Stop codon readthrough contexts influence reporter expression differentially depending on the presence of an IRES [version 1; peer review: 1 not approved]

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Abstract
Background: Previously we reported the discovery of stop codon readthrough in AMD1 mRNA followed by ribosome stalling at the end of a conserved Open Reading Frame (AMD1 tail). To explain the severe suppression of reporters fused to AMD1 tail we proposed a mechanism invoking ribosome queueing. To test this hypothesis, we placed the reporter stop codon in the context of readthrough permissive sequences in a dual reporter vector with downstream reporter expression governed by EMCV IRES. In accordance with our hypothesis, we observed a striking disproportional reduction of upstream reporter activity in response to increased readthrough levels.

Methods: Here we employ dual luciferase assay and western blotting to explore the effects of AMD1 tail and control sequences on reporter expression in dual and monocistronic reporter vectors.

Results: With the dual reporter system, the disproportionate reduction of upstream reporter activity is not specific to AMD1 tail and occurs as long as the readthrough stop codon context is present at the end of the reporter’s ORF. The decreased reporter activity that appears to be induced by the readthrough sequence occurs only in reporters containing EMCV IRES. Monocistronic reporters with the same readthrough context sequence exhibit only a modest reduction in reporter activity. Furthermore, in monocistronic vectors, the disproportionate reduction of reporter levels greatly increased when AMD1 tail was translated as a result of readthrough. Such readthrough-mediated reduction was not observed when AMD1 tail was substituted with unrelated sequences in agreement with our original hypothesis.

Conclusions: While our findings provide little new information regarding the functional role of AMD1 tail, they raise caution for the use of viral IRES elements in expression vectors for studying mechanisms of mRNA translation. These findings may also be

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Reviewer Status ×

Invited Reviewers

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version 1
22 Sep 2020

report

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pertinent to the natural properties of readthrough permissive sequences and of IRES elements, though these require a separate investigation.

**Keywords**
Translation control, AMD1, stop codon readthrough, IRES, OPRL1, ribosome stalling
**Introduction**

Recently we discovered that a proportion of ribosomes translating the *AMD1* mRNA read through its annotated stop codon and continue translating before stalling at the end of a 125-codons-long conserved open reading frame (ORF), referred to as *AMD1* tail (Yordanova et al., 2018). Ribosome stalling at the end of *AMD1* tail and its dependence on stop codon readthrough (RT) has been also confirmed in a more recent study (Wangen & Green, 2020). During our initial investigation of this phenomenon we found that fusing the product of *AMD1* translation, i.e. *AMD1* extension, to the C-terminus of reporters leads to nearly complete disappearance of reporter activity (Yordanova et al., 2018). After ruling out extracellular targeting or a protein destabilisation effect of the *AMD1* extension, we proposed a mechanism where ribosome stalling/queuing at the end of *AMD1* tail results in inhibition of the upstream main ORF translation. A prediction of this mechanism is that increasing the readthrough efficiency at the main ORF stop codon should accelerate the *AMD1* tail inhibitory effect by enhancing queue formation.

To test this prediction, we employed RT promoting sequences of varying efficiency from *LDHB*, *AQP4* and *OPRL1* genes (Loughran et al., 2014) to titrate ribosomes translating the *AMD1* tail (Yordanova et al., 2018). By increasing the RT efficiency at a reporter's stop codon to 2.5, 6 and 17% with *LDHB*, *AQP4* and *OPRL1* contexts, respectively, we saw a disproportionately large drop of reporter levels, i.e. beyond what would be expected due to protein degradation if *AMD1* extension had a destabilization effect as proposed for other products of 3'UTRs translation (Arribere et al., 2016). These experiments were performed with a bicistronic vector wherein the termination codon of the Renilla luciferase (Rluc) reporter was placed in varying RT promoting contexts upstream of *AMD1* tail. A firefly luciferase (Fluc) reporter was expressed via an EMCV IRES (Chamond et al., 2014) to monitor RNA levels.

We saw the potential of using this experimental approach of readthrough enabled ribosome titration to further explore the dynamics of *AMD1* tail translation and that of other test sequences.

These subsequent experiments revealed unexpected effects of readthrough contexts on reporter expression observed in an EMCV IRES containing vector but not in a vector without the EMCV IRES which we describe here.

