eIF4G2 balances its own mRNA translation via a PCBP2-based feedback loop

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ABSTRACT

Poly(rC)-binding protein 2 (PCBP2, hnRNP E2) is one of the most abundant RNA-binding proteins in mammalian cells. In humans, it exists in seven isoforms, which are assumed to play similar roles in cells. The protein is shown to bind 3′-untranslated regions (3′-UTRs) of many mRNAs and regulate their translation and/or stability, but nothing is known about the functional consequences of PCBP2 binding to 5′-UTRs. Here we show that the PCBP2 isoform f interacts with the 5′-UTRs of mRNAs encoding eIF4G2 (a translation initiation factor with a yet unknown mechanism of action, also known as DAP5) and Cyclin I, and inhibits their translation in vitro and in cultured cells, while the PCBP2 isoform e only affects Cyclin I translation. Furthermore, eIF4G2 participates in a cap-dependent translation of the PCBP2 mRNA. Thus, PCBP2 and eIF4G2 seem to regulate one another's expression via a novel type of feedback loop formed by the translation initiation factor and the RNA-binding protein.

Keywords: translational control; ribosomal scanning; αCP2

INTRODUCTION

The mechanism of translation initiation in eukaryotes was established about two decades ago when Marilyn Kozak's scanning model was complemented with a biochemical characterization of the initiation factors' activities (for reviews, see Hinnebusch 2011, 2014). Since then, this basement has not been shaken, although there are numerous cases that do not fit this model particularly well. eIF4G2 (also DAP5, Nat1) is one of such square pegs in a round hole of our knowledge of eukaryotic translation initiation. The protein seems to be present in all Chordata and many (but clearly not all) invertebrates. Despite its close homology with eIF4G1, eIF4G2 lacks binding sites for eIF4E and PABP (Imataka et al. 1997; Levy-Strumpf et al. 1997; Shaughnessy et al. 1997; Yamanaka et al. 1997) and thus it is believed to have a hand in cap-independent translation of certain cellular mRNAs (Henis-Korenblit et al. 2002; Hundsdoerfer et al. 2005; Lewis et al. 2008; Marash et al. 2008), although a stimulatory action of eIF4G2 on cap-dependent translation was also reported (Lee and McCormick 2006; de la Parra et al. 2018). The protein is essential for development in mouse (Yamanaka et al. 2000), zebrafish (Nousch et al. 2007), and *Drosophila* (Yoshikane et al. 2007), probably because of its participation in the translation of differentiation-associated proteins (Yoffe et al. 2016; Sugiyama et al. 2017), and its overexpression in ES cells leads to a spontaneous differentiation (Takahashi et al. 2005). Remarkably, to date, no organism is known whose eIF4G2 mRNA uses an AUG triplet as an initiator codon. In apparently most (if not all) vertebrates, it is GUG, while in Insecta, Mollusca, or simple Chordata, no translation initiation site can be assigned unambiguously. Such an evolutionarily conserved mRNA feature implies the

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existence of posttranscriptional regulation. It is now widely recognized that any mRNA is heavily packed with the bound proteins (Baltz et al. 2012; Castello et al. 2012; Beckmann et al. 2015; Hentze et al. 2018), and since every known case of posttranscriptional regulation relies on mRNA-binding proteins, we sought to identify the proteins that interact with the human eIF4G2 mRNA.

Poly(rC)-binding proteins (hnRNP K and PCBP1-4) are important actors in mRNA metabolism. They participate in splicing (Expert-Bezançon et al. 2002; Ji et al. 2016; Grelet et al. 2017), transcription (Choi et al. 2009), translational repression, and stabilization or destabilization of mRNAs via an interaction with their 3′-UTRs (Scoumanne et al. 2011; Han et al. 2013; Ren et al. 2016). The two probably most known cases of their translation-related activities are the stabilization of α-globin mRNA and the repression of ALOX15 mRNA translation during hematopoiesis (for review, see Ostareck-Lederer and Ostareck 2012). PCBP2 also participates in translation driven by the IRES element from poliovirus and other related viruses (Blyn et al. 1997; Gamarnik and Andino 1997; Graff et al. 1998; Walter et al. 1999; Sweeney et al. 2014; Asnani et al. 2016a). However, cases in which PCBP2 affects 5′-end-dependent translation via binding to 5′-UTRs, are not explicitly described. In humans, at least seven PCBP2 isoforms (a–g) are known to exist. These isoforms arise from alternative splicing, but all PCBP2-coding mRNAs seem to possess the same 5′-UTR. To date, no functional difference between the isoforms has been registered.

Here we show that the PCBP2 isoform f binds the 5′-UTR of the eIF4G2, and the PCBP2 isoforms e and f bind the 5′-UTR of the Cyclin I (CCNI) mRNAs in vitro and inhibit the translation of the corresponding reporter mRNAs, while the PCBP2 knockdown in 293T or Huh7 cells augments their translation. Strikingly, knockdown of the eIF4G2 gene in NIH/3T3, 293T, or Huh7 cells leads to a significantly decreased translation driven by the 5′-UTR of the PCBP2 mRNA as well as the PCBP2 protein level. Thus, eIF4G2 and PCBP2 mutually tune one another's translation.

RESULTS AND DISCUSSION

Proteins that bind the 5′-UTR of the eIF4G2 mRNA

First, we sought to identify proteins that interact with the eIF4G2 mRNA. The 5′-UTR and 3′-UTR of the human eIF4G2 mRNA (transcript variant 1) were cloned upstream and downstream from the firefly luciferase coding sequence, respectively, to create a pGL3-eIF4G2 plasmid that was used further for the reporter mRNA and the biotinylated bait synthesis. The authentic 5′ boundary of the 5′-UTR was defined on the basis of expressed sequence tags (EST) and cap-assisted gene expression analysis (CAGE) data (Severin et al. 2014). Since the evolutionarily conserved GUG initiator codon is most likely important for the regulation of eIF4G2 expression, we included it and started 30 nucleotides (nt) of the eIF4G2 coding region in the reporter. Additionally, the firefly luciferase AUG codon was mutated to exclude any contribution of the translation initiation downstream from the suboptimal authentic eIF4G2 start codon.

