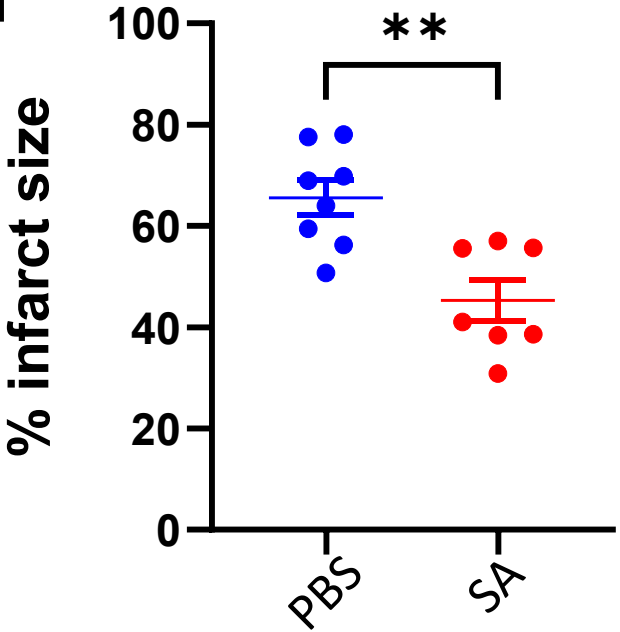


Figure 9

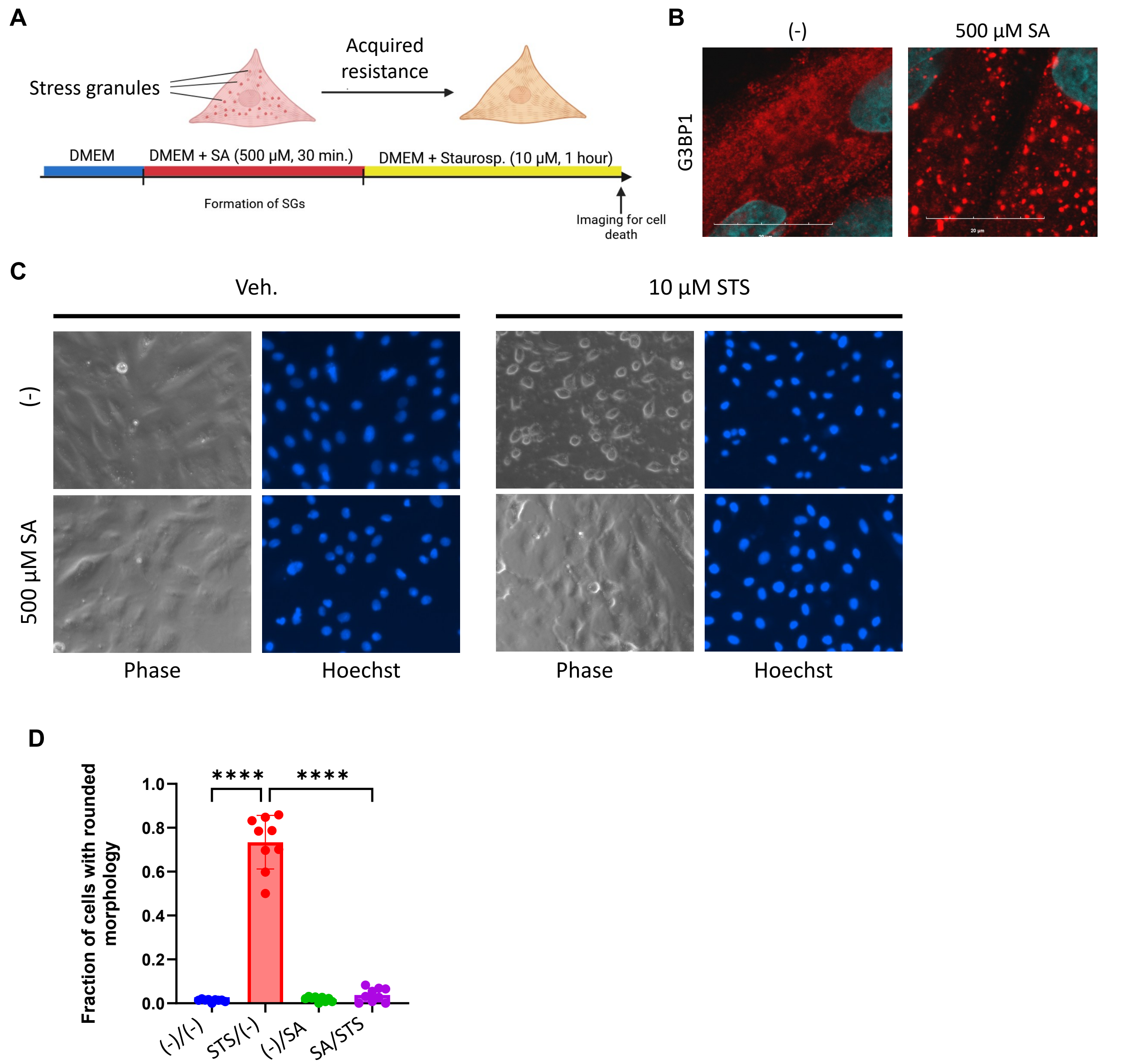


