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Current Biology

Regulation of the elongation phase of protein synthesis enhances translation accuracy and modulates lifespan.

--Manuscript Draft--

Manuscript Number:	CURRENT-BIOLOGY-D-18-00722R1
Full Title:	Regulation of the elongation phase of protein synthesis enhances translation accuracy and modulates lifespan.
Article Type:	Research Article
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Abstract:	<p>Maintaining accuracy during protein synthesis is crucial to avoid producing misfolded and/or non-functional proteins. The target of rapamycin complex 1 (TORC1) pathway and the activity of the protein synthesis machinery are known to negatively regulate lifespan in many organisms, although the precise mechanisms involved remain unclear. Mammalian TORC1 signaling accelerates the elongation stage of protein synthesis by inactivating eukaryotic elongation factor 2 kinase (eEF2K), which, when active, phosphorylates and inhibits eEF2, which mediates the movement of ribosomes along mRNAs, thereby slowing down the rate of elongation.</p> <p>We show that eEF2K enhances the accuracy of protein synthesis under a range of conditions and in several cell-types. For example, our data reveal it links mTORC1 signalling to the accuracy of translation. Activation of eEF2K decreases misreading or termination read-through errors during elongation, while knocking down or knocking out eEF2K increases their frequency. eEF2K also promotes the correct recognition of start codons in mRNAs.</p> <p>Reduced translational fidelity is known to correlate with shorter lifespan. Consistent with this, deletion of eEF2K ortholog or other factors implicated in translation fidelity in <i>Caenorhabditis elegans</i> decreases lifespan, and eEF2K is required for lifespan extension induced by nutrient-restriction. Our data uncover a novel mechanism linking nutrient supply, TORC1 signaling and the elongation stage of protein synthesis which enhances the accuracy of protein synthesis. Our data also indicate that modulating translation elongation and its fidelity affects lifespan.</p>

Dear Editor:

We thank you for your letter and the reviewers for their helpful comments on our paper entitled ‘Regulation of the elongation phase of protein synthesis enhances translation accuracy and modulates lifespan.’ (Manuscript No. CURRENT-BIOLOGY-D-18-00722).

We have now fully addressed the reviewers’ questions and wish to submit our revised paper for consideration by Current Biology.

We have conducted several additional experiments, some of which were performed using an additional model organism, *Drosophila melanogaster*. Data from these experiments are included in Figures 6C, S10, S18 and S19.

The additional work included lifespan studies in *Drosophila*, which required considerable time and efforts from our co-authors, especially Dr. Louise O’Keefe. We have added Dr O’Keefe as a co-author as she performed the *Drosophila* lifespan study (Figure S18).

All changes to the main text are highlighted in red. Our responses to the reviewers’ comments are provided in a separate ‘rebuttal letter’ where they are shown in bold/underlined text.

With kind regards,

Christopher G. Proud and colleagues

Dear Editor:

We thank you for your letter and the reviewers for their helpful comments on our paper entitled ‘Regulation of the elongation phase of protein synthesis enhances translation accuracy and modulates lifespan.’ (Manuscript No. CURRENT-BIOLOGY-D-18-00722).

We have now fully addressed the reviewers’ questions and wish to submit our revised paper for consideration by Current Biology.

We have conducted some additional experiments, some of which were performed using an additional model organism, *Drosophila melanogaster*. Data from these extra experiments are included in Figure S18. We also made a few minor corrections or changes to ensure clarity of the text. All changes to the main text are highlighted in red. Our responses to the reviewers’ comments are provided below in bold/underlined text.

With kind regards,

Christopher G. Proud and colleagues

Reviewer #1: In this manuscript, Xie et al investigated the correlation between translational quality and quantity primarily using a luciferase-based reporter system. The authors confirmed the previous finding that an increased elongation speed decreases the decoding accuracy. This concept potentially explains the beneficial effect of reduced nutrient signal pathways like mTORC1. The authors then provided evidence that this effect was mainly through eEF2K, a kinase negatively regulates the elongation factor eEF2 and the subsequent translocation. To validate the physiological significance of translation fidelity in longevity, the authors examined the lifespan of *C. elegans* after deleting tRNA synthetases or eEF2K.

Packed with comprehensive data sets, the entire manuscript is well-written with clear data presentation. While some results are quite convincing, others are less conclusive. For instance, it is unclear how reduced elongation speed modulates start codon selection. In vivo studies using *C. elegans* fall short of direct comparison with other genetic mutants. It seems to be premature to claim that the longevity effects of dietary restriction is solely due to eEF2K-controlled elongation. Since nutrient signaling mainly affects initiation, reduced translational output per se could influence the entire protein homeostasis. A careful consideration of alternative interpretation is needed to avoid overstatement. At least, the authors could tone down a bit about the main conclusion.

R: We thank this reviewer for his/her very helpful comments. We have therefore now added the following statement on page 12, third paragraph: “aaRSs [71] and eEF2K [72] (efk-1 in nematodes) are proteins that are required for or regulate, respectively, mRNA translation. While each has a specific function, they may exert pleiotropic effects, given the crucial importance of protein synthesis for cell physiology. Therefore, the decreased lifespan we observe in nematodes where *efk-1* or aaRSs were knocked down may be due to these effects.”

Earlier in the first paragraph on the same page (12), we also changed our statement to “These data indicate that eEF2K is required for the lifespan extension induced by nutrient restriction.”

Also, in the new Figure 6C, we observed a further reduction in the lifespan of *efk-1* worms when the methionyl-tRNA synthetase *mrs-1* was knocked down (but not for other aaRSs tested, i.e., *lrs-1*, *kars-1* and *nrs-1*). This indicates that the latter three affect lifespan in a manner linked to control of elongation, the only stage of translation where they are required, while this is not so for *mrs-1*, an enzyme which, unlike the others, is also essential for translation initiation. Similarly, we also observed a reduction in lifespan in *Drosophila* in which leucyl-tRNA synthetase had been knocked down during adulthood (Supplementary Figure S18). *Drosophila* is a widely-used and tractable model for studying lifespan, but lacks an ortholog of eEF2K, so we are unable to test its role in that organism. Nevertheless, the data are consistent with the notion that inaccuracies in translation elongation (e.g., due to insufficient levels of certain amino acyl-tRNAs) diminish lifespan. Taken together, these findings indicate that *efk-1* modulates lifespan by modulating fidelity during translation elongation.

Specific concerns:

1) Figure 1, for constructs with different codon optimality, the increased R218S and STOP Fluc for maxCFluc could be due to the much higher basal level of maxCFluc. If the Fluc level in Figure 1C is normalized by the one in Figure 1B, then no increase could be observed. Of course, it is technically challenging to set up a perfect system in this kind of experiment.

R: As we stated on page 5, second paragraph, “data (on Figure 1C) are always compared to the differing yields of active luciferase for the corresponding parent Min, Sta or Max vectors.” In other words, the Fluc levels in Figure 1C are already normalized to their corresponding WT controls (Min, Sta or MaxCFluc WT) in Figure 1B; we thus observed the highest ‘real’ rate of translation errors in MaxCFluc whereas it was the lowest in MinCFluc, supporting our model that faster translation elongation is associated with higher rates of translation errors.

Additionally, we have now measured the levels of the Rluc/Fluc mRNA under different conditions in A549 cells and MEFs (new Supplementary Figure S10A-D). mRNA levels do not differ between any of the constructs or conditions used, showing that the differences in Fluc activity we observed are not due to differences in mRNA levels (which might have arisen due to different efficiencies of transfection or transcription). Please see our responses to reviewer 2, questions 1 and 2 for further details.

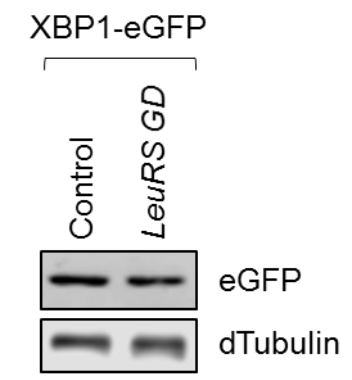
2) Figure 5, it's quite confusing how fast elongation (i.e., knocking down eEF2k) promotes non-AUG start codon selection. In the discussion part (page 11), the authors mentioned that impeded scanning by downstream ribosomes facilitates the selection of near-cognate start codons. If this is the case, how could the results in Figure 5 be reconciled? It seems to be opposite as expected.

R: We thank the reviewer for his/her comment and apology for our oversight. We have now modified the text (highlighted in red) on page 8 and 12 accordingly to discuss the data on Figure 5 and clarify our claims.

3) Figure 6 is rather descriptive without considering alternative interpretation. To support the authors' conclusion, it might be informative to show the relative quality of total proteins in these mutant *C. elegans*. Since both tRNA synthetase and eEF2K are general translation factors, their deficiency could have pleiotropic effect. The authors could try to measure unfolded protein response using fluorescence-based reporters.

R: To address the reviewer's helpful comment, we have now performed an additional experiment to monitor the levels of P-eIF2 α (a marker of ER stress/the integrated stress response, ISR) in WT and *efk-1* worms maintained under normal feeding (-/+ rapamycin) or under caloric restriction (new Supplementary Figure 18). We observed a similar levels of eIF2 α phosphorylation in *efk-1* and wildtype worms, which concurs with our observations in A549 cells where eEF2K knockdown by inducible shRNA did not induce phosphorylation of eIF2 α (Supplementary Figure 13). We therefore conclude that activation of the ISR is unlikely to be responsible for (or result in) the reduced lifespan of the (*efk-1*) worms. These new data are now discussed on page 10.

As mentioned before, we also observed a reduction in lifespan in *Drosophila* with ubiquitous knockdown of leucyl-tRNA synthetase during adulthood (Act>Tub-GAL80^{ts}>LeuRS GD). We also generated flies where LeuRS was knocked down specifically in the adult eye and included in those experiments a construct encoding an XBP1-eGFP fusion (XBP1 is a marker for another, independent, ISR pathway) to assess any effects of LeuRS knockdown on ER stress levels (Ryoo et al., 2007 EMBO J. 26(1): 242-252 Unfolded protein response in a *Drosophila* model for retinal degeneration). We observed no difference in eGFP levels by western analysis of adult heads (20 per sample) between control flies (GMR>XBP1-eGFP>+) and those with LeuRS GD knockdown (GMR>XBP1-eGFP>LeuRS GD). Thus we find no evidence of increased ER stress *in vivo* following knockdown of LeuRS.



Reviewer #2: Overall this a very interesting paper showing how translation fidelity is governed by TORC1 and how it may interact with lifespan in nematodes. The paper presents significant advance in the field and would ultimately be suitable for CB. I do believe though that the authors have to substantiate their main claims through the following:

1. The authors used diverse genetic and physiological manipulations to reveal effects of translation fidelity using the reporter gene. Increased luciferase activity is attributed to enhanced error rate. I was missing throughout an assessment of potential effects of their manipulation on mRNA stability. For example, in the constructs with fast codon it is possible that enhanced luminescence indeed results from enhanced error rate, but it is also possible that at least in part it results from high stability of the luciferase mRNA compared to that in the low efficiency codons' construct. I think the authors must include mRNA stability assays and ensure that their effects are not a result of differences in mRNA degradation rate and/or mRNA expression level.

R: We agree this is an important parameter and have now performed additional experiments to measure *Fluc* and *Rluc* mRNA levels in empty pICtest2 vector (contains *Rluc*) or Max/Sta/MinCFluc (WT, R218S or STOP)-transfected; untreated or IPTG-treated (to induce shRNA eEF2K) A549 cells, as well as in eEF2K^{+/+} and eEF2K^{-/-} MEFs. As shown in new

Supplementary Figure S10A-D, there were no changes in *Fluc* or *Rluc* mRNA levels upon shRNA-induced eEF2K knockdown in A549 cells, or between WT and eEF2K KO MEFs. Therefore, we conclude that the differences in Fluc activity that we observe do not reflect altered mRNA levels (which could have arisen due to a difference in transfection or transcription efficiency). This is now discussed on page 6.

2. Related to that - it is entirely possible that the enhanced luminescence in the fast codons construct or in the shRNA that targets the EEF2K mRNA all result from differences in translation efficiency (i.e. proteins produced per mRNA) and not from translation fidelity. Do they simply get high amounts of the luciferase or indeed higher fraction of functional protein due to translation error? This must be established unequivocally. A quantification of protein amount is needed to exclude the trivial effects of better codons on translation efficiency.

R: we agree with this helpful comment. As the reviewer suggested, we have now performed additional experiments (new Supplementary Figure S10E-K) to monitor Fluc protein levels in empty pICtest2 vector or Max/StaCFluc (WT, R218S or STOP)-transfected; untreated, 2-DG-treated, IPTG-treated (to induce shRNA eEF2K) and 2-DG + IPTG-treated A549 cells; as well as in untreated or 2-DG-treated eEF2K^{+/+} and eEF2K^{-/-} MEFs. Please note that MinCFluc was not detected by the Fluc antibody (Supplementary Figure S2D), probably because its levels of expression are very low and the antibody is not sensitive enough to detect it in immunoblots.

Full-length Fluc protein is 60 kDa. The “truncated” Fluc[STOP] proteins should have a molecular weight of 23.44 kDa, but they were not detected (Supplementary Figure S10E) perhaps either because our Fluc antibody recognizes an epitope located after the truncation point (amino acid residue L210). It is also possible that the truncated (and probably improperly folded) protein is degraded very quickly, as often happens to misfolded polypeptides.

As shown in new Supplementary Figure S10F-K, the levels of expression of Fluc from either MaxCFluc[WT] and MaxCFluc[R218S] or StaCFluc[WT] and StaCFluc[R218S], were unaffected by neither 2-DG treatment nor eEF2K knockdown (shRNA-induced, in A549 cells)/knockout (in MEFs). We therefore conclude that the differences in Fluc activity we observe are not due to differences in translational efficiency, and this is now discussed on page 6.

3. Regarding the use of the PEST motif: the motivation was to check if the recoding of the reporter could affect protein stability (and hence also the measured activity). I'm not sure why they expected, or rather suspected, that recoding of the gene would affect stability of the protein. A rationale for that would be helpful. On other hand, recoding of a gene could certainly affect mRNA levels through effects on RNA degradation. I would have examined this effect.

R: The PEST motif was added to make the protein turn over quickly so we can more accurately assess production of NEW Fluc protein without interference from pre-existing enzyme present at the start of the treatment, and rather than for the reason the reviewer suggests. We apologise for not having made this clear enough, and have now modified the text to clarify this on page 5.

4. The effect on lifespan is intriguing but might require some further analysis, and as is some more careful wording in the abstract and title. The real effect that is observed is that eEF2K is required for lifespan extension induced by nutrient restriction. The abstract is less clear about the conditional nature of the effect - on nutrient restriction. But how do we really know that the effect that eEF2K

exerts on life span is mediated by its effect on translation fidelity? Could the authors prove that by showing that if they somehow recover translation fidelity of the *efk-1* knockout worms they regain back the lifespan extension by caloric restriction? That would exclude a translation fidelity - independent effect of *efk-1* on life span.

