m^6A and eIF2α-ⓟ Team Up to Tackle *ATF4* Translation during Stress

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While m^6A modification of mRNAs is now known to be widespread, the cellular roles of this modification remain largely mysterious. In this issue of *Molecular Cell*, Zhou et al. (2018) show that m^6A modification unexpectedly contributes to the established uORF- and eIF2α-ⓟ-dependent mechanism of *ATF4* translational regulation in response to stress.

Studies of translational control have benefited enormously from development of a suite of tools that allow *in vivo* views of ribosome positions, providing information that was difficult to extract from preexisting genetic and *in vitro* approaches. These new methods include ribosome profiling, which measures genome-wide translation via the sequencing of mRNA fragments bound by 80S translating ribosomes (Ingolia et al., 2012), and QTI-seq (quantitative translation initiation sequencing), which precisely and quantitatively maps sites of translation initiation (Gao et al., 2015). Broad application of these methods has been powerful, often confirming defined translation mechanisms, but sometimes suggesting a need to revisit long-held assumptions about general and specific mechanisms of translational regulation. Zhou and colleagues integrate use of these and other global approaches to reveal an added layer to the regulation responsible for stress-responsive translation of the mRNA for transcription factor ATF4 (Zhou et al., 2018).

The 5′ leader of *ATF4* is known to contain two upstream open reading frames (uORFs). In the absence of stress, it is thought that the *ATF4* ORF is poorly translated due to the translation of uORF2, which is overlapping and out of frame with the *ATF4* ORF start codon (Vattem and Wek, 2004; Lu et al., 2004). Under an array of stresses, however—including starvation—the translation initiation factor eIF2α is phosphorylated (ⓟ), and the *ATF4* ORF is resultantly translated. A general model for uORF-based regulation, based on elegant studies of the Gcn4 transcription factor-encoding gene in budding yeast, proposes that eIF2α-ⓟ reduces the amount of ternary complex (TC) available to mediate translation initiation. This causes a general decrease in translation initiation. However, in the case of at least a subset of genes with ORF-repressive uORFs that are translated under non-stressed conditions, the ultimate effect is increased translation of the main ORF. This is due to "leaky scanning" by ribosomes that lack TCs, and therefore bypass uORFs (Hinnebusch, 2014). Consistent with this model, mutation of uORF2 enhances *ATF4* translation under non-stressed conditions (Vattem and Wek, 2004; Lu et al., 2004). Zhou and colleagues explored this mechanism in detail, comparing ribosome profiling and QTI-seq data for control and starved cells. They observed that, while 80S translating ribosome occupancy within the *ATF4* ORF indeed increased upon starvation, there was no associated decrease in ribosome occupancy of uORF2. This result was confirmed by reporter assay and led Zhou and colleagues to investigate the possibility that *ATF4* regulation involved additional mechanisms beyond the prevailing model.

Mass spectrometry of proteins associated with *ATF4* mRNA revealed enrichment of the RNA demethylase ALKBH5 in starved cells compared to control conditions. ALKBH5 can reverse N^6-methylation of adenosine (m^6A); therefore, the authors performed global mapping of the m^6A mRNA modification sites to investigate a possible link between this modification and *ATF4* translation. They noted that the *ATF4* mRNA showed several regions of m^6A modification, including one within uORF2. Intriguingly, this site in particular showed a decrease in m^6A upon starvation. m^6A modification is thought to be the most common internal mRNA modification, although the degree of its prevalence has only recently become clear. Some m^6A sites have been shown to be capable of driving transcript-specific translational regulation, poly(A) site choice, and mRNA stability, but no function is currently known for the large majority of known m^6A sites (Meyer and Jaffrey, 2017).

The authors considered the possibility that m^6A-based regulation contributed to the translational control of *ATF4*. They first tested this model by knocking down ALKBH5, which indeed revealed an absence of translational induction of the *ATF4* ORF upon starvation despite high levels of eIF2α-ⓟ. Consistently, inhibition of m^6A mRNA methylases enhanced *ATF4* translation upon starvation. Reporter assays demonstrated that blocking m^6A modification within uORF2 increased translation from the main ORF, an effect that did not occur in the absence of starvation, suggesting that this mechanism of regulation is interdependent with the classical one based on eIF2α-ⓟ (Figure 1). The authors propose that m^6A impedes scanning ribosomes, thus promoting translation initiation of uORF2 and decreasing the likelihood of re-initiation at the downstream and out-of-frame *ATF4* start codon. Genome-wide m^6A mapping in conjunction with QTI-seq identified cases in which starvation-induced decreased m^6A near start codons correlated with decreased initiating ribosome occupancy. Interestingly, in these cases, decreased initiating ribosome occupancy was not linked to a