Figure 10

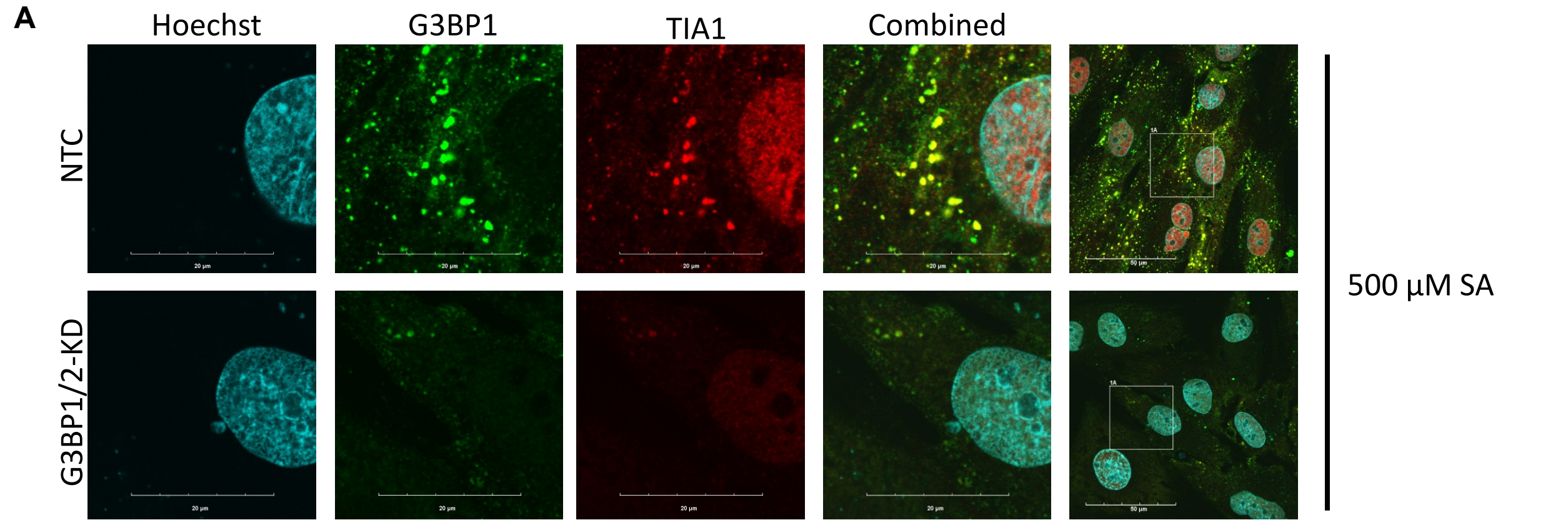
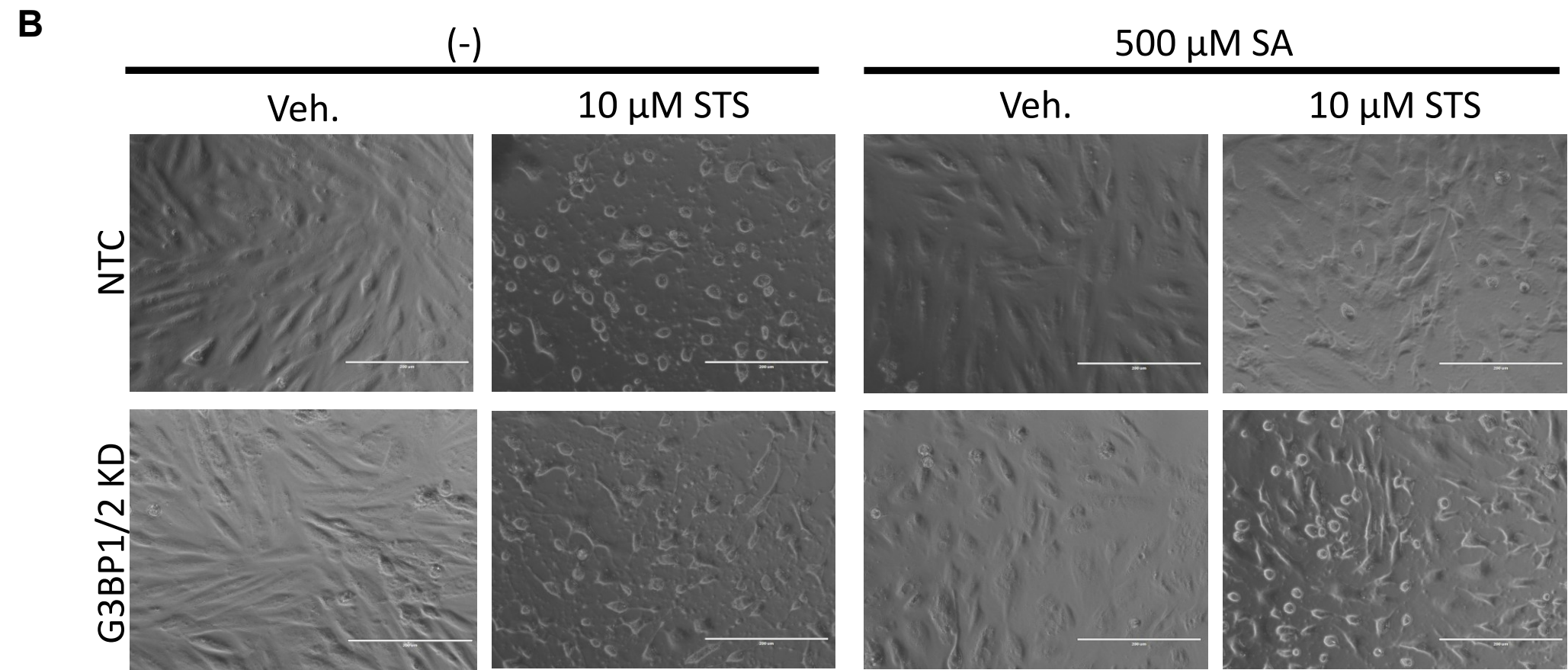
