Cancer-associated mRNAs regulated by the Helix-Loop-Helix motif of human EIF3A

By

Marina Volegova

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Committee in charge:

Professor Jamie Doudna Cate, Chair
Professor Donald Rio
Professor Elizabeth Purdom
Assistant Professor Nicholas Ingolia

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Abstract
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Marina Volegova
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Improper regulation of translation initiation, a vital check-point of protein synthesis in the cell, has been linked to a number of cancers. Overexpression of protein subunits of eukaryotic translation initiation factor 3 (eIF3) has been associated with increased translation of mRNAs involved in cell proliferation. In addition to playing a major role in general translation initiation by serving as a scaffold for the assembly of translation initiation complexes, eIF3 regulates translation of specific cellular mRNAs and viral RNAs. Mutations in the N-terminal Helix-Loop-Helix (HLH) RNA-binding motif of the EIF3A subunit in eIF3 interfere with Hepatitis C Virus Internal Ribosome Entry Site (IRES) mediated translation initiation in vitro. Here we show that the EIF3A HLH motif controls translation of a small set of cellular transcripts enriched in oncogenic mRNAs, including MYC. We also demonstrate that the HLH motif of EIF3A acts specifically on the 5'-UTR of MYC mRNA and modulates the function of EIF4A1 on select transcripts during translation initiation. In Ramos lymphoma cell lines, which are dependent on MYC overexpression, mutations in the HLH motif greatly reduce MYC expression, impede proliferation and sensitize cells to anti-cancer compounds. These results reveal the potential of the EIF3A HLH motif in eIF3 as a promising chemotherapeutic target.
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Chapter 1

Introduction

Eukaryotic translation initiation is a tightly controlled part of protein synthesis and its deregulation can lead to a wide variety of disorders, including cancer (Ruggero 2013). Overexpression of many translation initiation factors, such as the subunits of the mRNA cap-binding eukaryotic initiation factor 4F (eIF4F) complex, has been correlated with neoplasia and cancer incidence (Bhat et al. 2015). Overexpression of several subunits of eIF3 in particular is strongly associated with malignant transformation (Hershey 2015). Furthermore, upregulation of the translation machinery is not only involved in boosting global protein synthesis but has also been shown to regulate functionally related gene classes (Truitt & Ruggero 2016). Deregulation of the translation machinery can selectively re-program the downstream gene expression profile, which leads not only to tumorigenesis but also the diversification of cancer cell behaviors (Truitt & Ruggero 2016). To advance our understanding of the processes of cellular translation and to discover novel therapeutic approaches tailored to different cancer types, it is critical to understand the mechanisms behind how translation initiation factors control not only global mRNA translation but specifically regulate cellular pathway networks.

During canonical translation initiation, the eukaryotic small (40S) ribosomal subunit first associates with initiation factors eIF1, eIF1A, eIF3, and eIF5, which facilitate the loading of the eIF2 ternary complex (eIF2-GTP-Met-tRNA\text{Met}^\text{A})\text{t}, thus forming the mRNA-binding 43S pre-initiation complex (PIC) (Aitken & Lorsch 2012) (Fig. 1). The 43S PIC then binds to the mRNA-bearing eIF4F complex (eIF4A, 4B, 4G, 4E) in the 5' untranslated region (5'-UTR) of the mRNA to begin directional scanning for the start codon (Hinnebusch & Lorsch 2012; Hinnebusch 2017). The mRNA attachment to the 43S PIC is facilitated by eIF4F binding to the 7-methylguanosine (m\textsuperscript{7}G) cap at the 5' end via the cap-binding eIF4E, and the subsequent scanning of the 5'-UTR is enabled by the eIF4G scaffold recruiting the DEAD-box helicase eIF4A for local unwinding of the mRNA secondary structure (Hinnebusch 2014). eIF1 and eIF1A stabilize the required open conformation of the 43S PIC as the 5'-UTR is scanned base-by-base for the complementary triplet to the anticodon of the Met-tRNA\text{A}_{\text{t}} (Nanda et al. 2009; Hinnebusch 2014). Once the AUG codon is recognized, eIF1 and phosphate (P\text{t}) are released, eIF2-GDP dissociates along with eIF5, and eIF5B catalyzes subunit joining with the large 60S ribosomal subunit to form the 80S initiation complex (Hinnebusch 2014) (Fig. 1).

Throughout the initiation pathway, eIF3 – the largest of the translation initiation factors, comprised of 13 subunits (eIF3A-M) in mammals (Georges et al. 2015) – is wrapped around the 40S ribosomal subunit, interacting at both the mRNA entry and exit channels, promoting 43S PIC assembly and mRNA recruitment (Hinnebusch 2017). While most cellular mRNAs are thought to be translated by the canonical cap-dependent scanning mechanism, with eIF3 serving as a stabilizing scaffold for initiation factor and mRNA recruitment (Hinnebusch 2006), compelling new evidence indicates that eIF3 can also regulate alternative pathways of translation initiation in a transcript specific manner.
In a recent genome-wide study, eIF3 has been shown to either activate or repress translation of select cellular mRNAs, by interacting with RNA structural elements in the 5'-UTRs of these transcripts (A. S. Y. Lee et al. 2015). The study used photo-activatable crosslinking and immunoprecipitation (PAR-CLIP) (Hafner et al. 2010) followed by high throughput sequencing to uncover the interactions of eIF3 subunits with mRNAs in human cells. eIF3 subunits A, B, D, and G preferentially crosslinked to a small set of mRNAs, most often with one eIF3 binding site per transcript clustering primarily in the 5'-UTR region of the associated mRNAs. Subsequent functional validation showed that eIF3 binds to discrete structural elements in the 5'-UTR and can either upregulate or repress translation of the targeted transcript in a cap-dependent manner, distinct from eIF3 regulation of cap-independent viral Internal Ribosome Entry Site (IRES) mediated translation initiation (Fraser & Doudna 2007; Thakor et al. 2017). Both the activated and repressed mRNAs in the eIF3 PAR-CLIP dataset were significantly enriched for cancer-associated proliferation pathway transcripts, as well as those involved in apoptosis and differentiation, suggesting that eIF3 plays a major role in directing downstream gene expression programs that may be hijacked during carcinogenesis. In Chapter 2, we further explore the ability of eIF3 to activate and repress translation in a transcript-specific manner, particularly in relation to gene expression programs and pathway regulation.

In another alternative eIF3-mediated mode of translation initiation, eIF3 subunit D has been shown to bind the 5'- m7G cap of mRNAs in place of eIF4E (A. S. Y. Lee et al. 2016), allowing translation of a subset of transcripts to continue under cellular stress conditions when eIF4E is inactivated by phosphorylation or inhibitory 4E binding proteins (4E-BPs) (Wang et al. 1998; Gingras et al. 1999). In this study, building on the previously described global analysis of eIF3-dependent mRNAs, the authors found that one of the top eIF3-activated targets, c-JUN, was translated in a cap-dependent but eIF4F-independent manner and that of the four eIF3 RNA-binding subunits, only eIF3D protected the 5'- m7G cap (A. S. Y. Lee et al. 2016). The crystal structure of the eIF3d cap-binding domain, the highly phylogenetically conserved C-terminal region of the protein, was determined to be a complex cup-shaped fold that contains a positively-charged RNA-binding tunnel and is very similar to the DXO 5' - cap endonucleases involved in RNA quality control (A. S. Y. Lee et al. 2016; Jiao et al. 2013). The proposed model is for eIF3D-directed cap-dependent translation, where eIF3D is not only capable of binding in place of eIF4E, but eIF4F is also blocked by additional RNA structural elements in the 5'-UTR, suggesting an orchestrated switch as opposed to more passive replacement when eIF4E is inactivated during stress (A. S. Y. Lee et al. 2016).

Finally, several studies have shown that eIF3 can also promote translation under stress conditions by binding m6A-methylated 5'-UTRs in a cap-independent manner (Meyer et al. 2015; Wang et al. 2015). The reversible N6-methyladenosine base modification is observed across the entire length of mRNAs, but its function in the 5'UTR was recently found to stimulate translation independently of eIF4F (Dominissini et al. 2012; Meyer et al. 2015). In one study, the authors use in vitro reconstitution and toeprinting assays to show that eIF3 preferentially binds to m6A 5'-UTRs and that depletion of an m6A methyltransferase, METTL3, results in decreased ribosome occupancy for mRNAs with m6A-containing 5'-UTRs but not mRNAs with m6A in other
regions of the transcript (Meyer et al. 2015). Another transcriptome-wide study using PAR-CLIP investigated the human m\(^6\)A reader protein YTHDF1 and suggested that it may serve as a bridge between eIF3 and m\(^6\)A sites in all regions of a transcript and facilitate translation (Wang et al. 2015), although these findings are contested in Meyer et al., where YTHDF1 was not found to play a role if eIF3-dependent m\(^6\)A binding or translation (Meyer et al. 2015). Clearly, m\(^6\)A-mediated cap-independent initiation is another mode of translation where the molecular basis for eIF3-dependent translation regulation and its control of gene expression networks must be further characterized.

Structural analysis using cryo-electron microscopy (cryo-EM) has revealed that the core of eIF3, a five-lobed octameric complex, localizes to the solvent-exposed “backside” of the 40S ribosomal subunit and spans the mRNA entry and exit channels (Georges et al. 2015) (Fig. 2, Fig. 3). In the mammalian 43S pre-initiation complex (PIC), eIF3 subunits eIF3A and eIF3C have been shown to directly contact the 40S ribosomal subunit (Hashem, Georges, Dhote, Langlois, Liao, Grassucci, Hellen, et al. 2013), as well as participate in interactions with initiation factors eIF1, eIF1A, eIF2, eIF5, and eIF4F (Sokabe et al. 2011; Eliseev et al. 2018), thus coordinating the ordered assembly of the 48S initiation complex. However, in contrast to canonical translation initiation, translation of the Hepatitis C Virus (HCV) genomic RNA requires an Internal Ribosome Entry Site (HCV IRES) RNA structure within the 5'-UTR, which has been shown to bind to subunits eIF3A and eIF3C within the eIF3 complex by cryo-EM and native gel shift assays (Sun et al. 2013) (Fig. 4). Subsequent cryo-EM structures also revealed how an HCV-like viral IRES dramatically displaces eIF3 from binding to the 40S subunit, while maintaining binding to eIF3 through subunits eIF3A and eIF3C, which is consistent with the aforementioned biochemical data in the previous reconstituted system (Hashem, Georges, Dhote, Langlois, Liao, Grassucci, Pestova, et al. 2013) (Fig. 5). The importance of the eIF3A subunit in mediating RNA binding is underscored by \textit{in vitro} translation experiments in which mutations of three amino acids in a predicted RNA-binding Helix-Loop-Helix (HLH) motif abrogated eIF3 binding to the HCV IRES (Sun et al. 2013). While these experiments indicated that the HLH motif of eIF3A is critical for mediating HCV IRES binding by eIF3, the importance of this motif for cellular mRNA translation remained to be determined.