**Methods**

**Cloning**

Oligonucleotides were synthesized by IDT, Belgium. *AMD1* tail, *ODC1* PEST and *ACTB* sequences were obtained as gBlocks from IDT. gBlock and primer sequences including those that introduce *OPRL1*, *AQP4* and *LDHB* stop codon context sequences are provided in Extended data File 1 (Yordanova et al., 2020b). The amplicons were generated by standard one or multiple PCR reactions using Phusion High Fidelity DNA Polymerase (NEB) according to the manufacturer instructions. p2Luc (Grentzmann et al., 1998) was modified such that the second luciferase reporter (Fluc) is expressed under the control of the EMCV IRES. Due to the presence of an XbaI restriction site in *AMD1* tail, the first 65 nts of *AMD1* tail were omitted for cloning in the monocistronic vector. All constructs were transformed by 90 sec heat shock at 42°C in *E. coli* strain DH5-α and were verified by Sanger sequencing at Eurofins Genomics.

**Tissue culture and cell treatment**

Human Embryonic Kidney 293A cells (ATCC) were maintained as monolayer cultures, grown in DMEM (Sigma-Aldrich) supplemented with 10% FBS, 1mM L-glutamine and 1% penicillin/streptomycin at 37°C in an atmosphere of 5% CO₂. For the dual luciferase assay 4.5×10⁶ HEK293A cells were plated on 10 cm tissue culture dishes. After 24 h the cells were detached with trypsin, suspended in fresh media and transfected in four replicates with Lipofectamine 2000 reagent (Invitrogen), using the 1-day protocol in which suspended cells are added directly to the DNA complexes in 96-well plates. For each transfection, the following was added to each well: 25 ng plasmid DNA and 0.2 μl lipofectamine 2000 in 25 μl OptiMem (Gibco), 2×10³ cells in 50 μl DMEM, were added to the transfecting DNA complexes in each well. Transfected cells were incubated at 37°C in 5% CO₂ for 21 h and assayed using the dual luciferase assay. Data shown on the figures was obtained from three independent transfections each with four technical replicates.

**Dual luciferase assay**

Fluc and Rluc assay buffers were prepared as described in (Dyer et al., 2000). Relative light units were measured on a Veritas Microplate Luminometer fitted with two injectors (Turner Biosystems). Cells transfected in 96 well plate were washed once with 1x PBS and then lysed in 15 μl of 1x passive lysis buffer (PLB; Promega). Light emission was measured following injection of 50 μl of each luciferase substrate buffer. Raw data for the dual luciferase assays are available as Underlying data (Yordanova et al., 2020a).

**Protein isolation and western blot analysis**

Transfections for Western blotting analysis of constructs for Figure 1 were performed in 6 well plates scaled-up from the method described for 96 well plate transfections above. The following was added to each well: 1 μg plasmid DNA, 7 μl lipofectamine 2000 in 1 ml OptiMem. A total of 1×10⁵ cells in 3 ml DMEM, were added to the transfecting DNA complexes in each well. Transfected cells were incubated at 37°C in 5% CO₂ for 36 h for Western blotting. Cells were washed with 1x PBS and lysed in 1x PLB (Passive Lysis Buffer, Promega). Luciferase activities in the lysates were measured with the dual luciferase assay. Proteins were separated by 4–12% polyacrylamide gel electrophoresis on pre-made BoltTM 4–12% BisTris Plus gels (Thermo Fisher), transferred onto nitrocellulose membranes (Protran) and incubated with primary Rabbit Anti-Renilla Luciferase Polyclonal Antibody (1000x dilution) (MBL International) (RRID: AB_1520866) in 5% fat-free milk in PBST (1% Tween-20) overnight at 4°C. Incubation with IRDye® 800CW Goat anti-Rabbit IgG Secondary Antibody (10,000x dilution) (Abcam, ab216772) was for 0.5 h at room temperature.
Figure 1. OPRL1 readthrough context mediated inhibition of Rluc levels in EMCV IRES vector. (A) Schematic of the AMD1, ACTB and ODC1 PEST constructs where Rluc stop codon context is varied and Fluc expression is governed by the EMCV IRES. (B) Normalized (Rluc/Fluc) activities. (C) Absolute Rluc values. (D) Absolute Fluc values. (E) Upper panel, Anti-Rluc immunoblots of protein lysates from HEK293A cells transfected with the indicated constructs; termination products are indicated with a black arrowhead, readthrough products (seen only in ACTB constructs) are indicated with a red arrowhead; lower panel, normalised (Rluc/Fluc) activities from the protein lysates. See Methods for box plot elements.
Statistical analysis
Box plots were generated with a web tool BoxPlotR. Box plots elements: center lines show the medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles, outliers are represented by dots. n = 12 sample points. 2-tailed, paired samples t-test was performed in excel (version 2007) on samples as indicated.