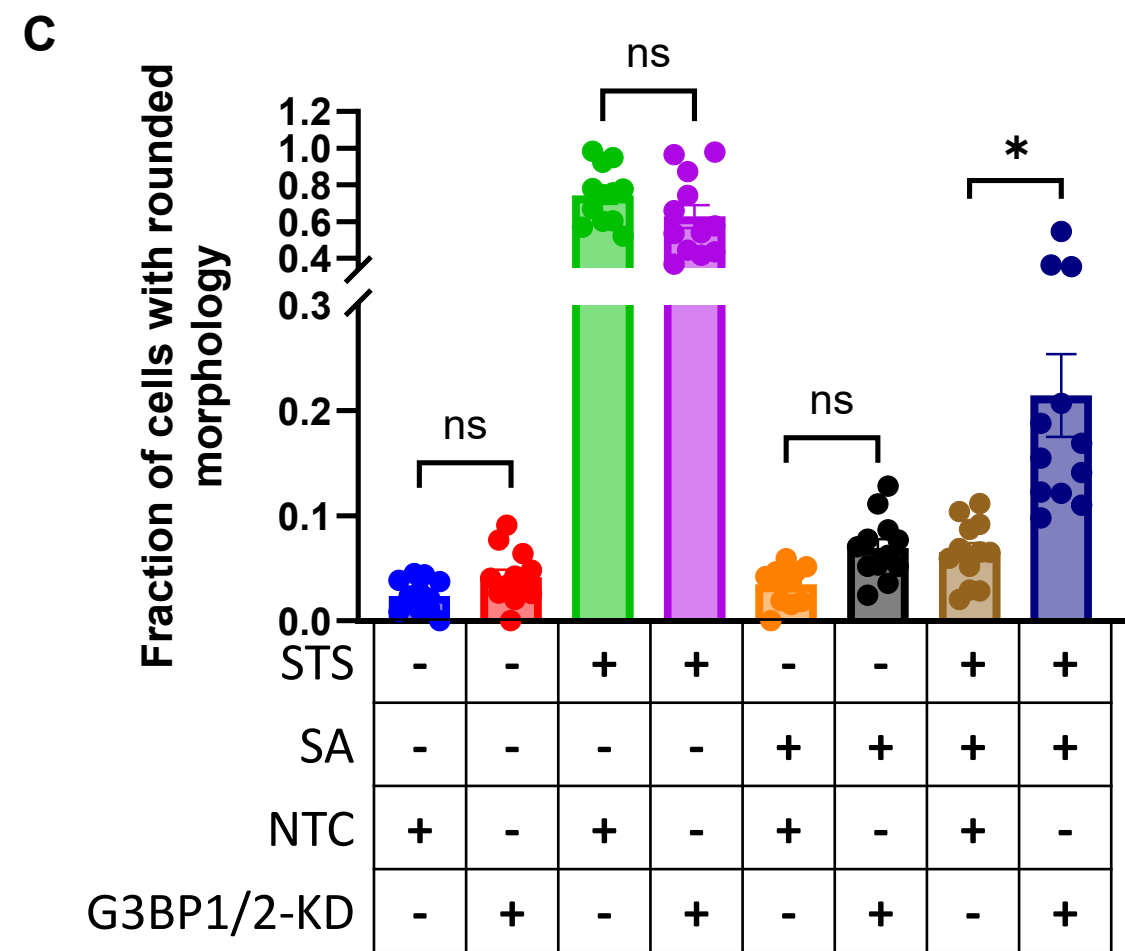
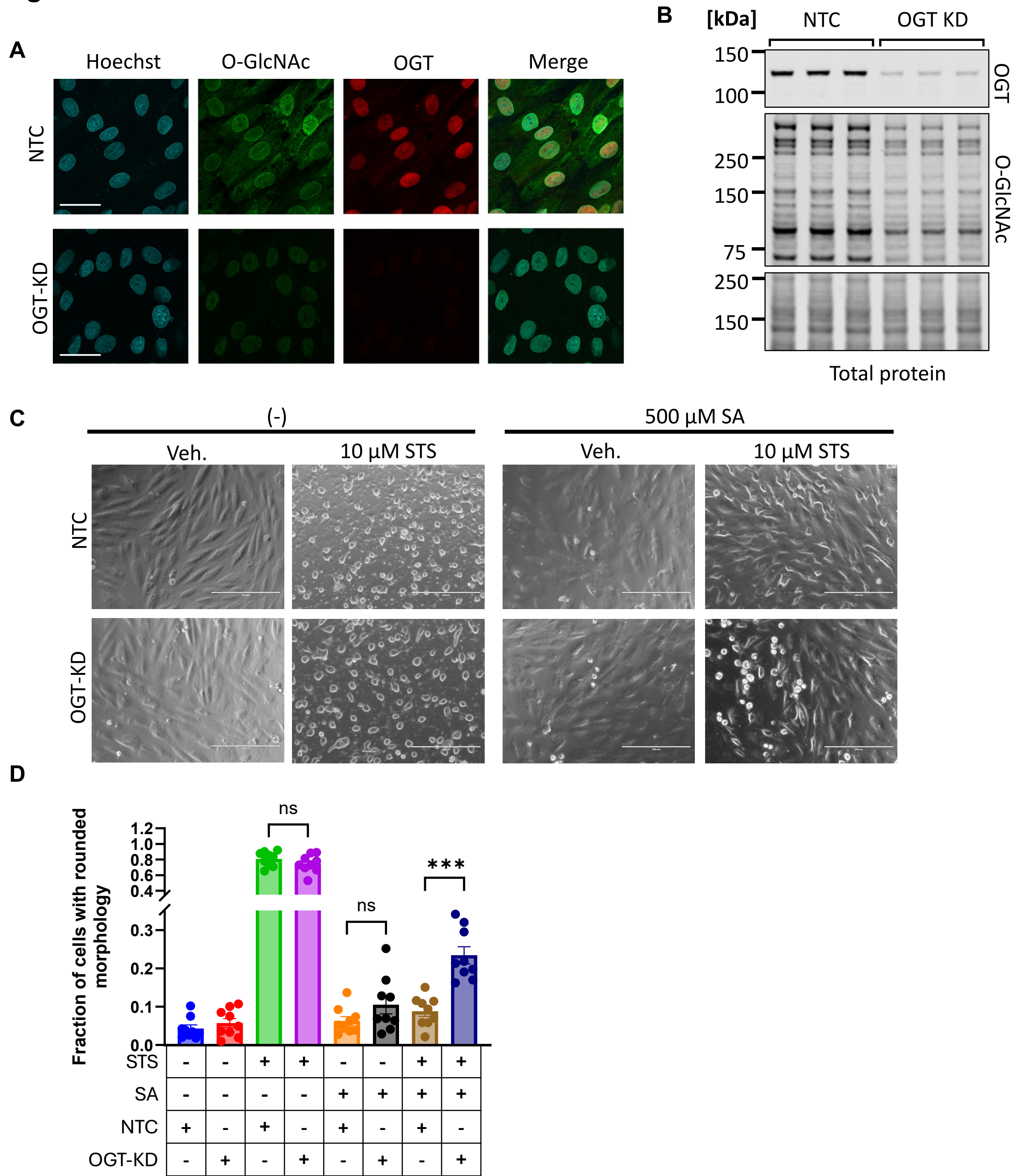