In vitro transcribed noncapped biotinylated RNA, corresponding to the human eIF4G2 5′-UTR, was used to pull down proteins from HeLa cytoplasmic extract (Fig. 1, left lane). The proteins specifically bound to the eIF4G2 5′-UTR were identified by mass spectrometry as hnRNP K, HuR, SERBP1, YBX3, PCBP2, and PTBP1. YBX1 and hnRNP Q are ubiquitously bound to any RNA tested (Fig. 1; also, data not shown). Similarly to the case of poliovirus IRES (Blyn et al. 1995, 1996, 1997; Andreev et al. 2012), PCBP2 manifests itself in the multiple bands, reflecting the diversity of its isoforms.

PCBP2f inhibits translation of the reporter mRNA bearing the eIF4G2 5′-UTR in vitro

All the identified proteins except for YBX3, the cDNA of which we have failed to amplify from a dozen different cDNA preparations, were cloned and expressed in *Escherichia coli*. We accidentally amplified cDNAs corresponding to two isoforms of the PCBP2, viz., PCBP2e and PCBP2f. Thus, both were studied. We addressed whether the addition of either protein affects translation of the eIF4G2 reporter mRNA in vitro in Krebs-2 cytoplasmic extract. While SERBP1, hnRNP K, HuR, PCBP2e, or PTBP1 exert no specific effect (data not shown) on the translation of a dozen of reporter mRNAs tested, including the eIF4G2 reporter, the other PCBP2 isoform, PCBP2f, inhibits the

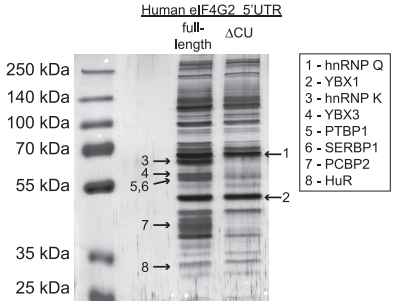


FIGURE 1. Identification of the proteins bound to the 5′-UTR of the eIF4G2 mRNA. In vitro transcribed biotinylated noncapped RNAs corresponding to either the human eIF4G2 5′-UTR or its deletion mutant lacking the polypyrimidine tract (199–274 nt of the eIF4G2 5′-UTR, eIF4G2ΔCU) were soaked in HeLa cytoplasmic extract, the complexes were purified via streptavidin-agarose chromatography, the bound proteins were resolved by SDS-PAGE, and identities of the specific bands were identified by LS-MS. Two major bands that bind to all mRNAs in our hands have been identified as YBX1 and hnRNP Q. PCBP2, PTBP1, YBX3, hnRNP K, and SERBP1 were found to interact with the polypyrimidine tract within the 5′-UTR of the eIF4G2 mRNA.

translation of the eIF4G2 reporter mRNA and this effect does not depend on the presence of the authentic 3′-UTR sequence (Fig. 2A). Notably, closely related PCBP1 also has no effect on the translation of the eIF4G2 reporter mRNA (data not shown).

The polypyrimidine sequence within the 5′-UTR of the eIF4G2 mRNA is necessary and sufficient for the PCBP2-mediated translation repression in vitro

To map the PCBP2-binding site within the 5′-UTR of the eIF4G2 mRNA, we have analyzed the ENCODE data set ENCSR339FUY, which represents the PCBP2 eCLIP data for HepG2 cells (ENCODE Project Consortium 2012). These data indicate that the polypyrimidine stretch in the middle of the eIF4G2 mRNA 5′-UTR is the PCBP2-binding site. As expected, the deletion of the 78-nt-long polypyrimidine sequence (of which 72 nt are C and U) resulted in a loss of PCBP2 binding (Fig. 1, right lane). Notably, this CU-rich region seems to be responsible for the binding of the whole bunch of the eIF4G2 mRNA-specific proteins, because hnRNP K, PTBP1, YBX3, and SERBP1 also fail to bind this deletion mutant (eIF4G2ΔCU). This may suggest that some of these proteins function as a complex. Indeed, hnRNP K and PCBP1/2 are often bound to the same mRNA targets (Kiledjian et al. 1995; Ostareck et al. 1997; Collier et al. 1998; Thiele et al. 2004; Lee et al. 2007; Thyagarajan and Szaro 2008).

In contrast to the wild-type eIF4G2 reporter mRNA, the translation of its deletion mutant is fully resistant to the PCBP2f addition in vitro (Fig. 2B). In a reciprocal approach, this CU-rich element was inserted into the late adenovirus tripartite leader (TPL) in either sense (TPL+) or antisense (TPL–) orientation. As we anticipated, translation of the TPL+ reporter has become susceptible to the PCBP2f-mediated inhibition in vitro (Fig. 2B).