Structural models for eIF3 bound to 43S and 48S pre-initiation complexes suggest that eIF3 controls the translation of the HCV IRES in a distinct manner compared to most cellular transcripts, differing from both the canonical scanning mechanism or eIF3-dependent regulation of specific transcripts. In addition to its displacement from the 40S subunit by the HCV IRES RNA, the HLH motif in eIF3A is spatially distant from the eIF3D cap-binding domain (Eliseev et al. 2018) and is more discrete than the proposed multi-subunit interfaces thought to recognize specific RNA secondary structures (A. S. Y. Lee et al. 2015) and m\(^6\)A modifications (Meyer et al. 2015; Erzberger et al. 2014). In Chapter 3, we describe the role of the human EIF3A HLH motif in regulating cellular translation initiation in cells and \textit{in vitro}. We found that the previously \textit{in vitro} characterized mutations in the EIF3A HLH motif affected the translational efficiency of transcripts involved in proliferative pathways, including the mRNAs encoding MYC, PRL3 and MET (Chen et al. 2018; Gherardi et al. n.d.; Ye et al. 2015). The selective enhancement of translation
initiation on cancer-associated transcripts by the EIF3A HLH motif is distinct from the previously described PAR-CLIP analysis and highlights a new mode of eIF3 translation regulation, as well as identifies a well-defined, discrete structural motif (Erzberger et al. 2014) that could be targeted for future drug development efforts and cancer therapeutics.
Figure 1. Eukaryotic translation initiation pathway. Adapted from (Hinnebusch 2014). The 43S pre-initiation complex (PIC) binds to the mRNA-bearing eIF4F complex (eIF4A, 4B, 4G, 4E). Upon AUG recognition, eIF1 and phosphate (Pi) are released, eIF2-GDP and eIF5 dissociate, and eIF5B catalyzes subunit joining with the 60S ribosomal subunit to form the 80S initiation complex.
Figure 2. Cryo-EM structure of the 43S pre-initiation complex. Adapted from (Hashem, Georges, Dhote, Langlois, Liao, Grassucci, Hellen, et al. 2013). A. The 43S pre-initiation complex viewed from the inter-subunit face. B. The rotated 43S pre-initiation complex viewed from the back.
Figure 3. Structure of eIF3 core. Adapted from (Georges et al. 2015). A-C. Three rotated views of the three-dimensional eIF3 core. D. Schematic representation of the eIF3 octamer architecture.
Figure 4. Cryo-EM reconstructions of eIF3 8-mer in its apo and HCV IRES IIIabc-bound form. Adapted from (Sun et al. 2013). HCV IRES IIIabc region (secondary structure schematic, left) binds to the eIF3 octamer core (in gray). The difference map shows the IRES interacting with eIF3 subunit A (IRES density in purple, right).
Figure 5. Cryo-EM structures of the canonical and IRES-bound 43S pre-initiation complex. Adapted from (Hashem, Georges, Dhote, Langlois, Liao, Grassucci, Pestova, et al. 2013). Cryo-EM structure of the canonical 43S pre-initiation complex (left), compared to cryo-EM structure of the CSFV ΔII-IRES•40S•DHX29 complex bound to eIF3 (right).
Chapter 2
Translation regulation of Ubiquitin Proteasome Pathway mRNAs by eIF3

The discovery that eIF3 can selectively activate or repress translation of certain transcripts by interacting with RNA structural elements in their 5’-UTRs provided a new outlook on the role of eIF3 in pathway regulation. The study used photoactivatable ribonucleoside-enhanced crosslinking and immunoprecipitation (PAR-CLIP) (Hafner et al. 2010) coupled with high throughput sequencing to identify the eIF3 binding sites on cellular mRNAs at a transcriptome-wide level, and subsequent validation experiments uncovered the ability of eIF3 to activate and repress translation in a transcript-specific manner (A. S. Y. Lee et al. 2015). The initial study explored the new mode of regulation by means of functional analysis of the cell proliferation regulator mRNAs c-jun and BTG1, which are respectively activated and repressed by eIF3. However, the PAR-CLIP dataset yielded a significant number of eIF3-dependent transcripts that clustered in other cell regulatory pathways, such as the Ubiquitin Proteasome Pathway (UPP) (Tu et al. 2012). To further analyze how eIF3 modulates translation of functionally related transcripts, we identified a subset of UPP-related mRNAs in the original PAR-CLIP dataset and subjected them to the same functional analysis to determine eIF3 dependency. Through a series of rigorous in vitro and in vivo assays, we assessed the contribution of mRNA features, cellular conditions, and a variety of stressors to the translation of UPP-associated transcripts, as well as interrogated the differential effect on ubiquitin encoding mRNAs, of which only one is found to be eIF3-dependent according to the PAR-CLIP dataset. We confirmed that eIF3 both activates and represses the UPP-associated transcripts identified by PAR-CLIP in a cap-dependent manner. Interestingly, we also found that translation of the eIF3-regulated ubiquitin variant transcript, UBB, is substantially correlated to eIF3 protein levels across different cell lines, providing further evidence that the interaction between eIF3 and the target 5’-UTR is specific and functionally relevant to translation control in the cell.

We selected the following UPP-associated transcripts from the PAR-CLIP dataset, based on a comprehensive literature analysis: USP1, PCNA, UBB, TRIM68, and PSMD14. USP1 is a ubiquitin specific protease that deubiquitinates the DNA replication processivity factor, PCNA, whose mRNA is also potentially regulated by eIF3; UBB is a polyubiquitin (polyUb) precursor polypeptide comprised of three ubiquitin moieties; TRIM68 is an E3 ubiquitin ligase implicated in viral response; and PSMD14 is a core component of the 19S regulatory cap complex of the 26S proteasome and is responsible for substrate deubiquitination during degradation (Das et al. 2017; Huang et al. 2006; Oh et al. 2013; Kobayashi et al. 2016; Wynne et al. 2014; Byrne et al. 2010; Luo et al. 2017). Transfection of human HEK293T cells with Renilla luciferase reporter mRNAs harboring target 5’-UTRs, either intact or with and the PAR-CLIP crosslinking site deleted, showed that the eIF3 binding sites in UBB, TRIM68, and PSMD14 mRNAs activate translation, whereas in USP1 mRNA it is somewhat repressive (Fig. 1A). In vitro translation of USP1 and PCNA reporter mRNAs in cellular extracts showed that eIF3 translationally represses USP1 but mildly activates PCNA, and both transcripts are cap-dependent, although deletion of the PAR-CLIP site significantly increases the cap dependence of USP1 (Fig. 1B-C). Since deubiquitinated PCNA cannot perform translesion synthesis of DNA (TLS),
downregulation of USP1 and subsequent accumulation of monoubiquitinated PCNA results in TLS and DNA mutagenesis (Huang et al. 2006). This suggests that in the event of eIF3 deregulation in cancer, suppression of USP1 by upregulated eIF3 would result in accumulation of monoubiquitinated PCNA and increased mutagenesis, further amplifying the carcinogenic effects of increased global translation due to overexpressed eIF3 (Hershey 2015; Zhang et al. 2007). These hypotheses should be tested in the future.

In human cells, ubiquitin is encoded by four genes: UBB and UBC code for polyubiquitin chains of 3 and 9 moieties respectively, while RPS27A and UBA52 encode ubiquitin-ribosomal protein fusions to S27A and L40 (Bianchi et al. 2015). UBB and UBC are more closely related in cellular function than RPS27A and UBA52, with the former pair coding for polyUb and the latter only coding for the ubiquitin fusion to their respective ribosomal proteins. We cloned all four 5'-UTRs into Renilla luciferase reporter mRNA constructs in order to compare the effect of cellular stress on the translation of eIF3-dependent UBB in comparison to the eIF3-independent ubiquitin transcripts. We transfected the 5'-UTR Renilla luciferase mRNAs into HEK293T cells in the presence of dithiothreitol (DTT), which induces ER stress (Oslowski & Urano 2011), and observed a relatively equal decrease in translation for all four mRNAs (Fig. 2A). When we tested the response to heat shock by transfecting and growing the cells at 42 ºC, we found that while all four mRNAs translated poorly compared to standard 37 ºC conditions, the UBA52 5'-UTR mRNA fared comparatively better (Fig. 2B). To take a closer look at the differences between UBB and UBC translation under specific inhibitor stress, we transfected the respective Renilla mRNAs in increasing concentrations of MG132 proteasome inhibitor (Oslowski & Urano 2011). In contrast to the UBB mRNA, which is downregulated during proteasomal stress, the UBC mRNA is substantially induced, which is consistent with observations that UbC rather than UbB is regulated in response to flux in ubiquitin pool dynamics in mice (Ryu et al. 2007) (Fig 3A). To address whether the ubiquitin 5'-UTRs can initiate in a cap-independent manner in vivo, we transfected standard m7G-capped and A-capped ubiquitin 5'-UTR Renilla mRNAs and found that all four ubiquitin 5'-UTRs are cap-dependent and therefore do not contain IRES-like elements (Komar & Hatzoglou 2011) (Fig. 2C). We also transfected the UBB and UBC Renilla mRNAs in the presence of 4Ei-10 cap-binding inhibitor (a gift from the Carsten Wagner lab) (Ghosh et al. 2009) and found that while the UBB mRNA was largely unaffected, translation of the UBC mRNA was curiously improved, potentially due to a decrease in competing cap-dependent mRNAs (Fig. 3B).

Ubiquitin expression downregulation by targeting UBB has been shown to inhibit tumor growth and thus has been proposed to have therapeutic value (Oh et al. 2013). We used our ubiquitin 5'-UTR Renilla mRNAs to transfect a variety of human cancer derived cell lines in order to assess relative ubiquitin transcript distributions (Fig. 4A). A549 (lung adenocarcinoma) and HepG2 (liver carcinoma) cell lines showed a different pattern of relative ubiquitin 5'-UTR translation compared to MCF7 (breast adenocarcinoma), SK-N-MC (neuroblastoma), and U2OS (osteosarcoma) cell lines (Fig. 4A). A549 and HepG2 cells have relatively higher translation of the UBC 5'-UTR, with a decrease in RPS27A translation (Fig. 4A, top row). MCF7, SK-N-MC, and U2OS cells share a different pattern of ubiquitin 5'-UTR translation distribution, with low UBC 5'-UTR translation, higher
RPS27A 5'-UTR translation, and varying UBB 5'-UTR translation (Fig. 4A, bottom row). Preferential UBC 5'-UTR translation is seen more clearly in synchronized HEK293T cells, where the translation levels in G1 synchronized cells show greatly increased translation of the UBC 5'-UTR relative to the S-phase (Fig. 4B). The highest translation of the UBB 5'-UTR is found in the MCF7 cells, which also have significantly higher levels of EIF3A, a major RNA-binding regulatory subunit of the eIF3 complex (Fig. 4C). Curiously, the outlying cell lines with the different pattern of ubiquitin translation, A549 and HepG2, have the lowest relative levels of EIF3A protein (Fig. 4C). High EIF3A levels are found in a variety of cancers and are thought to promote carcinogenesis both through increasing global protein synthesis and by stimulating translation of eIF3 dependent mRNAs (A. S. Y. Lee et al. 2015). In the case of ubiquitin variant 5'-UTR translation, these results show the preferential translation activation of UBB over other variants by eIF3 in the different cell lines.