Figure 11



**Loss of OGT activity impairs the dynamic
formation of protective stress granules in
ischemic cardiomyocytes**

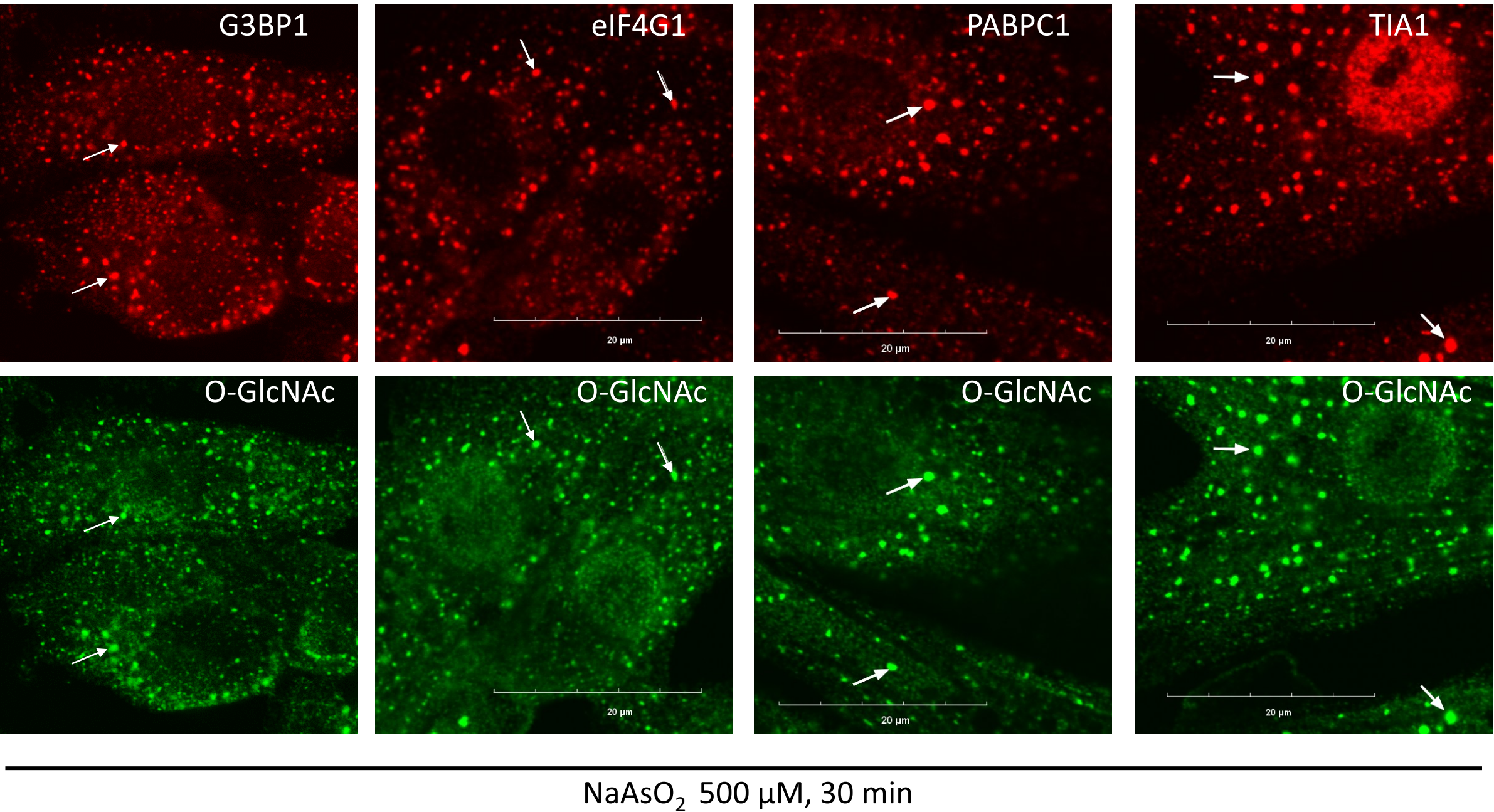
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O'Rourke, Natasha E. Zachara & Kyriakos N.
Papanicolaou

Supplemental material

[Supplemental figures 1-3](#)

