O-GlcNAc modification of eIF4GI acts as a translational switch in heat shock response

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Heat shock response (HSR) is an ancient signaling pathway leading to thermoprotection of nearly all living organisms. Emerging evidence suggests that intracellular O-linked β-N-acetylglucosamine (O-GlcNAc) serves as a molecular ‘thermometer’ by reporting ambient temperature fluctuations. Whether and how O-GlcNAc modification regulates HSR remains unclear. Here we report that, upon heat shock stress, the key translation initiation factor eIF4GI undergoes dynamic O-GlcNAcylation at the N-terminal region. Without O-GlcNAc modification, the preferential translation of stress mRNAs is impaired. Unexpectedly, stress mRNAs are entrapped within stress granules (SGs) that are no longer dissolved during stress recovery. Mechanistically, we show that stress-induced eIF4GI O-GlcNAcylation repels poly(A)-binding protein 1 and promotes SG disassembly, thereby licensing stress mRNAs for selective translation. Using various eIF4GI mutants created by CRISPR/Cas9, we demonstrate that eIF4GI acts as a translational switch via reversible O-GlcNAcylation. Our study reveals a central mechanism linking heat stress sensing, protein remodeling, SG dynamics and translational reprogramming.

Dynamic glycosylation of proteins with O-GlcNAc has been rapidly emerging as a cellular signaling mechanism1–3. The reversible O-GlcNAcylation is mediated by a single pair of enzymes: O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA). While the former installs O-GlcNAc on serine or threonine residues of proteins4, the latter removes the monosaccharide unit from proteins5. The dynamic cycling of O-GlcNAc modification occurs in a nutrient- and stress-responsive manner. For many ectotherms, O-GlcNAc levels are closely correlated with ambient temperature during their development6. Likewise, heat shock stress rapidly increases the levels of O-GlcNAcylation in mammalian cells7–9. Notably, O-GlcNAc modification has been shown to confer heat stress resistance in endotherms such as humans10. However, neither the identity of protein targets nor the physiological significance of O-GlcNAc modification in mammals and humans has been clearly defined.

HSR is an evolutionarily conserved pathway that results in rapid induction of genes encoding molecular chaperones essential for protection and recovery from cellular damages11–13. Upon heat shock stress, there is a marked inhibition of global protein synthesis accompanied by the selective induction of stress proteins such as Hsp70 (also termed Hsp72). This reprogramming of gene expression involves a coordination of transcription14, translation15 and post-transcriptional processes16,17. Additionally, a wide variety of stress stimuli trigger the assembly of SGs, discrete RNA–protein structures that contain nontranslating mRNAs18–21. The stress-induced O-GlcNAcylation is mediated by a single pair of enzymes: O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA). O-GlcNAc (Fig. 1a). Upon heat shock stress, a robust induction of Hsp70 was evident in MEFs. As expected, 48 h exposure to 4HT resulted in complete depletion of Hsp70 levels (Fig. 1b). These results suggest a crucial role for OGT in translational control of HSR.

Results
Deficient Hsp70 translation in cells lacking OGT. In mammalian cells, O-GlcNAc modification has been implicated in HSR, although the underlying mechanism remains to be resolved10,16. We took advantage of a mouse embryonic fibroblast (MEF) cell line, OgtF/Y (mER-Cre) MEFs, carrying an Ogt gene that can be excised by simple administration of 4-hydroxytamoxifen (4HT)19. As expected, 48h exposure to 4HT resulted in complete depletion of OGT and disappearance of background O-GlcNAc (Fig. 1a). Upon heat shock stress, a robust induction of Hsp70 was evident in MEFs before 4HT treatment. However, OGT depletion via 4HT pretreatment led to marked attenuation of Hsp70 induction (Fig. 1a). The impairment in Hsp70 expression was not due to the side effect of 4HT, as the same treatment had little effect in control MEFs bearing GFP (Supplementary Fig. 1a). Additionally, OGT depletion did not yet lead to apoptosis or necrosis, at least under these experimental conditions (Supplementary Fig. 1b,c). The OGT-dependent Hsp70 induction also held true in HeLa cells, as RNA interference (RNAi)-mediated OGT knockdown reduced Hsp70 levels (Supplementary Fig. 1d). The crucial role of OGT in HSR is not limited to Hsp70, because small chaperones such as Hsp25 also showed substantial reduction in stressed MEFs lacking OGT (Supplementary Fig. 1e). However, constitutively expressed chaperones such as Hsc70 were minimally affected.

Looking into the underlying mechanism, we found that OGT depletion had negligible effect on Hsp70 mRNA levels in heat-stressed MEFs (Fig. 1a). To assess the translational status of Hsp70 in these cells, we examined mRNA enrichment in polysome fractions separated by sucrose gradient sedimentation22. The stress-induced Hsp70 mRNA, but not the housekeeping messenger B2m, showed decreased polysome enrichment after OGT depletion (Fig. 1b). These results suggest a crucial role for OGT in translational control of HSR.