Overexpression of eIF3 and its individual subunits has long been demonstrated in a wide variety of cancers, but the exact mechanism beyond increased global protein synthesis have remained elusive. The PAR-CLIP study demonstrated how specific interactions between eIF3 subunits and the target mRNA 5'UTR can activate or repress translation, showing how eIF3 levels can influence carcinogenesis through regulation of specific transcripts (A. S. Y. Lee et al. 2015). Here, we further show how eIF3 is involved not only in modulating translation of specific mRNAs but also in regulating cellular pathways but targeting select subsets of functionally related transcripts. We validated the eIF3 dependency of target UPP associated mRNAs and assessed the differential stress response of eIF3-dependent and eIF3-independent ubiquitin transcript 5'-UTRs. These results indicate that eIF3 plays an important role in regulating the Ubiquitin Proteasomal Pathway through translation of UBB, and it remains to be determined how this mode of regulation relates to the separate control of the UPP via UBC, which our findings indicate is strongly associated with translation regulation of the cell cycle (Stumpf et al. 2013).

In the next chapter, we present a different mechanism of translation regulation by eIF3, focusing on a discrete structural motif in EIF3A. Although there is no overlap with the PAR-CLIP dataset, the new mode of regulation by EIF3A similarly targets functionally related subsets of transcripts, enriched for proliferative, carcinogenic mRNAs.
Figure 1. Deletion of eIF3 binding site in the 5'-UTR of select transcripts alters translational efficiency. A. Live cell transfection of wild type and eIF3-binding site deletion USP1, UBB, TRIM68, and PSMD14 5'-UTR Renilla luciferase reporter mRNAs. The schematic on the right shows the features and design of the mRNAs used. Luciferase signal is normalized to wild type 5'-UTR mRNA. Error bars represent standard deviation of biological triplicates. B. In vitro translation of wild type and eIF3-binding site deletion USP1 and PCNA 5'-UTR Renilla luciferase reporter mRNAs in HEK293T cell extracts. Luciferase signal is normalized to wild type 5'-UTR mRNA. Error bars represent standard deviation of technical triplicates. C. In vitro translation of wild type and eIF3-binding site deletion USP1 and PCNA 5'-UTR Renilla luciferase reporter mRNAs in HEK293T cell extracts, with or without competing m7G cap. Luciferase signal is normalized to wild type 5'-UTR mRNA. Error bars represent standard deviation of technical triplicates.
Figure 2. Differential response to stress by variant ubiquitin transcript 5' -UTRs. A. Live cell transfection of four ubiquitin 5'-UTR Renilla luciferase reporter mRNAs in the presence of DTT. Luciferase signal is normalized to wild type 5'-UTR mRNA. Error bars represent standard deviation of biological triplicates. B. Transfection of four ubiquitin 5'-UTR Renilla luciferase reporter mRNAs in heat shock conditions. Luciferase signal is normalized to UBB 5'-UTR mRNA. Error bars represent standard deviation of biological triplicates. C. Transfection of A-capped versus m7G-capped ubiquitin 5'-UTR Renilla luciferase reporter mRNAs. Luciferase signal is normalized to wild type 5'-UTR mRNA. Error bars represent standard deviation of biological triplicates.
Figure 3. Differential response to inhibitors by variant ubiquitin transcript 5’-UTRs. A. Transfection of UBB and UBC ubiquitin variant 5’-UTR Renilla luciferase reporter mRNAs in the presence of increasing concentrations of MG132 proteosome inhibitor. Luciferase signal is normalized to vehicle control (VH). Error bars represent standard deviation of biological triplicates. B. Transfection of UBB and UBC ubiquitin variant 5’-UTR Renilla luciferase reporter mRNAs in the presence of increasing concentrations of 4Ei-10 EIF4E cap-binding inhibitor. Luciferase signal is normalized to vehicle control (VH). Error bars represent standard deviation of biological triplicates.
Figure 4. Translation of ubiquitin 5'-UTR-containing mRNAs in different cell types and cell cycle stages. A. Transfection of ubiquitin homolog 5'-UTR Renilla luciferase reporter mRNAs in A549 (lung adenocarcinoma), HepG2 (liver carcinoma), MCF7 (breast adenocarcinoma), SK-N-MC (neuroblastoma), and U2OS (osteosarcoma) human cell lines. Luciferase signal is normalized to UBB 5'-UTR mRNA. Error bars represent standard deviation of biological triplicates. B. Transfection of ubiquitin homolog 5'-UTR Renilla luciferase reporter mRNAs in S phase and G1 phase synchronized HEK293T cells. Luciferase signal is normalized to UBB 5'-UTR mRNA. Error bars represent standard deviation of biological triplicates. C. Western blotting of EIF3A protein levels in human cancer derived cell lines. Numbers below indicate relative quantification, normalized to ACTB and HEK293T EIF3A protein levels.
Materials and Methods

Cell culture
All cell lines were obtained from UC Berkeley Cell Culture Facility (ATCC origin). HEK293T cells were cultured in DMEM (Invitrogen 11995-073) with 10% FBS (VWR Seradigm 97068-085) and Pen-Strep (10 U/mL at 37 °C and 5% CO₂. HBSS was used for washing (Invitrogen 14175-103). A549, HepG2, MCF7, SK-N-MC, and U2OS cells were cultured in DMEM with 10% FBS, 1 mM sodium pyruvate (Thermo 11360-070), 1x NEAA (Thermo 11140-050 100x), and 100 U/mL Pen-Strep (Thermo 15140-122). RNA transfections were performed using Opti-MEM Reduced Serum Media (Invitrogen 31985-088) and TransIT-2020 (MIR5404) and TransIT-mRNA (MIR2225) reagents according to the manufacturer’s protocol (Mirus) and luciferase signal was measured using Dual-Glo Luciferase Assay System (Promega E2920) at 4 hours post transfection without inhibitor treatment. Inhibitors (DTT, MG132 (proteasome inhibitor, a gift from Michael Rape lab), 4Ei-10 (cap-binding inhibitor, a gift from the Carsten Wagner lab) were added 1 hour post transfection and luciferase signal was measured 6 hours post transfection.

Cell synchronization
Cells were synchronized in S phase using the double-thymidine block (18 hours at 2mM thymidine in media, followed by 9 hours in fresh media, followed by 15 hours at 2mM thymidine and collection). Cells were synchronized in G1 phase using the thymidine-nocodazole block (24 hours at 2mM thymidine in media, followed by 3 hours in fresh media, followed by 12 hours in 100ng/mL nocodazole and collection). Cells were washed with 1x HBSS between each step and gently scraped for collection.

In vitro transcription
RNAs were transcribed from 1 µg of PCR-amplified templates using T7 RNA polymerase in 1x transcription buffer (30 mM Tris-Cl pH 8, 5 mM MgCl₂, 0.01% Triton X-100, 2 mM spermidine, 20 mM NTPs, 10 mM DTT) for 5 hrs at 37 °C. Reactions were treated with RQ1 DNase (Promega M6101) for 20 min at 37 °C, precipitated using 2x volume 7.5 M LiCl/50 mM EDTA at -20 °C for 30 min, washed 2x in 70% EtOH, and resuspended in RNase free water. RNAs were capped using the Vaccinia capping system (NEB M2080S) according to manufacturer’s protocol, in the presence of 100 U murine RNase inhibitor (M0314S), extracted with an equal volume of phenol:chloroform pH 6, precipitated at -20 °C overnight in 5x volume 2% LiClO₄ in acetone, washed 2x in 70% EtOH and resuspended in RNase free water. For A-capped RNAs, GTP was substituted by G(5')ppp(5')A RNA Cap Structure Analog (NEB S1406S). RNAs that were amplified without a poly-A tail were poly-adenylated using poly-A polymerase (NEB M0276) according to the manufacturer’s protocol.

In vitro translation
Cell extracts were prepared from HEK293T cell lines at ~80% confluency. Cells were washed and scraped in cold PBS, spun down for 5 min at 1000 g at 4 °C, and resuspended in an equal volume of hypotonic lysis buffer (10 mM HEPES 7.6, 0.5 mM MgOAc, 5 mM DTT, Halt protease/phosphatase inhibitor cocktail (Thermo 78440)) for 45 min. Extracts were homogenized ~20 times through a 27G needle, spun down for 1 min at 14,000 g at 4 °C, and the supernatant removed, avoiding the lipids on the top and
interface on the bottom. *In vitro* translation reactions with luciferase reporter mRNAs were carried out with 0.5x extract, energy mix (final 0.84 mM ATP, 0.21 mM GTP, 21 mM creatine phosphate (Roche 10621722001), 45 U/mL creatine phosphokinase (Roche 10127566001), 10 mM HEPES pH 7.5, 2 mM DTT, 2.5 mM MgOAc, 50 mM KOAc, 8 μM amino acids (Promega PRL4461), 255 μM spermidine, 1U/mL murine RNase inhibitor (NEB M0314)), and 400 ng total RNA. Reactions were incubated for 1 hr at 30 °C and luciferase signal was measured using Dual-Glo Luciferase Assay System (Promega E2920). For cap competition assays, m7G(5')ppp(5')G RNA Cap Structure Analog was added to the energy mix.

**Western Blotting**

The following antibodies were used for Western blot analysis using the manufacturers' suggested dilutions: anti-beta-Actin (Abcam ab8227), anti-eIF3A (Sigma SAB1402997-100UG), Protein levels in Western blots were quantified using ImageJ (Schneider et al. 2012).

**Cloning**

All 5’-UTR elements were cloned into pcDNA4-Rluc using HinDIII and EcoRI restriction sites. Deleted eIF3 binding site sequences are highlighted in red.

**USP1**

CTCGCGCGCACGACATGGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
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PCNA
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GCAGCTAGTACAGAGTGGTCGTTGTCTTTCTAGGTCTCAGCCGGTCGTCGCGACG
TTCCGCCCGCTCGCTCTGAGGCTCCTGAAGCCGAAACCAGCTAGACTTTCCTCCTTC
CCGCCCTGCCGTAGCCGCTCTCGCTCTGAGGCTCCTGAAGCCGAAACCAGCTAGACTTTC
CTCTCCGGGATTTGGTGTGGTCTCAGCCGCTAGACTTTCCTCCTTC

UBB
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TTCCCTATTTATATAGGAAGGAAAGGAAGGAAGGGAAGGAAGGGAATATTT
TTTTAGGAGCTTTTTCTTTACGCGTATGAGGAAATTTGGGCTCAGTTGAAAGACCTA
AACTGCGCTCTCGGGAGGTGTTGGGCGGCGGCGGCGGCGGACTATTTCGCGGCGGCGGCA
CGGCGCTCTAGTGAGGCTGATAAGTGACGCAACACTCGCTTGTGGCATAAATTTGGC
TCCGCCAGCCGGAGCATTAGGGCCGGCGGTTTTGGGTGTGGTCTTCGGCGGGAGCTCCGGGAAACCCGGCGCTGCCGCGCG
TTCTCTTTTTTTCGATCCGCCATCTGCGGTGGAGCCGCCACCAAA

UBC
AGGGGCCGCGGAGGGGCCGCCCGCCGCTTGGGGACCTCCTCGGGCGCTCCCGGCGGCTCCTCAG
GCAGCTGCTGATCAGAGTCGACGGCGAGGTGTTGGGCTCAGTTGAAAAGCCTA
AACTGCGCTCTCGGGAGGTGTTGGGCGGCGGCGGCGGCGGACTATTTCGCGGCGGCGGCA
CGGCGCTCTAGTGAGGCTGATAAGTGACGCAACACTCGCTTGTGGCATAAATTTGGC
TCCGCCAGCCGGAGCATTAGGGCCGGCGGTTTTGGGTGTGGTCTTCGGCGGGAGCTCCGGGAAACCCGGCGCTGCCGCGCG
TTCTCTTTTTTTCGATCCGCCATCTGCGGTGGAGCCGCCACCAAA