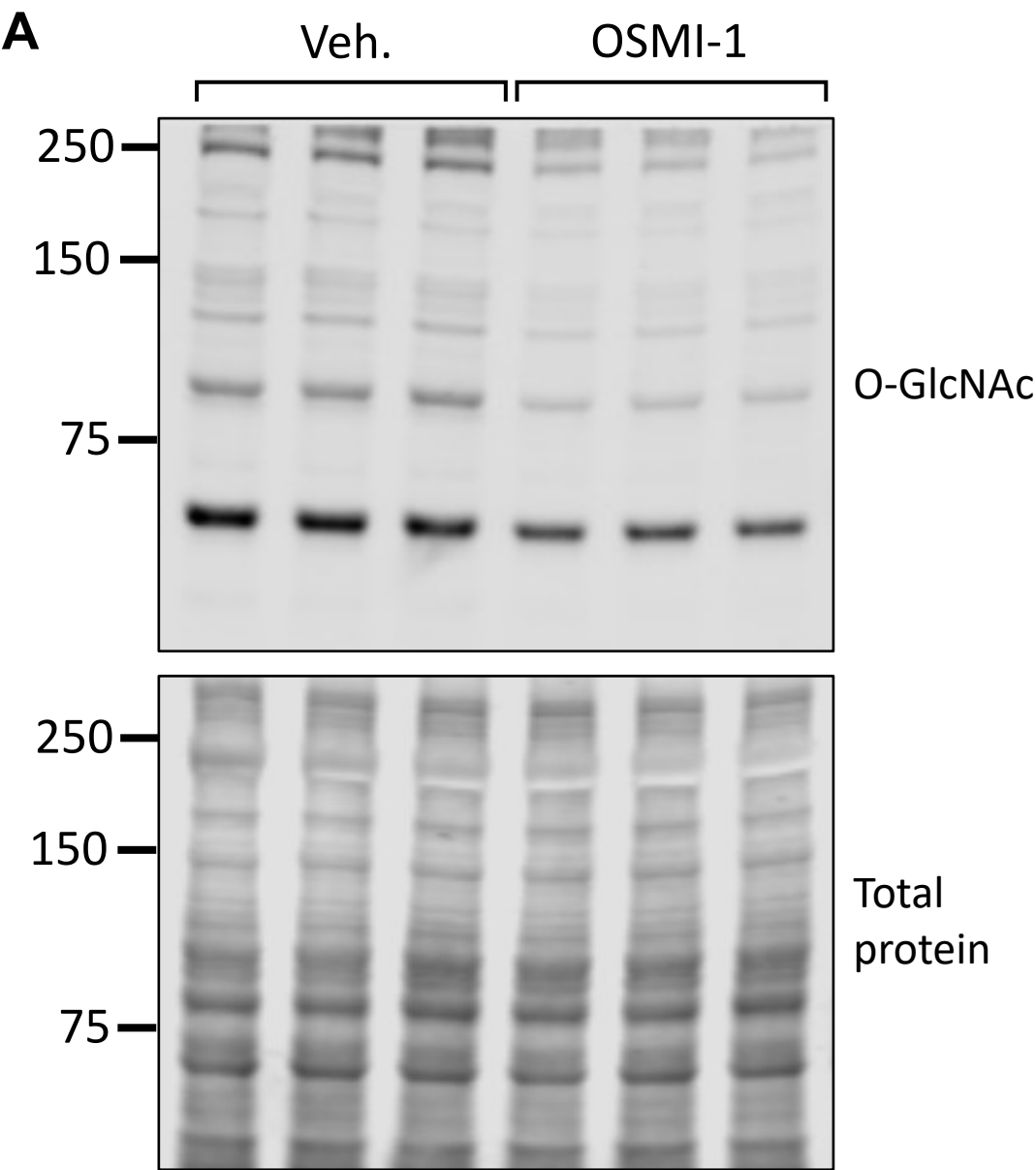
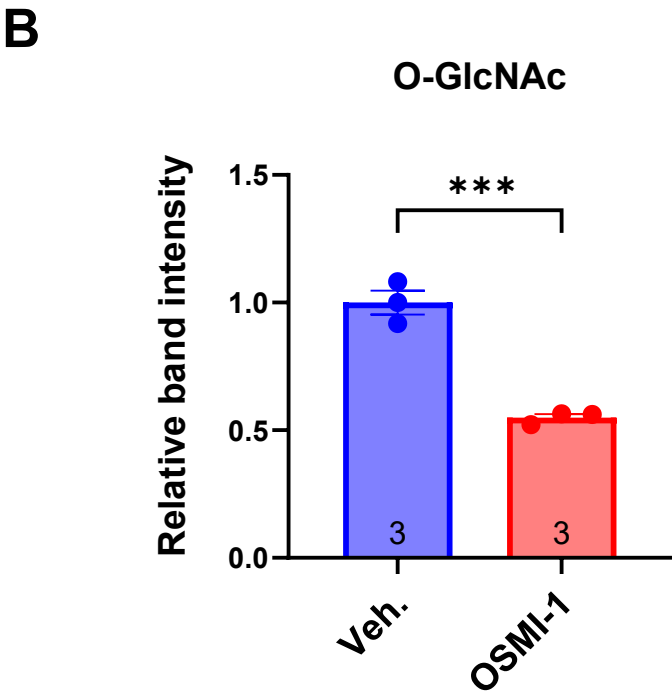