Results and discussion
Inhibitory effect of OPRL1 stop codon context in EMCV IRES vector
To further explore AMD1 tail translation in the bicistronic RT constructs we designed and tested two unrelated sequences using the dual luciferase reporter system from our previous work (Yordanova et al., 2018) (Figure 1A). The AMD1 tail was replaced either with a fragment of ACTB coding sequence of equivalent length (381 nt) or with that of mouse ornithine decarboxylase 1 (ODC1) C-terminal PEST encoding region (492 nt). ACTB was selected to represent a neutral sequence optimized for efficient translation that is not expected to affect the reporter levels. ODC1 PEST codes for a degradation signal (Loetscher et al., 1991) and was selected to control for the effects that a degron could have on the reporter when placed downstream of an RT context. In agreement with our previous work, high-level readthrough of AMD1 tail resulted in a significant drop in reporter levels in a construct where AMD1 tail is placed downstream of the Rluc stop codon in the OPRL1 RT context (Figure 1B, compare UGA with OPRL1). We have attributed this effect to the inhibition of translation by ribosome stalling in AMD1 tail (Yordanova et al., 2018). However, when we replaced AMD1 tail with the neutral ACTB (no decrease in reporter expected) or the ODC1 PEST (<20% decrease expected) sequences, they exhibited similar inhibitory behaviour (Figure 1B, compare UGA and OPRL1).

One possible explanation for the observed inhibition of reporter levels is that it results from the addition of OPRL1 context sequence at the end of the reporter’s ORF. Both 5’ and 3’ nucleotides of OPRL1 context contribute to RT efficiency. To explore the role of the 5’ OPRL1 context, we tested constructs wherein ribosome access to the sequence beyond the stop codon is prevented. For this we substituted UGA in the OPRL1 context with two UAA codons (Figure 1B, OPRL1 UAA). In addition, we tested constructs wherein OPRL1 5’ signal was truncated to only its two last codons instead of six (Figure 1B, trOPRL1). We have recently determined that just two codons 5’ of the OPRL1 stop signal are sufficient for maximal readthrough (Loughran, personal communication).

The termination products of OPRL1 and OPRL1 UAA constructs have the exact same amino acid composition, with both having the six OPRL1 derived amino acids at the C-terminus. For the OPRL1 UAA construct the reporter levels are indicative of the availability/activity of the termination product only, while for the UGA construct both the readthrough product and the termination product contribute to the reporter levels. OPRL1 UAA constructs exhibit very similar reporter levels as the OPRL1 constructs (Figure 1B) suggesting that the observed reduction was due to the occurrence of the six OPRL1 codons just upstream of the stop codon and did not depend on downstream translation. Nonetheless, it should be noted that there is a small difference in the reporter levels between OPRL1 and OPRL1 UAA which is most significant for AMD1 (p=10^-15, t-test), less significant for ODC1 PEST (p=10^-6, t-test), and even less significant for ACTB (p=10^-4, t-test). This could be either due to reduced stability of the readthrough proteins in which case any AMD1 tail destabilisation effect must exceed that of ODC1 PEST degron or else it could be due to AMD1 tail translation having an inhibitory effect on the reporter’s translation.

Shortening the 5’sequence of OPRL1 context by deleting four of the six codons largely recovered reporter levels with all three test sequences (Figure 1B, trOPRL1) supporting the idea that these four codons contribute to the observed effect. Like with the full 5’ RT context in OPRL1, the truncated form in trOPRL1 constructs exhibited similar reporter levels with all three test sequences. These findings would appear to argue against an AMD1 tail specific inhibitory effect in the RT constructs that we reported in our original work (Yordanova et al., 2018).

To investigate if OPRL1 5’ context was interfering with reporter’s activity or whether it affected the protein levels, we performed western blotting which showed that the amount of detectable reporter protein was significantly reduced in the presence of the OPRL1 context for all three test sequences (Figure 1E, compare UGA and trOPRL1 vs OPRL1 and OPRL1 UAA). This indicates that in these reporters the OPRL1 5’ context does not simply interfere with Rluc activity. As expected, RT product was detected only with ACTB constructs.