R: We would first like to thank the reviewer for his/her very helpful comment. As we now state on page 12, third paragraph: “aaRSs [71] and eEF2K [72] (*efk-1* in nematodes) are proteins that are required for or regulate, respectively, mRNA translation. While each has a specific function, they may exert pleiotropic effects, given the crucial importance of protein synthesis for cell physiology. Therefore, the decreased lifespan we observe in nematodes where *efk-1* or aaRSs were knocked down may be due to these effects.”

Additionally, we have made substantial efforts to address this point by knocking down aaRSs in *efk-1* worms (please see our first response to reviewer 1 and the new Figure 6C), and have shown that aaRSs (with the exception of *mrs-1*, which is needed for initiation as well as elongation) and *efk-1* play mutually redundant roles in the maintenance of lifespan in *C. elegans*. This is consistent with our conclusion that *efk-1* and translation elongation modulate lifespan by affecting translation fidelity.

We have also modified the Abstract to clarify the nature of the effect.

There are several serious inaccuracies in citing the literature:

1. The comment in the Introduction on protein translation "... lacks an editing function" is a great mistake. A huge literature on kinetic proof reading and other modes of editing exist on translation.

R: Our original statement was meant to explain that bacteria have a way of editing out misincorporated amino acids after peptidyl transfer while eukaryotes do not. To clarify things, we have now state that “misincorporated amino acids are subject to ‘editing’ in bacteria [4] but eukaryotes do not appear to have an analogous function for editing after decoding [5]”. We apologise for the confusion.

2. The introductory comment "Because tRNA competition is the main determinant of misreading errors in translation [9]" refers to a 2007 paper in bacteria. I'm not sure that this statement holds as stated so generally here. I'm not aware of a proven role of tRNA in fidelity that is so widely accepted and that applies generally. This statement should be modified or much better supported.

R: As suggested, we have now modified the text to “Errors in amino acylation of tRNAs and codon decoding can each affect accuracy. Given that >99% of amino acids are incorporated during elongation, the vast majority of translation accuracy errors are made during this stage of translation [11]”.

3. Likewise the citation "In mammalian cells, faster elongation rates compromise the accuracy of codon:anticodon recognition [8, 30]" appears to be inaccurate too: while ref #8 does refer to a mammalian system, ref 30 appears to be focused on yeast and bacteria.

R: We apology for this unfortunate mistake. We have now modified this to “In yeast, bacterial [32] and mammalian [10] cells, faster elongation rates compromise the accuracy of codon:anticodon recognition.”

Finally, the figures are very condensed, with too many western blots. At least some can be summarized by graphs and moved to supplementary.

R: Thank you for this suggestion: we have now moved all the SDS-PAGE/WB images in the 'old' Figure 2 to the supplementary data (new Supplementary Figures S3-S6), and have thereby simplified Figure 2 which now only contains data for the luciferase assays.

**Regulation of the elongation phase of protein synthesis enhances translation accuracy
and modulates lifespan**

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Running title: slowing elongation enhances fidelity and lifespan

Keywords: eEF2K, translation fidelity, mTOR, lifespan, *Caenorhabditis elegans*

Word Count: 5422

Summary:

Maintaining accuracy during protein synthesis is crucial to avoid producing misfolded and/or non-functional proteins. The target of rapamycin complex 1 (TORC1) pathway and the activity of the protein synthesis machinery are known to negatively regulate lifespan in many organisms, although the precise mechanisms involved remain unclear. Mammalian TORC1 signaling accelerates the elongation stage of protein synthesis by inactivating eukaryotic elongation factor 2 kinase (eEF2K), which, when active, phosphorylates and inhibits eEF2, which mediates the movement of ribosomes along mRNAs, thereby slowing down the rate of elongation.

We show that eEF2K enhances the accuracy of protein synthesis under a range of conditions and in several cell-types. For example, our data reveal it links mTORC1 signalling to the accuracy of translation. Activation of eEF2K decreases misreading or termination read-through errors during elongation, while knocking down or knocking out eEF2K increases their frequency. eEF2K also promotes the correct recognition of start codons in mRNAs.

Reduced translational fidelity is known to correlate with shorter lifespan. Consistent with this, deletion of eEF2K ortholog or other factors implicated in translation fidelity in *Caenorhabditis elegans* decreases lifespan, **and eEF2K is required for lifespan extension induced by nutrient-restriction**. Our data uncover a novel mechanism linking nutrient supply, TORC1 signaling and the elongation stage of protein synthesis which enhances the accuracy of protein synthesis. **Our data also indicate that modulating translation elongation and its fidelity affects lifespan.**

Introduction

The production of properly-made and -folded proteins is crucial for normal cell function and organismal survival. Conversely, misfolded proteins can lead to a number of common and serious diseases, including several neurodegenerative disorders [1].

With respect to protein production, maintaining accuracy during protein synthesis (mRNA translation) is crucial for the faithful decoding of genetic information to generate fully-functional proteins. However, mRNA translation has a higher intrinsic error rate than DNA polymerase [2, 3]. **Misincorporated amino acids are subject to ‘editing’ in bacteria [4] but eukaryotes do not appear to have an analogous function for editing after decoding [5].** It has been shown that in mammals, as an evolutionary phenomenon, high levels of translational accuracy correlate positively with longer lifespan [6, 7]. It therefore follows that cells require mechanisms to optimise the accuracy of protein synthesis without compromising the efficiency of protein production. Interestingly, one of the major pathways that modulates accelerated aging in eukaryotic organisms, target of rapamycin complex 1 (TORC1) signaling [8], also activates protein synthesis and other anabolic processes [9]. Recently, Conn and Qian [10] showed that active mTORC1 signaling impairs translational accuracy (also termed ‘fidelity’). **Errors in amino acylation of tRNAs and codon decoding can each affect accuracy. Given that >99% of amino acids are incorporated during elongation, the vast majority of translation accuracy errors are made during this stage of translation [11].**

During mRNA translation, amino acyl-tRNAs are recruited into the ribosomal A-site by eukaryotic elongation factor 1A (eEF1A). After achieving an appropriate codon:anticodon match, the corresponding amino acid is added to the growing polypeptide by forming a peptide bond and concomitant transfer of the nascent chain to the A-site tRNA. There is no known connection between mTORC1 signaling and regulation of this step in elongation.

The ribosome then undergoes translocation, whereby it moves the equivalent of one codon along the mRNA, thereby bringing the peptidyl-tRNA into the P-site and the next codon into the now-vacant A-site. This step requires a second elongation factor, eEF2 [12], whose activity is inhibited by phosphorylation, which is in turn controlled by mTORC1 signaling [13].

The phosphorylation of eEF2 on Thr56 decreases its affinity for the ribosome, thereby inactivating it [14, 15]. eEF2 is phosphorylated by a dedicated protein kinase, eEF2 kinase (eEF2K), which belongs to the atypical α -kinase family and is subject to tight regulation [16]. For example, it is inactivated by anabolic and mitogenic signaling pathways such as mTORC1 and the classical ERK MAP kinase pathway [17]. Thus, activation of these anabolic or mitogenic signaling pathways turns off eEF2K, ensuring that eEF2 and elongation are active. Consistent with this, when initiating ribosomes are stalled using harringtonine [10], TSC2 null cells (in which mTORC1 signaling is constitutively activated [18]) exhibit earlier ribosomal runoff, indicating a faster rate of translation elongation (reflecting disinhibition of eEF2), while rapamycin – which inhibits mTORC1 - slows down elongation [13]. Conversely, eEF2K is activated by Ca^{2+} -ions [19], at low pH [20, 21] and under various stress conditions [22-24].

Given that mTORC1 controls the activity of eEF2K and thus elongation, we asked whether the effects of mTORC1 signaling on fidelity involve the regulation of eEF2 by eEF2K. Surprisingly, given that eEF2 is not involved in the step of elongation where cognate

aminoacyl-tRNAs are selected, we find activation of eEF2K enhances translational fidelity indicating that, its activity nonetheless governs the accuracy of elongation.

Inhibition of (m)TORC1 as well as controlled nutritional restriction are known to extend lifespan in a variety of organisms [25-28]. We have therefore also explored the impact of this regulatory system on lifespan; deletion of the eEF2K ortholog reduces the lifespan of *C. elegans*. Taken together, our data reveal a crucial role for eEF2K in reducing errors in translation when cells are under stress, and that reducing translation elongation improves translation fidelity and extends lifespan.

Results

The speed of translation elongation dictates translation accuracy

The rate of translation elongation can be affected by the codon composition of the translated region of mRNAs, since some codons are used less often than others and hence there are fewer tRNAs with the corresponding anticodon, it takes longer to achieve a codon:anticodon match for such codons. This can therefore slow down the decoding of the mRNA [29, 30]. To assess whether rates of elongation affect the stringency of translation initiation in yeast, we created constructs encoding firefly luciferase (Fluc, see Table 1 for a list of specific terms used in this study) using the slowest (yeast MinCFluc) and fastest (yeast MaxCFluc) possible codons to test this idea [31]. We made the slowest [human (h) hMinCFluc] and fastest [human (h) hMaxCFluc] constructs according to predicted human decoding times (Figure 1A, S1 and S2, please see STAR Methods for details), in a vector which also contains a cistron encoding Renilla luciferase (Rluc); Rluc activity levels allow the Fluc data to be normalised to control for factors such as transfection efficiency or overall translational activity. All data are therefore given as ‘relative Fluc activity’, i.e., Fluc/Rluc ratio as a % of the corresponding control conditions. We named the original wild-type ‘standard’ Fluc ‘StaCFluc’. hMaxCFluc produced the highest amount of active Fluc protein, whereas hMinCFluc produced much less (Figure 1B, S2D and E), showing that codon composition strongly affects the overall efficiency of protein expression and thereby demonstrating that elongation rates limit the overall rate of translation of this mRNA.

In yeast, bacterial [32] and mammalian [10] cells, faster elongation rates compromise the accuracy of codon:anticodon recognition, although it has recently been reported that rates of translation elongation do not correlate with translation fidelity in yeast [33, 34]. The above constructs allow us to assess the extent to which the rate of elongation affects the accuracy (fidelity) of mRNA translation, and explore what factors affect this. To do this, we created two Fluc reporter missense mutants for use in mammalian cells. In the first, the arginine codon (AGA) at position 218 was changed to a serine codon (AGT) yielding the ‘Fluc[R218S]’ vectors. As Arg218 is a key residue for binding of substrate to luciferase [35], correct decoding at this position generates catalytically-inactive protein. The level of active luciferase (in which this codon has actually been misread as encoding arginine) therefore serves as an indicator of misreading of the serine codon to incorporate arginine, i.e., lack of fidelity during elongation [36]. In the second variant, the leucine codon (CTT) at position 210 of the corresponding amino acid sequence was replaced by a stop codon (TGA, yielding the Fluc[STOP] vector), allowing us to detect read-through errors during translation [36], i.e., another failure in its accuracy. As

changes in steady-state protein levels could, in principle, also result from altered rates of protein degradation, we made the same mutations in a StaCFluc reporter that encodes Fluc followed by a PEST motif (SSGTRHGFPPPEVEEQAAGTLPMSQESGMDRHPAACASARINV). This motif causes the destabilization and rapid degradation of newly-synthesized Fluc ($t_{1/2} \approx 2$ h) [37] (vectors termed Fluc[R218S-PEST] and Fluc[STOP-PEST], respectively, see Figure S1). **This accelerates the turnover of Fluc allowing us to more accurately assess new Fluc protein production without interference from already-existing enzyme present at the start of the treatment.**

Importantly, we observed the highest levels of these translation errors (higher luciferase activity) in HEK293 cells transfected with the hMaxCFluc mutants; while the lowest levels of FLuc, and therefore of errors, were seen in cells transfected with the hMinCFluc mutants (Figure 1C; note that data are always compared to the differing yields of active luciferase for the corresponding parent Min, Sta or Max vectors). These results show that faster translation elongation leads to impaired translational fidelity.

eEF2K promotes translational fidelity

Since eEF2K regulates the rate of elongation, we wished to explore its role in modulating translational fidelity. As noted above, eEF2K is activated under diverse stress conditions [20-24]. Using A549 (human lung adenocarcinoma) cells, we first studied the effects of (i) energy deprivation, mimicked by treating cells with 2-deoxyglucose (2-DG), a glucose analog which can be phosphorylated by hexokinase but cannot be metabolised through glycolysis; consequently, it depletes cells of ATP [38]; (ii) nutrient deprivation [23] (cells were transferred to D-PBS); and (iii) extracellular acidosis, which activates eEF2K [20, 21]. We also treated cells (iv) with the mTOR inhibitor AZD8055 [39], which alleviates inhibitory inputs into eEF2K (Figure S3-S7). As expected, each of these conditions inhibited mTORC1 signaling, as shown by decreased rpS6 phosphorylation (downstream of mTORC1), and evoked activation of eEF2K, as indicated by increased eEF2 phosphorylation (Figure S3-S7). Notably, in A549 cells, 2-DG treatment rapidly induced the phosphorylation of eEF2 (within 30 min) **which** persisted for at least 16 h (Figure S7).

To test the role of eEF2K in translational accuracy, we made use of A549 or HCT116 cells that inducibly express an shRNA that targets the *EEF2K* mRNA (Figure 1D-F and Figure S3) [21]. We transfected them with wild-type (WT) and mutant StaCFluc. In some cases, cells were treated with IPTG to induce the shRNA against *EEF2K*. (i-iv). Cells were then cultured for 16 h in the presence or absence of 2-DG. Knocking-down eEF2K by inducible shRNA did not affect the Fluc/Rluc activity ratio from the Min/Sta/MaxCFluc[WT] or StaCFluc[WT-PEST] vectors (Figure 1E and F, Figure S8-S10). However, knockdown of eEF2K did increase expression of active StaCFluc from the StaCFluc[R218S-PEST] and StaCFluc[STOP-PEST] vectors (Figure S9C-E), as well as Min/Sta/MaxCFluc[R218S] and Min/Sta/MaxCFluc[STOP] (Figure 1D-F and Figure S9) either under basal conditions [cells cultured in DMEM containing low glucose (5.5 mM)] or when cells were treated with 2-DG.

We also used CRISPR/Cas9 genome editing to generate eEF2K knockout (KO) MDA-MB-231 (human breast cancer) cells. Whereas incubation in 2-DG slowly increased p-eEF2 levels in wild-type cells (Figure S11A), no eEF2 phosphorylation was seen in KO cells (Figure S11B). We observed an increase in the expression of active StaCFluc from StaCFluc[R218S]-

transfected eEF2K-KO cells as compared to control cells (Figure S11C), similar to our observations for A549 and HCT116 cells where *EEF2K* was knocked down by inducible shRNA (Figure 1D-F, Figure S9). *Fluc* mRNA abundance (Figure S10A-D) as well as Fluc protein expression levels (Figure S10E-K) were very similar in WT and IPTG-treated A549 cells and in eEF2K^{+/+} and eEF2K^{-/-} MEFs; thus, the differences in Fluc activity observed (Figure 1, S8 and S9) were not due to a difference in transfection, transcription or translation efficiency. Levels of total Fluc from the WT or R218S vectors were very similar, indicating that the differences in activity reflect mistranslation in the case of the latter (Figure 10E). [Note that MinCFluc (Figure S2D) and Fluc[STOP] (Figure S10E) were not detected by the Fluc antibody, in the former case as levels were too low and in the latter probably because either the epitope lies after the stop codon or the truncated Fluc is rapidly degraded].

