The mRNA encoding the JUND tumor suppressor detains nuclear RNA-binding proteins to assemble polysomes that are unaffected by mTOR

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Running title: Non-eIF4E translation is supported by nuclear protein

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Key words: complex 5'-UTR, non-canonical cap-binding complex, nuclear RNA helicase, polyribosome, post-transcriptional control element (PCE), RNA-binding protein, JunD proto-oncogene AP-1 transcription factor subunit (JUND), tumor suppressor, ribonucleoprotein (RNP), mechanistic target of rapamycin (mTOR)

ABSTRACT

One long-standing knowledge gap is the role of nuclear proteins in mRNA translation. Nuclear RNA helicase A (DHX9/RHA) is necessary for the translation of the mRNAs of JunD proto-oncogene AP-1 transcription factor subunit (JUND) and HIV-1 genes, and nuclear cap–binding protein 1 (NCBP1)/CBP80 is a component of HIV-1 polysomes. The protein kinase mTOR activates canonical messenger ribonucleoproteins (mRNPs) by post-translationally downregulating the eIF4E inhibitory protein, 4E-BP1. We posited here that NCBP1 and DHX9/RHA (RHA) support a translation pathway of JUND RNA that is independent of mTOR. We present evidence from reciprocal immunoprecipitation experiments indicating that NCBP1 and RHA both are components of mRNPs in several cell types. Moreover, tandem affinity and RT-qPCR results revealed that JUND mRNA is a component of a previously unknown ribonucleoprotein complex. Results from the tandem IP indicated that another component of the JUND-containing ribonucleoprotein complex is NCBP3, a recently identified ortholog of NCBP2/CBP20. We also found that NCBP1, NCBP3, and RHA, but not NCBP2, are components of JUND-containing polysomes. Mutational analysis uncovered two dsRNA-binding domains of RHA that are necessary to tether JUND–NCBP1/NCBP3 to polysomes. We also found that JUND translation is unaffected by inhibition of mTOR, unless RHA was down-regulated by siRNA. These findings uncover a non-canonical cap-binding complex consisting of NCBP1/NCBP3 and indicate that RHA substitutes for eukaryotic translation initiation factor 4E (eIF4E) and eIF4G in activating mTOR-independent translation of the mRNA encoding the tumor suppressor JUND.

Introduction

Nuclear proteins engender the translational utilization of mRNAs, but the
mechanisms are controversial (1–5). Nuclear proteins are co-transcriptionally bound to nascent RNAs through recognition of the 5′-cap structure. The 5′-cap structure is bound by nuclear cap-binding protein (NCBP) 2/CBP20 and NCBP1/CBP80 heterodimeric nuclear cap-binding complex (CBC) (6–10). The CBC is necessary for the processing and nuclear export as well as translational utilization of mature transcripts (11–15). The mature transcripts retain the canonical CBC (consisting of NCBP1/NCBP2) to support a pioneer round of ribosome scanning for premature termination codons that trigger nonsense mediated decay (16).

Subsequently, CBC bound at the 5′-RNA cap is exchanged to cytoplasmic cap-binding protein eukaryotic translation initiation factor 4E (eIF4E). eIF4E engages eIF4G and other components of pre-initiation complexes (PIC) to initiate canonical cap-dependent translation. The eIF4E-dependent messenger ribonucleoproteins (mRNPs) activate polysome assembly and steady state protein synthesis (17). These mRNPs are activated by the serine-threonine kinase, mTOR through post-translational downregulation of 4E-BP1 (18). Treatment with rapamycin or small molecule inhibitors of mTOR (e.g. Torin-1) downregulates eIF4E activity (18).

The traditional view has been that eIF4E is necessary for cap-dependent protein synthesis (16, 17). Small subsets of cap-dependent mRNAs maintain polysomes during the downregulation of eIF4E, including JUND (19, 20). Recently, alternatives to eIF4E have been shown to be assembling translation RNPs on select mRNAs. Examples are the DAP5/p97 isoform of eIF4G (21–25); FXR1a/PARN (26, 27); tRNA synthetase (28); and eIF3d (29). NCBP1 is a component of HIV-1 polysomes and polysomes of select histone mRNAs (30–33). Immunoprecipitations (IPs) of HIV-1 polysomes identified that late mRNAs are enriched in NCBP1 coprecipitates while undetected in eIF4E immunoprecipitates. These results posited a non-eIF4E- pathway for cap-dependent translation of select mRNAs (31).

Recently an additional cap-binding protein, NCBP3 was identified to serve redundantly with NCBP2 under physiological conditions (9). The observation that co-depletion of NCBP2/NCBP3 curtailed NCBP1 association with 5′-caps validated that NCBP1 alternatively forms CBC with NCBP2 or NCBP3 (9). Further evidence demonstrated NCBP3 is essential to mount a precise and appropriate stress response (34). NCBP3-deficient mice suffered from severe lung pathology and increased morbidity after influenza A virus challenge, suggesting NCBP3 supports expression of stress-responsive genes (34). Given NCBP2 and NCBP3 function redundantly to support nuclear processing of mRNAs and that NCBP3-deficient cells evoke a reduced antiviral response posited the noncanonical CBC (consisting of NCBP1/NCBP3) is important for translation of stress response genes (31). The gap in knowledge remains to determine involvement of the noncanonical CBC in translation of select mRNAs.

AP-1 transcription factors are stress-response proteins (JUND, JUNB, c-Jun) (35). JUND, a negative regulator of cell proliferation, is among the subset of host mRNAs selectively translated during post-translational downregulation of eIF4E (19). Previous investigations identified translation of JUND and retroviral transcripts is dependent upon nuclear RNA helicase A (DHX9/RHA) (36). DHX9/RHA (RHA) binds to a specific class of post-transcriptional control element (PCE) in JUND and several retroviral 5′-UTRs to stimulate polysome formation (36–38). RHA downregulation or ectopic expression of the two N-terminal RNA binding domains (RBDs) were found to attenuate the synthesis of JUND and HIV-1 virion proteins (36, 38). The RHA-cofactors necessary for assembling polysomes remain to be identified. Given the mutual engagement of NCBP1 and RHA to select mRNAs, we posited NCBP1 and RHA are components of the same RNP that is facilitating cap-dependent translation of JUND.