The reduction of Rluc levels observed in the presence of OPRL1 context could be due to a protein destabilising effect of the OPRL1 derived peptide at the reporter’s C-terminus. However, western blotting revealed that the amount of detectable reporter protein was significantly reduced in both the termination and the RT product (as seen for ACTB constructs, Figure 1E), which argues against a C-terminal degron activity. In addition, it has been shown recently that the Venezuelan equine encephalitis virus (VEEV) RT stop codon context promotes ribosome stalling and it has been proposed that such stalling could be a general feature of RT promoting sequences (Lashkevich et al., 2020). If so, the observed reduction of reporters containing OPRL1 context could be attributed to slow peptide release and/or reporter mRNA degradation upon activation of ribosome quality control (RQC) pathways (Ikeuchi et al., 2018; Joazeiro, 2019)

Variation of EMCV IRES governed Fluc expression levels
While Rluc activities normalized over Fluc values report a very similar picture for all three sequences tested (Figure 1B), this is not the case for the absolute values of these reporters. Addition of the last six codons of OPRL1 to UAA reporters greatly reduced Rluc levels for ACTB and ODC1 PEST but not for AMD1 (Figure 1C, OPRL1 UAA). The most likely explanation for this is that any reduction in Rluc for the AMD1 reporters was masked by the increased stability of its corresponding mRNA as can be judged from its Fluc activity.
(Figure 1D). With the exception of AMD1 extensions, stop codon contexts that supported efficient termination (as in UGA and OPRL1 UAA) had three-fold lower Fluc levels as compared to those promoting readthrough (OPRL1 and trOPRL1) which might be expected due to mRNA decay pathways such as Nonsense Mediated Decay (NMD) or No Go Decay (NGD) (Shoemaker & Green, 2012) sensing reduced translation. In case of AMD1, enabling readthrough with OPRL1 context did not lead to further stabilization of mRNA (Figure 1D, OPRL1 vs OPRL1 UAA). The relatively high Fluc expression levels observed when AMD1 tail is placed specifically downstream of OPRL1 UAA context is intriguing and will need to be investigated further as it may shed light onto the properties of AMD1 tail and its function in the regulation of AMD1 expression.

Upstream reporter reduction depends on the RT promoting context and its efficiency

To determine whether these observations are specific to the OPRL1 stop codon context we next tested the other two RT promoting contexts from our previous study (Yordanova et al., 2018), AQP4 and LDHB. AMD1, ACTB and ODC1 PEST constructs where the Rluc stop codon was substituted with RT contexts from LDHB, AQP4 and OPRL1 of 2.5, 6.5 and 17% RT efficiency respectively (Loughran et al., 2017) and the corresponding controls where the RT UGA codon was substituted with two UAA codons were tested alongside (Figure 2A). Like in our original AMD1 study, increasing RT efficiency with LDHB, AQP4 and OPRL1 resulted in reduction of reporter levels (Figure 2B). The drop in reporter levels was disproportionately larger than what is expected to result from degradation of the RT product. However, as in the case of OPRL1 RT context, constructs with LDHB and AQP4 contexts exhibited the same trend when AMD1 tail was substituted with ACTB and ODC1 PEST (Figure 2C). With all three RT promoting contexts, a certain degree of recovery of reporter levels was observed upon substitution of UGA with UAAUAA. These results suggest that the observed reporter reduction depends on both, the RT context upstream of the stop codon as well as the readthrough efficiency but not on the extension sequence downstream of the stop codon.

Uncoupling of OPRL1 RT context and AMD1 tail translation effects on reporter levels in a monocistronic vector

The results described so far argue that the changes in Rluc reporter levels align with RT efficiency independently of translation downstream of the test sequence. These findings...
were unexpected because earlier studies with *OPRL1* RT context did not provide evidence for such effects on reporter levels which were found to be not substantially different in the presence or absence of *OPRL1* context (Loughran et al., 2017). The main difference in the experimental approach in the previous study compared to our current analysis is the absence of the EMCV IRES in the constructs used previously.

Therefore, to clarify these contradicting observations, we next tested the sequences shown in Figure 1A in a monocistronic vector that encodes Rluc and has no EMCV IRES (Figure 3A). Cells were transfected with these constructs along with a separate Fluc expressing vector to control for transfection efficiency. Because Fluc is expressed from a separate vector in this setup we do not account for RNA stability levels.