Amino acyl-tRNA synthetases (aaRSs) play a crucial role in protein synthesis and in maintaining its fidelity by generating correctly charged tRNAs (reviewed in [40, 41]). Errors during elongation may arise from competition of cognate and near-cognate aminoacyl-tRNAs at the ribosomal A-site; therefore, lower levels of the cognate aminoacyl-tRNA is expected to give near-cognate aminoacyl-tRNAs a higher chance being accepted at a given codon [42]. Consistent with this, and as expected from the nature of the mutation in this vector, expression of StaCFluc[R218S] was greatly enhanced in cells where expression of the seryl aaRS (SARS) was knocked-down by siRNA (Figure 1G-I). The observed magnitude of the change may be an underestimate since SARS knock-down led to a reduction in the expression of StaCFluc[WT].

Inhibition of mTORC1 signaling improves translation fidelity through the activation of eEF2K

Active mTORC1 signaling increases global protein synthesis but impairs translational fidelity [10]. Because mTORC1 is an upstream negative regulator of eEF2K [17, 43], we asked whether eEF2K mediates the effect of mTORC1 on translational fidelity. For this purpose, we incubated A549 cells (Figure 2A, S3A and C) as well as eEF2K^{+/+} and eEF2K^{-/-} MEFs (Figure 2B, S3B and D) in the presence or absence of rapamycin or AZD8055 [39]. As expected, rapamycin or AZD8055 treatment abolished the phosphorylation of rpS6 at Ser240/Ser244, indicating mTORC1 was inhibited, and increased the phosphorylation of eEF2 at Thr56 (Figure S3). Genetic deletion of eEF2K totally blocked phosphorylation of eEF2 (Figure S3D).

Rapamycin or AZD8055 treatment reduced expression of active StaCFluc from StaCFluc/StaCFluc-PEST [R218S] and [STOP] vectors but not from the WT vector, indicating lower rates of misreading or termination readthrough errors. Consistently, knocking-down eEF2K using inducible shRNA in A549 cells (Figure 2A and S12) or knockout of eEF2K in MEFs (Figure 2B) significantly attenuated the ability of mTOR inhibitors to decrease error rates. Thus, the ability of mTORC1 signaling to modulate error rates requires eEF2K, an effector of mTORC1 signaling. The data show that mTORC1 regulates translation fidelity by affecting the rate of translation elongation via eEF2K, thus providing a clear molecular mechanism to explain the findings of Conn and Qian regarding the impact of mTORC1 signaling on translation fidelity [10].

eEF2K is a general modulator of translation fidelity

We also wished to test whether eEF2K is involved in maintaining the quality of translation under other conditions. We therefore incubated A549 cells, which had been transfected with

vectors for StaCFluc[WT] or mutants, under stress conditions known to activate eEF2K, i.e. incubation in medium buffered at low pH (pH 6.7, Figure 2C and S4), in D-PBS (which lack amino acids, Figure 2D and S5) or under hypoxia (Figure 2E-F and Figure S6). All these treatments, except for glycolytic stress induced by 2-DG [44], inhibited signaling through mTORC1 independently of AMPK, as shown by the decreased rpS6 phosphorylation and increased electrophoretic mobility of 4E-BP1 (reflecting its dephosphorylation) (Figures S3-S6). Phosphorylation of eIF2 α (Figure S13), a key regulator of translation initiation, was not affected, although it was induced by cycloheximide (used as a positive control for the P-eIF2 α antibody; Figure S13).

Inducing the shRNA reduced eEF2K levels and attenuated eEF2 phosphorylation (Figure 2E-F and Figure S3-S6). Incubation in D-PBS modestly decreased StaCFluc-PEST[WT] (Figure 2D) while hypoxia (5% oxygen) greatly reduced StaCFluc[WT] levels (Figure 2E and F). Cells cultured in medium buffered at low pH (6.7), in D-PBS or under hypoxia exhibited reduced levels of active Fluc from the StaCFluc, StaCFluc-PEST R218S or STOP vectors (Figure 2C-F), indicating enhanced translational accuracy. While knock-down of eEF2K did not affect the expression of StaCFluc/StaCFluc-PEST[WT], it did increase levels of active (mistranslated) StaCFluc/StaCFluc-PEST R218S under these conditions (Figure 2C-F and Figure S14). These data provide further evidence that eEF2K serves as a “guardian” of translation fidelity under diverse stress conditions.

Slower elongation, not lower overall protein synthesis, enhances fidelity

The treatment of cells with 2-DG, AZD8055, D-PBS or low pH decreased global protein synthesis. Although knocking down eEF2K did not affect overall protein synthesis rates under any of the conditions tested (Figure S15), it did increase the proportion of translationally-inactive monosomal fractions concomitantly with a decrease in translationally-active polysomal fractions, as analysed on sucrose density gradients (Figure 3). This presumably reflects faster rates of elongation in the knockdown cells (as fewer ribosomes are engaged in polysomes at any given time, due to ribosomal ‘run-off’). Hence, the positive effect of eEF2K on translation fidelity reflects slower translation elongation rather than lower overall rates of protein synthesis.

The AMPK pathway is crucial for maintaining translation fidelity

Stress conditions can evoke activation of eEF2K via the AMP-activated protein kinase (AMPK). Genetic knockout of AMPK completely (2-DG, Figure 4A) or partially (AZD8055, D-PBS, low pH, Figure 4B) **prevented** the increased phosphorylation of eEF2, indicating AMPK plays a role in mediating these effects. In line with our previous studies [21, 24], we also observed a reduction in levels of eEF2K protein in AMPK-null MEFs (Figure 4A and B); this may well also contribute to the lower eEF2 phosphorylation seen in these cells.

Importantly, compared to WT MEFs, AMPK-null MEFs transfected with the Fluc vector exhibited higher levels of Fluc activity from the StaCFluc[R218S] and StaCluc[STOP] but not from the StaCFluc[WT] vectors (Figure 4C), illustrating that AMPK helps ensure translational accuracy under stress conditions. Given that AMPK activates eEF2K, and the above data linking eEF2K to translational accuracy, these effects are likely to be mediated via activation of eEF2K and **concomitant** inhibition of eEF2.

For experiments where cells were treated with 2-DG, they were transferred into medium containing lower glucose levels (5.5 mM). Even without 2-DG, this resulted in increased p-eEF2 levels (Figure S9 and S10). This likely explains why we observed an increase in Fluc activity in Fluc[R218S]/Fluc[STOP]-transfected, eEF2K knock-down A549 or HCT116 cells (Figure 1E, Figure S8 and S9); as well as in AMPK^{-/-} MEFs (Figure 4C) when compared to the corresponding control cells.

Reducing cellular levels of eEF2 also improves the fidelity of translation

Our data indicate that stimulation of eEF2K, and thus lower eEF2 activity, promote translational accuracy. It follows that reducing eEF2 protein levels should also improve fidelity. Accordingly, A549 cells were transfected with siRNA against eEF2 and, 24 h later, with the vectors encoding Fluc or mutants. After a further 48 h, by which time eEF2 levels had been reduced substantially (Figure 4D), luciferase activity was measured. Knockdown of eEF2 decreased the levels of active luciferase from the StaCFluc[R218S] and StaCFluc[STOP] vectors but had no effect on WT StaCFluc expression (Figure 4E). Taken together, these data indicate that the AMPK-eEF2K-eEF2 pathway is crucial for translation quality control when cells are subjected to stress conditions.

eEF2K modulates start codon selection during translation initiation

The process of start site selection in eukaryotes differs fundamentally from aminoacyl-tRNA choice during elongation; in particular, during initiation, the 40S subunit (with its associated factors) is already equipped with the initiator methionyl-tRNA and is seeking the correct codon, while in elongation the converse is true: a correct match has to be achieved between the codon already in the A-site and the relevant aminoacyl-tRNA. Chu *et al.* [31] reported that in yeast, when rare (infrequently used) codons are positioned immediately after the translation start codon, the impaired speed of translation elongation restricts the “liberation” of 40S ribosome from the start codon and hence loading of the next 40S subunit. Under these circumstances, translation elongation can affect initiation. However, it was not known whether this also applies in mammalian cells. Because eEF2K regulates the rate of elongation, we asked whether eEF2K affected the stringency of translation start-site selection.

We previously developed “pICtest2” dual luciferase reporters with non-AUG start codons as well as an inferior Kozak consensus (non-optimal initiation start site context; [45]). To test the effect of eEF2K on start-site recognition, we selected two near-cognate non-AUG start codon variants (GUG and CUG).

We first compared the relative expression levels of luciferase expressed from these plasmids in A549 cells. Non-AUG start codon plasmids exhibited 7.7% (GUG) or 17.3% (CUG) of Fluc activity compared to the WT Fluc (AUG start codon with optimal Kozak consensus; Figure 5A).

Treating the cells with 2-DG to increase eEF2 phosphorylation tended to reduce the expression of Fluc. Knocking down eEF2K significantly increased the expression of active Fluc from GUG or CUG as start codons but did not affect expression of Fluc with AUG as start codon (Figure 5B). This implies that increased phosphorylation of eEF2, and thus slower elongation, also aid the fidelity of start codon recognition, by augmenting the ability of ribosomes to distinguish between AUG and near-cognate start codons (Figure 5C).

eEF2K does not affect chaperone or proteasome activity

It was important to assess whether changes in chaperone or proteasome activity contributed to the increase in active Fluc that we observe when knocking down eEF2K. *In vitro* Fluc-refolding assays revealed that there was a slight decrease in refolding ability when denatured recombinant Fluc was incubated with lysates from eEF2K knock-down A549 cells that had been pre-treated with 2-DG, pH 6.7 medium or AZD8055 (Figure S16) as compared to lysates from control cells with normal eEF2K levels. The reduced refolding capacity might reflect higher levels of client misfolded proteins resulting from impaired translation fidelity in the knockdown cells. The rise in active Fluc levels upon eEF2K knockdown cannot therefore be explained by lower protein folding/chaperone capacity; indeed, these data may indicate that the measured effects of eEF2K to promote translation fidelity are a slight underestimate.

To study the effect of altered eEF2K levels on the ubiquitin-proteasome system, we treated A549 and HCT116 cells with the proteasome inhibitor MG132. Increasing concentrations of MG132 evoked phosphorylation of eEF2, indicating activation of eEF2K, in parallel with an increase in polyubiquitinated proteins (Figure S17A-C). Knocking down eEF2K did not lead to obvious changes in polyubiquitin signals or WT StaCFluc expression in A549 or HCT116 cells (Figure S17B and C). We also applied a cell-based proteasome assay to measure its chymotrypsin-like activity in these cells (Figure S17D-G). We observed decreased proteasome activity in 2-DG treated A549 cells (Figure S17D) and increased proteasome activity in A549 cells that had been incubated in D-PBS (Figure S17E). However, under no conditions tested did knockdown of eEF2K affect proteasome activity (Figure S17D-G).

Taken together, these data imply that the increases in active Fluc seen in the absence of eEF2K are not due to changes in protein folding or proteasome activity.

*aaRS orthologs and the eEF2K ortholog *efk1* control lifespan in *C. elegans**

mRNA translation accuracy strongly correlates with lifespan of organisms during evolution [6, 7]. Mammals with high rates in translation fidelity lives longer than those with high levels of translation inaccuracy [7]. We argued that inadequate levels of aminoacyl-tRNA synthetases (aaRSs) are expected to impair fidelity by limiting the availability of specific amino acyl-tRNAs [40, 41], leading to misincorporation of amino acids at the codons normally recognised by those charged tRNAs (due to use of a near-cognate but incorrect aminoacyl-tRNA). Similar findings were recently reported in yeast [33]. To test this, we knocked down selected aminoacyl-tRNA synthetases in *C. elegans*, a short-lived nematode worm which is widely used to the control of lifespan. [As, unlike eEF2K, these are essential genes they cannot be completely knocked out.] Depletion of the leucyl (*lrs-1*), arginyl (*rrs-1*), asparaginyl (*nrs-1*) or methionyl-tRNA (*mrs-1*) synthetases significantly decreased lifespan when worms were maintained at 25°C on normal food, implying that impaired translation fidelity does indeed shorten lifespan (Fig. 6A). *Drosophila* is a widely-used and tractable model for studying lifespan [46], but lacks an ortholog of eEF2K. We found that when leucyl-tRNA synthetase (LeuRS) was knocked down during adulthood in *Drosophila*, there was a marked reduction in lifespan (Figure S18), analogous to our findings in *C. elegans* (Fig. 6A).

C. elegans has an ortholog of eEF2K; its target residue in eEF2 and the adjacent sequence are conserved in *C. elegans* eEF2 [23]. As expected [47, 48], caloric restriction (achieved by using heat-killed [HK] *E. coli* as food) extended the lifespan of wild-type N2 worms (Figure 6B; see also [23, 47]). *efk-1* knockout worms (*Ok3609*) (previously characterized in [49]) had only a slightly reduced lifespan on normal diet, but, importantly, showed no extension upon caloric restriction (Figure 6B). **These data indicate** that eEF2K is required for lifespan extension induced by nutrient restriction. **Interestingly, depletion of *mrs-1* but not of other aaRSs (*lrs-1*, *kars-1* and *nrs-1*) further reduced lifespan of *efk-1* knockout worms under normal (OP50) feeding (Figure 6C). This likely reflects the fact that the latter three enzymes are only required for elongation, which is controlled by eEF2K, while *mrs-1* is also needed for translation initiation.**

It has previously been shown that inhibition of TOR in *C. elegans* extends lifespan [50]. Here, we observed that rapamycin-treated *efk-1* knockout worms still outlived the rapamycin-treated WT worms (Figure 6D), implying that *efk-1* positively contributes to longevity in a TOR-independent fashion in nematodes. **As a readout of endoplasmic reticulum (ER) stress [51], we monitored the levels of P-eIF2 α in WT and *efk-1* knockout worms under normal feeding in the absence or presence of rapamycin; or under caloric restriction. Caloric restriction greatly reduced levels of P-eIF2 α , while rapamycin also slightly decreased the levels of P-eIF2 α in both WT and *efk-1* knockout worms (Figure S19). However, levels of P-eIF2 α were similar in *efk-1* knockout worms (Figure S19), which corroborates our observations that eEF2K knockdown in A549 cells did not induce phosphorylation of eIF2 α (Figure S13). Therefore, the observed impairment of lifespan as the result of *efk-1* knockout is unlikely to be caused by an increase in ER stress.**

Discussion

The faithful translation of mRNAs into proteins plays crucial roles in normal cell functions and organismal health-span. During translation, amino acid misincorporation is estimated to happen at a rate between every 1 in 1,000 to 10,000 codons [3, 52]. Over time, even low error rates in the synthesis of certain proteins, such as disease-associated ones, e.g., SOD1 (whose misfolding causes amyotrophic lateral sclerosis), can lead to accumulation of defective proteins even affecting the faithful transmission of genetic information [6]. In the 1960-1970s, Orgel [53-55] proposed that increased rates of translation errors directly affect ageing and lifespan. Although this theory was challenged in the 1970-80s [56-59], it was recently demonstrated that mRNA translation accuracy imposes strong evolutionary pressure [6, 7]. Indeed, mammals with low levels of translation inaccuracy live longer than those with high rates of translation errors [7].

