Limited effects of m$^6$A modification on mRNA partitioning into stress granules

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The presence of the m$^6$A modification in mammalian mRNAs is proposed to promote mRNA recruitment to stress granules through the interaction with YTHDF proteins. We test this possibility by examining the accumulation of mRNAs in stress granules in both WT and ΔMETTL3 mES cells, which are deficient in m$^6$A modification. A critical observation is that all m$^6$A modified mRNAs partition similarly into stress granules in both wild-type and m$^6$A-deficient cells by single-molecule FISH. Moreover, multiple linear regression analysis indicates m$^6$A modification explains only 6% of the variance in stress granule localization when controlled for length. Finally, the artificial tethering of 25 YTHDF proteins on reporter mRNAs leads to only a modest increase in mRNA partitioning to stress granules. Since most mammalian mRNAs have 4 or fewer m$^6$A sites, and those sites are not fully modified, this argues m$^6$A modifications are unlikely to play a significant role in recruiting mRNAs to stress granules. Taken together, these observations argue that m$^6$A modifications play a minimal, if any, role in mRNA partitioning into stress granules.
Stress granules are cytoplasmic molecular condensates composed of non-translation messenger ribonucleoproteins (mRNPs). Stress granules form when there is an increase in the pool of non-translation mRNPs, which often occurs when cells undergo a variety of stress conditions including oxidative stress, hypoxia, and heat shock that downregulate translation initiation. Stress granules are of interest since they are thought to play roles in a variety of diseases such as viral infection, cancer, and neurodegenerative disorders. Moreover, their investigation may provide insights into other RNA and protein condensates such as the nucleolus, P-bodies, and germ granules.

Recent studies have elucidated the composition of stress granules in a variety of stress conditions. One of the major conclusions from these studies is the mRNAs that are preferentially enriched in stress granules are biased towards poorer translation efficiency and longer length. Despite this length bias, mRNAs of the same length can show different recruitment into stress granules suggesting that there can also be sequence-specific information that affects mRNPs partitioning into stress granules.

Length-independent effects of stress granule recruitment have been argued to occur by the m6A modification increasing mRNA partitioning into stress granules through the binding of YTHDF proteins. However, this possible mechanism has not been directly tested by inhibiting m6A modification and examining the effect on mRNP partitioning into stress granules. Herein, we compared the localization of poly-m6A mRNAs in wildtype and ΔMETTL3 mES cells in stress granules and discovered that the poly-m6A mRNAs are enriched similarly in stress granules in both cell types. These results suggest m6A plays a little role in recruiting endogenous mRNA to stress granules. Moreover, we observed tethering up to 25 YTHDF proteins to a reporter mRNA has only a modest increase in the reporter mRNA partitioning into stress granules. Therefore, in an artificial reporter system, YTHDF proteins can recruit RNAs to stress granules, but this requires significantly many more proteins than what is normally observed for the number of m6As that are seen for endogenous mRNAs. This argues m6A modification plays little role in the recruitment of endogenous mRNAs to stress granules.

**Results**

Observations that argue m6A targets mRNAs to stress granules. In previous work, three main observations were used to argue that m6A modification targets mRNAs to stress granules by providing binding sites for YTHDF proteins, whose intrinsically disordered regions (IDRs) then promote mRNPs entering stress granules. First, YTHDF proteins were shown to undergo self-disordered regions (IDRs) then promote mRNPs entering stress granules providing binding sites for YTHDF proteins, whose intrinsically disordered regions (IDRs) then promote mRNPs entering stress granules. Therefore, in an artificial reporter system, YTHDF proteins can recruit RNAs to stress granules, but this requires significantly many more proteins than what is normally observed for the number of m6As that are seen for endogenous mRNAs. This argues m6A modification plays little role in the recruitment of endogenous mRNAs to stress granules.