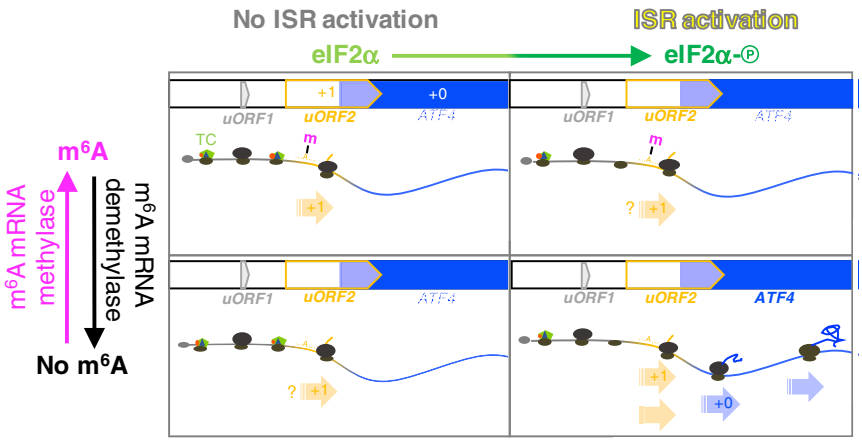

Figure 1. An Integrated Model for *ATF4* Translation during the Integrated Stress Response
During non-stressed conditions (left), methylation of uORF2 (top) aids the repression of *ATF4* ORF translation by impeding scanning ribosomes. Under these conditions, ternary complexes (TCs) are non-limiting and allow efficient translation of uORF2, which is thought to prevent *ATF4* ORF translation. Upon activation of the integrated stress response (right), eIF2α is phosphorylated, reducing available TCs. This, combined with removal of an m^{6}A site within uORF2 (bottom), enables efficient translation of the *ATF4* ORF. Surprisingly, this condition also results in increased translation of uORF2, suggesting a more complex mechanism than previously thought. Question marks denote that the level of uORF2 translation with either m^{6}A modification or eIF2α-Ⓟ alone is not directly investigated in this study.

decrease in translation of the gene, suggesting that methylation may change the dwell time of initiating ribosomes rather than decreasing the number of initiation events. Furthermore, Zhou and colleagues note a correlation between increased local m^{6}A modification and usage of non-canonical start codons during amino acid starvation.

The added complexity to *ATF4* regulation reported here is fascinating given that *ATF4* is arguably the best-characterized model for uORF-based regulation in mammals. This work paves the way to answering a number of interesting questions about uORF-based regulation in general, as well as about this specific and important case. The interplay between eIF2α phosphorylation and mRNA methylation in *ATF4* regulation warrants further study. While it seems clear that both contribute to *ATF4* translation, the authors propose that the m^{6}A stalling mechanism promotes translation of uORF2, and that removal of m^{6}A at uORF2 results in increased translation of the *ATF4* ORF *and* uORF2, a model that is distinct from that proposed to explain regulation based on eIF2α phosphorylation. Exactly how the low TC-based model resulting in leaky scanning contributes to this model is an intriguing open question. Reconciling these models and determining the precise mechanism of starvation-induced translation initiation at the *ATF4* ORF is clearly an important future direction.

Locally slowed ribosome scanning is an appealing model to explain suboptimal start codon usage and, as the authors propose, may be used to explain the translation initiation now known to occur at some—but not all—non-AUG codons (Brar, 2016). A similar model to the one proposed here, but based on mRNA structure rather than modification, has also been proposed to explain the translation initiation seen at sub-optimal start codons in multiple frames in repeat-associated non-ATG (RAN) translation, a phenomenon associated with several neurodegenerative diseases (Kearse et al., 2016). The general idea that *cis*-context within an mRNA molecule can determine its level and mechanism of translation is not a new one, but it is an area of study that has expanded rapidly in recent years (Brar, 2016). New methods, including those that have enabled genome-wide mapping of m^{6}A, have also resulted in identification of an array of other mRNA modifications, many of which were previously thought to be limited to noncoding RNAs, although in most cases, the roles for these modifications in mRNA remain mysterious (Gilbert et al., 2016). It is likely that further study will reveal that specific combinations of *cis*- and *trans*-control are broadly used to enable necessary specificity in translational regulation, akin to the types of regulation that are well established to provide specificity to transcription.

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