Decades of studies have shown that caloric restriction can extend lifespan in invertebrates [48] and in rodents [60]. More recent studies have implicated key cellular components linked to the control of translation and that are regulated (directly or indirectly) by nutrients in modulating lifespan. These include mTORC1 [25] and its downstream substrates or effectors S6K1 ([61], in mice), 4E-BP1 ([62], in *D. melanogaster*), eIF4E ([63], in *C. elegans*) and AMPK ([61], in mice). Inhibition of mTOR signaling (a pathway which activates protein synthesis) extends lifespan in diverse organisms from yeast to mammals [25-28, 50, 64, 65]. Furthermore, a range of conditions that slow down translation all extend lifespan in *C. elegans* [64].

Translation fidelity is assured by two main events: synthesis of the precisely paired aminoacyl-tRNAs by aaRSs and the stringent selection of proper aminoacyl-tRNAs by the ribosomes during elongation [41]. It has been shown that translation rates inversely correlate with co-translation folding efficiency [66, 67] as well as translational accuracy [10]. Conn and Qian demonstrated that rates of mRNA mistranslation in TSC2 null cells (where mTORC1 signaling is constitutively activated, [18]) are significantly higher than in WT cells [10]. They also showed that the impairment of translational fidelity in TSC2-null cells results from activation of mTORC1-S6K1 pathway and that TSC2 null cells exhibit faster translation elongation [10]. However, prior to this, it was not known whether translation elongation directly impacted on translation fidelity or, if so, how this was linked to the mTORC1 pathway.

Here, we demonstrate faster translation due to the presence of surrounding codons with high usage rates (“fast codons”) leads to higher levels of mistranslation (Figure 1A-C), while slower translation elongation reflecting low usage-rate codons (“slow codons”) correlates with reduced rates of translation errors. This provides further evidence that increased productivity of the translation machinery occurs at the expense of poorer accuracy. Here we demonstrate that, under various stress conditions, where eEF2K is activated and slows down translation [21, 23, 24], eEF2K aids translational accuracy. Conversely, when mTORC1 signaling is active and switches off eEF2K to accelerate elongation, this reduces translation fidelity.

A reduction in eEF2K levels or its inactivation reduces the accuracy of decoding. mTORC1 can directly or indirectly drive phosphorylation of several residues in eEF2K which impair its activity [68]; for example, S6K1 directly phosphorylates eEF2K at Ser366 and thereby inhibits its activity [43]. Importantly, we show that disabling eEF2K attenuates the ability of mTOR inhibitors to promote translational fidelity (Figure 2A-E), thereby providing a molecular mechanism for the ability of mTORC1 to modulate the accuracy of protein synthesis [10]. Thus, surprisingly, although eEF2 does not itself mediate the step in elongation where aminoacyl-tRNAs are recruited to the A-site, where a correct codon:anticodon match must be achieved, it does nevertheless link mTORC1 signaling and thus multiple stress conditions that activate eEF2K to the accuracy of elongation. These conditions include impaired glycolysis (Figure 1E and F), low pH (Figure 2C), nutrient deprivation (Figure 2D) and hypoxia (Figure 2E-F). Since levels of global protein synthesis were unaffected when eEF2K was knocked down (Figure S15), translation fidelity appears to be affected mainly by the speed of elongation rather than the overall rate of protein production. Thus, our data point to eEF2K-mediated regulation of elongation helping to ensure accuracy rather than affecting overall rate of protein production, at least in the cells and under the conditions tested here.

AMPK is activated upon lack of metabolic energy and lies upstream of mTORC1 and eEF2K, suppressing or stimulating them, respectively [18, 69]. We show that the activation of AMPK also plays a positive regulatory role in translation fidelity; AMPK-null MEFs exhibit impaired translation accuracy. This may reflect loss of the ability of AMPK to inhibit mTORC1 and stimulate eEF2K and/or the lower eEF2K protein levels in AMPK null cells ([21, 24], Figure 4A and B).

We also show that activation of eEF2K helps reduce initiation at codons similar to canonical AUG start sites, i.e., CUG/GUG (Figure 5). Initiation involves ‘scanning’ by 40S ribosomal subunits and associated factors to seek appropriate start codons; this is facilitated by the fact that the relevant methionyl-initiator tRNA is already associated with them. **Although** the

presence of secondary structure downstream of a start codon favours initiation from non-AUG codons [70] and contributes to neurodegenerative diseases such as ALS (amyotrophic lateral sclerosis) and FXTAS (fragile X-associated tremor/ataxia syndrome) [71], **our data actually suggest that impaired elongation, which would slow down ribosomes within the open reading frame, actually favours the “correct” choice of an AUG start codon; perhaps because faster elongation (in cells where eEF2K has been knocked down) allows less time for selection of the optimal initiation codon (AUG) pair at the start site and acceptance of otherwise less favoured ones such as GUG and CUG (Fig 5C).**

A further key finding of this study is that *efk-1*^{-/-} is required for the lifespan extension that normally occurs under nutritionally-restricted conditions. Furthermore, since ablation of several aminoacyl (leucyl, arginyl, asparaginyl or methionyl)-tRNA synthetases also decreases lifespan in nematodes, our data clearly demonstrate that factors implicated in regulating translational accuracy influence longevity (Figure 6). However, it should be noted that, **aaRSs [72] and eEF2K [73] (*efk-1* in nematodes) are proteins that are required for or regulate, respectively, mRNA translation. While each has a specific function, they may exert pleiotropic effects, given the crucial importance of protein synthesis for cell physiology. Therefore, the decreased lifespan we observe in nematodes where *efk-1* or aaRSs were knocked down may be due to these effects.** Also, although the correlation between slower elongation and greater lifespan holds true in nematodes and human cells, since TORC1 signaling is not known to regulate eEF2 in *C. elegans*, additional mechanisms must exist in them to account for the link between mTORC1 and longevity in nematodes.

Our data show that eEF2K promotes the fidelity of translation elongation and initiation, thereby providing a link between inhibition of mTORC1 signaling (which activates eEF2K in mammals) and the enhanced accuracy of protein synthesis. These data provide a mechanism for the very well-known association between impaired mTORC1 signaling or slower protein synthesis and greater lifespan.

Declaration of Interests

The authors declare no competing interests.

Author contributions

JX conducted most of the experiments. VdSA performed the *C. elegans* lifespan study. **LO performed the *Drosophila* lifespan study.** TvdH, RL, RVL, MJC and XW also contributed data. All authors helped design the experiments. KBJ, XW and CGP provided supervision. All authors interpreted and analysed the data. JX, XW and CGP wrote the manuscript.

Acknowledgements

The authors would like to thank Dr. Daniel J. Peet (University of Adelaide, Australia) for the use of Edwards Instrument Hypoxia Workstation. We would like to thank South Australian Health and Medical Research Institute (SAHMRI) for financial support. We would also like to acknowledge CGC - Caenorhabditis Genetic Center [funded by: National Institutes of Health

- Office of Research Infrastructure Programs (P40 OD010440)] for their donation of nematode worms.

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Figure legends

Figure 1. Accelerated translation speed correlates with a reduction in translation fidelity.

A) The heat-map illustrates the calculated decoding speed of the designed Min/Sta/MaxCFluc constructs (a colour bar is shown below for reference). **B)** HEK293 cells were transfected with empty pICtest2 or pICtest2 vectors encoding Min/Sta/MaxCFluc. After 48 h, Fluc activity was quantified using luciferase assay. **C)** HEK293 cells were transfected with the MinCFluc WT, R218S or STOP ; StaCFluc WT, R218S or STOP; MaxCFluc WT, R218S or STOP constructs. After 48 h, relative Fluc activity was then analysed using luciferase assay. The relative Fluc expression levels when using R218S or STOP constructs were then normalized and presented as % of WT control. Results are given as means \pm standard deviation [S.D.]. *, $0.01 \leq P < 0.05$; **, $0.001 \leq P < 0.01$; ***, $P < 0.001$, as determined by two-way ANOVA followed by Dunnett's test. **(A)** or Tukey's test (in **C**). $n = 4$ in **B** and **C**. **D)** A549 cells were treated with 1 μ M of IPTG for up to 120 h (5 days) to induce the expression of an shRNA against *EEF2K*. **E** and **F)** IPTG (1 μ M) was added to A549 cells 5 days before the experiment to induce expression of an shRNA against *EEF2K*. A549 cells were transfected with Sta(**E**)/Max(**F**)CFluc WT, R218S or STOP constructs for 24 h, cells were then cultured in DMEM containing low glucose (5.5 mM) for 1 h, before treatment with 2-DG (10 mM) for 16 h. Fluc activity was quantified using luciferase assay and normalized to mock-treated control. Results are given as means \pm S.D.; $n = 3$ independent experiments each performed in triplicate. **(G)** A549 cells were transfected with scrambled or SARS siRNA for 72 h, lysed and subjected to immunoblotting analysis with indicated antibodies. **(H)** Quantification of SARS from **G**. **(I)** A549 cells were transfected with scrambled or SARS siRNAs. After 24 h, the cells were transfected with StaCFluc WT or R218S vectors. 48 h later, luciferase activity was quantified. Results are given

as means \pm S.D.; $n = 4$ independent experiments each performed in triplicate. See also Figure S1, S2 and S8-S11.

Figure 2. eEF2K activation enhances translation fidelity. **A)** A549 cells were transfected with StaCFluc WT, R218S or STOP constructs. After 24 h, cells were treated with vehicle (DMSO, as a control), rapamycin (200 nM) or AZD8055 (1 μ M) for 16 h. After 16 h, Fluc activity was determined using luciferase assay. **B)** eEF2K^{+/+} and eEF2K^{-/-} MEFs were transfected with StaCFluc WT, R218S or STOP and then incubated with vehicle (DMSO), rapamycin (200 nM) or AZD8055 (1 μ M). After 16 h, Fluc activity was determined using luciferase assay. **C)** A549 cells were transfected with StaCFluc WT, R218S or STOP constructs for 24 h, and were then incubated in pH (7.4 or 6.7) buffered medium for 16 h, before luciferase assay analysis. **D)** A549 cells were transfected with Fluc-PEST WT, R218S or STOP constructs, followed by the incubation in growth medium or DPBS for 3 h, before luciferase assay analysis. **E)** A549 or **F)** HCT116 cells were transfected with StaCFluc WT, R218S or STOP constructs for 24 h, and were then incubated in 20% or 5% O₂ for 16 h, before luciferase assay. For experiments performed in A549 (**A**, **C**, **D** and **E**) and HCT116 (**F**) cells, IPTG (1 μ M) was added to cells 5 days before StaCFluc transfection to induce the expression of shRNA against *EEF2K*. Fluc activity is expressed as means \pm S.D.; $n = 4$ (**A**, **B**, **C** and **D**) or 3 (**E** and **F**) independent experiments performed in triplicate. See also Figure S3-S7 and S12-S17.

Figure 3. eEF2K knock down reduces polysome/monosome (P/M) ratio in A549 cells. **A)** Polysome profile analysis of lysates from A549 cells treated with vehicle (DMSO) control, rapamycin (200 nM) or AZD8055 (1 μ M) for 16 h. **B)** Polysome:monosome (P/M) ratio quantification of data in **A**. **C)** A549 cells were cultured under low (5.5 mM) glucose DMEM for 1 h before the addition of 2-DG (10 μ M) for 16 h. Cells were then subjected to polysome profile analysis. **D)** P/M ratio (polysome/monosome) quantification of **C**. **E)** A549 cells were cultured either in growth media or DPBS for 3 h before polysome profile analysis. **F)** P/M ratio (polysome/monosome) quantification of **E**. **G)** A549 cells were cultured in pH (7.4 or 6.7) buffered medium for 16 h before polysome profile analysis. **H)** P/M ratio (polysome/monosome) quantification of **G**. For all experiments, IPTG (1 μ M) was added to A549 cells 5 days before experiment to induce the expression of shRNA against *EEF2K*.

Figure 4. AMPK helps to promote translation fidelity. **A)** AMPK^{+/+} and AMPK^{-/-} MEFs were pre-incubated in low glucose (5.5 mM) DMEM for 1 h, before treatment with 2-DG (10 mM) for 16 h. **B)** AMPK^{+/+} and AMPK^{-/-} MEFs were cultured for 3 h with 1 μ M AZD8055 or vehicle (DMSO), or in medium buffered at pH 7.4 or 6.7 for 16 h; or growth medium (as control) or DPBS. Cells were then lysed and lysates were subjected to immunoblotting analysis with indicated antibodies. **C)** AMPK^{+/+} and AMPK^{-/-} MEFs were transfected with StaCFluc WT, R218S or STOP constructs. After 24h, cells were then placed in low glucose (5.5 mM) DMEM for 1 h, before treated with 2-DG (10 mM) for 16 h. Fluc activity was measured and normalized to vehicle-treated control. **D)** A549 cells were transfected with scrambled siRNA or siRNA against *EEF2* for 24 h; cells were then transfected with WT/R218S/STOP StaCFluc. After 48 h cells were lysed followed by SDS-PAGE/Western blotting analysis. **E)** Cells were treated as in **D**, and luciferase activity was measured. Results are as means \pm S.D.; $n = 4$ independent experiments performed in duplicate. *, $0.01 \leq P < 0.05$; **, $0.001 \leq P < 0.01$; ***, $P < 0.001$, as obtained by two-way ANOVA.

Figure 5. eEF2K stimulation increases the stringency of initiation codon selection. A) A549 cells were transfected with pICtest2 vectors encoding StaCFluc with different start codons (previously reported in [1]; sequences indicated). Fluc activity was then measured and is expressed as percentage of control (GCCAUGG) (means \pm S.D.; $n = 4$ independent experiments conducted in triplicate). *, $0.01 \leq P < 0.05$; ***, $P < 0.001$, as obtained by one-way ANOVA followed by Dunnett's test. **B)** A549 cells were transfected with pICtest2 reporter vectors as in **A**, and after 48 h were treated with 2-DG as in Fig. 1D-F. Fluc activity was measured and is expressed as percentage of control (means \pm S.D.; $n = 4$ independent experiments, each performed in triplicate). **C)** Schematic representation of how slow elongation speed favours the correct selection of start codon as well as of cognate codon:anticodon (tRNA) pair.