Results

NCBP1-RHA and eIF4E-eIF4G are mutually exclusive RNP

To address the hypothesis that NCBP1 and RHA are components of a mutual RNP, reciprocal co-immunoprecipitations (co-IPs) were performed with specific antisera in three or more replicate experiments. HEK293 cytoplasmic (cyto) lysates were incubated with specific antisera or isotype-specific IgG. Analysis of the
immune complexes by Western blots (WB) revealed that the antiserum to NCBP1 co-precipitated RHA, NCBP2, NCBP3, and cytoplasmic poly-A binding protein (PABPC1). In the course of seven independent experiments, neither eIF4E nor eIF4G were detectable in the NCBP1-RHA immune complexes (Fig. 1A). Antiserum to eIF4E co-precipitated eIF4G, PABPC1 and DDX3 (Fig. 1B) (39, 40). The eIF4E-complexes were devoid of detectable NCBP1, RHA, NCBP2, or NCBP3 (Fig. 1B), validating that NCBP1-RHA and eIF4E-DDX3 are components of mutually exclusive mRNPs (Fig. 1).

Antiserum to NCBP2 or NCBP3 independently coprecipitated NCBP1 (Fig. 1A), corroborating the previous finding that NCBP1 independently enriches NCBP2- or NCBP3-bound 5′-capped-mRNAs (9). The RHA immune complexes coprecipitated NCBP1 and NCBP2 or NCBP3 and the results of seven replicate experiments confirmed lack of detectable eIF4E and eIF4G, recapitulating different mRNPs. Reciprocal IPs validated each of the indicated interactions (Fig 1A). NCBP3 coprecipitated DDX3, but not other components of the eIF4E RNP. The observed NCBP3-DDX3 interaction may be attributable to DDX3 in transitional interaction between NCBP-bound and eIF4E-bound 5′-caps. In summary, the immune complexes of NCBP1-RHA did not bind DDX3, whereas eIF4E did not bind RHA. The results suggest there are multiple NCBP3 complexes.

To ascertain whether NCBP1-RHA RNP is conserved in other cell types, we investigated cyto lysates of several other cell lines such as simian fibroblasts (COS), HTLV-1-transformed SLB-1 human T lymphocytes and CEMx174 human T lymphocytes. The studies assessed coprecipitation of NCBP1 and RHA with or without NCBP2, NCBP3 or eIF4E. The results from three independent experiments demonstrated that NCBP1-RHA complexes selectively interact with NCBP3 or NCBP2 and are mutually exclusive of eIF4E in these cell lines (Fig. 2A and B). These findings were not surprising since prior studies have demonstrated NCBP1, NCBP3 or NCBP2 and RHA exhibit high level amino acid sequence conservation across Mammalia and translation of viral mRNAs requires RHA of bovine, feline, human, and simian origin (36).

**NCBP1-NCBP3-RHA mRNP exclusive of NCBP2, are components of JUND polysomes**

Next, experiments employed tandem IPs to document whether RHA and NCBP1, NCBP3 or NCBP2 are components of the same RNP in polysomes (Fig. 3A, B). Exogenous expression of pFLAG-RHA was performed and the HEK293 cells were collected in the lysis buffer and designated as Input cyto lysate (Fig. 3B). Next the Inputs were subjected to sucrose gradient centrifugation and polysome fractions were collected (Fig. 3A) and subjected to WB (Fig. 3C). WB by several antiserum verified FLAG-RHA, NCBP3, NCBP1 and NCBP2 in the Input cyto lysate (Fig. 3C, lane 1). Noticeably, FLAG-RHA, NCBP3 and NCBP1 were enriched in polysomes, whereas NCBP2 was absent (Fig. 3C, lane 2). These results exposed functional differences between canonical CBC (NCBP1-NCBP2) and non-canonical CBC (NCBP1-NCBP3). The lack of NCBP2 detection in polysomes corroborated previous demonstration of CBC activity in pioneer round, but not steady state translation (41).

Next, the Input and polysome samples were incubated with FLAG antiserum conjugated to protein G magnetic beads. Immune complexes were washed and collected by competitive elution with 3x FLAG peptides (250 µg/ml) as depicted in Fig. 3B. The WB of the cyto IP verified the enrichment of FLAG-RHA and the coprecipitation of NCBP1, NCBP2 and NCBP3 (Fig. 3C, lane 3). Moreover, the WB of the polysome IP verified the enrichment of FLAG-RHA and the coprecipitation of NCBP1 and NCBP3. Importantly, NCBP2 was not detectable in the polysome IP (Fig. 3C, lane 4), corroborating the lack of NCBP2 in the WB of the collected polysomes (Fig. 3C, lane 2). There was no detectable enrichment of the proteins in the protein G negative control (Fig. 3, lane 5). Next, the eluates were subjected to tandem IP with NCBP1 antiserum conjugated to protein G magnetic beads and analyzed by WB (Fig. 3B). The results validated that the NCBP1 tandem IP captured FLAG-RHA from cyto and polysome samples (Fig. 3C, lane 6 and 7 [FLAG-RHA panel]). The tandem IP ascertained NCBP3 and NCBP2 enrichment in the cyto tandem IP (Fig. 3C,
However, the polysome tandem IP captured NCBP3, but failed to capture NCBP2 (Fig. 3C, lane 7). There was no enrichment of these proteins in the IgG isotype control (Fig. 3C, lane 8 and 9). We concluded that NCBP3 is the mutual component of FLAG-RHA-NCBP1 RNPs in polysomes.