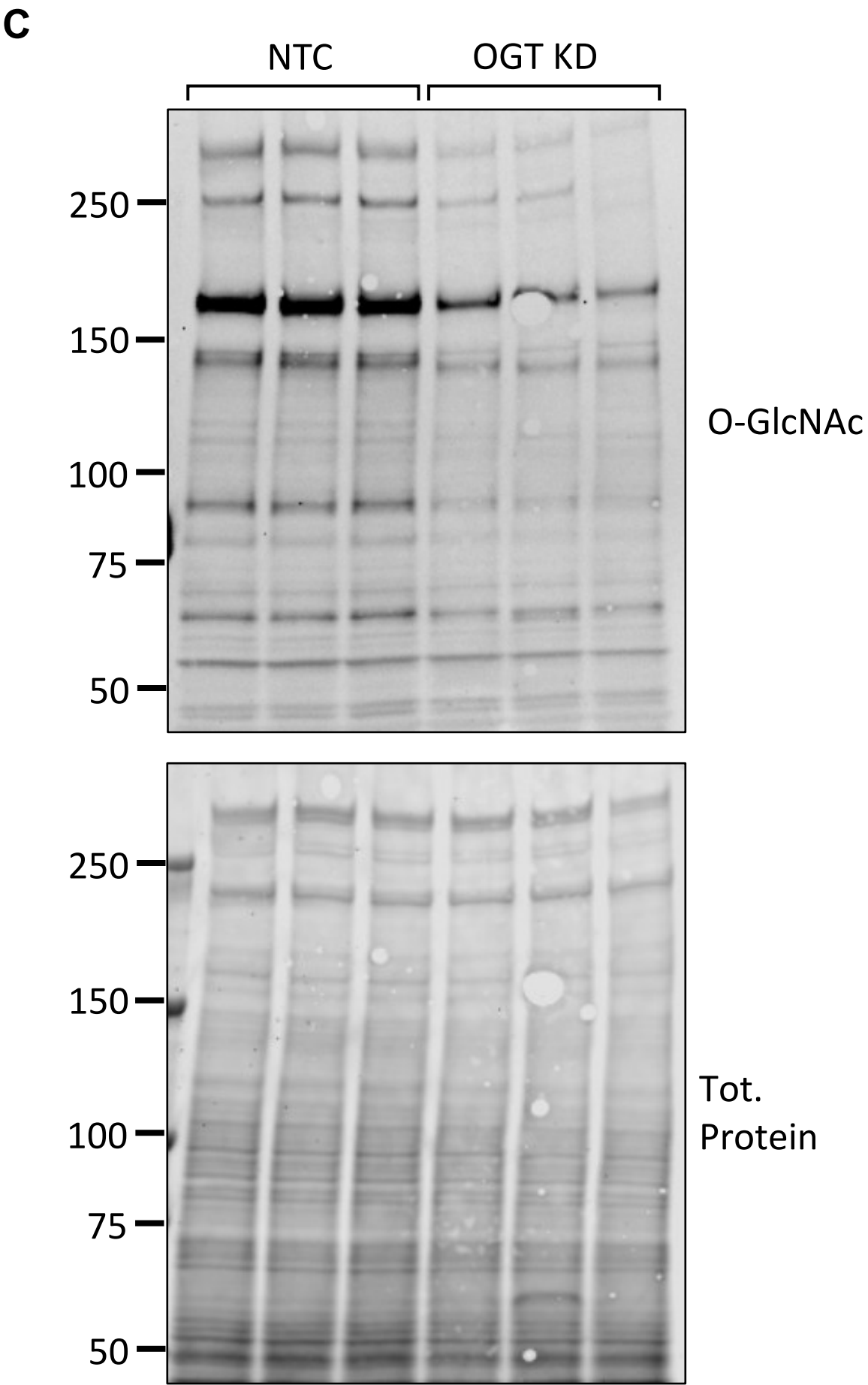
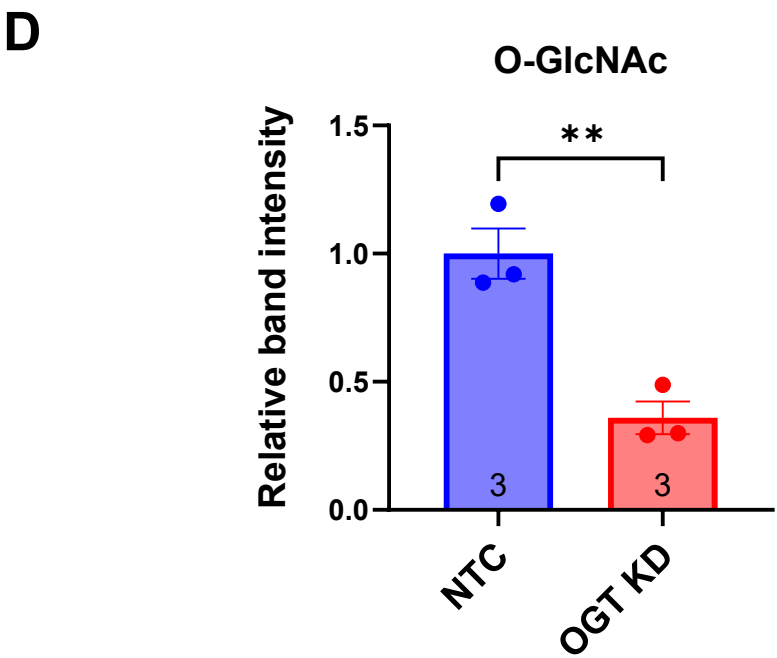
[\(Supplemental table 3\)](#)