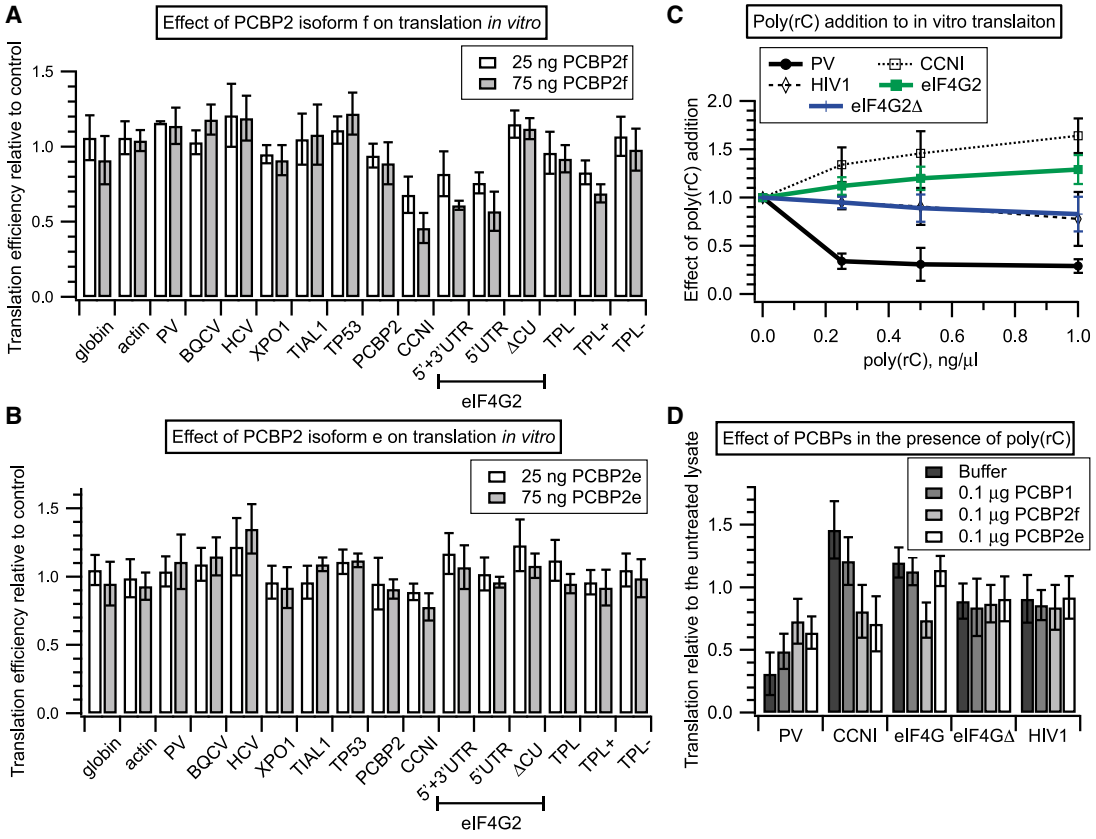


FIGURE 2. PCBP2 specifically inhibits translation in Krebs-2 cytoplasmic extract. In vitro transcribed m^7G-capped and polyadenylated mRNAs bearing the indicated 5′-UTRs and encoding Fluc or Nluc in the case of the CCNI reporter were translated for 40 min in Krebs-2 cytoplasmic extract in the presence of the indicated amounts (25 ng or 75 ng) of the recombinant PCBP2 isoforms, and then the luciferase activity was measured. Bars show the translation level relative to control reactions which contained PCBP2 storage buffer instead of the protein. The eIF4G2 reporter contained either the authentic eIF4G2 mRNA 3′-UTR (labeled 5′+3′-UTR) or SV40 3′-UTR (labeled 5′-UTR). (*A*) PCBP2e isoform. (*B*) PCBP2f isoform. Data are presented as mean with SD, five replicates at least. (*C*) Supplementation of translation reactions with the increasing amounts of poly(rC) inhibits the translation of the poliovirus reporter but augments the translation of the CCNI and eIF4G2 reporters. Note that the eIF4G2 reporter mutant that lacks the polypyrimidine stretch (eIF4G2ΔCU) is unresponsive to the poly(rC) addition. (*D*) Translation in Krebs-2 S30 lysate supplemented with 0.5 ng/μl poly(rC) and the indicated amounts of either PCBP1 as control and PCBP2f of PCBP2e. Data from panels *C* and *D* are presented as mean with SD, four replicates.

Inhibition of Cyclin I translation by PCBP2 in vitro

To address whether inhibition of translation by PCBP2 binding to 5′-UTRs is a more widespread phenomenon that is not limited to the case of the eIF4G2 mRNA, we have further analyzed the eCLIP data (data sets ENCSR339FUY and ENCSR115GAA) published by the ENCODE Project (ENCODE Project Consortium 2012) and the iCLIP data from Flynn et al. (2015). We have selected the human CCNI, TIAL1, XPO1, and the murine PCBP2 5′-UTRs (we have failed to amplify the human PCBP2 5′-UTR sequence, thus highly homologous murine PCBP2 mRNA 5′-UTR was used) for further analyses on the basis of their deduced interaction with PCBP2. We also noticed that the eCLIP data suggest that PCBP2 is bound to the 3′-UTR of the TP53 mRNA. Thus, we cloned the corresponding 5′-UTRs upstream of the firefly luciferase coding sequence, and also the 5′-UTR and 3′-UTR of the TP53 mRNA in the case of the TP53 reporter upstream and downstream, respectively. The 5′-UTR of the CCNI mRNA was cloned upstream of a much brighter NanoLuc (Nluc) because, in our hands, the CCNI-Fluc reporter is translated very poorly compared to other mRNAs. Next, we translated all these reporters in vitro with the addition of either PCBP2e or PCBP2f. Among the corresponding reporters, only that with the 5′-UTR of the CCNI mRNA is sensitive to PCBP2f in vitro (Fig. 2A) and, unlike the eIF4G2 case, this mRNA is also responsive to PCBP2e (Fig. 2B).

With respect to translation initiation, PCBP2's better-characterized role is to activate translation of the poliovirus and other related IRESes (Blyn et al. 1997; Gamarnik and Andino 1997; Graff et al. 1998; Walter et al. 1999; Sweeney et al. 2014; Asnani et al. 2016a,b), presumably acting as an RNA chaperone. Although translation of the poliovirus mRNA is strongly dependent on PCBP2 (Blyn et al. 1997; Gamarnik and Andino 1997; Sweeney et al. 2014), no effect of the PCBP2 addition is observed in the case of the poliovirus IRES-containing reporter (Fig. 2A, B). This, however, replicates earlier reports when the poliovirus mRNA was translated in HeLa cytoplasmic extract, and no effect of the PCBP2 addition could be seen until the lysate had been depleted of this protein (Blyn et al. 1997). PCBP2 is one of the most abundant RNA-binding proteins in a variety of cells (Hein et al. 2015; Uhlen et al. 2015), and it is apparently present at a saturating level already. To address this apparent contradiction, we supplemented the translation reactions with the increasing amounts of poly(rC). In accordance with the earlier report (Walter et al. 1999), this results in a dose-dependent inhibition of the poliovirus IRES-driven translation (Fig. 2C), while the translation of the eIF4G2 and CCNI reporters is stimulated by the poly(rC) addition. The PCBP2-insensitive HIV1 and eIF4G2ΔCU reporters are expectedly unresponsive to the poly(rC) addition. The supplementation of the depleted lysate with either recombinant PCBP1, PCBP2f, or PCBP2e stimulates the translation driven by the poliovirus IRES, which recapitulates its dependence on the poly (rC)-binding proteins. Accordingly, the translation of the eIF4G2 and CCNI reporter mRNAs is inhibited by the PCBP2f addition, and the CCNI reporter also responds to the PCBP2e addition.