Figure 6. Factors that increase translation fidelity affects lifespan in *C. elegans*. A) Lifespan of WT (N2), *lrs-1*, *mrs-1* and *rrs-1* knockdown worms under standard growth conditions. Mean survival: N2 = 13 days; N2/*lrs-1* = 9 days; N2/*mrs-1* = 7 days; N2/*rrs-1* = 7 days; N2/*nrs-1* = 11 days). N2 vs. N2/*mrs-1*: $p = 0.0075$; N2 vs. N2/*lrs-1*: $p = 0.0023$; N2 vs. N2/*rrs-1*: $p = 0.0075$; N2 vs. N2/*nrs-1*: $p = 0.0291$. **B)** The lifespan of N2 and *efk-1* knockout worms when fed with normal food or under caloric restriction [fed with heat-killed (HK) *E.coli*]. Mean survival: N2/OP50 (fed normally with *E. coli* OP50) = 11 days; N2/HK = 12 days; *efk-1*/OP50 = 9 days; *efk-1*/HK = 9 days. N2/OP50 vs. N2/HK: $p = 0.0146$; N2/HK vs. *efk-1*/HK: $p = 0.0358$; N2/OP50 vs. *efk-1*/OP50: $p = 0.0006$. **C)** Lifespan of *efk-1*, *efk-1 + lrs-1*, *efk-1 + mrs-1*, *efk-1 + nrs-1* and *efk-1 + kars-1* knockdown worms under standard growth conditions. Mean survival: *efk-1* = 11 days; *efk-1 + lrs-1* = 11 days; *efk-1 + mrs-1* = 7 days; *efk-1 + nrs-1* = 11 days; *efk-1 + kars-1* = 12 days). *efk-1* vs. *efk-1 + mrs-1*: $p = 0.0006$. **D)** The lifespan of N2 and *efk-1* worms when fed with normal food (OP50) containing 100 μ M rapamycin (Rap) where indicated. Mean survival: N2/OP50 = 12 days; N2/OP50/Rap = 18 days; *efk-1*/OP50 = 10 days; *efk-1*/OP50/Rap = 14 days. *efk-1*/OP50 vs. *efk-1*/OP50 + Rap: $p < 0.0001$; N2/OP50 vs. *efk-1*/OP50: $p = 0.0006$. See also Figure S18 and S19.