Suppl. Figure 1



Supplemental Figure 1. Immunocytochemistry of stress granule markers and O-GlcNAcylation in cardiomyocytes. Primary neonatal ventricular myocytes (NRVMs) were seeded in ibidi chamber slides (2.5×10^5 cells/ chamber). To induce stress granule formation, cells were exposed to 500 μ M and 30 min. later were fixed (4% pre-warmed paraformaldehyde in PBS), permeabilized and processed for dual immunocytochemistry for one of the following stress granule markers G3BP1, eIF4G1, PABPC1 and TIA1 (all raised in rabbit and used at a dilution 200:1) in combination with RL2 O-GlcNAc antibody (raised in mouse, used at a dilution 200:1). Secondary antibodies were anti-mouse Alexa Fluor 488 and anti-rabbit Alexa Fluor 546. Images were acquired on an Olympus FV3000RS confocal system using the 100 $\times$ objective. Arrows indicate selected stress granules ($\sim 1 \mu$ m condensates in diameter) showing strong dual signals for all the stress granule marker and O-GlcNAc. Scale bar, 20 μ m.

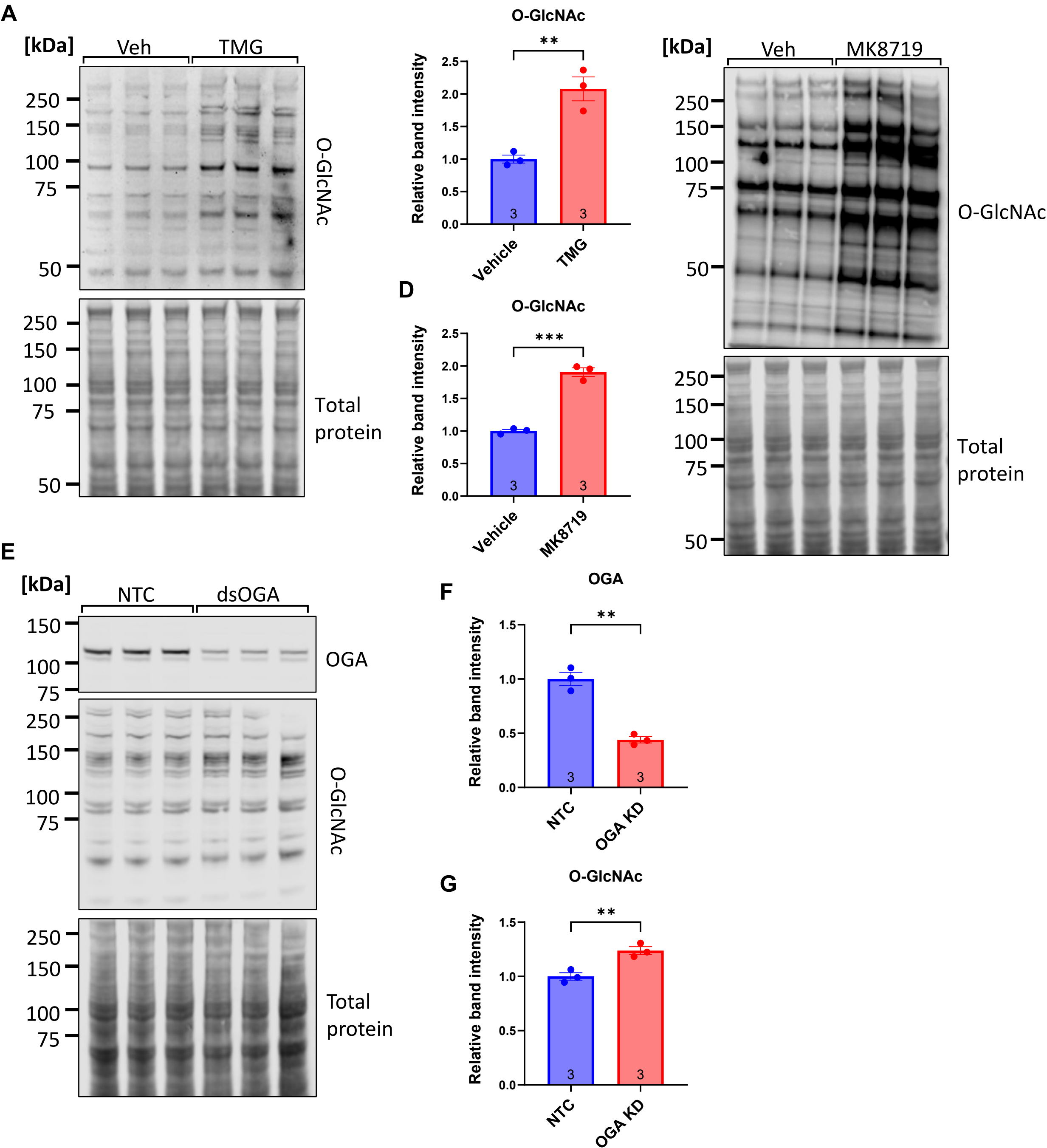
Suppl. Figure 2



Supplemental Figure 2. Pharmacologic inhibition, or siRNA-mediated targeting of OGT significantly reduce O-GlcNAc levels in H9c2 cells.

(A-B) Cells (1×10^6 /well) were treated with Vehicle (0.1% v/v DMSO), or OSMI-1 (25 μ M) for 6 h and were subsequently collected and proteins extracted for western blot for O-GlcNAcylation (antibody RL2, used at a dilution of 1000:1). Total protein loading was assessed using Revert700 and used as normalizing loading control. Results of the densitometry are shown in B. **(C-D)** Cells (1×10^6 /well) were transfected with 20 nM siRNA serving either as a non-targeting control (NTC) or directed against rat OGT. After 48 h, the cells were harvested and extracted for western blot. O-GlcNAcylation and total protein loading were assessed as above, and the normalized band densitometry are reported in D. Data are mean $\pm$ SEM from n = 3 biol. replicates/group. Comparisons were performed using an unpaired two-tailed t-test. ** P < 0.01, *** P < 0.001

Suppl. Figure 3



Supplemental Figure 3. Pharmacologic inhibition, or siRNA-mediated targeting of OGA significantly increase O-GlcNAc levels in H9c2 cells.

(A-B) Cells (1×10^6 /well) were treated with Vehicle (0.1% v/v DMSO), or TMG (25 μ M) for 6 h and were subsequently collected and proteins extracted for western blot for O-GlcNAcylation. Total protein loading was assessed using Revert700. Normalized relative O-GlcNAc levels obtained by densitometry (performed in ImageStudio Lite) are reported in B. **(C-D)** Cells were treated with Vehicle (0.1% v/v DMSO), or MK8719 (25 μ M) for 6 h and were subsequently collected and proteins extracted for western blot for O-GlcNAcylation as above. The relative results of O-GlcNAcylation are reported in panel D. **(E-G)** Cells were transfected with 20 nM siRNA serving either as a non-targeting control (NTC) or directed against rat OGA. After 48 h, the cells were harvested and proteins extracted for western blot. OGA, O-GlcNAcylation and total protein loading were assessed as above, and the normalized densitometry results are reported in panels E (for OGA) and G (for O-GlcNAc). Data are mean $\pm$ SEM from n = 3 biol. replicates/group. Comparisons were performed using an unpaired two-tailed t-test. ** P < 0.01, *** P < 0.001