RPS27
CTCGACCTCTTTTTAAAAATTCTCTTTAGCCACGTTGATTGTACGGGAAAAGCCTTTT
TAAAACATTTTTACGTTGCTAAAAACCTACAGCTTTGCGAAGGATCTCCGGGACAT
GTTGAGGAAAATAGGGCAGGCTAGGAGGAAAGCATGTCAGTTGCTCTTCAGTCTG
GCACCCGCTTTTGAGTCTGCTGGACGCGCTAGCTGAGGATTTTGGTGTTGTTGCTGCT
AGGGTTGTTGCTCTTTCCGGGAGGCTCCTCCGGGAAACCAGCCTGCGCGCGC
TTCTCTTTTTGATTCCCGCATCTGCGGTGGGAGCCGCCCAAAA

UBA52
GCTCGTGCACAGCGCTTTTGCGCGGCGGATTAGGTGGTTTCCCGGTTTCCGCTATCTCTTTTCTTCAGCGAGCGGCGAGCTGGTGGTGGCGGCGGTCGTGCGGACGCAAACA
Chapter 3
A New Mode of Translation Regulation by EIF3A

Introduction

Eukaryotic translation initiation is tightly controlled and its deregulation can lead to a wide variety of disorders, including cancer (Ruggero 2013). During canonical translation initiation, the eukaryotic small (40S) ribosomal subunit first associates with initiation factors eIF1, eIF1A, eIF3, and eIF5, and is subsequently loaded with the eIF2 ternary complex (eIF2-GTP-Met-tRNA\textsubscript{Met}). It then binds to the mRNA-bearing eIF4F complex (eIF4A, 4B, 4G, 4E) to begin directional scanning of the 5'-untranslated region (5'-UTR) for the start codon (Hinnebusch & Lorsch 2012). Distinct from the canonical scanning mechanism, recent evidence indicates that eIF3 regulates alternative pathways of translation initiation. For select cellular mRNAs, eIF3 can either activate or repress translation by interacting with RNA structural elements in the 5’-UTRs of these mRNAs (A. S. Y. Lee et al. 2015). Additionally, eIF3 can bind the 5’-cap of mRNAs using EIF3D (A. S. Y. Lee et al. 2016), allowing translation of select transcripts to continue under cellular stress conditions when eIF4E is inactivated. Finally, eIF3 can also promote translation under stress conditions by binding m6A-methylated 5’-UTRs in a cap-independent manner (Meyer et al. 2015; Wang et al. 2015). In all these cases, the molecular basis for eIF3-dependent translation regulation and its control of gene expression networks remain unclear.

Structural analysis using cryo-electron microscopy (cryo-EM) revealed that the core of eIF3, a five-lobed octameric complex, localizes to the solvent-exposed “backside” of the 40S subunit and spans the mRNA entry and exit channels (Georges et al. 2015). In the mammalian 43S pre-initiation complex (PIC), eIF3 subunits EIF3A and EIF3C directly contact the 40S subunit (Hashem, Georges, Dhote, Langlois, Liao, Grassucci, Hellen, et al. 2013), as well as participate in interactions with eIF1, eIF1A, eIF2, eIF5, and eIF4F (Sokabe et al. 2011; Eliseev et al. 2018), thus coordinating the ordered assembly of the 48S initiation complex. By contrast with canonical initiation, translation of the Hepatitis C Virus (HCV) genomic RNA requires an Internal Ribosome Entry Site (HCV IRES) RNA structure in the 5'-UTR, which binds to subunits EIF3A and EIF3C within the eIF3 complex (Sun et al. 2013). Recent cryo-EM structures revealed how an HCV-like viral IRES displaces eIF3 from binding the 40S subunit, while still binding to eIF3 through subunits EIF3A and EIF3C (Hashem, Georges, Dhote, Langlois, Liao, Grassucci, Pestova, et al. 2013) (Fig. 1A). The importance of the EIF3A subunit in mediating RNA binding is underscored by in vitro translation (IVT) experiments in which mutation of amino acids 36-39 (KSKK > NSEE) in a predicted RNA-binding HLH (Helix-Loop-Helix) motif abrogated eIF3 binding to the HCV IRES (Sun et al. 2013). While these experiments indicated that the HLH motif of EIF3A is critical for mediating HCV IRES binding by eIF3, the importance of this motif for cellular mRNA translation remains to be determined.

Beyond the role of EIF3D in binding the mRNA m7G cap (A. S. Y. Lee et al. 2016), the mechanisms responsible for eIF3-mediated regulation of specific cellular mRNAs remain unclear. Structural models for eIF3 bound to 43S and 48S pre-initiation complexes suggest that eIF3 controls the translation of the HCV IRES in a distinct manner compared
to cellular transcripts, whether involving canonical scanning or eIF3-dependent regulation of specific transcripts. In addition to its displacement from the 40S subunit by the HCV IRES RNA in the cryo-EM reconstruction, the HLH motif in EIF3A is spatially distant from the EIF3D cap-binding domain (Eliseev et al. 2018) and is more discrete than the proposed multi-subunit interface thought to recognize specific RNA secondary structures (A. S. Y. Lee et al. 2015) and m6A modifications (Meyer et al. 2015; Erzberger et al. 2014). We therefore probed the role of the EIF3A HLH motif in regulating cellular translation initiation in cells and in vitro. We found that mutations in the EIF3A HLH motif affected the translational efficiency of transcripts involved in proliferative pathways, including the mRNAs encoding MYC, PRL3 and MET. MYC is a well-known transcription factor strongly associated with cancer initiation and is found to be deregulated in over half of human cancers, whereas PRL3 and MET are implicated in cancer metastasis through regulating oncogenic effector pathways, such as PI3K/Akt/mTOR and HGF/SF signaling, respectively (Chen et al. 2018; Gherardi et al. n.d.; Ye et al. 2015). The selective enhancement of translation initiation on cancer-associated transcripts by the EIF3A HLH motif highlights a new mode of eIF3 translation regulation and identifies a well-defined, discrete structural motif (Erzberger et al. 2014) that could be targeted for future drug development efforts.

Results

Mutation of the EIF3A HLH motif causes selective translation repression of proliferative mRNAs

We first introduced a 3 amino-acid mutation in the HLH RNA-binding motif in EIF3A (KSKK > NSEE, hereafter termed EIF3A HLH*), previously shown to disrupt HCV IRES-mediated translation initiation (Sun et al. 2013), into HEK293T cells using a lentiviral delivery and integration system under hygromycin selection. We then knocked down endogenous EIF3A expression using an shRNA targeting the native mRNA 3’-UTR, expressed by a second lentiviral system under dual hygromycin/puromycin selection (table S1). We also generated control (CT) cell lines in the same manner but with no mutation in the exogenous EIF3A sequence. HLH* cell lysates showed a dramatic (~80%) decrease in encephalomyocarditis virus (EMCV) IRES-mediated translation, consistent with the effects previously seen in vitro with reconstituted eIF3 and the HCV IRES (Fig. 1B) (Sun et al. 2013).

We then used the CT and HLH* cell lines to determine the effect of the EIF3A HLH mutation on the translational efficiency of cellular mRNAs. To assess the extent of mutant EIF3A expression, RNA deep sequencing (RNAseq) data were aligned to wild-type and HLH* EIF3A sequences, revealing that >80% of aligned HLH reads mapped to the HLH* sequence, and confirming robust expression of exogenous over endogenous EIF3A (Fig. 1C). At the transcriptional level, HLH* EIF3A had little effect on mRNA expression (fig. S1). However, bioinformatic analysis of ribosome profiling data, normalized to the RNAseq data, revealed a small number of transcripts differentially regulated at the translational level in cells expressing HLH* EIF3A, 80 negatively regulated and 25 positively regulated (p<0.01 and >3x change, Fig. 1D, fig. S2A). Functional classification of these genes showed strong enrichment of oncogenic transcripts (Fig. 1E), including those encoding PRL3, MYC and MET, all of which were negatively affected by HLH*
EIF3A (Fig. 1D). Consistent with the ribosome profiling results, Western blot analysis showed a decrease in protein levels for MYC, MET, and PRL3 (Fig. 1F). Notably, in experiments using transient expression of HLH* EIF3A, MYC protein levels were also suppressed at 72 hours post-transfection, when transcript levels for the mRNA encoding HLH* EIF3A dropped to less than 50% of wild-type EIF3A mRNA levels (fig. S1A). Taken together, these results indicate that HLH* EIF3A lowers the translation of certain oncogenic transcripts such as MYC that are important for cell proliferation, in a dominant negative manner.

In longer timeframes than those used to collect samples for the ribosome profiling and RNAseq experiments, we observed that the shRNA cell lines expressing HLH* EIF3A had reverted MYC protein expression to those seen in CT cells, and proliferated like CT cells with increased passage number. We therefore engineered HEK293T cells to express CT or HLH* EIF3A from transduced lentiviral vectors, as in the shRNA cell lines, but instead using CRISPRi to suppress expression of endogenous EIF3A (table S1). In the CRISPRi cell lines, we observed the same decrease in MYC, MET, and PRL3 levels by Western blot as in the shRNA cell lines (Fig. 1G). Although the CRISPRi cell lines exhibited a moderate global decrease in translation, in contrast to the shRNA-based cell lines as determined by metabolic labeling (fig. S1B), we continued to use the CRISPRi cell lines for subsequent biochemical and luciferase reporter-based experiments since they were more stable with passage number compared to the shRNA cell lines, as assessed by growth rate and relative MYC levels as a function of passage number.

**HLH* EIF3A causes transcript-specific defects in translation initiation factor recruitment**

To test the effect of HLH* EIF3A on translation initiation, we used the CRISPRi-based CT and HLH* cell lines to prepare cytoplasmic extracts for in vitro translation experiments. We programmed these extracts with full-length GAPDH and MYC mRNAs, and stalled translation reactions with either cycloheximide or GMPPNP, and fractionated them on sucrose gradients (Fig. 2, A and B). Cycloheximide stalls 80S ribosomes immediately after initiation, whereas GMPPNP stalls 48S pre-initiation complexes at the start codon (Sun et al. 2013; Dmitriev et al. 2003). In Western blots of the sucrose gradient fractions, we observed a defect in EIF3A and EIF5B distribution in the HLH* EIF3A in vitro translation reactions programmed with either GAPDH or MYC mRNA (Fig. 2, C – F). These results are consistent with those using reconstituted eIF3 and the HCV IRES, which showed that the EIF3A HLH motif is important for eIF3 association with the 40S subunit, and for EIF5B association with pre-initiation complexes and the 80S ribosome (Sun et al. 2013), and suggest these defects are general. We did not observe a defect in the distribution of Met-tRNAi in contrast to the previously observed Met-tRNAi distribution defect in in vitro reconstituted HCV IRES mediated translation with reconstituted eIF3 (Sun et al. 2013) (fig. S3A-C). Notably, in vitro translation reactions using HLH* EIF3A extracts programmed with MYC mRNA exhibited a specific defect in EIF4A1 incorporation into preinitiation complexes (Fig. 2, E and F). The defect in EIF4A1 distribution occurred in both cycloheximide and GMPPNP stalled reactions, suggesting that HLH* EIF3A selectively destabilizes 48S pre-initiation complexes on some mRNAs (Fig. 2, fig. S4A).
**HLH* EIF3A sensitizes translation extracts to eIF4A1 inhibitor RocA**

To address the destabilizing effect of HLH* EIF3A on EIF4A1 incorporation into 48S pre-initiation complexes, we used Rocaglamide A (RocA) to inhibit EIF4A1 function in CT and HLH* extracts. RocA causes stalling, premature upstream initiation, and decreased translation by locking EIF4A1 onto poly-purine sequences in the 5'-UTR of mRNAs (Iwasaki et al. 2016). *In vitro* translation of *Renilla* luciferase reporter mRNAs harboring the HCV IRES or *MYC* 5'-UTR showed a marked translation defect in extracts from the HLH* cells (Fig. 3A). By contrast, a *Firefly* luciferase reporter mRNA with the *HBB* 5'-UTR used as a control was unaffected by HLH* EIF3A, indicating that the HLH* mutation is specific to EIF3A HLH-sensitive mRNAs in a 5'-UTR dependent manner (Fig. 3A). Notably, HLH* EIF3A further sensitizes the HCV IRES and the *MYC* 5'-UTR to RocA in the *in vitro* translation reactions (Fig. 3B). The RocA-dependent decrease in translation occurred in addition to the HLH*-specific defect (Fig. 3A). Importantly, neither of these mRNAs were sensitive to RocA in the CT lysate, consistent with previous results (Iwasaki et al. 2016).