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Heat shock stress induces O-GlcNAcylation of eIF4G. We next attempted to identify protein targets subjected to O-GlcNAc modification in response to heat shock stress. We initially focused on ribosomal proteins, whose O-GlcNAcylation has been reported previously\(^{23,24}\). However, translating ribosomes exhibited very low basal levels of O-GlcNAc modification and showed negligible changes in response to heat shock stress (Supplementary Fig. 2a,b). We noticed that the most conspicuous O-GlcNAc species in ribosome fractions were of high molecular weight, and they disappeared in OGT-depleted MEF cells after 4HT pretreatment (Supplementary Fig. 2b,c). A close inspection of these high-molecular-weight species revealed a distinct band of approximate 250 kDa that emerged shortly after heat shock stress (Fig. 2a). Notably, the heat-stress-induced O-GlcNAc species appeared earlier than Hsp70 and vanished during stress recovery. This dynamic feature was reproducible in HeLa cells (Fig. 2a), suggesting that the reversible O-GlcNAc modification is a general phenomenon in mammalian cells upon heat shock stress.

Given the putative role of O-GlcNAcylation in translational control of HSR, we surveyed several translation initiation factors that are relatively large in size. eIF4G emerged as one of the candidates because of high scores of predicted O-GlcNAcylation and the similar size of 250kDa on gels (Supplementary Fig. 3a). To experimentally validate this finding, we immunoprecipitated endogenous eIF4G from HeLa cells with or without heat shock stress. With similar input levels, eIF4G obtained from heat-stressed cells showed a marked increase in O-GlcNAc signal (Fig. 2b). We also examined the eIF4G paralog eIF4GII\(^1\), which appeared to be barely modified in spite of the same size and sequence homology (Supplementary Fig. 3b). The stress-induced O-GlcNAc modification of eIF4G was further confirmed in MEF cells (Fig. 2c). Notably, OGT depletion after 4HT treatment completely abolished O-GlcNAc signals associated with eIF4G. Therefore, heat shock stress triggers rapid O-GlcNAc modification of eIF4G.

We next applied tandem mass spectrometry (MS) to map the positions of O-GlcNAc modification on endogenous eIF4G purified from heat-stressed MEF cells. With a peptide coverage of 67.5%, MS revealed only one modification site with high confidence, Ser68 (Fig. 2d). Notably, Ser68 in the mouse eIF4G is well conserved in the human eIF4G (annotated as Ser61). The Ser61 of human eIF4G has previously been mapped as a putative modification site\(^2\). To validate the identified modification site, we cloned the full-length eIF4G from HeLa cells and fused YFP and Flag to the N terminus. Stress-induced O-GlcNAc modification was well preserved in transfected YFP-flag-eIF4G (Fig. 2e). Introducing an S68A mutation completely abolished O-GlcNAc signals from purified eIF4G fusion proteins (Fig. 2e). By contrast, S1285A mutation minimally affected the total O-GlcNAc levels of transfected eIF4G. Therefore, the Ser68 is the primary site of eIF4G that undergoes dynamic O-GlcNAcylation in response to heat shock stress.

eIF4G O-GlcNAcylation repels PABP1. The N-terminal third of eIF4G contains binding sites for poly(A)-binding protein 1 (PABP1) and eIF4E\(^{22,23}\). We found that eIF4G bound to eIF4E irrespective of O-GlcNAc modification (Fig. 3a). Notably, heat stress diminished the interaction between eIF4G and PABP1 in wild-type cells. However, in MEF cells lacking OGT, comparable amount of PABP1 molecules were pulled down by eIF4G before and after stress (Fig. 3a). Given the close proximity of Ser68 and the PABP1 binding site of eIF4G, the stress-induced PABP1 dissociation is likely to be due to stress-induced O-GlcNAcylation of eIF4G. To test this possibility, we conducted reciprocal immunoprecipitation by purifying either eIF4E or PABP1 followed by eIF4G detection. While eIF4E readily pulled down both modified and unmodified eIF4G, much less eIF4G was coprecipitated with PABP1 and showed no O-GlcNAc signals (Fig. 3b). Notably, heat shock stress also diminished the coprecipitation between PABP1 and eIF4E. As an independent validation, we treated MEF cells with thiamet G, an inhibitor of OGA. Consistent with the dynamic nature of eIF4G O-GlcNAcylation, we observed an increase of O-GlcNAc signals during the early stage of stress recovery (3h) but an evident decrease at the late stage (6h) (Supplementary Fig. 3c). In the presence of thiamet G, however, the O-GlcNAc modification of eIF4G was sustained. Notably, much less PABP1 was
coprecipitated with eIF4GI (Supplementary Fig. 3c). Therefore, stress-induced O-GlcNAcylation modification of eIF4GI remodels the assembled eIF4F complex by dissociating PABP1.