mRNA partitioning to stress granule unchanged by m6A loss. In principle, the differential localization of the Fem1b, Fignl1, Grk6, and Polr2a mRNAs could be due to m6A differences or to other features of each mRNA. We analyzed the partitioning of several poly m6A mRNAs to stress granules in wildtype and ΔMETTL3 mES cells as assessed by m6A immunoprecipitation followed by m6A mapping (Supplementary Fig. 1B, Batista et al.) and by qRT-PCR (Supplementary Fig. 1C). Nanog is a positive control that was previously shown by Batista et al. to be m6A depleted by m6A mapping and qRT-PCR (Supplementary Fig. 1C), which we reconfirmed with our analysis (Supplementary Fig. 1C). In addition, consistent with the role of m6A in reducing mRNA stability, we see a substantial increase in m6A modified mRNAs in ΔMETTL3 mES cells compared to wild-type mES cells (Supplementary Fig. 1D). This was also observed for Nanog mRNAs by Batista et al. (Supplementary Fig. 1D). Ten of these 11 mRNAs have multiple m6A mapped sites distributed in different mRNA regions (Supplementary Fig. 1B and Batista et al., which would be expected to increase any possible contribution from m6A modification. A critical observation is that we observed no significant differences between WT and ΔMETTL3 mES cells in the stress granule accumulation of all 11 m6A mRNAs tested (Fig. 1, Supplementary Fig. 2A–B, Supplementary Movies 1–2). These results argue that m6A modification does not play a major role in the targeting of these mRNAs to stress granules.

We also looked at 3 other RNAs where the modification did not change between WT and ΔMETTL3 cells or was unmodified to begin with (Fem1b, Fignl1, and Polr2A) (Supplementary Fig. 1C, Supplementary Fig. 2C, D). We see no changes in the localization of these mRNAs, and Fem1b and Fignl1 mRNA levels did not change as drastically between ΔMETTL3 and WT cells compared to the other m6A modified RNAs (Supplementary Fig. 1D).

Length, not m6A, correlates with stress granule enrichment. These results demonstrate that m6A modifications in mRNAs are not a strong contributor to mRNA partitioning in stress granules. However, prior work has described a correlation between the number of m6A sites on mRNAs and enrichment in stress granules and mRNP granules, respectively. However, this correlation may be fortuitous since the number of m6A sites and stress granule enrichment are both correlated with mRNA length (Fig. 2A).
To computationally examine if m6Am modification contributes to mRNA partitioning into stress granules independent of length, we performed multiple regression analyses where we compared the effect of mRNA length on stress granule partitioning with or without an additional contribution of m6Am modification. We found that a linear regression model based on mRNA length alone showed an $R^2$ score of 0.41 for predicted stress granule enrichment vs. observed stress granule enrichment (Fig. 2B). A linear regression model based on the mapped m6A sites per transcript and m6A ratio showed an $R^2$ score of 0.23 for predicted stress granule enrichment vs. observed stress granule enrichment (Fig. 2C).

While mRNA length was a better predictor of stress granule enrichment than m6A modification, this does not rule out the possibility that m6A plays an additional role in stress granule enrichment in concert with length. To parse the relative contributions of length and m6A to stress granule enrichment, we built a multiple linear regression model using length, the mapped m6A sites per transcript, and the m6A ratio as predictors of stress granule enrichment (Fig. 2D). When we considered the combination of m6A mapped m6A sites per transcript and m6A ratio and RNA length, our model improved from an $R^2$ value of 0.41 to 0.47, suggesting that m6A modification could explain a maximum of an additional 6% of the variance in stress granule enrichment (Fig. 2D). We see similar results when we considered the individual contribution of m6A ratio or m6A mapped sites, which increased the $R^2$ value from 0.41 to 0.44 or 0.45.

Fig. 1 There is no difference in the fraction of polymethylated m6A mRNAs in arsenite-induced stress granules between wildtype and ΔMETTL3 mES cells. Representative images of wildtype and ΔMETTL3 mES cells stressed for 1 h with arsenite and costained with single-molecule FISH probes against Bahcc1, Dlx30, Tnrc6c, Mtf2, Aff1, Lrp5, Rictor, and Zeb1 (red) and antibody against PABP protein (green). The nuclei are stained with DAPI (blue). Scale bar is 1 µM. Standard deviations are derived from three biological replicates. ns denotes not significant respectively (unpaired two-tailed Student’s t test, P > 0.05). Source data are provided for this Figure (see data availability).
respectively (Supplementary Fig. 3A–C). These results suggest that m^6A modification provides a minimal impact on mRNA partitioning into stress granules.