To assess the effect of RocA on translation pre-initiation complex formation, we used *in vitro* translation reactions programmed with full-length *GAPDH* or *MYC* mRNAs and inhibited the reactions with both GMPPNP and RocA. Western blots of fractionated reactions showed a further decrease in EIF4A1 recruitment in the HLH* *in vitro* translation extracts programmed with *MYC* mRNA compared to GMPPNP alone (Fig. 3, C – E, compare to fig. S4, A and B). RocA did not affect EIF4A1 distributions in *GAPDH*-programmed reactions or in CT extracts programmed with *MYC* mRNA, showing that the combined defect of the HLH* EIF3A and RocA on EIF4A1 association with 48S pre-initiation complexes is also transcript-specific. No significant difference was observed for Met-tRNAi distribution, suggesting it is not perturbed by RocA (fig. S4C).

**EIF3A HLH motif interacts with mRNAs in counterpoint to EIF4A1**

In the yeast *Saccharomyces cerevisiae*, the EIF3A N-terminal domain (NTD), which includes the HLH motif, has been shown to enhance re-initiation upon translation of upstream open reading frames uORF1 and uORF2 of *GCN4* (Gunišová et al. 2016; Munzarová et al. 2011; Szamecz et al. 2008), likely by interacting with mRNA at the mRNA exit channel of the 40S subunit (Aitken et al. 2016). Notably, in addition to being tuned to levels of active eIF2, control of *GCN4* translation by the uORFs in its 5'-UTR also requires scanning in an eIF4A-dependent manner (Watanabe et al. 2010). Translational control of the functional ortholog of Gcn4 in mammals – stress response transcription factor ATF4 – also requires uORFs (Vattem & Wek 2004). Briefly, after translation of ATF4 uORF1 under normal conditions, ribosomes re-initiate and continue scanning, encountering inhibitory uORF2, which causes dissociation before the start codon (Vattem & Wek 2004). Under stressed conditions with low eIF2, a portion of ribosomes scans through uORF2 and initiates at the start codon. Deletion of uORF1 in the 5'-UTR lowers translation under normal conditions, while deletion of uORF2 elevates it (Vattem & Wek 2004). Unlike *MYC*, we did not identify ATF4 as an EIF3A HLH*-sensitive mRNA (Fig. 1D, table S2-S4). However, we wondered if ATF4 might become sensitive to HLH* EIF3A in the presence of RocA. We transfected CT and HLH* CRISPRi cells with *Renilla* luciferase reporter mRNAs harboring the ATF4 5'-UTR and saw a modest decrease in
luciferase signal in the HLH* cells, normalized to Firefly luciferase reporter mRNA with the HBB 5'-UTR (fig. S5A). Surprisingly, reporter translation increased in the presence of RocA in HLH* but not CT cells, for the mRNAs containing the WT ATF4 5'-UTR (WT) or the ATF4 variant with a mutated start codon in uORF1 (ΔuORF1). RocA-mediated repression was also relieved in HLH* cells relative to CT cells for the ATF4 variant with a mutated start codon in uORF1 (ΔuORF1) (Fig. 3F). Treatment with thapsigargin (Tg), which induces an ATF4-dependent stress response by decreasing active eIF2 levels, had similar effects on translation from these ATF4 reporter mRNAs in the presence of HLH* EIF3A (fig. S5, A and B). By contrast, the combination of RocA and HLH* EIF3A still exhibited a synergistic inhibitory effect on the translation of mRNAs with the MYC 5'-UTR in transfected cells (fig. S5B) as observed in vitro (Fig. 3B). Thus, while HLH* EIF3A does not appear to affect ATF4 uORF translation appreciably (Fig. 3F), it can counterbalance EIF4A1 regulation of certain transcripts, as reflected in the reversal of RocA sensitivity of mRNAs harboring the ATF4 5'-UTR element.

**HLH* EIF3A suppresses MYC-induced proliferation of Burkitt’s lymphoma cells**

MYC overexpression is responsible for the transformation of Ramos Burkitt’s lymphoma cells (Habel & Jung 2006), which prompted us to assess the effect of HLH* EIF3A on proliferation of these MYC-addicted cells. We observed a substantial decrease in MYC and MET protein levels in Ramos Burkitt’s lymphoma cell lines expressing the HLH* but not CT EIF3A (Fig. 4A, fig. S6A), more than the mean global decrease in translation in the HLH* Ramos cell lines compared to the CT control cell lines (fig. S6B). To assess the combined effect of HLH* EIF3A and EIF4A1 inhibition, CT and HLH* Ramos cells were cultured overnight in the presence of increasing concentrations of RocA. The HLH* Ramos cells were much more sensitive to RocA compared to CT cells (Fig. 4C). This is consistent with the synergistic sensitization of MYC 5'-UTR observed in in vitro translation extracts (Fig. 3B), and given Ramos cell addiction to MYC overexpression (Habel & Jung 2006). The sensitization effect of HLH* also occurred in the presence of chemotherapeutic agent doxorubicin (Fig. 4D).

**Discussion**

Mammalian eIF3 has been shown to regulate translation initiation of specific mRNAs in a variety of ways: by binding RNA secondary structures in mRNA 5'-UTRs that activate or repress translation, through EIF3D binding to the m^7G cap, and through m^6A-dependent interactions with specific mRNAs (A. S. Y. Lee et al. 2015; A. S. Y. Lee et al. 2016; Meyer et al. 2015; Wang et al. 2015). Viral genomic RNAs also target eIF3 to promote translation initiation (Sizova et al. 1998; Ji et al. 2004). Structural studies of HCV IRES binding and incorporation into translation preinitiation complexes revealed that the IRES displaces eIF3 from the 40S ribosomal subunit (Hashem, Georges, Dhote, Langlois, Liao, Grassucci, Pestova, et al. 2013). The HCV IRES-driven mode of interaction also requires an HLH RNA binding motif in the EIF3A subunit of eIF3 that is critical for IRES binding and function. Mutation of the putative RNA-binding loop in the HLH motif of EIF3A disrupted eIF3 binding to the IRES and also to the 40S ribosomal subunit (Sun et al. 2013). Here, using a combination of cell engineering and ribosome profiling, we show that mutating the loop in the HLH motif of EIF3A affects the translation of a discrete set of
cellular mRNAs. The set of cellular transcripts identified as functionally dependent on the EIF3A HLH motif does not overlap with the eIF3-dependent mRNAs identified previously that require RNA secondary structures (A. S. Y. Lee et al. 2015), rely on EIF3D cap-binding (A. S. Y. Lee et al. 2016), or m^6^A recognition (Meyer et al. 2015; Wang et al. 2015), suggesting that the HLH motif in EIF3A contributes to translation of these mRNA using a different mechanism.

In contrast to the HCV IRES, the cellular transcripts sensitive to mutations in the HLH motif of EIF3A are not enriched for putative viral-like RNA secondary structural elements in their 5'-UTRs to which eIF3 could bind (Mathews 2014), and are not enriched for uORFs (Wethmar et al. 2014; S. Lee et al. 2012). Since the mutation of the HLH motif in EIF3A also disrupts direct binding to the 40S ribosomal subunit (Sun et al. 2013), translation of the cellular mRNAs identified here may be those most dependent upon the interaction of eIF3 with the 40S subunit. Structural and biochemical evidence has shown eIF3 interacts with the 40S subunit at both the mRNA entry and exit sites within pre-initiation complexes (Aitken et al. 2016). Specifically, the N-terminal domain of EIF3A binds the 40S subunit at the mRNA exit site, while the C-terminal domain projects towards the mRNA entry tunnel. In yeast, mutations in the N-terminal region of eIF3a that weaken mRNA binding to 48S pre-initiation complexes sensitize these to mRNA interactions at the mRNA entry channel, remote from where eIF3a interacts with the 40S subunit. Conversely, mutations in the C-terminus of eIF3a that affect mRNA interactions with the mRNA entry channel also influence mRNA interactions with the mRNA exit site on the opposite side of the 40S subunit. These results reveal a long-distance connection between the two mRNA binding regions in the 48S pre-initiation complex important for mRNA recruitment (Eliseev et al. 2018). The HLH motif in human EIF3A resides in the N-terminal region that binds at the mRNA exit site and, in yeast, is proposed to generally stabilize recruitment of mRNAs to the 43S pre-initiation complex (Aitken et al. 2016). By analogy to the yeast system, the 3-amino acid mutation in the HLH motif in human EIF3A that disrupts eIF3 interactions with the 40S subunit (Sun et al. 2013) would be predicted to globally decrease favorable interactions of PICs with cellular mRNAs in the mRNA exit channel (Fig. 5A). Global elimination of eIF3-induced mRNA interactions at the mRNA exit site in complexes harboring HLH* EIF3A would then result in decreased translation initiation on transcripts with the least stable interactions in the mRNA entry channel (Fig. 5A), possibly explaining the observed specificity in translation efficiency in HLH* EIF3A expressing cells.

In addition to uncovering cellular mRNAs that may be most sensitive to interactions with the 43S pre-initiation complex at the mRNA entry channel, we found that the HLH motif in EIF3A acts as a counterbalance to the action of EIF4A1 during translation initiation. The in vitro translation reactions using HLH* cell extracts demonstrated that the HLH* mutations destabilize interactions between EIF3A, the 40S subunit and EIF5B in a transcript-independent manner (Fig. 2), as seen before using a reconstituted system (Sun et al. 2013). We also found that the HLH mutation destabilizes interactions of initiation complexes with EIF4A1 in a transcript-specific manner, possibly due to weakening eIF3 binding to pre-initiation complexes (Fig. 2) (Yourik et al. 2017). The interaction of the HLH motif in EIF3A and EIF4A1 with the mRNA at the exit and entry points of the initiation
complex, respectively, likely affects the dynamics of mRNA scanning to the start codon in a transcript-specific manner, as well as affecting mRNA recruitment (Aitken et al. 2016; Yourik et al. 2017). This transcript specificity is enhanced in the presence of RocA, which locks EIF4A1 onto poly-purine stretches in the 5'-UTR, thereby stalling scanning and mRNA unwinding (Iwasaki et al. 2016). In the context of an HLH-sensitive transcript, RocA exacerbates the translational defect due to HLH* EIF3A (Fig. 3B) by further depleting EIF4A1 from 48S pre-initiation complexes (Fig. 2, E and F, Fig. 3, D and E). By contrast, for transcripts that are not highly reliant on the HLH motif in EIF3A, such as ATF4, RocA repression is alleviated by HLH* EIF3A. Taken together, these results suggest that loosening and tightening of mRNA contacts at either end of the mRNA channel in the 48S pre-initiation complex can lead to combinatorial increases or decreases in translation in a transcript-specific manner. The mRNA entry and exit channels are spatially separated (Hashem, Georges, Dhote, Langlois, Liao, Grassucci, Hellen, et al. 2013; Eliseev et al. 2018; Aitken et al. 2016), implying that HLH* EIF3A loosening of mRNA contacts at the mRNA exit channel of the pre-initiation complex must propagate to the mRNA entry channel to affect the EIF4A1–mRNA interaction, connecting the functions of EIF3A and EIF4A1 during mRNA scanning (Fig. 5).