In the absence of EMCV IRES, the *OPRL1* UAA context resulted in a similar drop in reporter levels with *AMD1*, *ACTB* and *ODC1* PEST (Figure 3B and 3C, *OPRL1* UAA). This is consistent with the *OPRL1* context destabilising effect as revealed in the EMCV IRES vector (Figure 1); however, the similarities between constructs with and without EMCV IRES end there. With *OPRL1* constructs that support efficient readthrough, *ACTB* had reporter levels only slightly reduced consistent with it being a neutral sequence. The small but significant drop might be due to reduced Rluc activity in the fusion product. The more significant drop with *ODC1* PEST is consistent with its degron properties. *AMD1* exhibited the greatest reduction of more than 10-fold. Though slightly higher than those of *OPRL1*, the reporter levels generated with the truncated context in *trOPRL1* showed the same trend, i.e. *ACTB* had almost no reduction, small reduction with PEST and the largest reduction of about 5-fold with *AMD1*. The different effects that *AMD1*, *ACTB* and *ODC1* PEST had on reporter levels in the constructs with readthrough, *OPRL1* and *trOPRL1*, suggest that these effects are related to the nature of the translated sequence downstream of the stop codon in the RT context. Critically, for *AMD1* the reduction of Rluc activity in *ORRL1* RT in comparison with *OPRL1* UAA construct greatly exceed what would be expected if this was due to degradation of the readthrough product only, supporting our earlier claim (Yordanova et al., 2018).

**Figure 3.** Effect of *OPRL1* readthrough context on Rluc levels in a vector without an IRES. (A) Schematic of the *AMD1*, *ACTB* and *ODC1* PEST constructs where Rluc stop codon context is varied. Fluc is expressed by a separate vector. (B) Normalized (Rluc/Fluc) luciferase activities. The leftmost blue box represents reporter levels with the empty Rluc expressing vector. (C) Normalized (Rluc/Fluc) activities were calculated for the constructs from (B) as percentages of the corresponding UGA construct. See Methods for box plot elements.
Reporter expression levels from the empty Rluc vector were in the range of those from OPR1 and OPRL1 UAA constructs for ACTB and ODC1 PEST. This is consistent with the previous study, which showed no change in reporter expression levels in the presence of OPRL1 context (Loughran et al., 2017) (Figure 3B).

Conclusions
In our investigation of ribosome stalling following stop codon readthrough in the human AMD1 gene, we proposed a ribosome queuing model to explain downregulation of reporter genes fused with AMD1 tail (Yordanova et al., 2018). To test the model, we varied readthrough context at the AMD1 stop codon and observed disproportionately high inhibition of upstream reporters in response to increased readthrough efficiency as predicted by the model. Here we report that the observed reduction of upstream reporter levels is due to the RT context rather than due to AMD1 tail translation, contrary to our initial interpretations of the experiments presented in Figure 3C of the original publication. We also found that this inhibition is observed only in the reporter vector where the downstream reporter is under the control of an EMCV IRES.

This result helped us to uncouple the inhibitory effects of RT contexts and AMD1 tail translation on reporter’s expression. In a vector not using EMCV IRES initiation, reporter expression is reduced further when AMD1 tail is translated (due to readthrough). This reduction is not observed when AMD1 tail is replaced with unrelated sequences supporting our original claim that translation of AMD1 tail has an inhibitory effect on expression of upstream ORFs.

While the nature of molecular mechanisms responsible for the reported effects remains to be elucidated, our work extends the list of unexpected properties of IRES elements (Payne et al., 2013; Shikama et al., 2010) and thus reinforces the need for caution in interpretation of data obtained with IRES containing reporters.

Data availability
Underlying data
Figshare: Stop codon readthrough contexts influence reporter expression differentially depending on the presence of an IRES. https://doi.org/10.6084/m9.figshare.1283762 (Yordanova et al., 2020b).

This project contains the following underlying data:
- dataFigure_1.csc. (Raw data dual luciferase assay for Figure 1.)
- dataFigure_2. (Raw data dual luciferase assay for Figure 2.)
- dataFigure_3. (Raw data dual luciferase assay for Figure 3.)
- western_700. (Original unannotated western blot image.)

Extended data
Figshare: Stop codon readthrough contexts influence reporter expression differentially depending on the presence of an IRES. https://doi.org/10.6084/m9.figshare.1283762 (Yordanova et al., 2020b).

This project contains the following extended data:
- Extended_Data_File_1.csv. (List of test sequences and primers.)