Depletion of PCBP2 stimulates translation of the eIF4G2 and Cyclin I reporters in cultured cells

In order to address how PCBP2 affects the translation of the selected mRNAs in cultured cells, we knocked down the PCBP2 gene in 293T or Huh7 cells (Fig. 3A,B). Sequences of the siRNAs have been designed so that all seven reported PCBP2 isoforms are targeted. In 293T cells, the translation of only two mRNAs shows statistically significant (P-values <0.01) up-regulation compared to the β-globin or β-actin reporters, namely, eIF4G2 and Cyclin I. Similar to the in vitro data, the 3′-UTR of the eIF4G2 mRNA does not contribute to the PCBP2-sensitivity. Importantly, the translation of the eIF4G2 reporter lacking the CU-tract is evidently unaffected. It should be noted that in our hands the PCBP2 knockdown in 293T cells inevitably leads to a drop (20%–25%) in a translatability of known PCBP2-unresponsive reporters; see, e.g., β-globin or β-actin (see Fig. 3A). This is also true for a couple of other siRNAs targeting PCBP2 (data not shown). In Huh7 cells, the drop is not observed. The set of the TPL-based reporters behaved in cells less pronounced than in the in vitro translation experiments. This is perhaps due to a very high activity of the TPL in promoting translation in cells or, at least partially, due to ribosomal shunting (Yueh and Schneider 1996) when a scanning ribosome bypasses PCBP2 bound to the 5′-UTR. Similar results were obtained with another siRNA against PCBP2 (data not shown).

In many cases, mRNA-binding proteins affect mRNA turnover. Since northern blotting or RT-qPCR are not able to analyze a transfected RNA stability (Barreau et al. 2006; Thomson et al. 2013), we opted for kinetics analyses of transfected mRNAs expression (Dmitriev et al. 2007; Andreev et al. 2009). The idea behind this approach is that accumulation of a reporter protein should slow down for a less stable mRNA compared to that for a more stable mRNA. Clearly, despite clearly different efficiencies, all the investigated mRNAs are translated with similar kinetics, arguing against a significant difference between their stabilities in Huh7 (Fig. 3D) or 293T cells (data not shown).

eIF4G2 participates in translation of PCBP2 mRNA

Analysis of ribosome footprint profiling of mES (Sugiyama et al. 2017) and our own profiling of 293T and NIH/3T3 cells (which will be published elsewhere) revealed that the translation of the PCBP2 mRNA is inhibited upon the

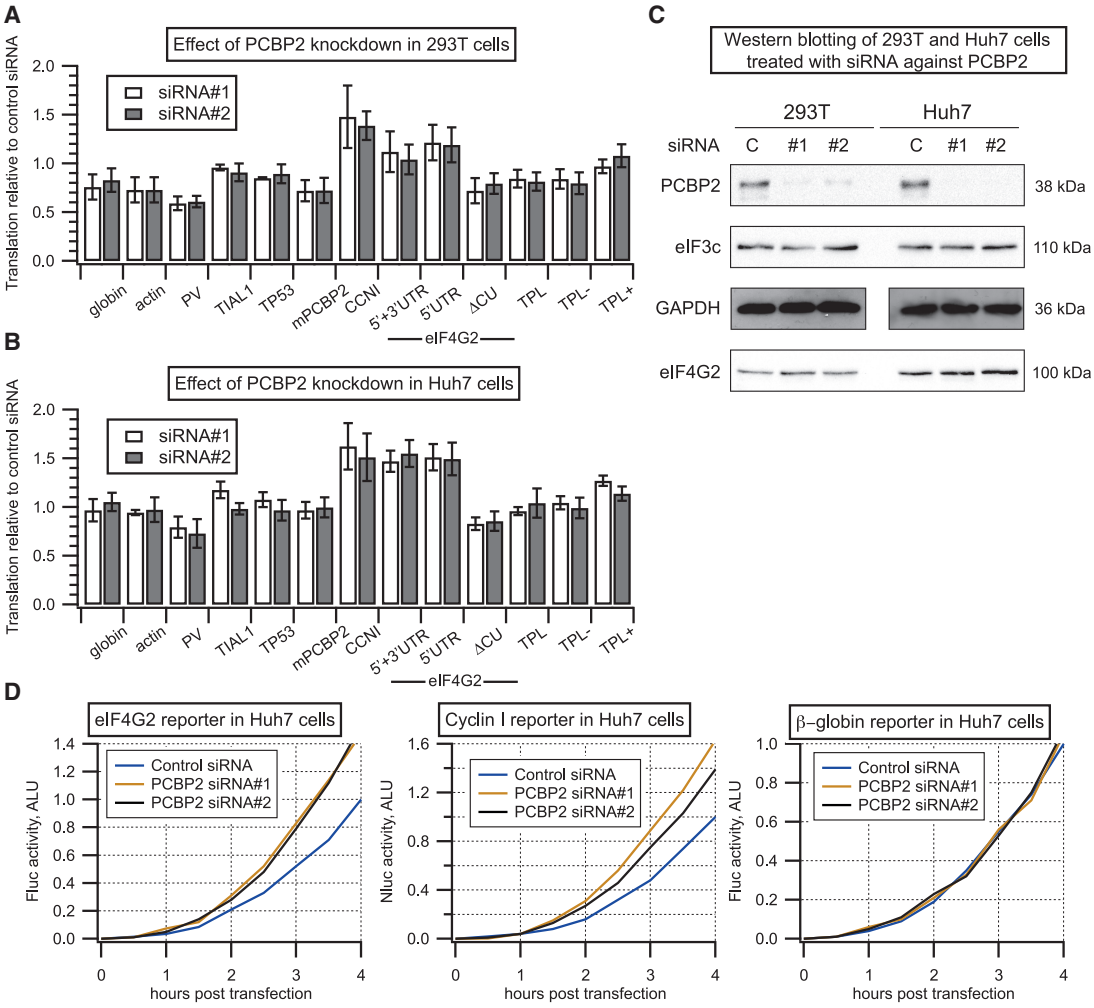
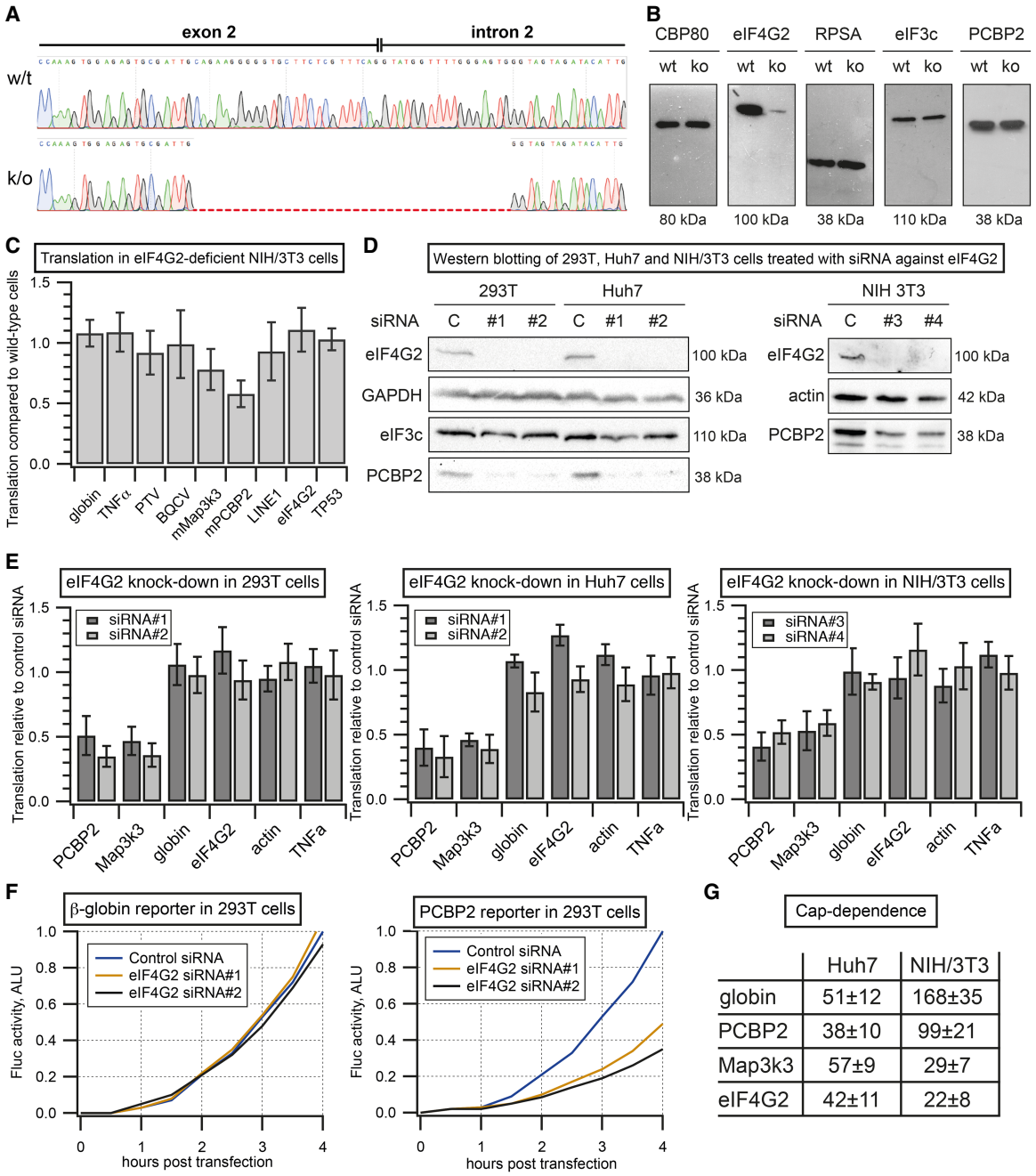