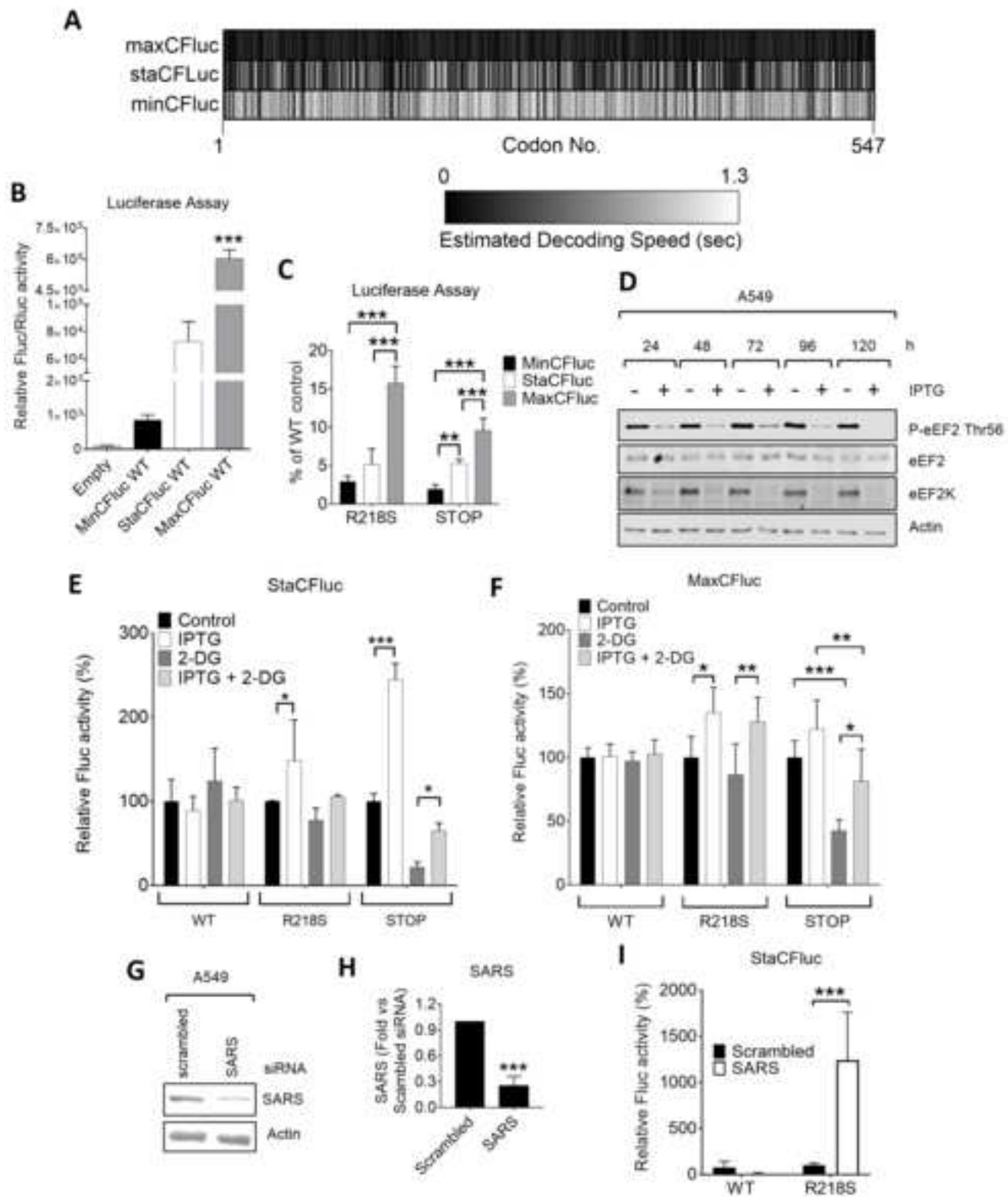


Figure 1

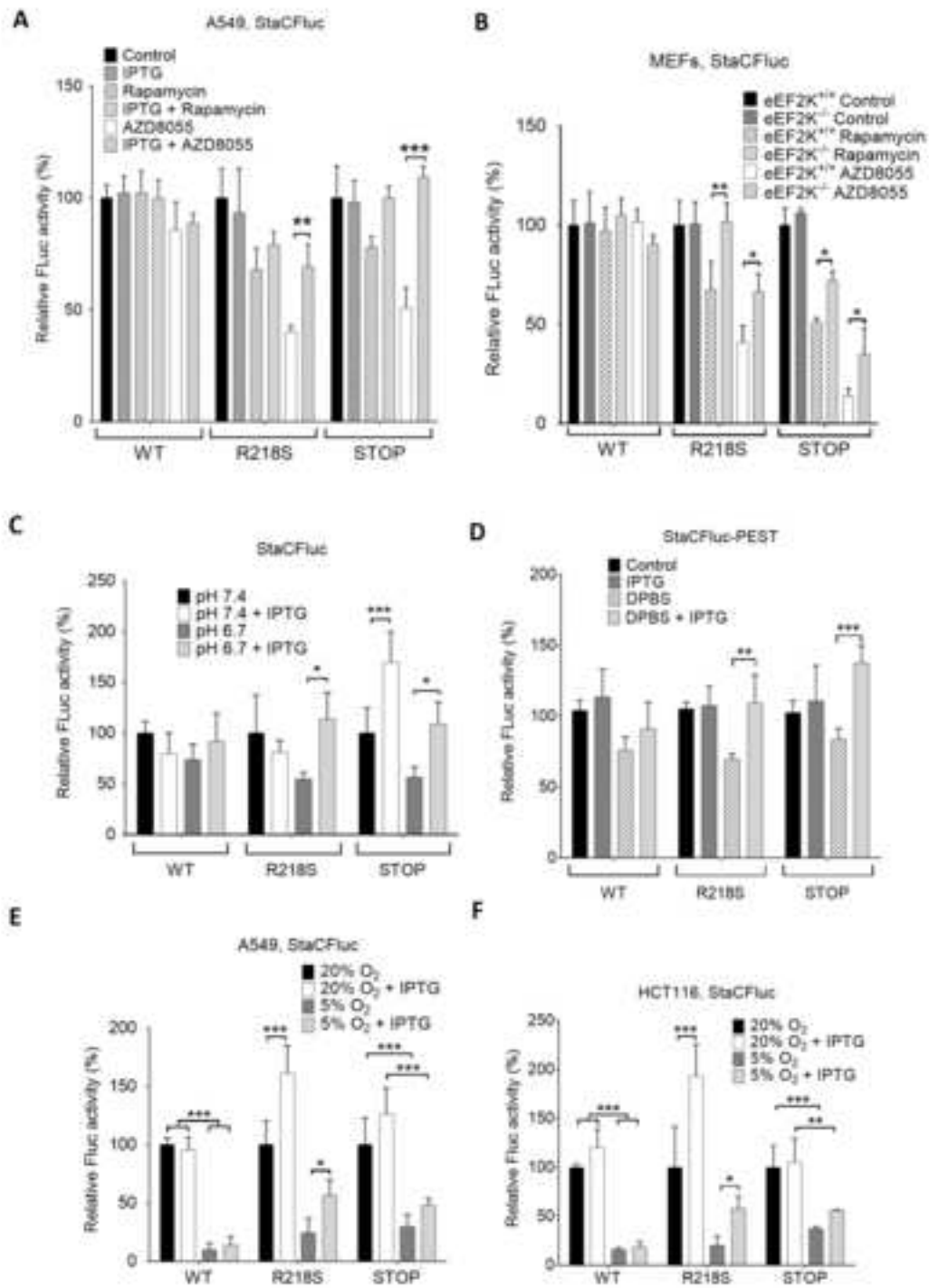


Figure 2

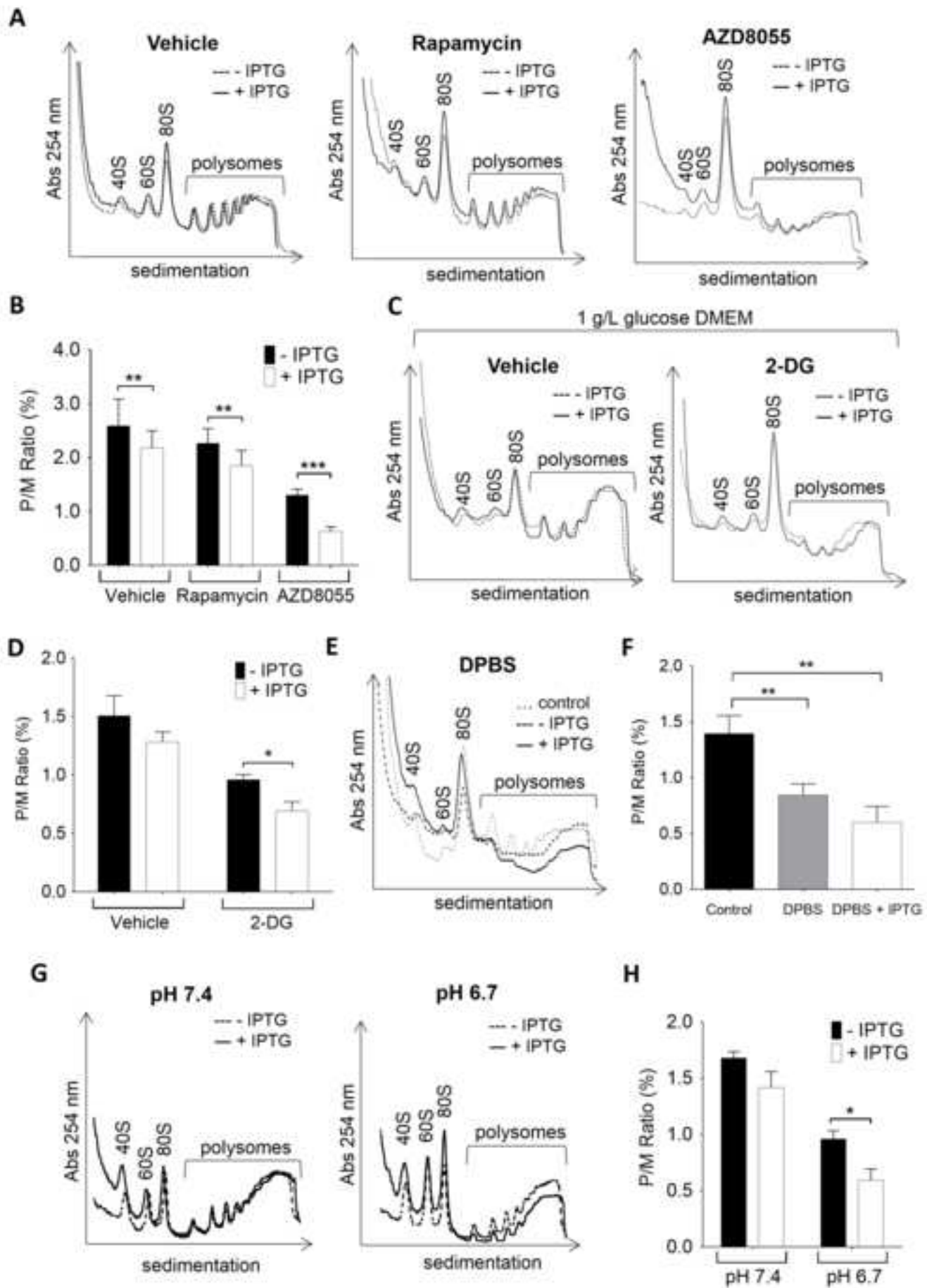


Figure 3

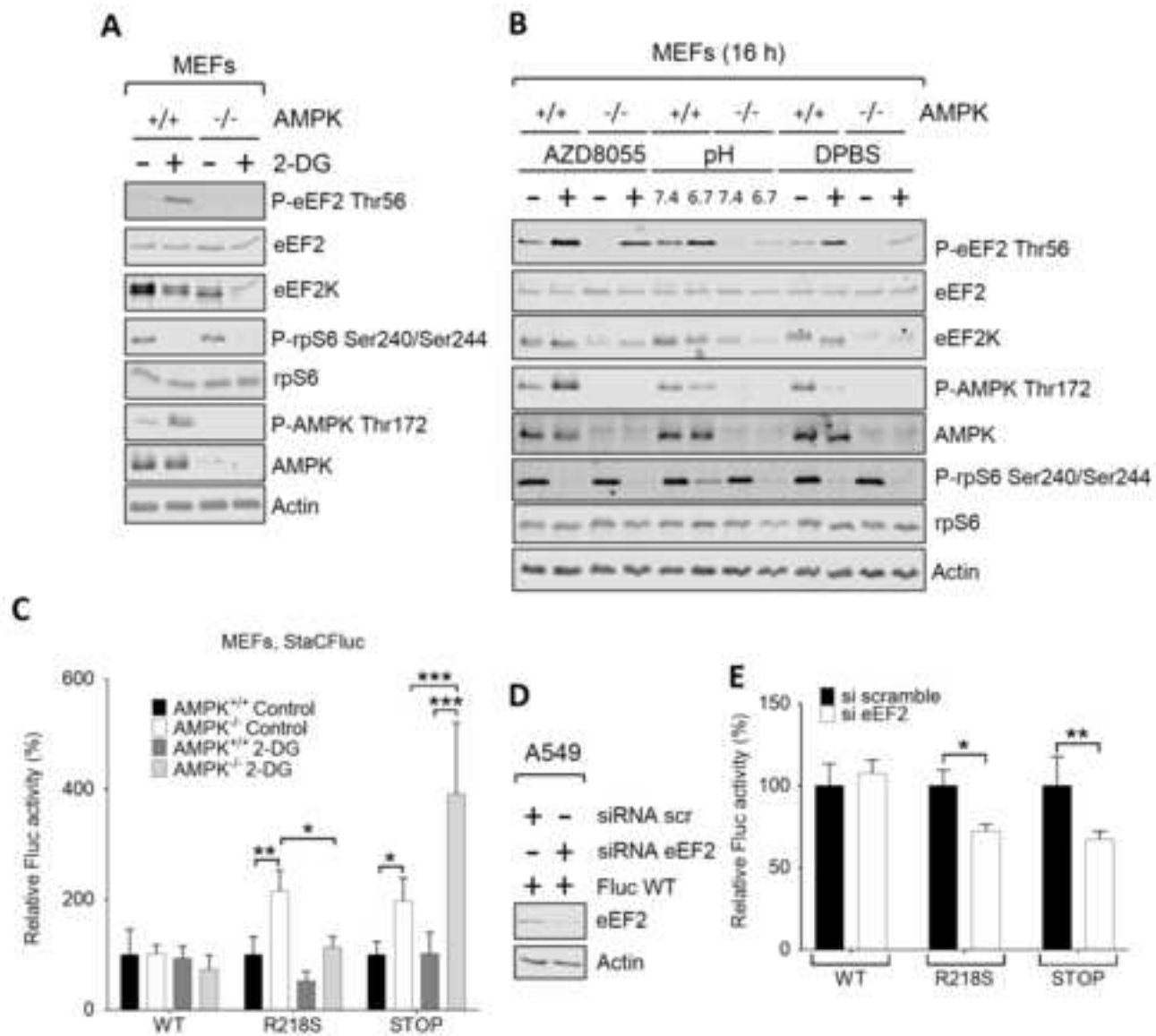


Figure 4

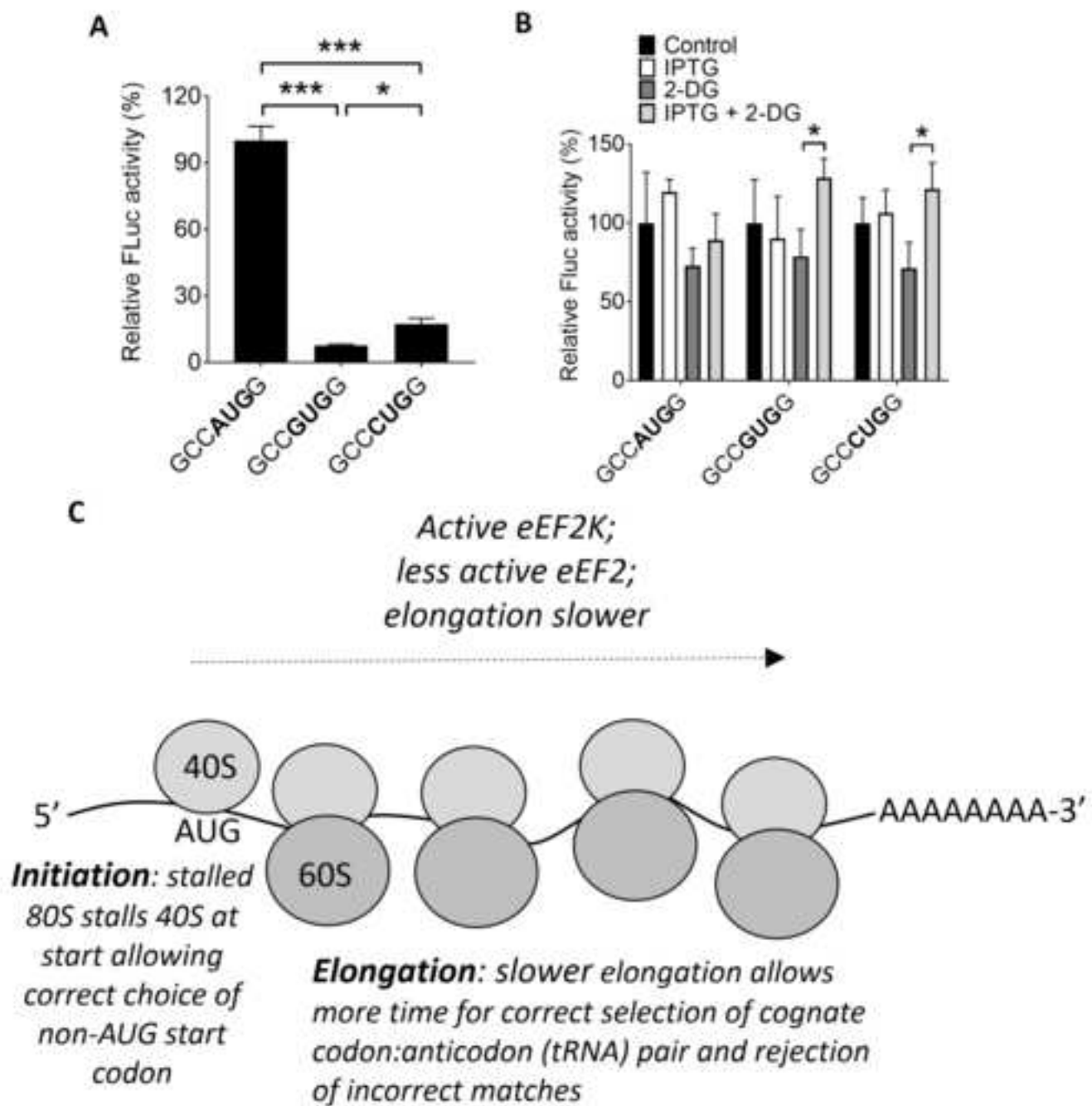


Figure 5

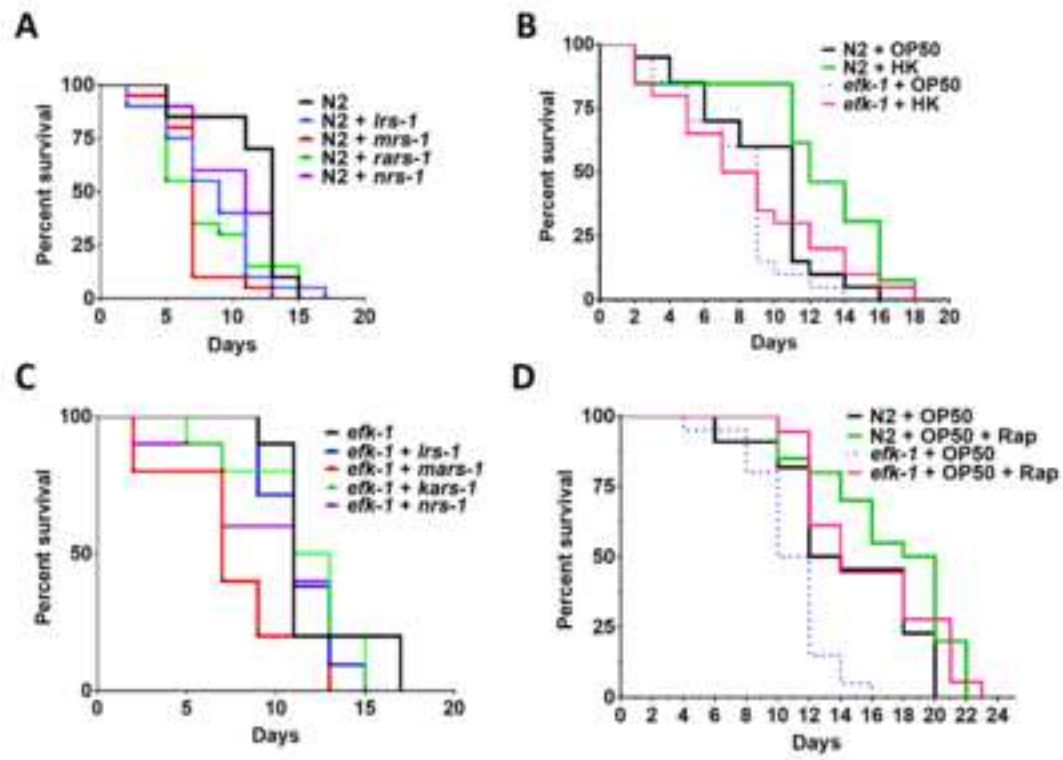


Figure 6

STAR Methods

Chemicals and reagents

All chemicals and reagents were from Sigma-Aldrich unless otherwise stated. The Bradford assay reagent was from Bio-Rad. Rapamycin was from Merck. AZD8055 was provided by Selleck Chemicals. [³⁵S]Methionine-cysteine was purchased from MP Biomedicals.

Generation of eEF2K knockout MEFs

Mice were maintained at Biomedical Research Facility, University of Southampton, in line with the United Kingdom Animals (Scientific Procedures) Act 1986. The generation of eEF2K knockout mice was previously described [S1]. Mouse embryonic fibroblasts (MEFs) from these mice and their wild-type (WT) counterparts were prepared from embryos at embryonic day 13.5.

Cell culture, treatment and lysis

A549 and HCT116 cells expressing inducible short hairpin RNA (shRNA) against eEF2K were generously provided by Janssen Pharmaceutica (Beerse, Belgium). Cells were maintained in high glucose (4.5 g/L) Dulbecco's modified Eagle medium (DMEM) containing 10% (vol/vol) fetal bovine serum (FBS) and 1% penicillin-streptomycin. To induce the knockdown of eEF2K, cells were cultured with 1 M isopropyl-β-D-thiogalactopyranoside (IPTG) for 5 days prior to use. For 2-deoxyglucose (2-DG) treatment, cells were pre-cultured in low glucose (1 g/L) DMEM for 1 h before the addition of 10 mM 2-DG for the indicated periods of times. For pH-buffered medium, pH was adjusted by adding different concentrations of NaHCO₃. For culturing cells in Dulbecco's Phosphate Buffered Saline (DPBS) or rapamycin/AZD8055 treatment, medium was changed just before the experiment. Hypoxia experiments were performed using a humidified hypoxia workstation (Edwards Instruments, Sydney, Australia) as previously described in [S2]. After treatment, cells were lysed in ice-cold lysis buffer containing 1% (v/v) Triton X-100, 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM NaH₂P₂O₇, 1 mM β-glycerophosphate, 1 mM Na₃VO₄ and protease inhibitor cocktail (1X, Roche). Lysates were spun at 16,000 x g for 10 min, the supernatants were kept and total protein concentration was quantified by the Bradford assay (BioRad Laboratories).

SDS-PAGE and Western-blot analysis

Laemmli sample buffer was added to cell lysates and the mixture was boiled at 100°C for 4 min before loading on to the SDS-PAGE gel (Protean 3 mini-slab gel system, BioRad Laboratories). Proteins were then transferred to nitrocellulose membranes. Membranes were blocked in phosphate-buffered saline (PBS) containing 0.1% Tween-20 (PBST) and 5% skimmed milk for 1 h, washed thrice in PBST, followed by incubation with primary antibody overnight at 4°C. Membranes were then washed twice in PBST and incubated with fluorescently tagged secondary antibody for 1 h, washed again thrice in PBST and fluorescent signals were visualized using Li-Cor Odyssey® imaging system (Millennium Science). See below for a list of primary antibodies used in this study. Abbreviations: CST: Cell Signaling Technology, Danvers, USA; EG: Eurogentec, Seraing, Belgium; P-: phosphorylated; SA: Sigma-Aldrich, Castle Hill, Australia; SCB: Santa Cruz Biotechnology, Dallas, USA; WB: western blotting.

<i>I^{ty}</i> antibody	<i>From</i>	<i>Cat. No.</i>	<i>Dilution</i>
P-eEF2 Thr56	EG	Customized	1:1000
eEF2	CST	2332S	1:1000
eEF2K	EG	Customized	1:1000
P-rpS6 Ser240/Ser244	CST	2215S	1:5000
rpS6	SCB	SC74459	1:2000
P-S6K1 Thr389	CST	9205S	1:500

S6K1	SCB	SC230	1:1000
4EBP1	CST	9452S	1:1000
P-PKB Ser473	CST	9271S	1:1000
PKB	CST	9272S	1:1000
P-Erk1/2 Thr202/Tyr204	CST	9101S	1:1000
ERK1/2	CST	9102S	1:1000
P-ACC Ser79	CST	3661S	1:1000
ACC	CST	4190S	1:1000
P-AMPK α Ser172	CST	2535S	1:1000
AMPK	CST	2532S	1:1000
Ubiquitin	CST	3936S	1:1000
SARS	SA	AV40654-100UL	1:1000
P-eIF2 α Ser51	CST	9721S	1:1000
eIF2 α	CST	5324S	1:1000
Firefly luciferase	SA	L0159	1:1000
β -Actin	SA	A5441	1:2000
α -tubulin	SA	T6074	1:1000

Plasmids and transfection

Fluc (firefly luciferase) DNA constructs used in this study were described previously in [S3]. Cells were transfected using Lipofectamine 3000 (Life Technologies) according to the manufacturer's instructions.

Point mutagenesis

Point mutations were introduced by PCR mutagenesis using pfu polymerase (Promega) with the primers listed below in the session of "primers" (*1. Mutagenesis*).

Polysome analysis

Polysome analysis was performed as previously described [S4]. Briefly, cells were lysed in 'polysome lysis buffer' [10 mM NaCl, 10 mM MgCl₂, 10 mM Tris-HCl, pH 7.5, 1% (v/v) Triton X-100, 1% sodium deoxycholate, 36 U/ml RNase inhibitor, 1 mM dithiothreitol] and layered in a 20-50% (w/v) sucrose gradient in 30 mM Tris-HCl, pH 7.5, 100 mM NaCl and 10 mM MgCl₂, followed by centrifugation at 234 000 x g for 150 min. The absorbance at 254 nm across the sucrose gradient was continuously monitored using a UA-6 UV/VIS detector (Isco). Data was analysed using the WinDaq Waveform Browser software (DataQ instruments, Inc.).

Luciferase assay

Fluc activity was measured with luciferase reporter assay systems (Promega) on a GloMax®-discover multimode detection system (Promega) following the manufacturer's instructions.

Real-time RT-PCR amplification analysis

Total RNA was extracted using TRIzol (Life Technologies). cDNA was synthesized using Super-Script® III First-Strand (synthesis system for RT-PCR) from Life Technologies with oligo(dT)₂₀. Subsequently, RT-PCR was performed using primers as previously published in [S5]. Samples were analysed in triplicate (for each experiment) with SYBR green dye mix (PrimerDesign) on a CFX Connect Real-Time system (BioRad). The comparative threshold cycle (C_T) method was used to determine the amount of specific mRNAs compared with level of *B2M* (human or mouse cells) or *RP49* (*Drosophila*) RNA as the house-keeping genes, respectively.