While adding m^6A modifications improved our \( R^2 \) metric from 0.41 to 0.47, the \( R^2 \) metric does not give any insight into to what degree m^6A might enhance stress granule enrichment. To examine the degree to which transcript length and the number of m^6A sites contribute to stress granule enrichment, we simulated data representing transcripts of lengths up to 7.5 kb and ranging from 0 to 7 m^6A sites and analyzed the predicted...
stress granule enrichment values of the multiple linear regression model (Fig. 2E). We observed that transcript length showed a stronger influence on stress granule enrichment than m6A modifications (Fig. 2E), which can be visualized by the increase in stress granule enrichment along the y-axis. The number of m6A modifications did show some effect on stress granule enrichment (x-axis), and we note that as the number of m6A sites per transcript increases (y-axis), stress granule enrichment also is predicted to slightly increase.

It should be noted that this simulation is likely overestimating the contributions of m6A modifications for two reasons. First, we simulated transcripts containing up to 7 m6A sites, but >97% of transcripts are thought to have 4 or fewer m6A sites (Fig. 2F)\(^1\). Second, in this analysis using the number of m6A sites per transcript, we assumed that each m6A site is 100% modified, which is unlikely to be true since often specific m6A modification sites are modified at lower rates\(^2\). In this modeling, we found that each additional m6A modification on an RNA of a fixed 2.5 kb length would lead to a ~1.6% increase in stress granule enrichment (Fig. 2E). Thus, for the majority (>97%) of transcripts, which contain four or fewer m6A sites, we would predict m6A to maximally account for a 6.4% increase in enrichment.

Taken together, our computational analysis suggests there is a correlation between m6A modification and stress granule enrichment that could explain ~6% of the variance in stress granule partitioning. Our multiple linear regression model is limited by the fact that we compare m6A and stress granule enrichment partitioning. Our multiple linear regression model is limited by reasons. First, we simulated transcripts containing up to 7 m6A sites, but >97% of transcripts are thought to have 4 or fewer m6A sites (Fig. 2F)\(^1\). Second, in this analysis using the number of m6A sites per transcript, we assumed that each m6A site is 100% modified, which is unlikely to be true since often specific m6A modification sites are modified at lower rates\(^2\). In this modeling, we found that each additional m6A modification on an RNA of a fixed 2.5 kb length would lead to a ~1.6% increase in stress granule enrichment (Fig. 2E). Thus, for the majority (>97%) of transcripts, which contain four or fewer m6A sites, we would predict m6A to maximally account for a 6.4% increase in enrichment.

YTHDF proteins minimally recruit RNAs to stress granules.

To experimentally examine if YTHDF proteins could have any impact on the recruitment of mRNAs to stress granules, we tethered 25 YTHDF proteins on luciferase reporter RNAs and examined its localization to stress granules using the LN-BoxB system\(^23\). By tethering 25 proteins to a single mRNA, which is an extreme condition, this assay should reveal if YTHDF proteins can have any effect on mRNAs partitioning into stress granules. YTHDF1 and YTHDF2 fused to GFP-LN transgene were transfected into U-2 OS cells stably expressing a luciferase reporter containing 25-BoxB stem-loops (Fig. 3A). We observed the tethering of YTHDF1-GFP-LN and YTHDF2-GFP-LN is functional because their expression reduced 25-BoxB-luciferase reporter levels, but not 0-BoxB-luciferase reporter levels (Fig. 3B), which is consistent with these YTHDF proteins enhancing mRNA degradation when associated with mRNAs\(^17\). We quantified the localization of the reporter mRNA to stress granules by single-molecule FISH after stress for 60 minutes.