To validate JUND is a component of the FLAG-RHA-NCBP1-NCBP3 RNPs, replicate tandem IP samples were extracted with TRIzol-LS and the coprecipitating RNA was collected and subjected to RT-qPCR using JUND and GAPDH specific primer pairs. The JUND transcripts were readily detectable in the FLAG-RHA-NCBP1-NCBP3 RNP, whereas GAPDH transcripts were less than the minimum detectable (< MD) (Fig. 3D). Both JUND and GAPDH were < MD in the IgG controls (Fig. 3D). These results validated FLAG-RHA-NCBP1-NCBP3 are mutual components of JUND polysomes.

**RHA tethers NCBP1-NCBP3 to polysomes through RNA**

NCBP1-NCBP3 may be tethered to RHA through RNA or via protein-protein interaction, which would be sensitive or resistant to RNase treatment, respectively. To examine the nature of the interaction between RHA and NCBP1-NCBP3, reciprocal IPs were performed using cyto lysates treated with RNaseA. The cyto lysate was incubated with RHA or NCBP1 antiserum conjugated to protein G magnetic beads with or without RNaseA (1 µg/ml) for 2 h. Immune complexes were washed and collected in SDS sample buffer. The RHA IP and NCBP1 IP coprecipitated RHA, NCBP1, NCPB3 and NCPB2 (Fig. 4A, lane 1 and 3). However, RNaseA treatment of the RHA IP eliminated NCBP1, NCPB3 and NCPB2 (Fig. 4A, lane 2). RNaseA treatment of the NCPB1 IP eliminated RHA coprecipitation, establishing that the interaction is RNA-dependent (Fig. 4A, lane 4). We concluded NCBP1 is tethered to RHA through RNA.

Since RHA binding to PCE RNA requires the N-terminal double-stranded (ds) RBDs and is eliminated by mutation of surface-exposed lysine residues (K54A, K55A, K236E) (42) (Fig. 4B), we postulated the same residues are critical for RHA interaction with NCBP1-NCBP3 or NCBP1-NCBP2. To examine this, HEK293 cells were transfected with FLAG-RHA-WT or FLAG-RHA modified by the following substitutions: K54A K55A (KK); K236E; or K54A, K55A, K236E (KKK) and subjected to reciprocal IPs using antiserum to FLAG or NCBP1.

The antiserum to FLAG identified WT, KK and K236E coprecipitated NCBP1 (Fig. 4C, lane 2, 5 and 8), whereas KKK was not detectable (Fig. 4C, lane 11). The longer exposure of the blot identified NCBP1 was barely detected in the KKK coprecipitate (3% of WT) (Fig. S1 and Table S1). Likewise, the coprecipitation of NCBP2 and NCBP3 was eliminated by the KKK mutation (Fig. 4C, lane 11).

Reciprocal IP was performed using antiserum to NCBP1. NCBP1 coprecipitated FLAG-WT, KK or K236E, but not KKK (Fig. 4D). NCBP3 and NCBP2 interactions were maintained. The coprecipitation of ribosomal proteins rpS6 and rpL5 by FLAG or NCBP1 was diminished, but not abrogated by the lysine mutations (Fig. 4C and D). The results demonstrated that NCBP1-NCBP3 and NCBP1-NCBP2 are tethered through RNA to the N-terminal dsRBDs of RHA.

**RHA is necessary for the assembly of NCBP1-NCBP3-JUND polysomes and mTOR-resistant JUND translation**

Next, we sought to establish whether RHA-PCE interaction is necessary for the assembly of NCBP1-NCBP3 polysomes. HEK293 cells were transfected with siRNAs targeting NCBP3 or RHA or with non-targeting (NT) control siRNA. Twenty-four hours post-transfection, cell lysates were analyzed by WB and total cellular RNA was analyzed by RT-qPCR using gene-specific primers. WB showed that NCBP3 and RHA proteins were downregulated by the specific siRNA, whereas NCBP1 and GAPDH protein expression was unaffected (Fig. 5A). The RT-qPCR recapitulated the downregulation of ncbp3 and dhx9/rha by the specific siRNAs relative to GAPDH (Fig. 5B). The cyto lysates were subjected to sucrose density sedimentation and A254 spectrometry to identify the ribosomal RNA profile. The results of three independent experiments identified magnitude of polysomes was modestly diminished by RHA downregulation compared to the siNT control (Fig. 5C). The siNCBP3 treatment did not diminish polysomes and reproducibly increased the robustness of the
60S and 80S peaks (Fig. 5C). The results indicate NCBP3 has a generalized effect on ribosome recruitment.

The distribution of mRNAs in the gradient fractions was determined by RT-qPCR. The downregulation of RHA significantly reduced JUND polysomes, whereas no change was observed in GAPDH polysomes (Fig. 5D). The results recapitulated previous metabolic labeling assays showing RHA downregulation reduces the synthesis of JUND, but not GAPDH protein (36). The downregulation of NCBP3 significantly increased PIC (48S and 60S mRNPs) and diminished polysomes (Fig. 5D). The JUND PIC increased from 2% to 15%, whereas heavy polysomes decreased from 69% to 58% of the RNA copies, accounting for the redistribution to PIC. The GAPDH PIC increased from 12% to 22% and light polysomes decreased from 20% to 10% of the RNA copies, accounting for the redistribution in RNA copies. The increase in PIC was 4-fold greater for JUND than GAPDH polysomes (Fig. 5D). The results do not exclude the possibility of redundant activity by RHA-NCBP1-NCBP2 as a result of NCBP3 downregulation. Indeed, NCBP2 and NCBP3 were shown to function redundantly to support nuclear processing of mRNAs (9). We concluded that RHA-NCBP1-NCBP3 activity is important for JUND polysome assembly at steady state. The prospect of RHA-NCBP1-NCBP3 RNP activity during mTOR downregulation was an important open issue.