To test whether the molecular insights gained using HLH* EIF3A could have physiological implications in cancer, we generated HLH* EIF3A expressing Ramos Burkitt’s lymphoma cell lines, which are addicted to MYC overexpression for their proliferation (Habel & Jung 2006). We observed that HLH* EIF3A resulted in a dramatic decrease in MYC protein levels and a severe growth defect. The fact that HLH* EIF3A is sufficient to lower MYC levels in these lymphoma cells and increase their sensitivity to chemotherapeutic compounds suggests that eIF3 could serve as a potential target for future cancer therapeutic strategies. Our model for how the HLH motif in EIF3A confers specificity on the translation of specific mRNAs involved in various pathways leading to cell proliferation makes it an intriguing target for treating a wide range of cancers. For example, by combining HLH* EIF3A with RocA treatment, we envision targeting both locations of mRNA engagement with the 48S pre-initiation complex, the mRNA entry and exit channels, to achieve selective inhibition of translation. Alternatively, targeting the HLH motif in EIF3A could be used in conjunction with drugs that target completely different pathways, e. g. doxorubicin, a drug thought to target DNA topoisomerases (Nitiss 2009) and is commonly used in the treatment of Burkitt’s lymphoma. Taken together the EIF3A HLH motif is an attractive new target for drug development to be employed in combination cancer therapy approaches.
Figure 1. EIF3A HLH motif regulates translation of cancer-associated RNAs. A. Schematic of eIF3 binding to the 40S ribosomal subunit in canonical and viral IRES-mediated initiation complexes based on cryo-EM reconstructions (Hashem, Georges, Dhote, Langlois, Liao, Grassucci, Hellen, et al. 2013; Hashem, Georges, Dhote, Langlois, Liao, Grassucci, Pestova, et al. 2013). B. In vitro translation of IRES-Renilla mRNA in CT and HLH* extracts. Error bars indicate the standard deviation of biological triplicates. C. Representative alignment of RNAseq CT and HLH reads to wild type (wt) or HLH* (mut) eIF3A sequences. D. Translational efficiency scatter plot of statistically significant transcripts (p-value < 0.01). Upregulated transcripts highlighted in blue (>3x increase),
downregulated in pink (>3x decrease). E. Functional classification of regulated transcripts based on literature analysis. F. Representative Western blot validation of top cancer associated hits in shRNA 293T cell lines. Levels of protein normalized to ACTB or HSP90 control given below gels. G. Representative Western blot in CRISPRi 293T cell lines. Levels of protein normalized to ACTB or HSP90 control given below gels.
Figure 2. General and transcript-specific defects in initiation factor recruitment

A, B. Representative sucrose gradient profile of in vitro translation reactions stalled with cycloheximide or GMPPNP and programmed with full length mRNA. Fractions were tracked by absorbance at 254 nm as shown, with the top of the gradient on the left.

C, D. Western blot analysis of initiation factor distribution in translation reactions stalled with cycloheximide.

E, F. Western blot analysis of initiation factor distribution in translation reactions stalled with GMPPNP. Red boxes indicate fractions with decreased levels of initiation factors of interest.
Figure 3. EIF3A HLH motif interacts with specific mRNA 5'-UTR elements. A. *In vitro* translation of HCV IRES and MYC 5'-UTR Renilla mRNAs in CT and HLH* extracts. The schematic on the right shows the features and design of the mRNAs used. Globin HBB 5'-UTR *Firefly* mRNA was used as a control. Statistical significance measured by student’s t-test, ***P < 0.0001. Error bars represent the standard deviation between technical triplicates. B. *In vitro* translation of HCV IRES and MYC 5'-UTR luciferase mRNAs in the presence of EIF4A1 inhibitor RocA. Statistical significance measured by student’s t-test, *P < 0.01; **P < 0.001; ***P < 0.0001. Error bars represent the standard deviation between technical triplicates. C-E. *In vitro* translation reactions programmed with full length *GAPDH* and *MYC* mRNAs inhibited with both GMPPNP and RocA and fractionated on 10-25% sucrose gradients. Western blotting of the sucrose gradient fractions, with red boxes indicating fractions of interest for EIF4A1 levels. Yellow asterisk indicates background signal in gel, which does not interfere with initiation factor
distribution analysis. F. EIF3A HLH* motif effects on RocA-mediated repression. The schematic on the right represents the ATF4 uORF variant 5'-UTRs (WT, ΔuORF1, ΔuORF2) fused to the Renilla luciferase ORF for transfection into CRISPRi CT and HLH* cells. Live cell transfection was performed instead of in vitro translation in order to observe the effect of RocA stress. Relative Luciferase Units (RLU) percentage was normalized to internal globin HBB 5'-UTR Firefly mRNA control signal. Statistical significance was measured by student’s t-test, *P<0.01, **P<0.001, ***P<0.0001. Error bars represent the standard deviation between technical triplicates.
Figure 4. HLH* EIF3A effects in Ramos Burkitt’s lymphoma cells. A. Western blot analysis of HLH* sensitive proteins in Ramos shRNA lentiviral cell lines. Levels of protein normalized to ACTB control given below gels. Asterisk notes that no detectable PRL3 was found in Ramos cells. B. CT and HLH* Ramos cells cultured in the presence of increasing concentrations of RocA. Error bars represent standard deviation of three biological replicates. C. Doxorubicin treatment of Ramos CT (R-CT) and HLH* (R-HLH) cell lines.
Figure 5. Model of EIF4A1 and EIF3A dynamic interactions with mRNA entry and exit sites. A. Schematic of EIF4A1 and eIF3 interacting with the mRNA at the entry and exit mRNA sites of the 40S ribosomal subunit, respectively. B. Schematic representing the displacement of EIF3 HLH* from the 40S subunit that leads to general defects in initiation factor recruitment and mRNA translation. C. Schematic representing the displacement of EIF3 HLH* from the 40S subunit in the presence of select transcripts, such as MYC, and the concomitant displacement of EIF4A1 at the entry site.
Table 1. Cell lines used in this study and the method of engineering.

<table>
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<th>Cell Line</th>
<th>Endogenous KD</th>
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</tbody>
</table>
Figure S1. Phenotypic and bioinformatic analyses of the shRNA lentiviral 293T cell lines. A. Transient transfection of CT and HLH* (HLH) lentiviral vectors into HEK293T cells was
analyzed by PCR-amplification and sequencing of the HLH mutation region (left) and western blotting for MYC protein levels (right). Levels of protein normalized to ACTB control given below gels. The arrows indicate the location of nucleotide base signal reflecting the HLH* mutations on the chromatogram. B. 35S-Met/Cys metabolic labeling of the shRNA lentiviral HEK294T and CRISPRi cell lines. Total protein is stained by Coomassie (left). C. Scatter plot of CT versus HLH* (HLH) transcriptional changes using average FPKM values of three biological replicates, showing transcripts meeting a p-value cutoff of 0.01. Three transcripts in pink are downregulated >3x, and one transcript in blue is upregulated >3x. D. Volcano plots of transcriptional fold change against FDR-corrected p-value, showing transcripts meeting an FDR cutoff of 0.05. Four transcripts in black show a >2x change in expression. E. RNAseq reproducibility scatter plots of CT and HLH biological replicates, showing non-zero transcripts used for statistical analysis. Highlighted in purple are transcripts with a >3x change in expression.
Figure S2. Volcano plot of translational efficiency fold change against FDR corrected p-value. A. Transcripts meeting a p-value cutoff of 0.01 in black. Out of those, transcripts with >3x fold change are highlighted in magenta. B. Ribosome profiling reproducibility scatter plots of CT and HLH* (HLH) biological replicates, showing non-zero transcripts used for statistical analysis. Highlighted in purple are transcripts with a >3x change in expression.
Figure S3. Met-tRNA<sub>i</sub> distribution in sucrose gradient fractions of in vitro translation reactions. A. Sucrose gradient profiles of cycloheximide (top) and GMPPNP (bottom) stalled in vitro translation reactions fractionated on 10-25% sucrose gradients. B. Northern blotting of Met-tRNA<sub>i</sub> in the presence of cycloheximide. C. Northern blotting of tRNA<sub>i</sub> in the presence of GMPPNP.
Figure S4. Initiation factor distribution in GMPPNP and GMPPNP/RocA stalled in vitro translation reactions. A. Western blot analysis of initiation factors in GMPPNP stalled in vitro translation reactions resolved by sucrose gradient fractionation, expanded from Fig. 2 to include additional factors. B. Western blot analysis of initiation factors in GMPPNP/RocA stalled in vitro translation reactions, expanded from Fig. 3 to include additional initiation factors. Boxes indicate fractions of interest for EIF4A1 levels. Yellow
asterisk indicates background signal in gel, does not interfere with initiation factor
distribution analysis. C. Northern blot analysis of Met-tRNA\textsubscript{i} distribution in
GMPPNP/RocA stalled \textit{in vitro} translation reactions.
Figure S5. MYC 5’-UTR response to RocA and ATF4 5’-UTR response to thapsigargin. A. Live cell transfections of mRNAs containing the ATF4 5’-UTR fused to the Renilla luciferase ORF in the presence of thapsigargin and RocA. Relative Luciferase Units (RLU) percentage was normalized to internal HBB 5’-UTR-Firefly luciferase mRNA control signal. B. Live cell transfections of ATF4 uORF variant 5’-UTRs (WT, ΔuORF1, ΔuORF2) fused to Renilla mRNAs in the presence of thapsigargin. Control samples are identical to those plotted in Fig. 3F. C. Live cell transfection of MYC 5’-UTR – Renilla luciferase mRNA was performed to observe the effect of RocA-induced stress. Relative Luciferase Units (RLU) percentage was normalized to internal HBB 5’-UTR-Firefly luciferase control signal.
Figure S6. Phenotypic analyses of Ramos Burkitt’s lymphoma cell lines. A. Western blot validation of MYC suppression in Ramos HLH\* cells. The numbers 1-4 represent separate cell lines transduced in parallel. Bottom panels show Western blot validation of additional cancer-associated negatively regulated transcript DEPTOR in HEK293T shRNA and Ramos shRNA cell lines. Levels of protein normalized to ACTB or HSP90 control given below gels. B. $^{35}$S-Met/Cys metabolic labeling of the Ramos cell lines. Total protein is stained by Coomassie (left).
Supplementary Tables
Included in a single Excel .xlsx file:
Table S1. RNAseq data (Supplementary Tables, tab 1, “RNAseq nonzero”)
Table S2. Ribosome profiling data (Supplementary Tables, tab 2, “RP (RSnonzero match)“)
Table S3. Babel statistical analysis of RNAseq and ribosome profiling data (Supplementary Tables, tab 3, “Babel_TE_FC“)
Table S4. Statistically significant transcripts from Babel analysis (Supplementary Tables, tab 4, “Babel_p<0.01”)
Materials and Methods