PABP1 is believed to bring the 5′ cap and 3′ end of mRNA into a closed loop and stimulate the cap-dependent translation initiation. It is conceivable that, by opening the mRNA loop, O-GlcNAcylation of eIF4GI not only represses cap-dependent translation, but also facilitates cap-independent translation by remodeling the eIF4F complex. Since eIF4GI bears an inherent mRNA binding capacity, we speculated that stress-induced O-GlcNAcylation led to preferential binding of eIF4GI to stress mRNAs for selective translation. To illustrate the mRNA-binding properties of eIF4GI with or without O-GlcNAc modification, we enriched eIF4GI-associated mRNAs by zero-distance cross-linking followed by real-time quantitative qPCR (RT-qPCR) (Fig. 3c). In control MEF cells, heat shock stress led to sixfold enrichment of Hsp70 (Hspa1a) mRNA to eIF4GI, but not β-actin (Actb) mRNA. Surprisingly, and perhaps counterintuitively, OGT depletion resulted in about 25-fold enrichment of Hspa1a mRNA in association with eIF4GI (Fig. 3c). Since eIF4GI lacking O-GlcNAc modification does not support Hspa1a mRNA translation (Fig. 1), the stable interaction between the unmodified eIF4GI and mRNA is suggestive of nonproductive trapping. As an independent validation, we compared the mRNA-binding properties of YFP-flag-eIF4GI and the S68A mutant in transfected MEF cells. As expected, reduced PABP1 binding upon heat shock stress was only observed for the wild-type eIF4GI but not the S68A mutant (Supplementary Fig. 3d). In agreement with the endogenous eIF4GI results, a large amount of mRNA was significantly enriched in the S68A mutant in comparison to the wild type (Fig. 3d). This unexpected result suggests that stress-induced O-GlcNAcylation of eIF4GI releases stress mRNAs that otherwise are trapped.

eIF4GI O-GlcNAcylation promotes SG disassembly. We noticed that, upon heat shock stress, less eIF4GI was recovered from the soluble fraction in MEF cells lacking OGT (Fig. 3a, input). Fractionation analysis further confirmed this feature (Fig. 4a). In addition to eIF4GI, eIF4E and PABP1 also reduced their solubility in OGT-null cells upon heat shock stress. This feature is reminiscent of stress granule (SG) formation in the cytoplasm. Immunofluorescence indeed revealed more cytoplasmic puncta signified by SG markers in OGT-knockout cells than in wild-type MEFs after heat shock stress (Fig. 4b). In wild-type cells, heat-stress-induced SGs were transient and quickly resolved during stress recovery. In OGT knockout cells, however, the SG puncta largely remained even after the cessation of stress for 2 h (Supplementary Fig. 4a). To substantiate this finding further, we knocked down OGT in HeLa cells and observed a sustained formation of SGs during stress recovery (Supplementary Fig. 4b). Therefore, lack of O-GlcNAcylation results in delayed SG sustained formation during stress recovery.

The unanticipated role of O-GlcNAc in SG disassembly is in sharp contrast to the results of a previous study that reported that OGT...
was essential for SG assembly. To directly address this issue, we assessed SG formation in MEF cells with or without OGT knockout after exposure to sodium arsenite, a potent SG inducer used in the previous study. Sodium arsenite treatment readily triggered SG formation in both wild-type and OGT knockout cells (Supplementary Fig. 5a). In addition, HeLa cells with OGT knockdown also exhibited a comparable number of, if not more, SG puncta upon sodium arsenite exposure (Supplementary Fig. 5b). We conclude that OGT is dispensable for SG formation. In the case of heat shock stress, O-GlcNAcylation of eIF4GI appears to be essential in SG disassembly, presumably by promoting selective translation of Hsp7033. Indeed, adding back Hsp70 into OGT knockout cells via recombinant adenoviruses prevented the sustained SG formation induced by heat stress (Supplementary Fig. 6).

**Fig. 3** | eIF4GI O-GlcNAcylates repels PABP1. a, Og⁻/⁻(mER-Cre) MEFs were heat stressed (HS) at 42 °C for 1 h and recovered at 37 °C for 2.5 h. Cell lysates were immunoprecipitated (IP) using an anti-eIF4GI antibody followed by immunoblotting. Representative results are shown from three independent experiments. Uncropped blots are shown in Supplementary Fig. 9. Right: a schematic of eIF4F complexes. 4E, eIF4E; 37 °C for 2.5 h. Cell lysates were immunoprecipitated using an anti-eIF4E or anti-PABP1 antibodies followed by immunoblotting. Representative results are shown from three independent experiments. Uncropped blots are shown in Supplementary Fig. 9. Left: HeLa cells were heat stressed at 42 °C for 1 h and recovered at 37 °C for 2.5 h. Cells were then irradiated with UV at 365 nm and cell lysates were immunoprecipitated using an anti-eIF4GI antibody followed by immunoblotting. Representative results are shown from three independent experiments. Uncropped blots are shown in Supplementary Fig. 9. b, c, Og⁻/⁻(mER-Cre) MEFs were treated with 100 μM 4-thiouridine (4SU) followed by heat stress at 42 °C for 1 h and recovered at 37 °C for 2.5 h. Cells were then irradiated with UV at 365 nm and cell lysates were immunoprecipitated using an anti-eIF4GI antibody. Immunoprecipitates were treated with proteinase K followed by RNA extraction and RT-qPCR. Basal levels of both transcripts in wild-type cells (–HS, –4HT) are set to 1. Error bars, mean ± s.e.m.; n = 3 independent experiments. d, MEF cells were transfected with plasmids expressing YFP-flag-eIF4GI wild type (WT) or mutant (S68A) for 24 h. Transfected cells were treated with 100 μM 4SU followed by heat stress at 42 °C for 1 h and recovered at 37 °C for 2.5 h. Cells were then irradiated at 365 nm UV and cell lysates were immunoprecipitated using an anti-Flag antibody. Immunoprecipitates were treated with proteinase K followed by RNA extraction and RT-qPCR. YFP was used as a negative control. Basal levels of both transcripts in wild-type cells (–HS, –4HT) are set to 1. Error bars, mean ± s.e.m.; n = 3 independent experiments.