Data are available under the terms of the Creative Commons Attribution 4.0 license (CC BY 4.0).

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Open Peer Review

Current Peer Review Status: ✗

Version 1

Reviewer Report 05 October 2020

https://doi.org/10.21956/wellcomeopenres.17828.r40566

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Yordanova and colleagues report follow-up studies of their Nature paper in which they found that translational readthrough at the AMD1 stop codon, leading to translation of a C-terminal extension (AMD1 tail), inhibited translation of the coding region. The authors had proposed that the inhibitory mechanism was due to ribosome stalling at the downstream stop codon and queuing of ribosomes on the mRNA. A more recent paper using ribosome profiling from another group confirmed that ribosomes translate the AMD1 tail, but found no evidence to support ribosome queuing. To evaluate their model further, these authors now test the prediction that increasing readthrough past the stop codon should increase the queuing and potentiate the inhibitory effect of the AMD1 tail. Using dicistronic constructs, they found that increasing the readthrough at the end Rluc results in lower Rluc/Fluc expression, but the effect does not depend on the AMD1 tail nor does it even require that readthrough onto the tail. They then tested another set of stop codon elements that allow readthrough and found that a higher level of readthrough efficiency resulted in more inhibition, but again, the effect was not dependent on the AMD1 tail. However, when they tested these stop codon contexts and tail sequence in monocistronic constructs, they then found that the AMD1 tail may be important for inhibition.

The data are interesting but unconvincing in support of any clear conclusions. The results do suggest that the impact of the dicistronic constructs does not depend on the AMD1 tail, and there are modest differences in the impacts of the AMD1 tail vs the other tails in the monocistronic constructs, but the other tails have intermediate effects. There are multiple uncertain assumptions and other findings that are not well explained.

Specific comments:

- Abstract: “Monocistronic reporters with the same readthrough context sequence exhibit only a modest reduction” is confusing since there is a strong inhibitory effect with the AMD1 tail in the OPRL1 monocistronic construct and the other two do inhibit moderately.

- The authors report as “personal communication” that the truncated ORFL1 stop codon is sufficient for maximal readthrough, but this finding is quite important for interpreting many of the results and so the data should be presented.
The authors state that the LDHB, AQP4, and OPRL1 RT sequences cause 2.5%, 6%, and 17% readthrough, but the paper cited (Loughran et al 2014) reports the latter OPRL1 cause 31% readthrough. A later paper revised that number to 17%. Might these numbers vary depending on the precise construct and context of the experiment? If so, it could impact the interpretation of some of the data (e.g. in Figure 2).

Page 5. The argument “against a C-terminal degron activity” mediated by the ORFL1-derived peptide is not convincing. The fact that both the termination and RT products are lower in the OPRL1 and ORPL1UAA constructs does not rule out the possibility that the ORFL1 peptide (more so than the truncated version) is a degron that acts to degrade the short and longer proteins.

In Fig. 1D, the variation in Fluc expression is hard to explain by any simple model. Among other confusing results, how do the authors explain the differing effects on Fluc expression of trOPRL1 in the AMD1 construct vs. in the ACTB and ODC1 PEST constructs? It may be true that the EMCV IRES is unreliable as they propose, but it is not clear it is really the problem nor how it might be misleading. There could be effects on IRES structure and function or on mRNA degradation of the dicistronic RNAs that results from upstream ORF, but they don’t provide direct evidence for any model. The authors generally interpret changes in the Fluc levels as being measures of mRNA, but that may not be incorrect, even for the monocistronic constructs. Some additional data, such as measuring RNA levels might help evaluate these alternatives. Alternatively, other methods might be needed.

Interpretation of the UAA constructs depends on the UAAUAA always being a strong terminator, which seems likely, but is unproven. The UAAUAA does reduce expression of the larger proteins in Fig. 1E but what are the other bands in the immunoblot that appear larger than the main protein? Are they background artifacts or other readthrough products?

How many independent replicates of the transfections were done? Presumably at least several, but given the vagaries of transfection assays, coupled with the possibility that the Fluc may not be a good internal control, it is important to know that the data presented are highly reproducible.
Yes

**Are the conclusions drawn adequately supported by the results?**
Partly

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** I have been studying protein synthesis regulatory mechanism for >30 years. I discovered and characterized the mechanism of one of the early examples of ribosomal stalling in eukaryotes and have remain interested in this area. I think I am quite well qualified to evaluate this report.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.