Therefore, we evaluated the effect of mTOR downregulation on the assembly of JUND polysome components, RHA-NCBP1-NCBP3. The small molecule mTOR inhibitor, Torin-1 activates the allosteric inhibitor of eIF4E, hypophosphorylated (hypo-) 4E-BP1. Torin-1 titration experiments were first carried out to establish treatment time and optimal dose to activate hypo-4E-BP1, while maintaining ribosome profiles and no change in cell viability. The titration experiments identified cells exposed to 50 nM Torin-1 for 18 h maintained consistent polysome profiles and upregulated hypo-4E-BP1 in several replicate experiments with the cell viability remaining similar to mock-treated cells. Immunoblotting of the cell lysates documented RHA, NCBP1, NCBP3 and other proteins maintained steady-state at 50 nM Torin-1 for 18 h (Fig. 6A and Fig. S2).

To evaluate the capacity for cells to recover from the Torin-1 treatment, medium was exchanged and cells were cultured without Torin-1 for 1 h or 18 h. Immunoblotting validated recovery of hyper-phosphorylated 4E-BP1 in the cells subject to short-term (1 h) and long-term (18 h) culture (Fig. S3). We concluded the treatment conditions employed were appropriate to maintain polysome profiles and cell viability for 18 h.

Next, HEK293 cells were transfected with RHA-specific or NT siRNAs (mock) for 24 h and then exposed to 50 nM Torin-1 for 18 h. The WB on cyto lysates validated RHA was downregulated by siRNA (Fig. 6B, lane 2 and 4) and hypo-4E-BP1 was upregulated by Torin-1 treatment (Fig. 6B, lane 3 and 4). Tubulin was unaffected by the treatments. The results identified JUND proteins reduced in response to the RHA downregulation (Fig. 6B, compare lane 1, 2 and 4), but not by Torin-1 treatment (compare lane 1 and 3). Conversely, GAPDH protein was reduced by Torin-1, but not by RHA downregulation. The results verified JUND translation is resistant to mTOR inhibition.

The cyto lysates were subjected to sucrose density sedimentation and A254 spectrometry. As expected, ribosomal profiles were diminished by the Torin-1 treatment (Fig. 6C). Next, RT-qPCR was performed on the input lysate and the RNA isolated from the gradient fractions. The results verified that RHA downregulation significantly reduces JUND polysomes (Fig. 6D, upper panel), but not GAPDH polysomes (Fig. 6D, lower panel). Torin-1 treatment significantly downregulated GAPDH polysomes (Fig. 6D, lower panel), but not JUND polysomes (Fig. 6D, upper panel). As expected, the combination of Torin-1 treatment and RHA downregulation significantly reduced both JUND and GAPDH polysomes (Fig. 6D). The distribution across the sucrose gradients of RHA, NCBP1, NCBP3 and other proteins was evaluated by immunoblotting (Fig. S4). The results validated that Torin-1 eliminated eIF4E from polysome fractions, whereas RHA, NCBP3 and NCBP1 remained.

Next, replicate polysome fractions were combined and proteins were coprecipitated by NCBP1 or eIF4E antiserum. The immunoblots validated NCBP1 coprecipitated RHA and
NCBP3 polysomes in the presence or absence of the Torin-1 treatment, whereas neither NCBP2 nor eIF4E were detectable (Fig. 6E). The eIF4E immunoprecipitates enriched eIF4G and these polysomes were eliminated by Torin-1 (Fig. 6F). None of the candidate proteins were detected in the IgG isotype controls (Fig. 6E and F).

The IP samples were extracted with TRIzol-LS and the co-precipitating RNAs were collected and subjected to the RT-qPCR. In results of three independent experiments, JUND transcripts were readily detectable in immunoprecipitated NCBP1 polysomes, whereas GAPDH transcripts were not (<MD) (Fig. 6E, below WB). In contrast, the eIF4E polysomes enriched GAPDH transcripts, but not JUND (<MD) (Fig. 6F, below WB). The IgG IPs did not enrich either JUND or GAPDH (<MD) (Fig. 6E and 6F). These findings validated the assembly of JUND polysome components, RHA-NCBP1-NCBP3 is mTOR-resistant.

Discussion

This study has identified a specialized translation pathway for cap-dependent translation of JUND that does not utilize eIF4E or eIF4G, nor NCBP2. Strong evidence is provided that NCBP1 and RHA are components of the same RNP that is facilitating cap-dependent translation of JUND. Moreover, JUND polysomes are composed of RHA-NCBP1-NCBP3 and assemble at steady state and during mTOR inhibition. Current evidence indicates the RHA-NCBP1-NCBP3 cap-dependent translation mRNP is not regulated by eIF4E levels and appears to be a constitutively active pathway, rather than a rescue pathway.

In addition to JUND, several retroviruses contain the RHA-responsive element, PCE and RHA-NCBP1-NCBP3 are likely involved in the translation control of these mRNAs. PCE does not support internal ribosome entry and PCE translation activity has been shown to be cap-dependent (37). Prior research established the RHA-PCE RNA interaction neutralizes structural barriers within the 5'-UTR that repress ribosome scanning to promote efficient translation (36). Our new findings provide strong evidence that RHA is necessary for the assembly of JUND polysomes at steady-state and during mTOR inhibition (Fig. 7). Moreover, NCBP1-NCBP3 and NCBP1-NCBP2 are tethered to RHA through JUND by the N-terminal dsRBDS of RHA. The molecular basis by which RHA-PCE interaction subverts canonical exchange of the nuclear cap-binding proteins to eIF4E remains an open issue.

JUND is a member of the Jun family of transcription factors that dimerize with c-Jun, Fos or other family members to form Activator protein-1 (AP-1) (29, 35). Recently, the 5'-UTR of c-jun was shown to assemble non-eIF4E translation initiation complexes (29). In preliminary studies, we observed c-jun RNA copies are enriched in RHA-NCBP1-NCBP3 immune complexes (5.3×10^4 and 3.1×10^4, minus and plus Torin-1, respectively) at levels similar to JUND in Fig. 6E. Next, we replicated the tandem IP experiment and identified the enrichment of c-jun-RHA-NCBP1 mRNPs in cyto and polysomes (11×10^4 and 39×10^4 copies). Further experimentation is warranted to validate the c-jun 5'-UTR recapitulates activity of JUND PCE and possibly retroviral PCEs, which is necessary for RHA interaction (36, 43–45) and mTOR-resistant translation.