**eIF4GI O-GlcNAcylation releases stress mRNAs from SGs.** Stress mRNAs such as *Hspa1a* are largely excluded from SG recruitment, likely because of their preferential translation under stress conditions. Having observed defective Hsp70 synthesis and the marked mRNA trapping by eIF4GI in the absence of O-GlcNAc modification, we reasoned that these stress mRNAs were likely to be retained within SGs. To test this possibility, we used fluorescence in situ hybridization (FISH) to detect the cellular location of *Hspa1a* induced by heat shock stress. In wild-type MEFs, FISH signals distributed uniformly in the cytoplasm, as well as nuclear foci that accounted for active Hsp70 transcription (Fig. 4c). In MEFs lacking OGT, however, the cytoplasmic FISH signals formed clear puncta overlapping with SG markers such as G3BP1. Therefore, in the absence of O-GlcNAcylation, *Hspa1a* mRNA is largely entrapped within SGs.

To confirm that it is the modification of eIF4GI that contributes to the mRNA triage in SG dynamics, we compared SG induction in MEF cells transfected with YFP-flag-eIF4GI or the S68A mutant. In spite of the presence of endogenous eIF4GI, overexpression of the nonmodifiable S68A mutant was sufficient for sustained SG formation in response to heat shock...
Hspa1a MEFs were heat stressed at 42 °C for 1 h and recovered at 37 °C for 2.5 h. Cells were then fixed with 3.7% formaldehyde followed by simultaneous G3BP1 experiments. Uncropped blots are shown in Supplementary Fig. 9.

These eIF4GI variants can be resolved by prolonged gel electrophoresis. PABP1

eIF4GI that undergoes –GlcNAcylation in response to heat shock stress. To address this question, we used the CRISPR/Cas9 system to genetically engineer the endogenous Eif4g1 gene. For instance, in a cell line bearing a single A insertion at Ser67 and Ser68 (Supplementary Fig. 7b), we established several MEF cell lines harboring different Eif4g1 mutations. As expected, the majority of mutants exhibited an insert/deletion (indel) that resulted in frame-shift of Eif4g1. For instance, in a cell line bearing a single A insertion before the Ser67 codon, very little full-length eIF4GI was detectable (Fig. 5b). Intriguingly, the short isoforms of eIF4GI became more abundant in these cells, presumably as a result of cellular adaptation. Since the physiological eIF4GI short isoforms lack the N-terminal region for O-GlcNAC modification, they offer a valuable tool for assessing the functionality of eIF4GI lacking both O-GlcNAC modification and PABP1 binding. Despite the absence of full-length eIF4GI, the induction of Hsp70 synthesis upon heat shock stress remained robust in these cells (Fig. 5b). However, these eIF4GI short isoforms neither changed their solubility nor supported SG formation upon heat shock stress (Fig. 5d,e). Thus, eIF4GI–PABP1 interaction is crucial for SG formation but dispensable for selective Hsp70 synthesis.

To confirm the critical role of PABP1 in SG induction, we assessed SG formation in MEF cells with PABP1 knockdown. Remarkably, even partial depletion of PABP1 nearly abolished SG assembly upon heat shock stress (Supplementary Fig. 8a). However, there was little effect on stress-induced Hsp70 synthesis (Supplementary Fig. 8b). The PABP1-independent Hsp70 translation is in agreement with the notion that the selective translation of stress mRNAs occurs preferentially during heat stress recovery.

Relying on a single guide RNA (sgRNA) targeting Ser67 and Ser68 (Supplementary Fig. 7b), we established several MEF cell lines harboring different Eif4g1 mutations. As expected, the majority of mutants exhibited an insert/deletion (indel) that resulted in frame-shift of Eif4g1. For instance, in a cell line bearing a single A insertion before the Ser67 codon, very little full-length eIF4GI was detectable (Fig. 5b). Intriguingly, the short isoforms of eIF4GI became more abundant in these cells, presumably as a result of cellular adaptation. Since the physiological eIF4GI short isoforms lack the N-terminal region for O-GlcNAC modification, they offer a valuable tool for assessing the functionality of eIF4GI lacking both O-GlcNAC modification and PABP1 binding. Despite the absence of full-length eIF4GI, the induction of Hsp70 synthesis upon heat shock stress remained robust in these cells (Fig. 5b). However, these eIF4GI short isoforms neither changed their solubility nor supported SG formation upon heat shock stress (Fig. 5d,e). Thus, eIF4GI–PABP1 interaction is crucial for SG formation but dispensable for selective Hsp70 synthesis.