***C. elegans* husbandry**

C. elegans strain N2 Bristol (wild isolate), *efk-1* (*Ok3609*) [CGC, #RB2588, previously described in [S6]] obtained from CGC (Caenorhabditis Genetic Center (CGC), University of Minnesota, Minneapolis, USA), were maintained at 15°C on nematode growth media (NGM) and propagated and manipulated on *E. coli* stain OP50 according to previously established procedures [S7, 8].

RNAi bacterial strains (*lrs-1*, *mrs-2*, *rrs-1* and *nrs-1*) were kindly provided by Crowder Lab [S9] and were grown overnight in LB broth with 50 µg/ml ampicillin and 10 µg/ml tetracycline at 37°C and then diluted 1:100 in LB with the same antibiotics. Strains were then grown at 37°C until reaching an OD of 0.4. A total of 100 µl of the RNAi bacteria plus 0.4 mM IPTG and ampicillin/tetracycline was added to each NGM plate. After 18h of bacterial growth at 23°, L1 nematodes were grown to young adulthood (L4 worms), unless otherwise noted, on the RNAi plates then assayed.

***C. elegans* lifespan assays**

Worms were tightly synchronized on OP50 seeded NGM plates [S10] and eggs were incubated at 15°C until they reached the L1 stage. L4 worms were transferred to NGM seeded with OP50 and incubated at 23°C until reached L4 stage. 80 worms of each strain were picked to several NGM plates (20 worms/plate), supplemented with rapamycin and 5'-fluorodeoxyuridine (FUdR), and maintained at 25°C. Animals were transferred to fresh OP50 [live or heat-killed (HK)] plates every 1-2 days. Worms that crawled off the plate, bagged, or ruptured were censored. For all experiments, ampicillin (100 µg/ml) was added to the medium to selectively prevent growth of *E. coli* OP50 carried over on transfer of worms. Kaplan Meier survival curves were generated and statistically compared using GraphPad Prism Software (GraphPad Software, Inc., San Diego, CA, USA).

Fly husbandry

Stocks used in this study were obtained from the Bloomington *Drosophila* Stock Center (NIH P400D018537): *w¹¹¹⁸*, *Actin-GAL4/CyO* (#4414), *tub-GAL80^{ts}* (#7017) or Vienna *Drosophila* Research Centre (www.vdrc.at): *LeuRS GD* knockdown (v34498) as described in Dietzl *et al.* [S11]. *D. melanogaster* stocks were maintained on fortified (F1) medium composed of 1% agar, 1% glucose, 6% fresh yeast, 9.3% molasses, 8.4% coarse semolina, 0.9% acid mix and 1.7% tegosept. Stocks were kept at 18°C, crosses were carried out at 18°C and adult flies were aged at 29°C for lifespan assays.

Fly lifespan assays

Adult flies were generated by crossing virgin females that were either *w¹¹¹⁸* (control) or *UAS-LeuRS GD* (*LeuRS* knockdown) to *Actin-GAL4/CyO;tub-GAL80^{ts}/TM6B* males. Crosses were carried out at 18 °C with care taken not to overcrowd vials. Adult flies were collected into vials (25 flies per vial) and shifted to 29 °C for ageing then turned onto new food every 2-3 days with the number of deaths recorded at every turn. Survival curve comparisons were graphed and analysed using GraphPad Prism with significance of difference survival curves determined using Log Rank (Mantel-Cox) test.

Sequence design for characterisation of the decoding system in mammalian cells

tRNA identities are from the genomic tRNA database [S12], based on a tRNAscan-SE Analysis of the Homo sapiens genome (hg18 - NCBI Build 36.1 Mar 2006). Anticodon modifications were introduced as reviewed in [S13] and symbols for modified nucleotides are as used in the Modomics database [S14].

1. The Decoding Scheme

All possible relationships between codons and tRNAs were examined and assigned to one of four possible categories: WC-cognate if all nucleotides of the anticodon can form Watson:Crick (WC) base pairs with the codon; wobble-cognate if the last two nucleotides of the anticodon can form WC base pairs and the first can form a wobble base pair; near-cognate if the middle nucleotide of the anticodon can form a WC base pair with the codon

and the first and third nucleotides can form wobble base pairs [following rules established in [S15]]; or non-cognate for all other tRNAs. The results are summarised in Supplementary Fig. 2A.

2. Modelling the human decoding system

From the scheme in Supplementary Fig. 2A, from individual tRNA expression levels in HEK293 cells as determined by microarray in [S16], and from a total tRNA concentration of 190 μ M [based on the value for baker's yeast [S17]], concentrations of the four classes of tRNAs were determined for each codon. The resulting concentrations were used as parameters for simulations of the decoding process for each codon. The rate parameters for WC-cognate, near-cognate and non-cognate tRNAs were as in our previous models.

For the wobble-cognate class, a new set of parameters was used based on the biochemical measurements reported in [S18]. This extends the previously used model to include a more realistic behaviour of wobble-decoding tRNAs (especially their significant rejection rate, which delays the decoding process beyond the near-cognate:cognate ratio).

3. Codon Decoding Speeds

The average of 1,000 individual stochastic simulation results were used to determine the average decoding time for each codon, as predicted by our model. The resulting average decoding times are shown in Supplementary Fig. 2B.

4. Luciferase sequence design

Based on the data in Supplementary Fig. 2B, sequences were designed encoding Firefly Luciferase protein, using only the fastest (hsa_maxCFLuc) or slowest (hsa_minCFLuc) possible codons. In all constructs, the luciferase protein is an engineered version lacking the C-terminal three amino acids of the wild-type protein as described in [S19], which removes the peroxisomal localisation signal [S20] leading to cytoplasmic expression.

Supplementary Fig. 2C compares the speed of the dis-optimised (left) and optimised (right) sequences with the original firefly luciferase (staCFLuc, middle). In this figure, the decoding speed of individual codons is plotted against the codon position. Speed of individual codons is plotted as grey circles, black lines display the smoothed local decoding speed.

5. Luciferase Sequences

>MaxCFLuc

```
ATTAAAGCTTGCCATGGAAGACGCTAAAAACATTAATAAAAGGACCTGCTCCTTTCTACCCTCTTGAA
GACGGAAGCTGCTGGAGAACAACCTTCACAAAGCTATGAAAAGATACGCTCTTGTTCTGGAAGCTATT
GCTTTCAGTACGCTCACATTGAAGTTAACACTTACGCTGAATACTTCGAAATGTCTGTTAGAC
TTGCTGAAGCTATGAAAAGATACGGACTTAACACTAACCACAGAATTGTTGTTTGCTCTGAAAAC
CTCTTCAATTCTTCATGCCTGTTCTTGAGCTCTTTTCATTGGAGTTGCTGTTGCTCCTGCTAACGAC
ATTTACAACGAAAGAGAAGCTTCTTAACCTCTATGAACATTTCTCAACCTACTGTTGTTTCGTTTCTA
AAAAAGGACTTCAAAAAATTCCTAACGTTCAAAAAAACTTCCTATTATTCAAAAAATTATTATTA
TGGACTCTAAAAGTACTACCAAGGATTCCAATCTATGTACACTTTCGTTACTTCTCACCTTCCTCC
TGGATTCAACGAATACGACTTCGTTCCCTGAATCTTTCGACAGAGACAAAAGCTATTGCTCTTATTAT
GAAGCTTCTGGATCTACTGGACTTCCTAAAGGAGTTGCTCTTCCTCACAGAAGCTGCTTGCCTTAGA
TTCTCTCACGCTAGAGACCCTATTTTCGAAACCAAATTATTCCTGACACTGCTATTCTTTCTGTTG
TTCTTTCCACCACGGATTTCGGAATGTTCACTACTCTTGGATACCTTATTTGCGGATTACAGAGTTGT
TCTTATGTACAGATTTCGAAGAAGAAGCTTTTCCTTAGATCTCTTCAAGACTACAAAATTCAATCTGCT
CTTCTTGTTCTACTCTTTTCTCTTTCTTCGCTAAATCTACTCTTATTGACAAATACGACCTTTCTAA
CCTTCACGAAATTGCTTCTGGAGGAGCTCCTCTTTCTAAAGAAGTTGGAGAAGCTGTTGCTAAAAG
ATTCCACCTTCCTGGAATTAGACAAGGATACGGACTTACTGAAACTACTTCTGCTATTCTTATTACT
CCTGAAGGAGACGACAAACCTGGAGCTGTTGGAAAAGTTGTTTCCTTTCTTCGAAGCTAAAGTTGTT
GACCTTGACACTGGAAAACTCTTGGAGTTAACCAAGAGGAGAAGCTTTGCGTTAGAGGACCTAT
GATTATGTCTGGATACGTTAACCAACCCTGAAGCTACTAACGCTCTTATTGACAAAGACGGATGGCT
TCACTCTGGAGACATTGCTTACTGGGACGAAGACGAACACTTCTTCATTGTTGACAGACTTAAATC
TCTTATTAATACAAAGGATACCAAGTTGCTCCTGCTGAACTTGAATCTATTCTTCTTCAACACCT
```

AACATTTTCGACGCTGGAGTTGCTGGACTTCTGACGACGACGCTGGAGAACTTCTGCTGCTGTT
GTTGTTCTTGAACACGGAAAACTATGACTGAAAAAGAAATTGTTGACTACGTTGCTTCTCAAGTT
ACTACTGCTAAAAAAGTTAGAGGAGGAGTTGTTTTGTTGACGAAGTTCTAAAGGACTTACTGGA
AACTTGACGCTAGAAAAATTAGAGAAATTCTTATTAAGCTAAAAAAGGAGGAAAAATAA **ACTAG**

TC

>MinCFluc

ATTAAGCTT**GCC**ATGGAGGATGCCAAGAATATCAAGAAGGGTCCCGCCCCCTTTTATCCCCTCGAG
GATGGTACCGCCGGTGAGCAGCTCCATAAGGCCATGAAGCGCTATGCCCTCGTCCCCGGTACCATC
GCCTTACCGATGCCCATATCGAGGTCAATATCACCTATGCCGAGTATTTGAGATGAGTGTCCGC
CTCGCCGAGGCCATGAAGCGCTATGGTCTCAATACCAATCATCGCATCGTCTGTAGTGAGAAT
AGTCTCCAGTTTTTTATGCCCGTCTCGGTGCCCTCTTTATCGGTGTCGCCGTGCCCCCGCCAATG
ATATCTATAATGAGCGGAGCTCCTCAATAGTATGAATATCAGTCAGCCACCGTCTGTCTTTGTCA
GTAAGAAGGGTCTCCAGAAGATCCTCAATGTCCAGAAGAAGCTCCCCATCATCCAGAAGATCATC
ATCATGGATAGTAAGACCGATTATCAGGGTTTTTCAGAGTATGTATACCTTTGTACCAGTCACTC
CCCCCGGTTTTAATGAGTATGATTTTGTCCCCGAGAGTTTTGATCGCGATAAGACCATCGCCCTCA
TCATGAATAGTAGTGGTAGTACCGGTCTCCCCAAGGGTGTCCGCTCCCCCATCGCACCGCCTGTG
TCAGATTTAGTCATGCCCGGATCCCATCTTTGGTAATCAGATCATCCCCGATACCGCCATCCTCAG
TGTCGTCCCCTTTCATCATGGTTTTGGTATGTTTACCACCCTCGGTTATCTCATCTGTGGTTTTTCGG
TCGTCTCATGTATCGTTTTGAGGAGGAGCTCTTCTCCGCAGTCTCCAGGATTATAAGATCCAGA
GTGCCCTCCTCGTCCCCACCCTCTTTAGTTTTTTTTGCCAAGAGTACCCTCATCGATAAGTATGATCT
CAGTAATCTCCATGAGATCGCCAGTGGTGGTGCCCCCTCAGTAAGGAGGTGGTGAGGCCGTGCG
CCAAGCGTTTTATCTCCCCGGTATCCGCCAGGGTTATGGTCTCACCGAGACCACAGTGCCATCC
TCATACCCCCGAGGGTATGATAAGCCCGGTGCCGTCCGTAAGGTCTGCCCTTTTTTTGAGGCCA
AGGTGTCGATCTCGATAACCGGTAAGACCCTCGGTGTCAATCAGCGCGGTGAGCTCTGTGTCCGCG
GTCCCATGATCATGAGTGGTTATGTCAATAATCCCAGGCCACCAATGCCCTCATCGATAAGGATG
GTTGGCTCCATAGTGGTATATCGCCTATTGGGATGAGGATGAGCATTTTTTTTATCGTCGATCGCCT
CAAGAGTCTCATCAAGTATAAGGGTTATCAGGTCCGCCCCGCGAGCTCGAGAGTATCCTCCTCCA
GCATCCCAATATCTTTGATGCCGGTGTCCGCGGTCTCCCCGATGATGATGCCGGTGAGTCCCCGC
CGCCGTGTCGTCCTCGAGCATGGTAAGACCATGACCGAGAAGGAGATCGTCGATTATGTGCCA
GTCAGGTACCACCGCCAAGAAGCTCCGCGGTGGTGTGCTTTTTGTGATGAGGTCCCCAAGGGTCT
TCACCGGTAAGCTCGATGCCCGCAAGATCCGCGAGATCCTCATCAAGGCCAAGAAGGGTGGTAAG
TAA **ACTAGTC**

>StaCFLuc

ATGGAAGACGCCAAAAACATAAAGAAAGGCCCGGCGCCATTCTATCCTCTAGAGGATGGAACCGC
TGGAGAGCAACTGCATAAGGCTATGAAGAGATACGCCCTGGTTCCTGGAACAATTGCTTTTACAG
ATGCACATATCGAGGTGAACATCACGTACGCGGAATACTTCGAAATGTCCGTTCCGGTTGGCAGAA
GCTATGAAACGATATGGGCTGAATACAAATCACAGAATCGTTCGATGCAGTGAAAACCTCTCTTCA
ATTCTTTATGCCGGTGTGGGCGCGTTATTTATCGGAGTTGCAGTTGCGCCC CGGAACGACATTTAT
AATGAACGTGAATTGCTCAACAGTATGAACATTTTCGCAGCCTACCGTAGTGTTTGTTCAAAAAG
GGGTTGCAAAAAATTTTGAACGTGCAAAAAAATTACCAATAATCCAGAAAATTATTATCATGGA
TTCTAAAACGATTACCAGGGATTTAGTTCGATGTACACGTTTCGTCACATCTCATCTACCTCCCGT
TTAATGAATACGATTTTGTACCAGAGTCTTTGATCGTGACAAAACAATTGCACTGATAATGAAT
TCCTCTGGATCTACTGGGTACCTAAGGGTGTGGCCCTTCCGCATAGAACTGCCTGCGTCAGATTCT
CGCATGCCAGAGATCCTATTTTTGGCAATCAAATCATTCCGATACTGCGATTTTAAGTGTGTTCC
ATTCCATCACGGTTTTTGAATGTTTACTACACTCGGATATTTGATATGTGGATTTTCGAGTCGTCTTA
ATGTATAGATTTGAAGAAGAGCTGTTTTTACGATCCCTTCAGGATTACAAAATTCAAAGTGC GTT
CTAGTACCAACCCTATTTTCATTCTTCGCCAAAAGCACTCTGATTGACAAAATACGATTTATCTAATT
TACACGAAATTGCTTCTGGGGGCGCACCTCTTTTCGAAAGAAGTCGGGGAAAGCGGTTGCAAAACGC
TTCCATCTTCCAGGGATACGACAAGGATATGGGCTCACTGAGACTACATCAGCTATTCTGATTACA
CCCGAGGGGGATGATAAACC GGGCGCGGTCCGTTAAAGTTGTTCCATTTTTTTGAAGCGAAGGTTGT
GGATCTGGATACCGGGAAAACGCTGGGCGTTAATCAGAGAGGCGAATTATGTGTCAGAGGACCTA
TGATTATGTCCGGTTATGTAAACAATCCGGAAGCGACCAACGCCCTTGATTGACAAGGATGGATGG
CTACATTCTGGAGACATAGCTTACTGGGACGAAGACGAACACTTCTTCATAGTTGACCGCTTGAAG