Cell culture
HEK293T cells were cultured in DMEM (Invitrogen 11995-073) with 10% FBS (VWR Seradigm 97068-085) and Pen-Strep (10 U/mL) at 37 °C and 5% CO₂. HBSS was used for washing (Invitrogen 14175-045). Ramos cells were cultured in RPMI 1640 (Thermo 11140-050) with 10% FBS, 1 mM sodium pyruvate (Thermo 11360-070), 1x NEAA (Thermo 11140-050 100x), and 100 U/mL Pen-Strep (Thermo 15140-122). Lentiviral cell lines were selected with hygromycin (Thermo 10687010, 250 μg/mL) and puromycin (Mirus MIR5940, 10 μg/mL). CT or HLH* EIF3A was cloned into nLV103-hygro, and the custom EIF3A shRNA vector was obtained from pLKO.1-puro bacterial stock. CRISPRi cell lines were made using catalytically dead Cas9 fused to BFP that was introduced into cells via lentivirus. Transduced cells were FACS sorted into 96-well plates for clonal amplification, manually screened for BFP expression, and the brightest colonies selected for subsequent introduction of lentiviruses encoding sgRNA in pSLQ1371_BLP1_ _Ef1A_puro_GFP, a gift from the Jonathan Weissman Lab, and CT or HLH* EIF3A in nLV103-hygro. Lentiviral vectors were transduced into HEK293T cells to generate viral particles at a ratio of 1 μg : 250 ng : 250 ng lentivirus carrying the gene of interest, dR8.91 packaging vector, and pMD2.G envelope vector, respectively, per each well in a 6-well format. The viral supernatant was harvested at 48 hours and transduced into relevant cell lines using 20 μg/mL polybrene. DNA and RNA transfections were performed using Opti-MEM Reduced Serum Media (Invitrogen 31985-088) and TransIT-2020 (MIR5404) and TransIT-mRNA (MIR2225) reagents according to the manufacturer’s protocol (Mirus).

Ribosome Profiling
Ribosome profiling libraries were prepared from three biological replicates per cell line according to previously described methods (Ingolia et al. 2013). RNAseq libraries were prepared from the same samples using TruSeq RNA Library Prep kit according to the manufacturer’s instructions (Illumina Part # 15026495). Sequencing data were analyzed using Bowtie v1.0.0 (Langmead et al. 2009) to remove rRNA reads, TopHat v2.0.14 (Trapnell et al. 2012; Kim et al. 2013) to align reads to the human GRCh38 genome, Cufflinks v2.2.1 and Cuffdiff v2.2.1 (Trapnell et al. 2012) to extract and merge raw read counts of the biological replicates, and R v3.2.2 package Babel v0.2-6: Ribosome Profiling Analysis to calculate FPKM, p-values and FDR (R Core Team (2015). R: A language and environment for statistical computing, R Foundation for Statistical Computing, Vienna, Austria https://www.R-project.org/). Translational efficiency (TE) was calculated as ribosome profiling FPKM / RNAseq FPKM and fold change was calculated as HLH* TE / CT TE. See tables S2–S4 for data (raw non-zero, Babel output). Bowtie2 (Langmead & Salzberg 2012) was used to generate indices from EIF3A CT and HLH* sequences for alignment with CT and HLH* RNAseq data (see Supplementary Methods). All data has been deposited in Gene Expression Omnibus.

5’UTR uORF and secondary structure computational analysis
We used the databases uORFdb (Wethmar et al. 2014) (uORFdb - a comprehensive literature database on eukaryotic uORF biology, http://www.compgen.uni-muenster.de/tools/uorfdb/) and TISdb (S. Lee et al. 2012) (Translation Initiation Site Database, http://tisdb.human.cornell.edu/) to analyze transcripts affected by HLH* EIF3A
for uORF presence. We used RNAstructure v6.0.1 Secondary Structure Web Server (Mathews 2014) (https://rna.urmc.rochester.edu/RNAstructureWeb/) to predict secondary structures of transcripts affected by HLH* EIF3A.

**Western Blotting**
The following antibodies were used for Western blot analysis using the manufacturers' suggested dilutions: anti-beta-Actin (Abcam ab8227), anti-ATF4 (Abcam ab184909), anti-DEPTOR (Sigma SAB4200214), anti-eIF1A (Abcam ab177939), anti-eIF2α (Bethyl A300-721A-M), anti-eIF3A (Sigma SAB1402997-100UG), anti-eIF4A1 (Abcam ab31217), anti-eIF5B (Bethyl A301-745A-M), anti-HSP90 (Abcam ab13492), anti-MET (Abcam ab51067), anti-MYC (Abcam ab32072), anti-PTP4A3 (Abcam ab50276 recognizes PRL-3), anti-RPS19 (Bethyl A304-002A), anti-Mouse IgG-HRP (A00160), anti-Rabbit IgG-HRP (NA934V). Protein levels in Western blots were quantified using ImageJ (Schneider et al. 2012).

**In vitro transcription**
RNAs were transcribed from 1 µg of PCR-amplified templates using T7 RNA polymerase in 1x transcription buffer (30 mM Tris-Cl pH 8, 5 mM MgCl2, 0.01% Triton X-100, 2 mM spermidine, 20 mM NTPs, 10 mM DTT) for 5 hrs at 37 °C. Reactions were treated with RQ1 DNase (Promega M6101) for 20 min at 37 °C, precipitated using 2x volume 7.5 M LiCl/50 mM EDTA at -20 °C for 30 min, washed 2x in 70% EtOH, and resuspended in RNase free water. RNAs were capped using the Vaccinia capping system (NEB M2080S) according to manufacturer’s protocol, in the presence of 100 U murine RNase inhibitor (M0314S), extracted with an equal volume of phenol:chloroform pH 6, precipitated at -20 °C overnight in 5x volume 2% LiClO4 in acetone, washed 2x in 70% EtOH and resuspended in RNase free water. RNAs that were amplified without a poly-A tail were poly-adenylated using poly-A polymerase (NEB M0276) according to the manufacturer’s protocol.

**In vitro translation**
Cell extracts were prepared from CRISPRi-engineered HEK293T cell lines at ~80% confluency. Cells were washed and scraped in cold PBS, spun down for 5 min at 1000 g at 4 °C, and resuspended in an equal volume of hypotonic lysis buffer (10 mM HEPES 7.6, 0.5 mM MgOAc, 5 mM DTT, Halt protease/phosphatase inhibitor cocktail (Thermo 78440)) for 45 min. Extracts were homogenized ~20 times through a 27G needle, spun down for 1 min at 14,000 g at 4 °C, and the supernatant removed, avoiding the lipids on the top and interface on the bottom. In vitro translation reactions with luciferase reporter mRNAs were carried out with 0.5x extract, energy mix (final 0.84 mM ATP, 0.21 mM GTP, 21 mM creatine phosphate (Roche 10621722001), 45 U/mL creatine phosphokinase (Roche 10127566001), 10 mM HEPES pH 7.5, 2 mM DTT, 2.5 mM MgOAc, 50 mM KOAc, 8 µM amino acids (Promega PRL4461), 255 µM spermidine, 1U/mL murine RNase inhibitor (NEB M0314)), and 400 ng total RNA. Rocaglamide A (RocA, a gift from the Nicholas Ingolia Lab) was added to a final concentration of 0.1 µM where indicated. Reactions were incubated for 1 hr at 30 °C and luciferase signal was measured using Dual-Glo Luciferase Assay System (Promega E2920). In vitro translation extracts for sucrose gradient fractionation were first treated with micrococcal nuclease (NEB
M0247S) and 0.8 mM CaCl₂ for 10 min at 25 °C. Treatment was stopped with 3.2 mM EGTA. Treated extracts were then mixed in a 2:1:1 ratio with the energy mix and 1 μg full-length MYC or GAPDH mRNA in water and incubated for 20 min at 30 °C prior to loading on gradients. Cycloheximide (100 μg/mL; Sigma 01810) or GMP-PNP (Sigma G0635) were added to the energy mix prior to the translation reaction. GMP-PNP was added at 0.21 mM instead of GTP.

Sucrose gradient fractionation

In vitro translation reactions were sedimented on 10-25% sucrose gradients (containing 20 mM HEPES pH 7.5, 150 mM KOAc, 2.5 mM MgOAc, 1 mM DTT, 0.2 mM spermidine, 100 μg/mL cycloheximide if reaction contained cycloheximide) for 3.5 hrs at 240,000 g at 4 °C using a SW41 rotor (Beckman Coulter). Gradients were fractionated using Teledyne Isco Tris Peristaltic Pump and fractions were collected and pooled according to the UV trace. Fractions were concentrated using Amicon 30 kDa spin columns (UFC503096) according to the manufacturer's instructions. For Northern blot analysis, fractions were treated with 1% SDS and 1% Proteinase K solution (20 mg/mL Proteinase K (Thermo 26160), 0.2 M Tris-HCl pH 7.5, 0.2 M NaCl, 1.5 mM MgCl₂) at 42 °C for 30 min. RNA was extracted using an equal volume of phenol:chloroform pH 6, precipitated at -20 °C overnight in 2x volume 100% EtOH, 2.7 M NaOAc, and 10 μg/mL GlycoBlue Coprecipitant (Thermo AM9515), washed 2x in 70% EtOH and resuspended in RNase free water.

Northern Blotting

Total RNA isolated from the sucrose gradient fractions was resolved using a 10% polyacrylamide gel in 0.5x TBE buffer buffer (1x TBE buffer contains 89 mM Tris, 89 mM boric acid, and 2 mM EDTA) and electroblotted onto a nylon (N+) membrane (GE Healthcare RPN203B) at 20 V for 90 min at 4 °C in 0.5x TBE buffer. The membrane was crosslinked and pre-hybridized in UltraHyb Hybridization Solution (Thermo AM8670) at 42 °C for 1 hour, then incubated overnight with 50 pmol Met-tRNAi specific probe (5′-TGGTAGCAGAGGATGGTTTCGAT-3′). The probe was labeled on the 5′ end with [γ-32P] ATP (Perkin Elmer) using T4 polynucleotide kinase (NEB M0201) according to the manufacturer’s protocol. Membranes were washed twice by 20 mL 6x SSC for 5 min at 42 °C and twice by 20 mL 2x SSC and twice by 20 mL 1x SSC (20x SSC contains 0.3 M sodium citrate in 3 M NaCl). Membranes were then wrapped in saran wrap, exposed to a phosphor screen overnight, and visualized by phosphor-imaging.

Cell viability assays

Ramos cells were seeded at 1 x 10⁶ cells/mL into 96-well plates in the presence or absence of drug (RocA, gift from Nicholas Ingolia Lab, 0 – 0.1 μM; Doxorubicin (Fisher BP25161), 0 – 4 μM), cultured for 24 hours, and cell viability was assessed using CellTiter-Glo assay according to the manufacturer’s protocol (Promega G7570).