AP-1 is a critical regulator of nuclear gene expression during T-cell activation, innate response to viral infections as well as anti-tumor immune response through type I interferons and pro-inflammatory cytokines (46). Dysregulation of AP-1 is hallmark of viral pathogenesis, neoplastic transformation and tumor progression (35, 46). Likewise, dysregulation of dhx9/rha is associated with productive viral infection (31, 37, 38, 45, 47–50) and tumor survival (51–54). The recent finding NCBP3 is essential to mount a precise antiviral response (34) posits dysfunction in a RHA-NCBP1-NCBP3 translation pathway contributes to deficient innate response. The dhx9/rha gene is within the 1q25 prostate/lung/breast cancer locus and gene mutation or over-expression is pervasive in human clinical samples (55). We speculate mutations in PCE or RHA residues identified in this report provide biomarkers for neoplastic transformation.

Experimental procedures

Cell culture and transfection

Dulbecco's Modified Eagle's Medium (DMEM) or RMPI culture medium were supplemented with 10% fetal bovine serum and 1×...
Antibiotic-Antimycotic solution (Gibco) and used to culture HEK293 and COS7 cells or SLB-1 and CEM×174 cells, respectively. The cell lines were obtained from ATCC and low-passage cultures were used in the experiments. In addition, mycoplasma testing was performed periodically and on a weekly basis. The cells were authenticated and documented free of mycoplasma based on morphological evaluation of cells plated at high and low culture densities under a microscope and the cell morphology images were maintained for comparisons.

Transfection of HEK293 cells (1×10^6 per 35 mm well) with siRNA (50 nM) targeting NCBP3 (9), RHA or non-targeting (NT) (36) siRNA used Lipofectamine 2000 (1 µl/10 nM siRNA) (Invitrogen) and Opti-MEM medium (500 µl). Fresh medium was exchanged after 6 h and 24 h later, supplemented with Torin-1 (50 nm) for 18 h. Plasmid transfections were performed using X-tremeGENE (Roche) (3 µl or 1.5 µl), plasmids (1 µg or 0.5 µg) in OptiMEM (500 µl) and cells that had been cultured overnight in 6-well (1×10^6 per well) or 12-well (2×10^5 per well) plates. After 24 h, transfected cells were washed twice in ice-cold 1× PBS and collected by 3-min low-speed centrifugation. Total cellular proteins and RNA were isolated in cell lysis buffer and TRIzol-LS (Ambion), respectively (56). Equivalent (20 µg) amount of cytoplasmic protein was subjected to WB with specific antibodies and protein-antibody complexes were detected by enhanced chemiluminescence (GE Biosciences), quantified by ImageJ (NIH) and densitometry results were compiled in Table S1. The antibodies used in this study are listed in Supporting Information, Tables S2 and S3.

As described previously, cDNA was generated using Omniscript (Qiagen), random primers (Invitrogen) and cellular RNA (2 µg), coprecipitated RNA samples (56) or tandem IP samples (15, 57), followed by RT-qPCR using gene-specific primers, which are provided in Supporting Information (Table S4). All experiments were performed in three or more biological replicates.

HEK293 cells (1×10^6 per 35 mm well) were cultured in medium containing different concentrations (0, 25, 50 or 100 nM) of Torin-1 or 0.2% DMSO for 0, 1, 5, 18 or 24 h. The cells treated with 50 nM Torin-1 for 18 h were washed twice and cultured in DMEM for 1 or 18 h. The cells were washed once with ice-cold 1× PBS and lysed in RIPA buffer. The soluble lysates were collected by centrifugation at 12,000 rpm for 2 min.

**Density sedimentation and analysis of RNPs**

Published protocols were used to perform density sedimentation and A254 spectrometry (58). Briefly, cells were transfected for 24 h and treated with Torin-1 (50 nM) for 18 h. The culture medium was supplemented with 0.1 mg/mL cycloheximide (CHX) for 10 min. Cells were washed twice with ice-cold 1× PBS containing 0.1 mg/mL CHX and collected in 5 mL of ice-cold 1× PBS with CHX by scraping. The cells were subjected to centrifugation at 1500 rpm for 4 min at 4°C and resuspended in 0.75 mL of low salt buffer (20 mM Tris—HCl [pH 7.5], 3 mM MgCl2, 10 mM NaCl, 2 mM DTT, 1× protease inhibitor cocktail EDTA-free, 5 µL/mL RNase Out) and allowed to swell on ice for 5 min. Cells were lysed on ice by the addition of 0.25 mL lysis buffer (0.2 M sucrose, 0.5% NP-40 and 0.1% Triton X-100 prepared in low salt buffer) and 10 strokes in a Dounce homogenizer (Kimble Chase). The lysates were depleted of nuclei by centrifugation at 13,000 rpm for 1 min at 4°C to collect cytoplasm. The lysates were used for the sucrose gradients and IPs. Equivalent amounts of RNA OD units across samples were layered on the top of 10-50% sucrose gradients and centrifuged using a SW41 rotor (Beckman Coulter) for 2 h 40 min at 35,000 rpm at 4°C. Polysome profiles were generated by continuous monitoring of RNA absorbance at 254 nm by the ISCO UA-6 absorbance detector unit (Brandel) and fractionated into 22 equivalent volume (0.5 mL) fractions by the ISCO Foxy R1 fraction collector. Brandel Peak Trace Software was used to generate the corresponding profile traces. The proteins were precipitated from the collected fractions by ProteoExtract protein precipitation kit (EMD Millipore) according to manufacturer’s instructions or by Trichloroacetic acid (TCA) precipitation. The fractions were supplemented with ice-cold TCA (final concentration of 20%), vortexed and incubated overnight at -20°C. Samples were centrifuged at 12,000 rpm for 15 min at room temperature and...
the pellets were washed twice with three volumes of ice-cold acetone. The precipitated proteins were resuspended in equivalent volume of low salt buffer and used for WB or tandem IP. The even fractions were used for protein precipitation and the odd fractions were used for RNA extraction in TRIzol-LS (Ambion).