FIGURE 3. Effect of the PCBP2 knockdown on the translation of the selected reporter mRNAs. All the reporters bear Fluc except the CCNI reporter, which encodes NanoLuc. The reporters bearing the eIF4G2 5′-UTR are labeled as in Figure 2. Indicated in vitro transcribed m^7G-capped and polyadenylated reporters were transfected into cells pretreated with the siRNA against PCBP2 or with control siRNA. Bars show the translation level relative to control cells. (*A*) 293T cells. The translation of the TIAL1 reporter is reproducibly up-regulated relative to the β-globin or β-actin reporters, yet this up-regulation is statistically insignificant (*P*-value >0.05). In the case of the CCNI/β-globin pair, *P*-values are less than 0.0005; in the case of the DAP5/β-globin pair, *P*-values are less than 0.01. For the TPL+/TPL and the TPL+/TPL– pairs, *P*-values are less than 0.05. At least five independent transfections have been performed. (*B*) Huh7 cells. For the DAP5/β-globin reporters' pair, *P*-values are less than 0.00005 ($n = 5$). For the CCNI reporter, *P*-values are less 0.01 ($n = 7$). For the TPL+/TPL and the TPL+/TPL– pairs, *P*-values are less than 0.005 ($n = 7$) for siRNA#1. For siRNA#2, *P*-values are 0.15 and 0.01 for the TPL+/TPL and the TPL+/TPL– comparisons, respectively ($n = 7$). All data are presented as mean with SD. Independent two-sample *t*-test was used for statistical analysis. (*C*) Western blot analysis of the PCBP2 knockdown in 293T and Huh7 cells. GADPH and eIF3c used as loading controls. eIF4G2 increase, if any, is marginal. (*D*) Kinetics of the selected reporters' translation in Huh7 cells. Data of a representative experiment are shown. Note that no deceleration of the eIF4G2 or CCNI reporter translation is observed in control cells.

eIF4G2 down-regulation in all three data sets. To address if this mRNA is really an eIF4G2 target and if the eIF4G2 dependence is provided solely by the 5′-UTR of the PCBP2 mRNA, we transfected several mRNA reporters, including one bearing the eIF4G2-dependent 5′-UTR of the mMap3k3 mRNA (Sugiyama et al. 2017) into NIH/3T3 cells where the eIF4G2 gene was knocked out via a CRISPR/Cas9 approach (Fig. 4A,B). Indeed, the mPCBP2 and mMap3k3 reporters are translated less efficiently in the eIF4G2-depleted cells (Fig. 4C). However, the PCBP2 protein level is not apparently altered (Fig. 4B). To address this apparent inconsistency, we knocked down the eIF4G2 gene in 293T and Huh7 cells (Fig. 4D) and found that the efficiencies of both mPCBP2 and mMap3k3 reporters' translation are markedly reduced (Fig. 4E) as well as the endogenous PCBP2 level (Fig. 4D). Kinetics of the reporters' expression is not altered in 293T cells (Fig. 4F) and Huh7 or NIH/3T3 cells (data not shown). Moreover, a transient eIF4G2 knockdown in NIH/3T3 cells also leads to a decrease in the PCBP2 protein level. Allegedly, our



	Huh7	NIH/3T3
globin	51±12	168±35
PCBP2	38±10	99±21
Map3k3	57±9	29±7
eIF4G2	42±11	22±8