We observed that tethering YTHDF1 or YTHDF2 proteins could increase the recruitment of the 25-BoxB-luciferase reporter mRNA to stress granules from an average of 11% to an average of 21 or 18% for YTHDF1-GFP-LN and YTHDF2-GFP-LN, respectively (Fig. 3C, D). This effect required the tethering of the YTHDF proteins to the reporter mRNA since no significant difference in stress granule recruitment was observed with the 0-BoxB-luciferase reporter control mRNA (Fig. 3C, D). In side-by-side experiments with the same cell line, the level of recruitment by YTHDF proteins was similar to the effect of tethering G3BP-GFP-LN (18%). Therefore, these experimental results support the idea that the recruitment of YTHDF proteins to mRNA can increase their partitioning into stress granules. However, it is important to note that the number of tethered YTHDF proteins is far more than the number of m6A sites observed for endogenous mRNAs (Fig. 2F), arguing that individual m6A modifications play at most a minor role (~6%) in the recruitment of endogenous mRNAs to stress granules (Fig. 2).

The YTHDF tethering assays should be interpreted with caution for a number of issues that may overemphasize the role of m6A recruiting mRNAs to stress granules. First, the Kd for BoxB elements binding to LN proteins is in the order of ~1.3 nM\(^24\), which is approximately 500-fold greater than the interaction between YTHDF2 proteins and m6A-modified mRNAs (~2.54 μM)\(^25\). Second, BoxB-containing RNAs do not have to compete with other mRNAs for binding to LN-fusion proteins in the tethering assay. In contrast, m6A-containing RNAs are likely competing with other m6A RNAs for binding to YTHDF proteins. Thus, this assay has many significant drawbacks that may artificially enhance the impact of m6A on mRNA recruitment to stress granules.

Several observations suggest that YTHDF and G3BP proteins behave similarly and share apparently contradictory properties. One shared property of YTHDF and G3BP proteins is that they can both affect the formation of stress granules when assessed by the overall area of stress granules\(^26-28\). Despite this role in stress granule formation, neither the interaction of either G3BP proteins (based on CRISPR knockout and stress granule transcriptome analysis\(^23\)) nor YTHDF proteins (as assessed by the absence of m6A modification), affect the partitioning of mRNAs into stress granules. We suggest the resolution of this apparent conflict is due to the difference between examining the partitioning of an individual mRNA into stress granules compared to examining the formation of stress granules in bulk. The targeting of any individual mRNA to a stress granule is a summation of many interactions and therefore, any individual interaction makes little or no difference\(^23\). In contrast, the overall assembly of a stress granule is a highly cooperative process, and if the average
interaction between many mRNAs is reduced by even a small percentage, the assembly curve can shift into the regime where stress granules are not formed.

**Discussion**

In summary, we present three observations indicating that m6A modification provides at most a minimal role in mRNP targeting to stress granules. Most importantly, in a direct test of the role of m6A modification, we observed that cells deficient in m6A modification do not show differences in the partitioning of thirteen m6A modified mRNAs into stress granules. We do see a small correlation of m6A modification with stress granule enrichment in linear regression analyzes, but in the absence of experimental data showing a role for m6A modification in stress granule targeting, it remains possible this correlation is indirect.
and to other shared properties. Finally, we observed tethering 25 YTHDF proteins to a reporter mRNA can increase stress granule accumulation. Taken together, these results argue m6A modifications have a small effect on mRNA partitioning, which we estimate as <10%. Modeling the impact of m6A modifications suggests the most optimal situation for m6A to make any difference in stress granule recruitment will be for mRNAs that have limited inherent targeting to stress granules and contain multiple numbers of highly modified m6A sites (Fig. 2E). For example, one additional m6A site on an mRNA with 2500 bases only increases partitioning in stress granules by 1.6%. Thus, our results, in general, argue that m6A is not an epigenetic RNA marker that can direct endogenous mRNA localization to stress granules (Ries et al.).