**IP and tandem IP**

Dynabeads Protein G (30 µL) (Invitrogen) were washed two times in 10 bed volume of IP lysis buffer (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 2 mM EDTA, 1% NP-40). The beads were incubated with the antiserum in 10 bed volume of IP lysis buffer containing 1 mM BSA for 45 min at room temperature. The antibodies used for IP are listed in Table S2. The bead-antibody complexes were washed once in 10 bed volume of IP wash buffer (20 mM Tris-HCl [pH 7.4], 300 mM NaCl, 0.5% NP-40) and incubated with 300 µg of cell lysate at 4°C for 2 h with rotation. The enriched immune complexes were washed four times in IP wash buffer and collected by boiling with 1× SDS sample buffer.

Tandem IP was performed on the cytoplasmic lysates from pFLAG-RHA-transfected HEK293 cell cultured in three 15-cm plates. Dynabeads Protein G (100 µL) (Invitrogen) was washed twice in 10 bed volume of lysis buffer (20 mM Tris-HCl [pH 7.5], 3 mM MgCl₂, 10 mM NaCl, 2 mM DTT, 1× protease inhibitor cocktail EDTA-free, 5 µL/mL RNase Out, 0.2 M sucrose, 0.5% NP-40 and 0.1% Triton X-100) and incubated with 10 µL of FLAG antiserum (1 mg/ml; Sigma) in 10 bed volume of lysis buffer containing 1 mM BSA for 45 min at room temperature. Five-hundred microgram of cytoplasmic lysate was diluted to 1 ml in the lysis buffer and the final concentration of NaCl was adjusted to 300 mM. The diluted lysate was mixed with Protein G-anti-FLAG complex and incubated at 4°C for 2 h. The RNP complexes captured by the anti-FLAG conjugated Protein G beads were subsequently washed twice (1 ml each) in ice-cold wash buffer I (20 mM Tris-HCl [pH 7.5], 300 mM NaCl, 0.1% NP-40) followed by two washes in ice-cold wash buffer II (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.1% NP-40). The samples were incubated with one bed volume of wash buffer II containing 250 µg/ml 3× FLAG peptide and 5 µL/mL RNase Out with gentle shaking at 4°C overnight. The suspension was cleared by magnetic force followed by centrifugation at 12,000 rpm for 5 min at 4°C. The cleared suspension was incubated with Protein G (50 µL)-anti-NCBP1 (4 µl) complex for 2 h at 4°C. The captured RNP complexes were washed four times in wash buffer II and divided into two equal parts for WB and RNA isolation. The proteins precipitated from polysome fractions were processed in a similar manner. Ten-percent of each sample volume was reserved from each step of the tandem IP for WB. The isolated proteins were resolved using commercial 4-15% gradient SDS-PAGE gels (Bio-Rad) and transferred to nitrocellulose membrane and immunoblotted.

**Statistical data analysis**

Three or more independent experiments were performed for each assay and results were combined to define the mean ± standard deviation (SD). Statistical significance was assessed using a two-tailed Student’s t test and a p value (*) of < 0.05 was considered to be significant. Bar graphs present mean and standard deviation of three independent experiments and the precise values from the individual experiments are denoted by small dots. Statistical significance: * p≤0.05, ** p≤0.005.

**Data availability:**
The raw RNA copies from the polysome analysis will be available upon request. Kathleen Boris-Lawrie: Department of Veterinary and Biomedical Sciences, University of Minnesota, 1971 Commonwealth Avenue, St. Paul, MN 55108; kbl@umn.edu; Tel. (612-625-2700); Fax. (612-625-5203). All other data are contained within the manuscript.

**Acknowledgments:**
The work was supported by NIH P50 NIGMS0103297 and T32GM068412.
Conflict of interests:
The authors declare that they have no conflicts of interest with the contents of this article.

The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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List of abbreviations:

Activator protein-1 (AP-1); cap-binding complex (CBC); immunoprecipitation (IP); co-immunoprecipitation (co-IP); double-stranded RNA-binding domains (dsRBD); pre-initiation complex (PIC); monosome (Mono); heavy polysomes (HP); light polysomes (LP); messenger ribonucleoprotein (mRNP); nuclear cap-binding protein (NCBP); poly-A binding protein 1 (PABPC1); post-transcriptional control element (PCE); RNA helicase A (RHA); Western blot (WB)
Figure legends:

Figure 1. NCBP1-RHA and eIF4E-eIF4G are components of mutually-exclusive RNPs
Reciprocal co-IP of selected proteins from HEK293 lysates with specific antisera (Bold). Bound proteins were eluted and analyzed by WB with the antisera indicated on the right. Isotype-specific IgG served as the negative control and input cell lysate served as the positive control. The results are summarized in the tables below each WB. (A) IP of NCBP1, RHA, NCBP2 or NCBP3. (B) IP of eIF4E, eIF4G or DDX3. The antiserum detected the specific proteins on the immunoblots, as shown relative to the pre-stained molecular weight markers (M). The same image of the molecular weight markers was used for each panel; (+): positive co-IP; (<MD): less than the minimum detectable co-IP; *Non-specific background. The WB were subjected to ImageJ densitometry quantification (Table S1).

Figure 2. NCBP1-RHA complexes exist in several cell types
IP of NCBP1 (upper panel) (A), RHA (lower panel) (B) or isotype IgG from lysates of HEK293, COS7, SLB-1 or CEM×174 cells depleted of nuclei. Immune complexes (Bold) were washed, collected in SDS sample buffer and analyzed by WB with the antisera indicated on the right. In each case, the input cell lysate served as the positive control and the isotype-specific IgG served as the negative control for background immunoreactivity. The results are summarized in the table below each WB. The antiserum detected the specific proteins on the immunoblots, as shown relative to the pre-stained molecular weight markers (M). The same image of the molecular weight markers was used for each panel; (+): positive co-IP; (<MD): less than the minimum detectable co-IP; *Non-specific background. The WB were subjected to ImageJ densitometry quantification (Table S1).