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Fig. 5 | The N terminus of eIF4GI acts a functional switch via O-GlcNAcylation. a, Left: N-terminal region of eIF4GI showing possible isoforms, with the number in parentheses indicating the position of the first amino acid. Both the O-GlcNAc modification site S68 and the PABP1 binding domain are highlighted. Right: eIF4GI isoforms obtained from heat-stressed MEF cells before and after immunoprecipitation (IP) using either O-GlcNAc or eIF4GI antibodies. Representative results are shown from three independent experiments. Uncropped blots are shown in Supplementary Fig. 9. b, Genomically engineered MEF cells bearing wild type (WT) or A insertion (Ins(A)) mutant eIF4GI were heat stressed (HS) at 42 °C for 1 h and recovered at 37 °C for indicated times. Whole-cell lysates were collected for immunoblotting. Representative results are shown from three independent experiments. Uncropped blots are shown in Supplementary Fig. 9. N: no heat shock. c, Genomically engineered MEF cells bearing the eIF4GI Ins(A) mutant or a deletion mutant (ΔSSA) were heat stressed at 42 °C for 1 h and recovered at 37 °C for indicated times. Whole-cell lysates were collected for immunoblotting. Uncropped blots are shown in Supplementary Fig. 9. d, Genomically engineered MEF cells bearing the eIF4GI Ins(A) mutant or a deletion mutant (ΔSSA) were heat stressed at 42 °C for 1 h and recovered at 37 °C for 2 h. Whole-cell lysates were fractionated into soluble (Sol; supernatant) and insoluble (Insol; pellet) fractions by centrifugation at 16,000 g for 15 min at 4 °C. Both fractions and total samples were collected for immunoblotting. Uncropped blots are shown in Supplementary Fig. 9. e, Genomically engineered MEF cells bearing the eIF4GI Ins(A) mutant or a deletion mutant (ΔSSA) were heat stressed at 42 °C for 1 h and recovered at 37 °C for 2.5 h. Cells were fixed with 3.7% formaldehyde followed by immunofluorescence staining. Scale bars, 10 μm. Right: SG index based on eIF4GI immunostaining. Error bars indicate mean ± s.e.m. for at least three independent experiments in which four fields of view with at least 30 cells per field of view were quantified. **P < 0.01; two-way ANOVA.

We next attempted to establish MEF cell lines with genomically frame-shifted mutants in eIF4GI that maintains the PABP1 binding capacity but cannot be modified by O-GlcNAc. Among hundreds of cell clones created by CRISPR/Cas9, two eIF4GI mutants lacking Ser68 (ΔSA and ΔSSA) were successfully selected (Supplementary Fig. 7b). Unlike the frameshifting Ins(A) mutant, the in-frame deletion mutants showed predominantly full-length eIF4GI (Fig. 5c). As expected, both ΔSSA and Ins(A) mutants showed no discernable O-GlcNAc signals upon heat shock stress (Supplementary Fig. 8c). With the intact PABP1 interaction, the eIF4GI ΔSSA mutant quickly lost solubility upon heat shock stress (Fig. 5d). This result indicates that other eIF4G homologs, such as eIF4GII and DAP5, could not compensate for the eIF4GI loss of function in selective translation of Hspa1a mRNA. Notably, lack of eIF4GI completely prevented heat-stress-induced SG formation (Supplementary Fig. 8e). We then reintroduced different variants of eIF4GI, including the shortest isoform, M197, which lacks the PABP1 binding site and a truncated variant, M457, which lacks the entire N-terminal region (Fig. 6a). To prevent downstream initiation of these transgenes, we blocked their N termini by fusing a YFP module. We also introduced synonymous mutations into these constructs rendering them resistant to sgRNA targeting P700. All the constructs except the S68A mutant restored stress-induced Hsp70 synthesis (Fig. 6a). However, adding back M197 or M457 mutants failed to trigger SG induction upon heat shock stress (Fig. 6b). Despite the relatively low expression levels in transfected cells, both M197 and M457 mutants rescued Hsp70 synthesis as fully as the wild type. Therefore, an intact PABP1 binding site is not essential for selective translation of Hsp70. In fact, a persistent PABP1 association (as in the case of the S68A mutant) prevents subsequent stress mRNA translation. Further supporting the crucial role of O-GlcNAcylation in the functional switch of eIF4GI, only the wild-type eIF4GI was able to rescue both SG formation and Hsp70 synthesis (Fig. 6a,b).

A model emerges from our data: the dynamic interaction between eIF4GI and PABP1 serves as a regulatory key to SG heat shock stress (Supplementary Fig. 8d). This result indicates that other eIF4G homologs, such as eIF4GII and DAP5, could not compensate for the eIF4GI loss of function in selective translation of Hspa1a mRNA. Notably, lack of eIF4GI completely prevented heat-stress-induced SG formation (Supplementary Fig. 8e). We then reintroduced different variants of eIF4GI, including the shortest isoform, M197, which lacks the PABP1 binding site and a truncated variant, M457, which lacks the entire N-terminal region (Fig. 6a). To prevent downstream initiation of these transgenes, we blocked their N termini by fusing a YFP module. We also introduced synonymous mutations into these constructs rendering them resistant to sgRNA targeting P700. All the constructs except the S68A mutant restored stress-induced Hsp70 synthesis (Fig. 6a). However, adding back M197 or M457 mutants failed to trigger SG induction upon heat shock stress (Fig. 6b). Despite the relatively low expression levels in transfected cells, both M197 and M457 mutants rescued Hsp70 synthesis as fully as the wild type. Therefore, an intact PABP1 binding site is not essential for selective translation of Hsp70. In fact, a persistent PABP1 association (as in the case of the S68A mutant) prevents subsequent stress mRNA translation. Further supporting the crucial role of O-GlcNAcylation in the functional switch of eIF4GI, only the wild-type eIF4GI was able to rescue both SG formation and Hsp70 synthesis (Fig. 6a,b).