We note three limitations of our observations. First, there is the formal possibility that the absence of m6A on mRNAs reduces their accumulation in stress granules and that the AMETTL3 mES cells have an additional phenotype that increases mRNA accumulation in stress granules by some other mechanism, although the absence of an increase in the partitioning of unmethylated mRNAs into stress granules in the AMETTL3 cells makes this unlikely (Supplementary Fig. 2D). Second, the location of the m6A modification on the mRNAs may play a role in stress granule location. For example, Anders et al. proposed stress-induced m6A deposited near the start codon on the 5′UTR direct mRNAs to stress granules during cellular stress with the help of YTHDF3 proteins. However, we do not see changes in stress granule localization for two mRNAs with 5′UTR m6A modifications, Mfy2 and Pik3r2, between wildtype and AMETTL3 mES cells (Fig. 1, Supplementary Fig. 2B), although we cannot fully rule out stress-induced methylation in the 5′UTR affecting some other mRNAs partitioning into stress granules. Finally, it is possible that m6A modification might affect mRNA partitioning into stress granules in a different cell type.

Given the limited effect of m6A modification on mRNAs partitioning into stress granules, how can one understand mRNA recruitment into stress granules? Our results with m6A modification are consistent with the model wherein RNA targeting to granules is a summative effect involving many interactions, and no single individual protein-RNA interaction dominates, including m6A-YTHDF interaction. This model explains why RNA length, which is likely highly correlated with valency, which is the number of interactions the mRNA can make with other RNAs and proteins, is so strongly correlated with enrichment in stress granules. This model also explains why length correlation with stress granule enrichment is a consistent metric in other cell types with different genes expressed. We suggest that this summative effect may be a general property of RNA targeting to RNP granules because length bias is also seen in the RNAs that accumulate in P-bodies during stress, P-granules, and BR-Bodies. Therefore, like stress granules, no single individual protein-RNA interactions will substantially affect RNP granule partitioning.

If the m6A modification does not alter RNA partitioning into stress granules, why does the knockdown of multiple YTHDF proteins lead to a decrease in stress granule formation? We suggest the explanation for this observation may be that the IDRs of the YTHDF proteins act as non-specific assembly factors by forming positive interactions with other components of stress granules once the granules are assembled by other specific interactions. This is similar to observations both in vitro and in cells that promiscuously interacting IDRs, which can interact with many other proteins, increase the assembly of condensates by shifting the assembly diagram towards increased assembly once
the promiscuous IDRs are concentrated in the condensate by specific interactions. Thus, we suggest that YTHDF proteins, once bound to mRNAs through m^A modifications, are recruited into stress granules and then through promiscuous interactions with other components of the stress granules, may enhance the assembly of these RNP granules.

**Methods**

**Multiple linear regression analysis.** Stress granule transcriptome data was obtained from Khoon et al. A reference transcriptome was acquired from GENCODE (GRCh38.p13). Reads were preprocessed using trim galore (version 0.6.5). Reads were mapped to the genome using salmon (version 1.8.0). Differential expression analysis was performed using DESeq2 (release 3.15) (Supplementary Data 1). m^A mapped sites, and m^A ratio were obtained from Xiang et al. and Molinie et al. The stress granule enrichment dataset was filtered to only consider the most highly expressed isoform for each gene. The m^A dataset used Refseq annotation, while our original dataset used Ensembl gene ID’s. In order to assign m^A peaks to genes in our stress granule isoform data, we matched Refseq annotations to gene names using pybiomart, counted the number of m^A peaks per gene, and merged the two datasets. Transcript lengths were also obtained using pybiomart.

Multiple linear regression analysis was performed using the scikit-learn package in python. Multiple models were constructed using transcript length, the number of m^A sites, the combination as features, and the observed fraction of molecules within stress granules as a response variable. ^2 values were obtained to assess the predictive power for each of these models by comparing predicted vs. observed stress granule enrichment. The visualization of our linear model was created by simulating transcripts of varying transcript lengths up to 7.5 kb in size and of varying numbers of m^A sites and running these data through our multiple linear regression model.

All visualizations in Fig. 2 were created using the matplotlib and seaborn packages in python. All code pertaining to this analysis is contained within the following GitHub repo (https://github.com/matheny/mes/) and linked to Zenodo (10.5281/zenodo.6585747).