Figure 3. NCBP1-NCBP3-RHA are components of the same RNP loaded to JUND polysomes
Tandem IPs were employed to isolate components of the same RNP from HEK293 cells transfected with pFLAG-RHA. Input cyto lysates were subjected to sucrose gradient centrifugation and polysomes were collected. (A) A254 spectrometry (red line) of sucrose gradient. (B) Outline of tandem IP. The polysome fractions were combined, precipitated and resuspended in low salt buffer (Input poly). Aliquots of the cyto (white tube) and polysome samples (blue tube) were incubated with FLAG antiserum conjugated to protein G beads. Beads were washed and incubated with 3× FLAG peptides to elute RNPs. A fraction of the eluate was reserved for WB and the remaining was incubated with NCBP1 antiserum conjugated to protein G beads. The beads were extracted in SDS buffer for WB analysis or with Trizol to isolate RNA for subsequent RT-qPCR with gene-specific primers. (C) WB of Input cyto and polysomes (lane 1 and 2), eluates of FLAG IP (lane 3 and 4), eluate of Protein G IP (control for FLAG IP, lane 5), eluates of NCBP1 IP (lane 6 and 7), eluates of IgG IP (control for NCBP1 IP, lane 8 and 9). The antiserum detected the specific proteins on the immunoblots, as shown relative to the pre-stained molecular weight markers (M). The same image of the molecular weight markers was used for each panel; *Non-specific band. (D) JUND and GAPDH copies by RT-qPCR. Results represent the mean of three independent experiments with standard deviation. <MD: Minimum detectable.

Figure 4. NCBP1 interaction with RHA requires N-terminal dsRBDs
(A) RHA or NCBP1 IP of CEM×174 cyto lysates treated with RNaseA (or left untreated). Coprecipitates were analyzed by WB using antiserum against RHA, NCBP3, NCBP1 or NCBP2. (B) Depiction of RHA with annotation of the domain structure and position of mutations K54, 55A; K236E. dsRBD I and II, red; DEIH helicase core, tangerine; helicase-associated 2 (HA2), grey; OB fold, brown and arginine-glycine-rich (RG-rich), gold. (C) FLAG IP and (D) NCBP1 IP of cyto lysates of HEK293 cells transfected with FLAG-tagged WT RHA or mutant RHA (K54, 55A [KK], K236E or K54, 55A and K236E [KKK] for 24 h followed by WB for the indicated proteins. The antiserum detected the specific proteins on the immunoblots, as shown relative to the pre-stained molecular weight markers (M). The same image of the
molecular weight markers was used for each panel; *Non-specific band. The WB were subjected to ImageJ densitometry quantification (Table S1).

**Figure 5. RHA downregulation reduces JUND polysomes**
Cyto lysates of HEK293 cells transfected with siRNA targeting NCBP3 (siNCBP3), RHA (siRHA) or non-targeting siRNA (siNT) were subjected to WB and RT-qPCR. (A) Representative immunoblot of the cyto lysates with antisera against RHA, NCBP3, NCBP1 or the loading control GAPDH. The antisera detected the specific proteins on the immunoblots, as shown relative to the pre-stained molecular weight markers (M). The same image of the molecular weight markers was used for each panel. (B) dhx9/rha and ncbp3 expression in cells treated with the indicated siRNA by RT-qPCR. The bar graph represents the expression of dhx9/rha and ncbp3 relative to GAPDH. (C) A_ spectrum of sucrose gradient showing rRNA distribution and designation of the fractions. RNP; PIC: Pre-initiation complex composed of 40S and 60S RNPs; Mono: monosome (80S); LP: light polysome (2-3 polysomes); HP: heavy polysome (4 or more polysomes). (D) RT-qPCR of RNA extracted from the fractions identified in (C) for expression of JUND and GAPDH. Copies were calculated relative to standard curves. The graphs present the distribution of the RNA copies across the gradients. Results represent the mean of three independent experiments (bar) with standard deviation. The colored circles indicate the values from the individual experiments. Statistical significance: * p ≤ 0.05, ** p ≤ 0.005.

**Figure 6. RHA is essential for mTOR-resistant translation of JUND**
(A) HEK293 cells were treated with mTOR inhibitor Torin-1 (0-100 nM) for 0-18 h and the cell lysates were analyzed by WB with the indicated antisera. (B) Cells transfected with siRNA targeting RHA (siRHA) or non-targeting control (siNT) and treated with 50 nM Torin-1 or 0.2% DMSO for 18 h were analyzed by WB with antisera to RHA, 4E-BP1, JUND, GAPDH or tubulin (loading control). (C) A_ spectrum of the sucrose gradient showing rRNA distribution in the fractions. RNP; PIC: Pre -initiation complex composed of 40S and 60S RNPs; Mono: monosome (80S); LP: light polysome (2-3 polysomes); HP: heavy polysome (4 or more polysomes). (D) JUND and GAPDH RNA copies were analyzed by RT-qPCR and calculated relative to standard curves. The graphs present the distribution of the RNA across the gradients. Results represent the mean of three independent experiments (bar) with standard deviation. The colored circles indicate the values from the individual experiments. Statistical significance: * p≤0.05, ** p≤0.005. (E and F) The LP and HP (polysome) fractions were combined, proteins precipitated and subjected to IP with NCBP1 or eIF4E antisera. Coprecipitates were subjected to WB with the indicated antisera or RNA extraction followed by RT-qPCR. Isotype-specific IgG were used as negative controls. The antisera detected the specific proteins on the immunoblots, as shown relative to the pre-stained molecular weight markers (M). The same image of the molecular weight markers was used for each panel. *Non-specific band. Table below each WB panel shows the number of copies of JUND and GAPDH relative to IgG control detected by RT-qPCR. <MD: Minimum detectable.