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O-GlcNAcylation acts as a functional switch for eIF4GI. To substantiate the finding that the O-GlcNAcylation of eIF4GI acts as a functional switch between SG induction and selective mRNA translation, we conducted rescue experiments. To deplete endogenous eIF4GI variants, we introduced indel mutations into Eif4g1 by targeting the conserved downstream region (P700) using CRISPR/Cas9 (Supplementary Fig. 7b). All surviving cells still contained residual eIF4GI but failed to induce robust Hsp70 synthesis in response to
O-GlcNAcylation of eIF4GI displaces PABP1, thereby dissolving SGs and permitting selective translation of stress mRNAs. Stress-induced O-GlcNAcylation of eIF4GI operates to remodel the closed mRNA loop. As a result, global protein synthesis remains repressed, whereas selective translation of stress mRNAs is permitted. Intriguingly, a wide range of viruses frequently target eIF4GI and PABP1, thereby subverting the host translation machinery8,19. For instance, the picornaviral 2A protease cleaves eIF4G40, whereas rotaviral NSP3 disrupts PABP1 and eIF4GI interaction6. Unlike the virus-mediated remodeling of eIF4F, which is long-lasting, stress-induced O-GlcNAc modification of eIF4G is dynamic and reversible. This physiological switch enables cells to produce an adaptive translatome by turning on different modes of translation initiation in response to stress. Perhaps the most surprising finding is that depleting O-GlcNAc modification or genetically eliminating the modification site of eIF4G1 leads to mRNA trapping in the insoluble fraction. This paradoxical result is in accordance with the sustained SG formation in the absence of eIF4G1 O-GlcNAcylation. SG formation is believed to be crucial for stress adaptation and is tightly coupled with the presence of mRNAs stalled in translation initiation18–21. Although both eIF4G1 and PABP1 are core SG markers, it is intriguing to find that their interaction contributes to SG dynamics, at least during heat shock stress. A recent study reported that PABP1 in yeast (Pab1) mediates liquid–liquid phase separation in vivo and in vitro upon elevated temperatures42. However, SGs appear to form through the crosslinking of nontranslating mRNAs, which provide a scaffold or platform for multiple RNA-binding proteins41. Our findings echo the importance of mRNA conformation in SG assembly and disassembly. We propose that persistent eIF4G1–PABP1 interaction, when it is not engaged for active translation, readily poises the looped mRNA for SG formation in the cytoplasm. Disrupting eIF4G1–PABP1 association by either O-GlcNAc modification or deleting the N-terminal region of eIF4G1 helps dissolve SGs. The identification of a single modification that toggles eIF4G between inhibitors and drivers of SG assembly reshapes our current perceptions of SG regulation. Collectively, dynamic O-GlcNAcylation of eIF4G1 acts as a major hub in translational control of many cellular processes, including transcription, protein degradation, basal metabolism and signaling pathways18. Our finding that dynamic O-GlcNAc modification acts as a functional switch for eIF4G imparts an additional layer of regulation in maintaining protein homeostasis.

The initiation factor eIF4G acts as a major hub in translational control of many cellular processes, stress response and viral infection12. The N-terminal region of eIF4G1, although largely unstructured, contains the PABP1 and eIF4E binding sites. PABP1 is known to enhance cap- and poly(A)-dependent mRNA translation via direct interaction with eIF4G120,23. In heat-stressed cells, however, PABP1 is rapidly dissociated from eIF4G1. This feature is in line with the finding that selective translation of Hsp70 mRNA is neither cap- nor PABP1-dependent14. However, very little is known how PABP1 is evicted from the eIF4F complex during heat stress. We provide evidence that N-terminal O-GlcNAcylation of eIF4G1 operates to remodel the initiation complex eIF4F. By displacing PABP1, the stress-induced O-GlcNAcylation of eIF4G1 disrupts the closed mRNA loop. As a result, global protein synthesis remains repressed, whereas selective translation of stress mRNAs is permitted. Intriguingly, a wide range of viruses frequently target eIF4G1 and PABP1, thereby subverting the host translation machinery8,19. For instance, the picornaviral 2A protease cleaves eIF4G40, whereas rotaviral NSP3 disrupts PABP1 and eIF4G interaction6. Unlike the virus-mediated remodeling of eIF4F, which is long-lasting, stress-induced O-GlcNAc modification of eIF4G is dynamic and reversible. This physiological switch enables cells to produce an adaptive translatome by turning on different modes of translation initiation in response to stress. Perhaps the most surprising finding is that depleting O-GlcNAc modification or genetically eliminating the modification site of eIF4G1 leads to mRNA trapping in the insoluble fraction. This paradoxical result is in accordance with the sustained SG formation in the absence of eIF4G1 O-GlcNAcylation. SG formation is believed to be crucial for stress adaptation and is tightly coupled with the presence of mRNAs stalled in translation initiation18–21. Although both eIF4G1 and PABP1 are core SG markers, it is intriguing to find that their interaction contributes to SG dynamics, at least during heat shock stress. A recent study reported that PABP1 in yeast (Pab1) mediates liquid–liquid phase separation in vivo and in vitro upon elevated temperatures42. However, SGs appear to form through the crosslinking of nontranslating mRNAs, which provide a scaffold or platform for multiple RNA-binding proteins41. Our findings echo the importance of mRNA conformation in SG assembly and disassembly. We propose that persistent eIF4G1–PABP1 interaction, when it is not engaged for active translation, readily poises the looped mRNA for SG formation in the cytoplasm. Disrupting eIF4G1–PABP1 association by either O-GlcNAc modification or deleting the N-terminal region of eIF4G1 helps dissolve SGs. The identification of a single modification that toggles eIF4G between inhibitors and drivers of SG assembly reshapes our current perceptions of SG regulation. Collectively, dynamic O-GlcNAcylation of
elf4GI serves as a common mechanism underlying selective mRNA translation and SG dynamics. Since aberrant elf4GI expression and dysregulated SG formation are associated with certain types of cancer and neurodegeneration\textsuperscript{13–15}, our findings raise the possibility that new strategies for converting functional variants of elf4GI could be effective for treating these diseases.