**Cell lines.** Wildtype and ΔMETTL3 mES cells were maintained in DMEM supplemented with 13% FCS (ES-009-B, Millipore Sigma), 1X non-essential amino acids (11-140-050, Gibco), 1X penicillin-streptomycin-glutamine (10378016, Thermo Fisher Scientific), 1 mM sodium pyruvate (11360070, Thermo Fisher Scientific), 1X EmbryoMax 2-mercaptoethanol (ES-007-E, Millipore Sigma), 10,000 units/ml ESGRO Leukemia Inhibitory Factor (ESG1108, Sigma-Aldrich), 1 mM PD0325901 (S1036, Selleck Chemicals), and 3 µM CHIR99021 (SML0146, Sigma-Aldrich) and maintained at 37 °C and 5% O_2 on 0.1% gelatin (G6144, Sigma-Aldrich) coated tissue culture plates. U-2 OS cells were maintained in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin/10% FBS at 37 °C and 5% O_2. U-2 OS cells stably expressing 0- and 25× ΔMETTL3 were generated by transducing with 500 µg of ΔMETTL3 plasmid and pLenti-EF-sh-Lenti-ΔMETTL3 (SML0146, Sigma-Aldrich) and maintained at 37 °C and 5% O_2 on 0.1% gelatin-coated tissue culture plates. U-2 OS cells were maintained in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin/glutamine at 37 °C and 5% O_2. U-2 OS cells stably expressing 0- and 25× ΔMETTL3 were generated by transducing with 500 µg of 0% or 25% ΔMETTL3 expression plasmid and pLenti-EF-ΔMETTL3 (SML0146, Sigma-Aldrich) and maintained at 37 °C and 5% O_2 on 0.1% gelatin (G6144, Sigma-Aldrich) coated tissue culture plates.

**Cell line maintenance.** mES cells were maintained in DMEM supplemented with 10% FCS (ES-009-B, Millipore Sigma), 1X non-essential amino acids (11-140-050, Gibco), 1X penicillin-streptomycin-glutamine (10378016, Thermo Fisher Scientific), 1 mM sodium pyruvate (11360070, Thermo Fisher Scientific), 1X EmbryoMax 2-mercaptoethanol (ES-007-E, Millipore Sigma), 10,000 units/ml ESGRO Leukemia Inhibitory Factor (ESG1108, Sigma-Aldrich), 1 µM PD0325901 (S1036, Selleck Chemicals), and 3 µM CHIR99021 (SML0146, Sigma-Aldrich) and maintained at 37 °C and 5% O_2 on 0.1% gelatin (G6144, Sigma-Aldrich) coated tissue culture plates. mES cells were maintained in DMEM supplemented with 10% FCS (ES-009-B, Millipore Sigma), 1X non-essential amino acids (11-140-050, Gibco), 1X penicillin-streptomycin-glutamine (10378016, Thermo Fisher Scientific), 1 mM sodium pyruvate (11360070, Thermo Fisher Scientific), 1X EmbryoMax 2-mercaptoethanol (ES-007-E, Millipore Sigma), 10,000 units/ml ESGRO Leukemia Inhibitory Factor (ESG1108, Sigma-Aldrich), 1 µM PD0325901 (S1036, Selleck Chemicals), and 3 µM CHIR99021 (SML0146, Sigma-Aldrich) and maintained at 37 °C and 5% O_2 on 0.1% gelatin (G6144, Sigma-Aldrich) coated tissue culture plates.

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tube. The reaction was incubated at 37 °C for 16 h in a PCR machine and fresh. In all, 3 μl of Taq DNA polymerase was added 8 h into reaction. Oligo sequences can be found in Supplementary Table 1. The reaction was purified using an oligo cleanup concentrator kit following the manufacturer’s protocol. Labeled oligonucleotides were then analyzed by absorption at 629 nm (which is the absorption max for Atto 653) or 554 nm (which is the absorption max for Atto 647). The concentration of labeled oligos was then determined following Beer’s law and diluted to a stock concentration of 12.5 μM.

Imaging parameters are adapted from previously in Khong et al. for Supplementary Fig. 2C–D and Fig. 3. Images were sampled on a GE wide-field DeltaVision Elite Microscope equipped with an Olympus UPlan-Sapo 100X/1.40-NA Oil Objective lens and a PCO Edge scCMOS Camera using appropriate filters with the help of SoftWoRx Imaging Software. Entire cells were imaged using an approximate number of Z sections (0.2 μm step size). Imaging parameters were adjusted to capture an approximate number of Z sections (0.2 μm step size). Imaging parameters were adapted from previously in Khong et al. for Supplementary Fig. 1E–F. Each data point in Fig. 3 represents one cell. Only cells that were adequately expressing the transgene GFP-adequately expressing the transgene GFP.