FIGURE 4. The eIF4G2 knockout leads to a decreased translation driven by the 5′-UTR of the PCBP2 mRNA in NIH/3T3 cells. (*A*) The eIF4G2 gene sequencing from the wild-type and the eIF4G2-deficient NIH/3T3 cells. The indel has occurred right at the junction of exon 2 and intron 2, which results in a deletion of the splice site. (*B*) Western blot analysis of the wild-type and eIF4G2-deficient NIH/3T3 cells. (*C*) Comparison of the translation efficiencies of the indicated reporter mRNAs in the eIF4G2-deficient vs. wild-type NIH/3T3 cells. Bars show translation in the cells lacking eIF4G2 relative to the wild-type cells. Note that here no further normalization has been performed and the eIF4G2-unresponsive mRNAs are indeed translated with similar efficiencies in either wild-type or knocked out cells. The decreases in the translation of the Map3k3 (*P*-value = 0.01, n = 5) and PCBP2 (*P*-value = 0.0001, n = 5) reporters are statistically significant. (*D*) Western blotting of 293T, Huh7, and NIH/3T3 cells transfected with siRNAs against eIF4G2. (*E*) Indicated in vitro transcribed m^7G-capped and polyadenylated reporters were transfected into 293T, Huh7, and NIH/3T3 cells pretreated either with the indicated siRNA against the eIF4G2 or with the control siRNA. Bars show the translation level relative to control cells. The decrease in the Map3k3 and PCBP2 translation efficiencies is statistically significant (*P*-values do not exceed 0.0001, n = 5). (*F*) Kinetics of the selected reporters' translation in 293T cells. A representative experiment is shown. Note that no deceleration of the PCBP2 reporter translation is observed. All transfection data are present as mean with SD. Independent two-sample *t*-test was used for statistical analysis. (*G*) Cap dependence of translation of the selected mRNA reporters in Huh7 and NIH/3T3 cells was calculated as a ratio of m^7G-capped to A-capped mRNA translation efficiencies. Data presented as mean with SD; at least three independent transfections.

eIF4G2-deficient NIH/3T3 cells have restored the PCBP2 level, which highlights the importance of this protein. Overall, these data show that eIF4G2 is important for the PCBP2 mRNA translation.

It was suggested that eIF4G2 affects its own cap-independent translation (Lewis et al. 2008; Yoffe et al. 2016). Arguably, such an interpretation could have arisen from inadequate use of bicistronic assay and hairpin-containing reporters (Jackson 2013; Terenin et al. 2017). Our data show that the translation of the m^7G-capped reporter mRNA with the 5′-UTR and 3′-UTR of the human eIF4G2 mRNA is not affected by the eIF4G2 knockout or knockdown, at least under commonly used growth conditions. Notably, both eIF4G2-dependent reporters used here (namely, mPCBP2 and mMap3k3) exhibit rather high cap dependence, i.e., stimulation of their translation by the m^7G-cap compared to the nonfunctional A-cap (Fig. 4G), indicating that eIF4G2 works on these mRNAs in a cap-dependent fashion as well. In line with this finding, de la Parra and colleagues just recently proposed that eIF4G2 is involved in a cap-dependent translation of about 20% of cellular mRNAs (de la Parra et al. 2018).

Conclusions

The pyrimidine-rich tract can be found in the 5′-UTRs of the eIF4G2 mRNAs from mammals, probably in lancelet, axolotl, at least from certain birds, yet it is not evident in mollusks, insects, or hydra, although homologs of the poly(rC)-binding proteins are present in all these organisms. Thus, the regulation of the eIF4G2 synthesis by PCBP2 may be quite an ancient mechanism. Most probably, PCBP2 hampers a ribosomal scanning and/or a ribosome attachment to an mRNA. Notably, the PCBP2 isoform f but not the isoform e inhibits eIF4G2 translation, while both inhibit CCNI translation. Thus, this study shows for the first time that not all seven PCBP2 isoforms are created equal and they do not have the same mRNA targets. Autoregulatory systems have been described that control translation of the eIF1 (Ivanov et al. 2010), eIF5 (Loughran et al. 2012), and PABP (de Melo Neto et al. 1995) mRNAs. This list is now expanded by the addition of eIF4G2, which controls its own translation via the mRNA-binding protein. However, the regulation swing is not exactly dramatic, and this feedback loop probably serves for fine-tuning of the eIF4G2 and PCBP2 expression. eIF4G2 is implicated in a cap-independent translation, but our data show that it participates in a cap-dependent translation as well, since at least some of its target mRNAs (PCBP2 and Map3k3) heavily rely on the m^7G-cap. In addition, our case raises the possibility that at least some of the reported effects of the eIF4G2 depletion may originate from changes in mRNA metabolism due to an altered pattern of mRNA-binding proteins rather than to a genuine eIF4G2 activity in translation initiation.

MATERIALS AND METHODS

Plasmids

HuR, PCBP1, PCBP2, and SERBP1 coding sequences were amplified with the appropriate primers using cDNA obtained from RKO cells. The primers' sequences are: HuRfw 5′-AAAAGATCTATGT CTAATGGTTATGAAGACCACAT-3′, HuRr 5′-AGACTCGAGTTT ATTTGTGGGACTTGTTGGTTTTGAA-3′, PCBP1fw 5′-TCGCCAT GGATGCCGGTGTGACTGAAA-3′, PCBP1r 5′-TTTCTCGAGTTT AGCTGCACCCCATGCCCTTCTCA-3′, PCBP2fw 5′-ACAGGATC CATGGACACCGGTGTGATTG-3′, PCBP2r 5′-CCGCTCGAGTC TAGCTGCTCCCCATGCCACCCGTCT-3′, SERBPfw 5′-ACAGGA TCCATGCCTGGGCACTTACAGGAA-3′, SERBPr 5′-TTACTCGA GTTTAAGCCAGAGCTGGGAATGCCT-3′.

The PCBP2 amplification resulted in two isoforms, namely, isoforms e and f (NP_001122384 and NP_001122385, respectively). All these sequences were cloned into the homemade pGEX-TEV plasmid, which is essentially pGEX-6p-1 (GE Healthcare) with a TEV cleavage site between the GST coding sequence and MCS. pET16b-hnRNPK (which encodes the human hnRNP K isoform a, NP_112553) was a gift from D. Ostareck (Ostareck et al. 1997). A plasmid for the PTBP1 expression was a gift from C.U.T. Hellen (Hellen et al. 1994).