**Methods**

Methods, including statements of data availability and any associated accession codes and references, are available at https://doi.org/10.1038/s41589-018-0120-6.

**References**


**Acknowledgements**

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**Author contributions**

X.Z. and S.-B.Q. conceived the project and designed the experiments. X.Z. performed the majority of experiments. X.E.S. assisted the experiments of elf4GI isoforms and different negative regulators. T.T. and J.B. performed the mass spectrometry. We thank the Protein and MS Facility of Cornell University for providing the mass spectrometry data. The Orbitrap Fusion mass spectrometer was supported by US National Institutes of Health S10 grant 1S01 OD017992-01. This work was supported by grants to S.-B.Q from the US National Institutes of Health (R01AG042400 and R01GM1222814) and a HHMI Faculty Scholar grant (55108556).

**Competing interests**

The authors declare no competing interests.

**Additional information**

Supplementary information is available for this paper at https://doi.org/10.1038/s41589-018-0120-6.

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Methods

Cell lines and reagents. Ogt\(^{mER-Cre}\) and Ogt\(^{GFP}\) (GFP) MEFs were kindly provided by Natasha E. Zachara (The Johns Hopkins University School of Medicine) and were maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 10% FBS. For heat shock treatment, MEF cells were incubated at 42 °C for 1 h followed by recovery at 37 °C for 4.5 hours.

Reverse primer for eIF4GI P700: 5′AAGCTTAGTACAAGTCAGGTGTGTTTAAG-3′; reverse primer for eIF4GI S68: 5′AGCACCACATCAGTGATATG (antisense); for PABP1, AGCACCACATCAGTGATATG (antisense); for eIF4GII, AGCACCACATCAGTGATATG (antisense); for v-CCTAGACTCGGGGGCCCACGGTTGCTG-3′. Primer sequences were synthesized by Sigma.

Single cells were then expanded and analyzed by PCR with puromycin. Single-cell suspensions of selected cells were transferred to 96-well plates by serial dilution. Four hours after transfection and filtered to eliminate cells, and target cells were infected in 5% to 38% acetonitrile in 0.1% formic acid at 300 nL/min. The Orbitrap Fusion was carried out using an Orbitrap Fusion Tribrid (Thermo-Fischer Scientific, San Diego, CA). Lysis buffer (50 mM Tris, pH 7.4, 1 mM MgCl\(_2\), 1 mM EDTA) was mixed with one volume 4 M urea solution and pellets were resuspended in 50 μL distilled water, 100 mM Ambic at room temperature in a Stratalinker 2400. Cells were collected with lysis buffer B and lyophilized.

For in-gel trypsin digestion, the gel pieces were reduced with 100 mM 2-mercaptoethanol, 0.1% bromophenol blue), pH 6.8, 2% SDS, 15% glycerol, 5% β-mercaptoethanol, 0.1% bromphenol blue), boiled for 5 min and analyzed by immunoblotting.

Higher Collision dissociation product ion triggered ETD (HCD-pd-ETD) MS/MS scans for precursor peptides with 2–8 charges above a threshold ion count of 50,000 with normalized collision energy of 32%. Product ion trigger list consisted of 138.0545 Da (HexNAc fragment). The ETD reaction time was set at 150 ms and the SA energy was set at 20%. All data were acquired using Xcalibur 3.0 software and Orbitrap Fusion Tune Application v. 2.1 (Thermo-Fisher Scientific).