**Figure 7. Model of the specialized translation of PCE-containing mRNAs and assembly of mTOR-resistant RNP complexes**
Model comparing nuclear assembly of cap-dependent mRNPs. Left, PCE-bearing mRNAs bind NCBP1-NCBP3 at the 5’-cap and RHA at the PCE and experience the exchange of PABPN to PABPC1. RHA-NCBP1-NCBP3 mRNPs facilitate assembly of JUND polysomes whether the elf4E-mRNPs are active or inhibited by 4E-BP1. Right, Canonical engagement of NCBP1-NCBP2 at the 5’-cap is depicted by mRNA lacking PCE. CBC: Cap-binding complex. Consequential to nuclear export, CBC undergoes exchange to elf4E, which engages elf4G and other factors to assemble polysomes. In common, both mRNPs experience the exchange of PABPN to PABPC1. The elf4E-elf4G-dependent polysome assembly is abrogated upon the inhibition of mTOR due to downregulated elf4E activity.
**Figure 1**

**Panel A**

| Protein | NCBP1 | RHA | NCBP2 | NCBP3 |
|---------|-------|-----|-------|-------|
| IP      | +     | +   | +     | +     |
| +       | IP    | +   | +     | +     |
| +       | +     | IP  | <MD   | NCBP2 |
| +       | +     | +   | <MD   | NCBP3 |
| <MD     | <MD   | <MD | <MD   | eIF4E |
| <MD     | <MD   | <MD | <MD   | eIF4G |
| <MD     | <MD   | <MD | +     | DDX3  |
| +       | +     | +   | +     | PABPC1 |

**Panel B**

| Protein | eIF4E | eIF4G | DDX3 |
|---------|-------|-------|------|
| IP      | +     | +     | +    |
| +       | IP    | +     | +    |
| +       | +     | IP    | +    |
| <MD     | <MD   | <MD   | <MD  | NCBP1 |
| <MD     | <MD   | <MD   | <MD  | NCBP2 |
| <MD     | <MD   | +     | NCBP3 |
| <MD     | <MD   | <MD   | RHA  |
| +       | +     | +     | +    | PABPC1 |
Figure 2

A

|          | HEK293 | COS7 | SLB-1 | CEM×174 | Co-IP |
|----------|--------|------|-------|---------|-------|
| IP       | IP     | IP   | IP    | IP      | NCBP1 |
| +        | +      | +    | +     | +       | RHA   |
| +        | +      | +    | +     | +       | NCBP3 |
| +        | +      | +    | +     | +       | NCBP2 |
| <MD      | <MD    | <MD  | <MD   | <MD     | eIF4E |

B

|          | HEK293 | COS7 | SLB-1 | CEM×174 | Co-IP |
|----------|--------|------|-------|---------|-------|
| IP       | IP     | IP   | IP    | IP      | RHA   |
| +        | +      | +    | +     | +       | NCBP1 |
| +        | +      | +    | +     | +       | NCBP3 |
| +        | +      | +    | +     | +       | NCBP2 |
| <MD      | <MD    | <MD  | <MD   | <MD     | eIF4E |
Figure 3

A

B

C

D

Table D

|                | IP: NCBP1 | IP: IgG |
|----------------|-----------|---------|
| JUND Cyto      | 19 ±1.5   | <MD     |
| JUND Poly      | 9 ±2.3    | <MD     |
| GAPDH Cyto     | <MD       | <MD     |
| GAPDH Poly     | <MD       | <MD     |

Eluted FLAG RNPs 75- by guest on May 6, 2020
Figure 4

A

| IP: RHA | NCBP1 |
|---------|--------|
| RNase A | - | + |
| - | - | + |

WB

| Lane | 1 | 2 | 3 | 4 |
|------|---|---|---|---|
| 150- | -RHA | 100- | -NCBP3 | 100- | -NCBP1 |
| 75- | 75- | 75- | -NCBP2 |
| 25- | 25- | 25- | 25- |

kDa M

B

dsRBDI | dsRBDII | DEIH helicase core | HA2 | OB-fold | RG-rich

C

FLAG-RHA

| Input | IgG | Input | IgG | Input | IgG | Input | IgG | Input | IgG |
|-------|-----|-------|-----|-------|-----|-------|-----|-------|-----|
| WT | K54,55A | K236E | K54,55A,236E |

WB

| Lane | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|------|---|---|---|---|---|---|---|---|---|----|----|----|
| 150- | -FLAG-RHA | 100- | -NCBP3 | 100- | -NCBP1 | 100- | -NCBP2 | 25- | -rpS6 | -rpL5 | - |
| 75- | 75- | 75- | 25- | 37- | 37- | 37- | 37- | 37- | 37- | 20- |
| 100- | 100- | 100- | 100- | 100- | 100- | 100- | 100- | 100- | 100- | 75- |

kDa M
Figure 5

A

B

C

D

[Images and graphs showing protein expression, RNA distribution, and statistical analysis]
Figure 7

The diagram illustrates the interaction between RHA and PCE in the presence and absence of NCBP1. In the presence of RHA-PCE interaction (left side), the CAP is retained in the nucleus. In the absence of PCE-RHA interaction (right side), the CAP is exchanged between the nucleus and cytoplasm.

The mTOR-resistant complex is formed when NCBP-RHA mRNP is present, whereas the mTOR-sensitive complex is formed with eIF4E-mRNP.
The mRNA encoding the JUND tumor suppressor detains nuclear RNA-binding proteins to assemble polysomes that are unaffected by mTOR
Gatikrushna Singh, Sarah E. Fritz, Bradley Seufzer and Kathleen Boris-Lawrie

J. Biol. Chem. published online April 20, 2020

Access the most updated version of this article at doi: 10.1074/jbc.RA119.012005

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