A plasmid with the BQCV IRES was a gift from P. Lidsky (UCSF). The late adenovirus TPL (Smirnova et al. 2016), poliovirus IRES (Andreev et al. 2012), PTV IRES (Pisarev et al. 2004), and rabbit β-globin 5′-UTR (Smirnova et al. 2016) reporters have been described previously. 5′ termini of the cloned 5′-UTRs were recreated on the basis of the appropriate CAGE and EST data (Severin et al. 2014). The 5′-UTRs of the human CCNI (5′ end corresponds to chr4 77997142), eIF4G2 (chr11, 10830470), TIAL1 (chr10, 121356188), TNFα (chr6, 31543342), and TP53 (chr17, 7590805) mRNAs were amplified from RKO or 293T cells cDNA (positions are given for hg19 assembly). The 5′-UTRs of the murine PCBP2 (chr15, 102300970, mm9 assembly) and Map3k3 (chr11, 105945908) mRNAs were amplified using NIH/3T3 cells' cDNA. In the eIF4G2ΔCU deletion, mutant nucleotides corresponding to 199–276 of the annotated transcript (NM_001418.3) or 10808760–10808837 of the genomic sequence (hg19) were substituted for HindIII site. Primers used for the amplification of the 5′-UTRs are as follows: mMap3k3fw 5′-AAAACGCGTTAATACGACTCACTATAGGGAGCGTCTTCTGG ACTTCAGGA-3′, mMap3k3r 5′-AATGCCTCTTGTTCATCCATG GT-3′, mPCBP2fw AAAACGCGTTAATACGACTCACTATAGGG CCCAGACCAGCAGAGGCAGCA, mPCBP2r 5′-ATTTCCATGGC TAGCAGTTACAGGGAGCTGGACTTT-3′, CCNIfw 5′-TCTTGGT ACCTAATACGACTCACTATAGGGCTCCCTCCCCAGCCTTCCC CGCGA-3′, CCNIr 5′-AACGAAATCTTCGAGTGTGAAGACCCTA GTGATTGCCTTTTCCAACA-3′, TNFa5fw 5′-CTTACGCGTTAAT ACGACTCACTATAGGAGCAGACGCTCCCTCAGCAAGGACA-3′, TNFa5r 5′-TGTCCATGGTGTCCTTTCCAGGGGAGA-3′, TP53_ 5fw 5′-CTTACGCGTTAATACGACTCACTATAGGAAAAGTCTAG AGCCACCGTCCA-3′, TP53_5r 5′-CCTCCATGGCAGTGACCCG GAA-3′, DAP5_5fw 5′-ATCTTACGCGTTAATACGACTCACTATA GGCAGTGAGTCGGAGCTCTATGGAGGTG-3′, DAP5_5r 5′-TT CTTTATGTTTTTGGCGTCTTCCGCGGCACCCCCTTCTGCAATC GCA-3′, TIALfw 5′-CTTACGCGTTAATACGACTCACTATAGGCC ATTTTGTTCATCCTCCTCCT-3′, TIALr 5′-TGTCCATGGTGGGT GCGACGGAGCGAT-3′. The eIF4G2 and CCNI reporters included parts of the corresponding coding regions. The β-actin, TP53,

and TNFα reporters included the authentic 3′-UTRs, and the eIF4G2 reporter contained either authentic or SV40 3′-UTR, as indicated. The following primers were used for amplification of the 3′-UTRs: TNFa3fw 5′-ACATCTAGAGGAGGACGAACATCCAACCTT-3′, TNFa3r 5′-AACTTGTTTATTGCAGCTTATAATGGCTAAGCAAACTTTATTTCTCGCCA-3′, TP53_3fw 5′-AACAACTAGTCATTCTCCACTTCTTGTTCCCCACT-3′, TP53_3r 5′-GTTGTTAACTTGTTTATTGCAGCTTATAATGGCACCCCTCAGACACACAGGTGGCA-3′, DAP5_3fw 5′-ACATCTAGAACCAGCCAAAGCCTTAAATT-3′, DAP5_3r 5′-TATGTTAACTTGTTTATTGCAGCTTATAATGGTCACTACATCAAGTATCACAATGTTTATT-3′, actin_3fw 5′-AACATCTAGAGCGGACTATGACTTAGTTGCGTTA-3′, actin_3r 5′-GTTGTTAACTTGTTTATTGCAGCTTATAATGGTAAGGTGTGCACTTTTATTCAACTGGTCTCAA-3′. To create the TPL+ and TPL− reporters, the polypyrimidine tract corresponding to nucleotides 199–276 of the human eIF4G2 mRNA was amplified with the primers bearing HindIII sites and inserted in the HindIII site of TPL (position 203 of the 235-nt-long TPL), in either sense (TPL+) or antisense (TPL−) orientation. All the UTRs were cloned in pGL3 vector (Promega) upstream of firefly luciferase (FLuc) coding sequence, with the exception of CCNI, in which 5′-UTR was cloned into pNL1.1 vector (Promega) upstream of NanoLuc luciferase (NLuc) coding sequence.

Analysis of ENCODE data

Data sets ENCSR339FUY, which includes two replicates of the PCBP2 eCLIP data in HepG2 cells, and ENCSR115GAA (mock input control) were analyzed. The aligned reads (ENCFF803QKO.bam, ENCSR339FUY.bam, ENCFF190ITO.bam) were sorted using the GenomicAlignments package (Lawrence et al. 2013). The iCLIP data obtained from Huh7 cells were taken from the GEO data set GSE59840.

RNA pull-down assay

RNAs were synthesized using MEGAscript T7 Transcription Kit (Ambion) as suggested by the manufacturer with an addition of 0.75 mM biotin-16-UTP (Jena Bioscience). Note that noncapped RNAs were used for the RNA pull-down. The latter was performed as described previously (Andreev et al. 2012) with minor modifications. A total of 300 pmol of the biotinylated RNA was added to a mixture that contained 200 µL (~30 mg of total protein) of HeLa cytoplasmic extract (CilBiotech, Belgium), 80 U Ribolock RNase inhibitor (Thermo Fisher), complete EDTA-free protease inhibitor cocktail (Roche) in buffer "B" [100 mM KCl, 20 mM Tris-HCl, pH 7.5, 1 mM $Mg(OAc)_2$, 1 mM DTT, and 0.05% NP40], up to a total volume of 400 µL. The mixture was incubated in a 2-mL tube with gentle agitation for 30 min at 30°C. Meanwhile, Streptavidin Sepharose High Performance (GE Healthcare) suspension (50 µL per reaction) was washed four times with buffer "A" [100 mM, 20 mM Tris-HCl, pH 7.5, 5 mM $Mg(OAc)_2$, 1 mM DTT, and 0.05% NP40], resuspended in 1300 µL of buffer "A," and added to the lysate with RNA. After 2 h of gentle shaking on ice, the resin was washed extensively with buffer "B," then resuspended in 80 µL of buffer "B" supplemented with 2 mM $CaCl_2$ and 450 U of micrococcal nuclease (Thermo Fisher). RNA was digested for 30 min at 37°C. Eluted proteins were separated by SDS-PAGE and their identities were determined by LC-MS.