Deviations were determined from three biological replicates in Supplementary Fig. 3. The images were blinded, and the single-molecule FISH spots were manually counted. Total numbers of spots outside and inside stress granules were determined. All images shown in the manuscript are deconvolved, and the brightness/contrast adjusted to best indicate data.

Images for Fig. 1 and Supplementary Fig. 1C–D were taken on a laser scanning confocal microscopy (Nikon A1R microscopy) with x100 objective (1.45 NA, Plan Apo I), a pixel size of 0.10 μm, and an integration time of 2.2 sec. Other imaging parameters were adjusted to capture fluorescence within the microscope’s dynamic range and kept the same between samples when looking at the same mRNAs by smFISH. Standard deviations were determined from three biological replicates in Supplementary Fig. 1E–F. Each data point in Fig. 3 represents one cell. Only cells that were adequately expressing the transgene GFP-Δ5 ('transgene GFP-Δ5'), G3BP-GFP-Δ5, YTHDF1-GFP-Δ5, and YTHDF2-GFP-Δ5 were counted (50,000 < Cell Total Cell Fluorescence <2,000,000 (determined by ImageJ)) in Fig.3. The images were blinded, and the single-molecule FISH spots were manually counted. Total numbers of spots outside and inside stress granules were determined. All images shown in the manuscript are deconvolved, and the brightness/contrast adjusted to best indicate data.

For Fig. 1 and Supplementary Fig. 1C–D were taken on a laser scanning confocal microscopy (Nikon A1R microscopy) with x100 objective (1.45 NA, Plan Apo I), a pixel size of 0.10 μm, and an integration time of 2.2 sec. Other imaging parameters were adjusted to capture fluorescence within the microscope’s dynamic range and kept the same between samples when looking at the same mRNAs by smFISH. Image analysis for Fig. 1 and Supplementary Fig. 1C–D was performed using Imaris (Version 9.7.0). Only single FISH spots in the cytoplasm were counted. smFISH in the nucleus was excluded from the analysis. We used Imaris to classify single FISH spots, stress granules and nuclei, and smFISH spots in stress granules and nuclei. Image analysis parameters for the same smFISH was done equally between smFISH spots, stress granules and nuclei, and smFISH spots in stress granules and nuclei. Image analysis parameters for the same smFISH was done equally between smFISH spots, stress granules and nuclei, and smFISH spots in stress granules and nuclei. Image analysis parameters for the same smFISH was done equally between smFISH spots, stress granules and nuclei, and smFISH spots in stress granules and nuclei. Image analysis parameters for the same smFISH was done equally between smFISH spots, stress granules and nuclei, and smFISH spots in stress granules and nuclei. Image analysis parameters for the same smFISH was done equally between smFISH spots, stress granules and nuclei, and smFISH spots in stress granules and nuclei. Image analysis parameters for the same smFISH was done equally between smFISH spots, stress granules and nuclei, and smFISH spots in stress granules and nuclei. Image analysis parameters for the same smFISH was done equally between smFISH spots, stress granules and nuclei, and smFISH spots in stress granules and nuclei. Image analysis parameters for the same smFISH was done equally between smFISH spots, stress granules and nuclei, and smFISH spots in stress granules and nuclei. Image analysis parameters for the same smFISH was done equally between smFISH spots, stress granules and nuclei, and smFISH spots in stress granules and nuclei.
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Author contributions
A.K., T.M., and R.P. conceptualized the study. A.K., T.M., and T.H. developed and designed the methodology. A.K. and V.B. performed experiments and collected data. A.K. and T.M. performed formal analysis. A.K. and R.P. wrote and edited the manuscript. A.K. and R.P. supervised the project. A.K. and R.P. provided funding.

Competing interests
R.P. is a founder and consultant for Faze Medicines. Other authors do not have competing interests.

Additional information
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