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Immunoprecipitation. Cells were washed twice with PBS and lysed in lysis buffer A (20 mM Tris–HCl, pH 7.5, 100 mM KC1, 1 mM Dithiothreitol, 0.5% EDTA, 10% glycerol, 0.5% NP-40). Lysates were spun at 16,000g for 15 min at 4 °C and supernatants were collected and incubated with primary antibody and agarose beads at 4 °C for overnight. Immunoprecipitates were washed four times with lysis buffer A. The washed beads were resuspended in SDS sample buffer (100 mM Tris, pH 6.8, 2% SDS, 15% glycerol, 5% β-mercaptoethanol, 0.1% bromphenol blue), boiled for 5 min and analyzed by immunoblotting.

Immunofluorescence staining. Cells grown on glass coverslips were fixed in 3% formaldehyde and permeabilized with 0.5% Triton X-100 in PBS. After blocking in 20% BSA in PBS 30 min, fixed cells were incubated with the primary antibody at 4 °C overnight, followed by 1 h incubation at room temperature with Alexa Fluor-labeled secondary antibodies. Cells were then washed with PBS and incubated for 5 min in PBS supplemented with Hoechst to co-stain the nuclei. After final wash with PBS, coverslips were mounted onto slides and viewed using a confocal microscope (Zeiss LSM710) at the Cornell University Institute of Biotechnology.

Stress granules were quantified using ImageJ (https://imagej.nih.gov/ij) as previously described. Briefly, images were randomly acquired in 4 different fields with at least 30 cells per field. Background intensity was subtracted from each image. The plug-in “Analyze Particles” was used to measure the total area of stress granules/total cell area. The stress granule index was determined by computing the total stress granule area in relation to the cell area for each field and then averaged across all fields.

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RNA FISH. RNA FISH was performed as previously described with small modifications. The same set of Stellaris FISH probes, single-labeled (quasar570) oligonucleotides, were synthesized to bind to mouse *Hspa1a* from Biosearch Technologies. MEF cells grown on glass coverslips were fixed in 3.7% formaldehyde and permeabilized with 0.5% Triton X-100 in PBS. Cells were then washed in PBSM 10 min and incubated 60 min at 37°C in prehybridization solution (20% formamide, 2 x SSC, 2 mg/mL BSA and 200 mM vanadyl ribonucleoside complex). After prehybridization, the hybridization buffer (Biosearch Technologies, SMF-HB1-10) containing probe (125 nM) plus anti-G3BP1 antibody were added to cells and incubated in the dark at 37°C for 16 h. Cells were then incubated with wash buffer A (SMF-WA1-60) plus anti-rabbit second antibody in the dark at room temperature for 1 h. Wash buffer A was aspirated, cells were washed twice with wash buffer B (SMF-WB1-20) and then coverslips were mounted onto slides with mounting medium containing DAPI and viewed using a confocal microscope (Zeiss LSM710) at the Cornell University Institute of Biotechnology.

Statistical analysis. Results are expressed as mean ± s.e.m. Comparisons between groups were made by two-way ANOVA and unpaired two-tailed Student t-test.

**Reporting Summary.** Further information on experimental design is available in the Nature Research Reporting Summary linked to this article.

**Data availability.** All data generated or analyzed during this study are included in this published article (and its Supplementary Information files) or are available from the corresponding author on reasonable request.

**References**
Experimental design

1. Sample size
   Describe how sample size was determined.

2. Data exclusions
   Describe any data exclusions.

3. Replication
   Describe the measures taken to verify the reproducibility of the experimental findings.

4. Randomization
   Describe how samples/organisms/participants were allocated into experimental groups.

5. Blinding
   Describe whether the investigators were blinded to group allocation during data collection and/or analysis.

Note: all in vivo studies must report how sample size was determined and whether blinding and randomization were used.

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See the web collection on statistics for biologists for further resources and guidance.
7. Software

Describe the software used to analyze the data in this study. Microsoft Excel, SigmaPlot, GraphPad Prism, ImageJ

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a third party. All materials are readily available

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species). anti-eIF2 alpha (9722) was purchased from Cell Signaling. Anti-O-GlcnAc (RL2, MA1-072), Alexa Fluor 488 or 546 labeled secondary antibodies (A21202, A10040) and Hoechst were purchased from Fisher Scientific; anti-eIF4GI (sc-11373), anti-eIF4E (sc-9976) from Santa Cruz; anti-Flag (M2) and beta-actin monoclonal antibody (F1804) from Sigma; anti-OGT (11576-2-AP) and anti-G3BP1 (13057-2-AP) from Proteintech; anti-Hsp70 (SPA801) anti-Hsp25 (SPA801) from Enzo Life Sciences; anti-eIF4GII for IF (2858), anti-HSP90 (4874), anti-RP56 (2217), anti-PARP (9542), and anti-eIF4E (9742), and anti-PABP1 (4992) from Cell Signaling; anti-eIF4GI C-terminal and anti-eIF4GII antibodies were kindly provided by Dr. Nahum Sonenberg (McGill University).

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used. MEF/Ogt F/Y(mER-Cre), MEF/Ogt F/Y(GFP), MEF, HeLa

b. Describe the method of cell line authentication used. Cell lines are not yet authenticated

c. Report whether the cell lines were tested for mycoplasma contamination. Cell lines are not yet test for mycoplasma contamination

d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use. No misidentified cell lines were used

11. Description of research animals

Provide all relevant details on animals and/or animal-derived materials used in the study. No animal were used

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants. The study did not involve any human research participants