Protein expression in *E. coli*

PCBP2e, PCBP2f, HuR, SERBP1, and PCBP1 were expressed in Rosetta-gami 2 cells (Novagen) at 15°C overnight after the induction by 50 µM IPTG (10 µM for PCBP1) at $OD_{600} \approx 0.5$. hnRNP K was expressed in Rosetta2 (DE3) similarly with the only exception that the expression was induced by 1 mM IPTG. HuR, PCBP1, PCBP2e, PCBP2f, and SERBP1 were expressed as GST-fusions and eluted from Glutathione Sepharose 4B (GE Healthcare) with a homemade TEV protease. A pure hnRNP K was obtained as a 200–300 mM imidazole elution fraction from Ni Sepharose High Performance (GE Healthcare). All proteins were dialyzed against A100 buffer (20 mM Tris-HCl, pH 7.5, 100 mM KCl, 0.5 mM EDTA, 1 mM DTT, 10% glycerol), with the exception of hnRNP K, which was purified and stored in buffers containing 20% glycerol, in order to prevent the protein aggregation. PTBP1 was expressed and purified as previously described (Hellen et al. 1994).

siRNAs

The siRNAs were designed using in-house software based on common principles (Reynolds et al. 2004; Fakhr et al. 2016) to improve efficacy and potency, and to exclude off-target effects. siRNAs were assembled using automated DNA/RNA synthesizer by 2′-TBDMS/phosphoramidite chemistry, purified by ion-exchange HPLC (Noll et al. 2011), and verified by ESI-MS. Duplexes were annealed in 50 mM KOAc water solution by heating to 80°C for 5 min followed by slow cooling to room temperature. Cells were transfected in 60 mm or 35 mm plates with the corresponding duplexes using Lipofectamine RNAiMAX (Invitrogen) at a final concentration in the medium 10 nM, 48 h prior to replating cells into a 24-well plate at ~30%–40% confluency; the latter coincided with another round of siRNA transfection. Twenty-four hours later (i.e., 72 h after the first siRNA transfection), mRNAs were transfected, as described below. Sequences of the siRNAs are (capital letters stand for unmodified ribonucleotides and lowercase denote 2′-OMe protected ribonucleotides): #1fw cuGAGAGAAuuAucAcuuudTsdT, #1rev AAAGUGAuAAUUCUCUcAGdTsdT, #2fw ucAucGGAAAGAAAGGAGAdTsdT, #2r, UCUCCUUUCUUUCCGAUGAdTsdT, #3fw GcGccAAAAucAAuGAGAudTsdT, #3r AUCUcAUUGAUUUUGGCGCdTsdT for the PCBP2 knockdown, and #1fw cuccuuAAAcuAAcuGAAAdTsdT, #1rev UUUcAGUuAGUUuAAGGAGdTsdT, #2fw cAAucAAAuucGucAAGAudTsdT, #2rev AUCUUGACGAAUUUGAUUGdTsdT for the eIF4G2 knockdown in human cells, and #3fw GcAcAAAcuGAucAGuuudTsdT, #3rev AAACUGAUcAGUUUUGuGCdTsdT, #4fw AuAcuuGAGuuGuuGcAAudTsdT, #4rev AUUGcAAcAACUcAAGuAUdTsdT for the eIF4G2 knockdown in murine cells. The control siRNA duplex was reconstituted from 5′-cAGcuGuAuucAuAAuuuATsdT-3′ and 5′-uAAAUuAUGAAuAcAGCUGdTsdT-3′ oligonucleotides.

mRNA transcription, in vitro translation and transfection

Templates for in vitro transcription were prepared via PCR, which enabled the introduction of the 50-nt-long poly(A) tail to all mRNAs used throughout the study. PCR products were purified with Wizard SV Gel and PCR Clean-Up System (Promega).

mRNAs were synthesized using T7 RiboMAX Large Scale RNA Production System (Promega) in reactions that contained either 3′-O-Me-m^{7}GpppG (ARCA) or ApppG (both from NEB) cap analogs. RNAs were purified via 2M LiCl precipitation. Translation in Krebs-2 ascites carcinoma cells S30 extract was performed essentially as described previously (Dmitriev et al. 2009). Recombinant proteins were added to the translation reactions prior to mRNA without any preincubation. Transfection was performed in a 24-well plate as previously described (Andreev et al. 2013), with minor modifications that included lesser mRNA per well (100 ng instead of 200 ng) and use of 5 ng of m^{7}G-capped β-globin-Nluc mRNA (instead of Rluc) as a reference for reporters coding for Fluc, or m^{7}G-capped β-globin-Fluc mRNA for reporters coding for Nluc. Relative translation efficiency was calculated for each mRNA as a reporter to reference ratio. For the kinetic analyses of transfected reporters' expression, mRNAs were transfected in several wells in parallel, and then translation was stopped by addition of cycloheximide up to 100 µg/mL at the indicated time points. Luciferases' activities were measured using Dual-Luciferase Reporter, Luciferase, or Nano-Glo Dual-Luciferase Assay Systems (all Promega) where appropriate.

Knocking out eIF4G2 gene in NIH/3T3 cells

gRNAs were designed at http://crispr.mit.edu/ and inserted into a pX458 vector (Ran et al. 2013). NIH/3T3 cells were transfected with the resulting vector, sorted for GFP presence, and seeded into a 96-well plate. The monoclonal cells were analyzed by PCR sequencing and western blotting against eIF4G2. The sequencing showed that the deletion occurred at the border of exon 2 and intron 2, resulting in a disruption of the splice site (Fig. 4A). Translation of the corresponding mRNA leads to termination just at the beginning of intron 2, resulting in a short MESAIG peptide synthesis.

Antibodies

eIF4G2 (Bethyl laboratories, A302-239A), CBP80 (Bethyl laboratories, A301-793A), eIF3c (Santa Cruz Biotechnology, sc-74507), PCBP2 (Abnova, H00005094-M05), anti-GADPH (Proteintech Group Inc, 10494-1-AP; or ZG003, Invitrogen), β-actin (Abcam, ab8229), and anti-RPSA antibodies were raised in mouse against full-length His_6-tagged human RPSA protein expressed in *E. coli*. Western blots were exposed to Kodak X-ray film or visualized via a Bio-Rad ChemiDoc XRS+ Molecular Imager.

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