Supporting Table S3. Reagent list

REAGENT	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal anti-O-GlcNAc	Zachara Lab	RL2
Rabbit monoclonal anti- MGEA5/OGA [EPR7154(B)]	Abcam	Cat# ab124807, RRID:AB_10971848
Rabbit polyclonal anti- MGEA5/OGA	Proteintech	Cat# 14711-1-AP, RRID:AB_2143063
Rabbit monoclonal anti- OGT (D1D8Q)	Cell Signaling Technology	Cat# 24083, RRID:AB_2716710
Rabbit polyclonal anti- OGT	Zachara Lab	AL24
Rabbit polyclonal anti-G3BP1	Proteintech	Cat# 13057-2-AP
Mouse monoclonal anti-G3BP1	Proteintech	Cat# 66486-I-Ig
Rabbit monoclonal anti-eIF4G1	Cell Signaling Technology	Cat# 2469
Rabbit polyclonal anti-PABPC1	Proteintech	Cat# 10970-1-AP
Rabbit polyclonal anti-TIA1	Proteintech	Cat# 12133-2-AP
Goat anti-Rabbit IgG1 Cross-Adsorbed Secondary Antibody, Alexa Fluor 546	Thermo	Cat# A11071
Goat anti-Mouse IgG1 Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	Thermo	Cat# A21121
Animal strains		
OGT flox mice (B6.129-Ogtm1Gwh/J)	Jackson Laboratory	Stock No. 004860
Myh6.MerCreMer transgenic mice (A1cr ^{flTg} (Myh6-cre/Esr1*)1Jmk/J)	Jackson Laboratory	Stock No. 005650
Rosa26.tdTomato.Lox.Stop.Lox reporter mice B6.Cg-Gt(ROSA)26Sor ^{tm9(CAG-tdTomato)Hze} /J or Ai9 (RCL-tdT)	Jackson Laboratory	Stock No. 007909
Chemicals and cell culture reagents		
OSMI-1	Sigma-Aldrich	SML1621; CAS No.: 1681056-61-0
Thiamet-G	Tocris	Cat# 4390; CAS No.: 1009816-48-1
NaAsO ₂	Sigma-Aldrich	S7400; CAS No.: 7784-46-5
GlutaMAX supplement	Thermo	Cat# 35050-061
Fetal Bovine Serum (FBS)	Thermo	
Tamoxifen	Sigma-Aldrich	T5648; CAS No.:10540-29-1
Dimethyl sulfoxide	Sigma-Aldrich	Cat# 472301; CAS No.: 67-68-5
MK-8719	MedChemExpress	Cat# HY-130121

Paraformaldehyde	Electron Microscopy Sciences	Cat# 15710
Tritonx100	Sigma-Aldrich	Cat# T9284
Tween 20	Promega	Cat# PRH5152
Vibrance anti-fading medium	Vector Laboratories	Cat# H-1700
Penicillin Streptomycin	Thermo	Cat# 15140-122
HEPES	Thermo	Cat# 15630-080
NEAA	Thermo	Cat# 11140-050
Fibronectin from bovine plasma	Sigma Aldrich	Cat# F4759
DMEM	Corning	Cat# 10-013-CV
Lipofectamine RNAiMax	Thermo	Cat# 13778075
ITS	Gibco	Cat# 51500056
BSA	Cell Signaling Technology	Cat# 9998
NGS	Cell Signaling Technology	Cat# 5425
OCT	Sakura	Cat# 4583
RIPA buffer	Sigma-Aldrich	Cat# R0278
Phenylmethanesulfonyl fluoride	Sigma-Aldrich	Cat# P7626
cOmplete, Mini, EDTA-free Protease Inhibitor Cocktail	Sigma-Aldrich	Cat# 11836170001
PhosSTOP	Sigma-Aldrich	Cat # 4906845001
DTT	Sigma-Aldrich	Cat# D9163-5G
LDS sample buffer	Thermo	Cat# NP0008
Staurosporine	Sigma Aldrich	Cat# S5921
OPTI-MEM	Thermo	Cat# 31985
NucBlue Live Cell Stain ReadyProbe	Thermo	Cat# R37605
Hoechst 33342	Thermo	Cat# H3570

Experimental models: Organisms/strains		
Timed pregnant female rats, outbred (for NRVM isolation)	Envigo	Hsd:Sprague Dawley Rat
H9c2 cells	ATCC	Cat# CRL-1446

Oligonucleotides		
Non targeting control (NTC)	IDT DNA	51-01-14-04
ds RNA sequence targeting rat OGT (rn.Ri.Ogt.13.2) Top: AAA GCA GUC AUC GAU UUU Bottom: UAG AUU UAA AAU CGA UGA	IDT DNA	Transcript: NM_017107
ds RNA sequence targeting rat OGA (rn.Ri.MGEA5.13.1) Top: CAA CUU CCC UUC UCU GAU AAA AAG Bottom: CAU UUU UAU CAG AGA AGG GAA GUU GGC	IDT DNA	Transcript: NM_131904 Targets exon 15
ds RNA sequence targeting rat G3BP1 (rn.Ri.G3BP1.1.13.1) Top: GAC AUG UGA UUA CCA AAC AAU UCA Bottom: UAG AAU UGU UUG GUA AUC ACA UGU CAA	IDT DNA	Transcript: NM_133565 Targets exon 12

ds RNA sequence targeting rat G3BP2 (rn.Ri.G3BP2.13.1) Top: CAG AGA AUC UUA AUU GCA AAA CCA Bottom: UUG GUU UUG CAA UUA AGA UUC UCU GAA	IDT DNA	Transcript: NM_001013989
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Software and algorithms		
Fiji/ImageJ	Schneider et al., 2012	https://imagej.nih.gov/ij/
ImageStudio Lite	LiCor	https://www.licor.com/bio/image-studio-lite/
Graphpad Prism	GraphPad Software	www.graphpad.com
CellProfiler	Stirling et al., 2021	https://cellprofiler.org/

Cell Culture Dishes		
μ-Slide 4 well ibiTreat	Ibidi	Cat# 80426
μ-Slide 8 well ibiTreat	Ibidi	Cat# 80806
Glass bottom cell culture dish	Nest	Cat# 801001