Metabolic labeling

Cells were seeded at 1 x 10⁶ cells/mL into 6-well plates and allowed to adhere and grow overnight. Media was changed to DMEM –Met/Cys (Thermo 21013024) for 30 min, then each well was incubated for 30 min at 37 °C with 5 μl/well EXPRE35S35S Protein Labeling Mix (PerkinElmer NEG072002MC), after which cells were lysed in RIPA lysis
buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.25 % deoxycholylic acid, 1 % NP-40, 1 mM EDTA). Lysates were boiled in SDS loading buffer, resolved on SDS-PAGE gels, and stained with Coomassie to visualize total protein. Gels were dried at 80 °C for 1 hour on a gel drier, exposed to a phosphor screen overnight, and 35S incorporation was visualized by phoshor-imaging.

**Cloning**

EIF3A was PCR-amplified from HEK293T cDNA and cloned into vector nLv-103 (hygromycin) (Smith et al. 2016) using PCR-based restriction free cloning. HLH mutations and shRNA target site mutations were introduced by PCR-based site-directed mutagenesis. Custom EIF3A shRNA lentiviral vector was obtained from Sigma-Aldrich MISSION TRC shRNA plKO.1-puro bacterial stock (Sigma SHCLNG). pMD2.G (Addgene #12259) envelope protein vector and pCMV-dR8.91 (Addgene # 2221) packaging vector were used to make viral particles for transduction. The same vectors were later used to package CRISPRi lentiviral plasmids. HCV IRES, MYC 5'-UTR, and ATF4 5'-UTR elements were cloned into pcDNA4-Rluc using HinDIII and EcoRI restriction sites; the HBB 5'-UTR was cloned into pcDNA4-Fluc, where the Renilla luciferase ORF sequence was replaced by the Firefly luciferase ORF. pCMV-SPORT6 containing MGC Human MYC cDNA (Cloned: 2985844) and enzyme stock was obtained from Dharmacon (MHS6278). Full-length MYC and GAPDH were amplified from cDNA and cloned into pCMV-SPORT6 using SacI and NotI restriction sites. pHR-EF1a-dCas9-HA-BFP-KRAB-NLS, a gift from the Jacob Corn Lab (Addgene plasmid #102244), was used to introduce catalytically dead Cas9 into HEK293T cell lines. EIF3A sgRNA sequences were obtained from the human CRISPRi library v2 (Horlbeck et al. 2016) and cloned into pSLQ1371_BLP1_Ef1A_puro_GFP, a gift from the Jonathan Weissman Lab, using BstX1 and Blp1 restriction sites. The EIF3A sgRNA top and bottom oligo were ordered from IDT (for final selected sgRNA: (top) 5'- TTGGCAGCCGCGAGAGGAGTTAAGAGG -3'; (bottom) 5'- TTAGCTTAAAACCTTCGCTTGCCCGCCAGCAAAG -3') and annealed by incubating at 95 °C for 5 min in annealing buffer (100 mM Potassium acetate, 30 mM HEPES-KOH pH 7.4, 2 mM Mg acetate) and slowly cooling to room temperature. 5 nM annealed oligo were ligated to 10 ng digested vector backbone using T4 ligase (Thermo A13726) according to the manufacturer’s protocol.

**EIF3A mRNA** (site of HLH mutations indicated in red)

ATGCGGGGCCCTTTTCCAGAGGCCCGGAAAATGCCCTCAAACGCAGCAACGAACTTTCTTGAGACACATAATTGGGTCTGTATCTTTTTGCCATGTCTTTATATATGTGTATTGAATAAGTAAAAACATAGAACATAGGAAAAGGACTAGACCAACCTTCTGCACAGATCTACGTCACTCACTAGTGCTTAGATATAGAGGACTCTATATCCAAACTCCGAGATGTTCTCTCCTAAATGCTGTGTAAGCTGGAAGAACGACTCGGATCTAGATAGACTACGAGTGATCTGTATCTCTGTGTTATCTCGAATTACGCAGACCTACGGTACGTCTCAGATGAGGATCTGAGCAGTAGGTTCTCCTAAATGCTGTGTAAGCTGGAAGAACGACTCGGATCTAGATAGACTACGAGTGATCTGTATCTCTGTGTTATCTCGAATTACGCAGACCTACGGTACGTCTCAGATGAGGATCTGAGCAGTAGGTTCTCCTAAATGCTGTGTAAGCTGGAAGAACGACTCGGATCTAGATAGACTACGAGTGATCTGTATCTCTGTGTTATCTCGAATTACGCAGACCTACGGTACGTCTCAGATGAGGATCTGAGCAGTAGGTTCTCCTAAATGCTGTGTAAGCTGGAAGAACGACTCGGATCTAGATAGACTACGAGTGATCTGTATCTCTGTGTTATCTCGAATTACGCAGACCTACGGTACGTCTCAGATGAGGATCTGAGCAGTAGGTTCTCCTAAATGCTGTGTAAGCTGGAAGAACGACTCGGATCTAGATAGACTACGAGTGATCTGTATCTCTGTGTTATCTCGAATTACGCAGACCTACGGTACGTCTCAGATGAGGATCTGAGCAGTAGGTTCTCCTAAATGCTGTGTAAGCTGGAAGAACGACTCGGATCTAGATAGACTACGAGTGATCTGTATCTCTGTGTTATCTCGAATTACGCAGACCTACGGTACGTCTCAGATGAGGATCTGAGCAGTAGGTTCTCCTAAATGCTGTGTAAGCTGGAAGAACGACTCGGATCTAGATAGACTACGAGTGATCTGTATCTCTGTGTTATCTCGAATTACGCAGACCTACGGTACGTCTCAGATGAGGATCTGAGCAGTAGGTTCTCCTAAATGCTGTGTAAGCTGGAAGAACGACTCGGATCTAGATAGACTACGAGTGATCTGTATCTCTGTGTTATCTCGAATTACGCAGACCTACGGTACGTCTCAGATGAGGATCTGAGCAGTAGGTTCTCCTAAATGCTGTGTAAGCTGGAAGAACGACTCGGATCTAGATAGACTACGAGTGATCTGTATCTCTGTGTTATCTCGAATTACGCAGACCTACGGTACGTCTCAGATGAGGATCTGAGCAGTAGGTTCTCCTAAATGCTGTGTAAGCTGGAAGAACGACTCGGATCTAGATAGACTACGAGTGATCTGTATCTCTGTGTTATCTCGAATTACGCAGACCTACGGTACGTCTCAGATGAGGATCTGAGCAGTAGGTTCTCCTAAATGCTGTGTAAGCTGGAAGAACGACTCGGATCTAGATAGACTACGAGTGATCTGTATCTCTGTGTTATCTCGAATTACGCAGACCTACGGTACGTCTCAGATGAGGATCTGAGCAGTAGGTTCTCCTAAATGCTGTGTAAGCTGGAAGAACGACTCGGATCTAGATAGACTACGAGTGATCTGTATCTCTGTGTTATCTCGAATTACGCAGACCTACGGTACGTCTCAGATGAGGATCTGAGCAGTAGGTTCTCCTAAATGCTGTGTAAGCTGGAAGAACGACTCGGATCTAGATAGACTACGAGTGATCTGTATCTCTGTGTTATCTCGAATTACGCAGACCTACGGTACGTCTCAGATGAGGATCTGAGCAGTAGGTTCTCCTAAATGCTGTGTAAGCTGGAAGAACGACTCGGATCTAGATAGACTACGAGTGATCTG...
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ACCGAAGAGGACCTCCACTCACTCAGATCAGACGTGCTGATGAGGACCGAGATCTCTTGAAATCTTCAA
GCTGGGAAGACTCAAGTGCCGGAGATTGCCAGGACGACCGGGTCCTTTCTGGATT
AACCCGCTCAATGAGTGTCGTGACCTCCAGGAGTTGGGCGTGCCCCCGAGACTGCTAGCCGAG
TAGTGGTCTGGTCCGAAAGGCCCTTGTGATGACTCGTCTGATAGGGTGCTTGGCGAGTG
CCCCGGGAGGTTCTCGTAGACCCGTGCATCATTGACCAAATCTCT
MYC 5'-UTR
GACCCCCAGCTGTGTCTGCTTGCTCAGCCGGCCACCCGGCCGGCCGGCCGTCCT
GGCTCCCTCCTGCTCAGAGGCAGAGGCGTTTCTCGAGAGGCCGCTGGGAGGAAA
AGAACCAGAAGGAGGAGGATCGCGCTGAGATTAAAGCCCGTTTTTCGCGGCTTTTACT
AACCCTCGCTGAGTAACTCCAGCAGGAGAGAGGCGGAGGGGCGGCGGCT
AGGGTGGAAGAGCCCGGCGAGCAGAGCTCCTGCGCGGGCGCTCTGGGAAGGAGGA
GATCCCGAGAGAATGGGGGGCTGCTCGGCGGCCAGCCTCCCTGCCGCTGATCCT
CACCAACGCGTCCGAGCCACTTTGCCGATCACCAGAACTTGTCCCATACGAGCG
GCCGGCACTTTGCACTGGAATTACAACCCCGAGCAAGGACGCAGACTCTCCCGA
CGCCGGGAGGCTATTTGCTGATCCATTTTGAGGACACTTCCCGCCCGTCGCGAGGACC
CGCTTCTGTGAAAGGGCTCTCCCTGGACGTCTTAGACGG
ATF4 5'-UTR (red ATG, uORF start codon)

EIF3A HLH* - AAAAGTAAAAA mutated to AACAGTGAAAGA
EIF3A shRNA target – GCGCCTTGAGAGTCTGAATAT
EIF3A shRNA target (mutated) – GCGACTAGAAAGCCTAAACAT
EIF3A HLH* fasta file sequence (for building Bowtie2 indices) –
ATGTTCTTTATGATGTTATGAACAGTGAAGACATAGAACATGGCAAAAG
HCV IRES (start codon indicated in red)
CTCCCTCTGTGAGGAACACTACTGTCTTACCGCAGAAAGGCTTCTAGCCATGCGGTATT
ATGAGTGTCGTCGAGCCTCCAGGAGCCCCCCTCCCCGGGGAGAGCCATAGTGGTCT
GGCGGaACCGGTGAGTGACACCAGGAAATTGCCAGGACGACCGGGTTCTTTCTGGATT
AACCCGCTCAATGAGTGTCGTGACCTCCAGGAGTTGGGCGTGCCCCCGAGACTGCTAGCCGAG
TAGTGGTCTGGTCCGAAAGGCCCTTGTGATGACTCGTCTGATAGGGTGCTTGGCGAGTG
CCCCGGGAGGTTCTCGTAGACCCGTGCATCATTGACCAAATCTCT

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TTTCTACTTTGGCCGCCCACAGATGATTTTCTCTGCGCTGCGGTGTGTTTCCCTCCTCTCCCGGCTCTAGGTCACGGCACCACCATGGCATTAGGCGGACAGCAGTCCTGCGGCAGCATTGCGCTCAGCGGCGGCACTCAGCCGGTTAAGGCATGGCCTTTCGTAACCCGACAAAAGACACCTTCTGAATTAAGCAGCATTCTCATTGCGATTCCAGCACAAGACACCGCAAACAGACACC

ATF4 ΔuORF1 – first ATG mutated to AGG
ATF4 ΔuORF2 – second ATG mutated to AGG

HBB 5′-UTR
ACATTTGCTTCTGACACAACTTGTTCTAGGAACACCTCACAACAGACACC
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