TCTTTAATTAATAACAAAGGATATCAGGTGGCCCCGCTGAATTGGAATCGATATTGTTACAACAC
CCCAACATCTTCGACGCGGGCGTGGCAGGTCTTCCCGACGATGACGCCGGTGAAGTCCCGCCGCC
GTTGTTGTTTTGGAGCACGGAAGACGATGACGGAAAAAGAGATCGTGGATTACGTCGCCAGTCA
AGTAACAACCGCGAAAAAGTTGCGCGGAGGAGTTGTGTTTGTGGACGAAGTACCGAAAGGTCTTA
CCGAAAACTCGACGCAAGAAAAATCAGAGAGATCCTCATAAAGGCCAAGAAGGGCGGAAAGTA
A

>Renilla luciferase

ATGACTTCGAAAGTTTATGATCCAGAACAAAGGAAACGGATGATAACTGGTCCGCAGTGGTGGGC
CAGATGTAAACAAATGAATGTTCTTGATTCAATTTATTAATTATTATGATTCAGAAAAACATGCAGA
AAATGCTGTTATTTTTTACATGGTAACGCGGCCTCTTCTTATTTATGGCGACATGTTGTGCCACAT
ATTGAGCCAGTAGCGCGGTGTATTATACCAGACCTTATTGGTATGGGCAAATCAGGCAAATCTGGT
AATGGTCTTATAGGTTACTTGATCATTACAAATATCTTACTGCATGGTTTGAAGTCTTAAATTTAC
CAAAGAAGATCATTTTTGTCGGCCATGATTGGGGTGCTTGTGTTGGCATTTCATTATAGCTATGAGC
ATCAAGATAAGATCAAAGCAATAGTTCACGCTGAAAGTGTAGTAGATGTGATTGAATCATGGGAT
GAATGGCCTGATATTGAAGAAGATATTGCGTTGATCAAATCTGAAGAAGGAGAAAAAATGGTTTT
GGAGAATAACTTCTTCGTGGAAACCATGTTGCCATCAAAAATCATGAGAAAGTTAGAACCAGAAG
AATTTGCAGCATATCTTGAACCATTCAAAGAGAAAGGTGAAGTTCGTCCGCAACATTATCATGGC
CTCGTGAAATCCCGTTAGTAAAAGGTGGTAAACCTGACGTTGTACAAATTGTTAGGAATTATAATG
CTTATCTACGTGCAAGTGATGATTTACCAAAAATGTTTATTGAATCGGACCCAGGATTCTTTTCCAA
TGCTATTGTTGAAGGTGCCAAGAAGTTTCCTAATACTGAATTTGTCAAAGTAAAAGGTCTTCATTTT
TCGCAAGAAGATGCACCTGATGAAATGGGAAAATATATCAAATCGTTCGTTGAGCGAGTTCTCAA
AAATGAACAAATGTCGTAA

Generation of CRISPR-directed eEF2K knockout MDA-MB-231 cells

The eEF2K knockout CRISPR targeting vector was the GeneArt® CD4 CRISPR Nuclease Vector (ThermoFisher Scientific, Scoresby, VIC, Australia). Guide ssDNA sequence was 5'-AGTGAGCGGTATAGCTCCAG-3'. Cells were transfected with the CRISPR vector by nucleofection (Lonza, Mt Waverley, Australia). After 72 h, CD4-positive cells were enriched using magnetic CD4 Dynabeads (ThermoFisher Scientific) and then sorted into individual wells of 96-well plates using a BD FACSFusion flow cytometer (Becton, Dickinson & Company, Wayville, SA, Australia). Positive clones were selected by immunoblotting analysis for the absence of P-eEF2, and were further confirmed by Sanger sequencing analysis (performed by the Australian Genome Research Facility, Adelaide, Australia).

Global protein synthesis measurements

Cells were preincubated in either DPBS or DMEM lacking glucose, NaHCO₃, methionine, and cysteine free (customized by Labtech International, Heathfield, UK), supplemented with 10% (v/v) FBS, 1% penicillin-streptomycin, 1 (for 2-DG treatment) or 4.5 (all other treatments) g/L glucose, and 44 mM NaHCO₃ (except for pH treatments) for 1 h. For pH treatments, pH was adjusted by adding different concentrations of NaHCO₃. Where indicated, 10 mM 2-DG or 1 μM AZD8055 was added to the cells. 10 μCi [³⁵S]methionine-cysteine were also added and cells were further incubated for either 1 (for DPBS treatment) or 16 (for all other treatments) h. Incorporated radioactivity was determined as described previously [S4].

***In vitro* refolding assay**

Recombinant Fluc (Promega, Sydney, Australia) was diluted in lysis buffer at 50 μg/ml and then denatured at 42 °C and 1000 rpm for 15 min. Denatured Fluc was added to cell lysates for a final concentration of 16.5 μg/ml. Refolding was conducted at room temperature and 1000 rpm for the indicated periods of time. Fluc activity was monitored with luciferase reporter assay system (Promega) on a Victor3 1420 multilabel counter (Perkin Elmer) following the manufacturer's instructions. Fluc activity in lysis buffer alone was also measured to exclude spontaneous refolding of denatured Fluc.

Proteasome activity measurement

Intracellular chymotrypsin activity was monitored using Proteasome-Glo™ chymotrypsin-like cell based assay system (Promega) on a Victor3 1420 multilabel counter (Perkin Elmer) following manufacturer's instructions.

Primers

1. Mutagenesis

MaxCFluc R218S Forward: 5'-CAGAAGCTTGCTTGCCTTAGTTTCTCTCACGCTAGAGAC-3'

MaxCFluc R218S Reverse: 5'-GTCTCTAGCGTGAGAGAACTAACGCAAGCAGTTCTG-3'

MaxCFluc STOP Forward: 5'-GACTTCCTAAAGGAGTTGCTTGACCTCACAGAAGCTTGCTTGCG-3'

MaxCFluc STOP Reverse: 5'-CGCAAGCAGTTCTGTGAGGTCAAGCAACTCCTTTAGGAAGTC-3'

StaCFluc R218S Forward: 5'-CTGCCTGCGTCAGTTTCTCGCATGCCAG-3'

StaCFluc R218S Reverse: 5'-CTGGCATGCGAGAACTGACGCAGGCAG-3'

StaCFluc STOP Forward: 5'-GTTACCTAAGGGTGTGGCCTGACCGCATAGAAGCTGCCTGC-3'

StaCFluc STOP Reverse: 5'-GCAGGCAGTTCTATGCGGTCAGGCCACACCCTTAGGTAAC-3'

MinCFluc R218S Forward: 5'-GCACCGCCTGTGTCAGTTTTAGTCATGCC-3'

MinCFluc R218S Reverse: 5'-GGCATGACTAAAAGTACACAGGCGGTGC-3'

MinCFluc STOP Forward: 5'-CCAAGGGTGTGCGCTGACCCCATCGCACCGC-3'

MinCFluc STOP Reverse: 5'-GCGGTGCGATGGGGTCAGGCGACACCCTTGG-3'

2. Cloning

MaxCFluc WT Forward: 5'-ATTAAGCTTGCCATGGAAGAC-3'

MaxCFluc WT Reverse: 5'-GACTAGTTTATTTTCCTCCTTTT-3'

MinCFluc WT Forward: 5'-ATTAAGCTTGCCATGGAGGAT-3'

MinCFluc WT Reverse: 5'-GACTAGTTTACTTACCACCCT-3'

StaCFluc WT Forward: 5'-ATTAAGCTTGCCATGGAAGACGCCAAAAAC-3'

StaCFluc WT Reverse: 5'-GACTAGTTTACTTTCCGCCCTTCTTGGC-3'

Colour coding: Optimal Kozak sequence in mammals (GCC), HindIII recognition site, SpeI recognition site and spacers* to "protect" the restriction sites.

3. qPCR

StaCFluc Forward: 5'-ATCCGGAAGCGACCAACGCC-3'

StaCFluc Reverse: 5'-GTCGGGAAGACCTGCCACGC-3'

MaxCFluc Forward: 5'-AAGACGGAAGCTGCTGGAGAA-3'

MaxCFluc Reverse: 5'-AGAGCTCCAAGAAGCAGGCAT-3'

MinCFluc Forward: 5'-TCATCGCATCGTCGTCTGTA-3'

MinCFluc Reverse: 5'-GGGGAGCTTCTTCTGGACAT-3'

Rluc Forward: 5'-TGTGCCACATATTGAGCCAG-3'

Rluc Reverse: 5'-CCAAACAAGCACCCCAATCA-3'

Human B2M Forward: 5'-GAGTATGCCTGCCGTGTGAAC-3'

Human *B2M* Reverse: 5'-CCAATCCAAATGCGGCATCTTC-3'.

Mouse *B2M* Forward: 5'-CTGCTACGTAACACAGTTCCACCC-3'

Mouse *B2M* Reverse: 5'-CATGATGCTTGATCACATGTCTCG-3'

Drosophila *LeuRS* Forward: 5'-CTTGCTCCAGAACATACGGC-3'

Drosophila *LeuRS* Reverse: 5'-CCCAGATTTCCGCCAGTCATG-3'

Drosophila *RP49* Forward: 5'-ATCGATATGCTAAGCTGTGCGAC-3'

Drosophila *RP49* Reverse: 5'-TGTCGATACCCTTGGGCTTG-3'

Statistical analysis

Statistical analysis were performed using a one-way or two-way analysis of variance with an unpaired student *t* test with the means of three independent experiments unless otherwise specified. GraphPad Prism software package was used to calculate *P*-values. *: $0.01 \leq P < 0.05$; **: $0.001 \leq P < 0.01$; ***: $P < 0.001$. For simplicity, not all statistical significances were shown.

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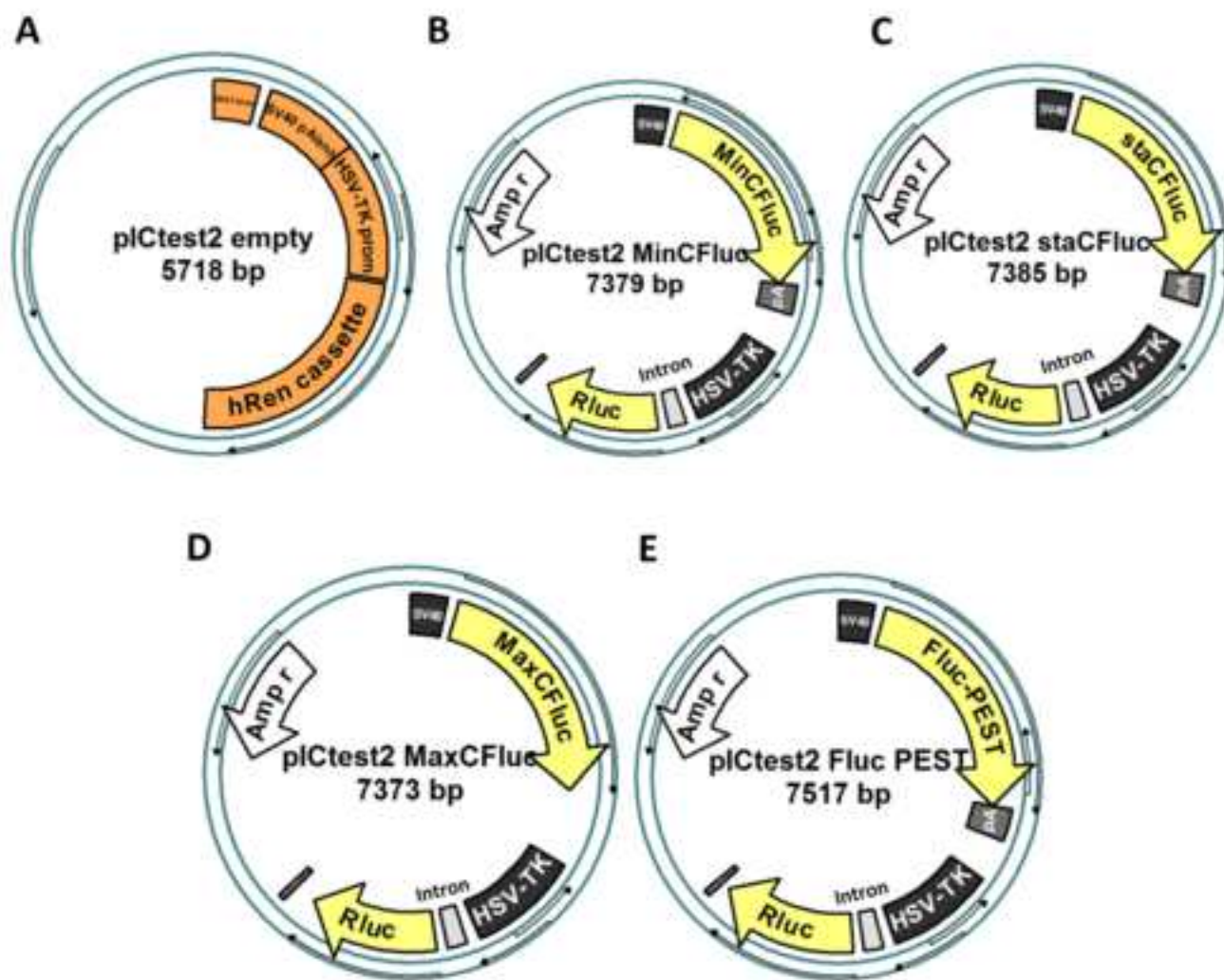


Figure S1, related to Figure 1A, B and C. Maps of reporter plasmids highlighting functional features. A) pICtest2 empty; B) pICtest2 MinCFluc; C) pICtest2 StaCFluc; D) pICtest2 MaxCFluc; E) pICtest2 Fluc-PEST.

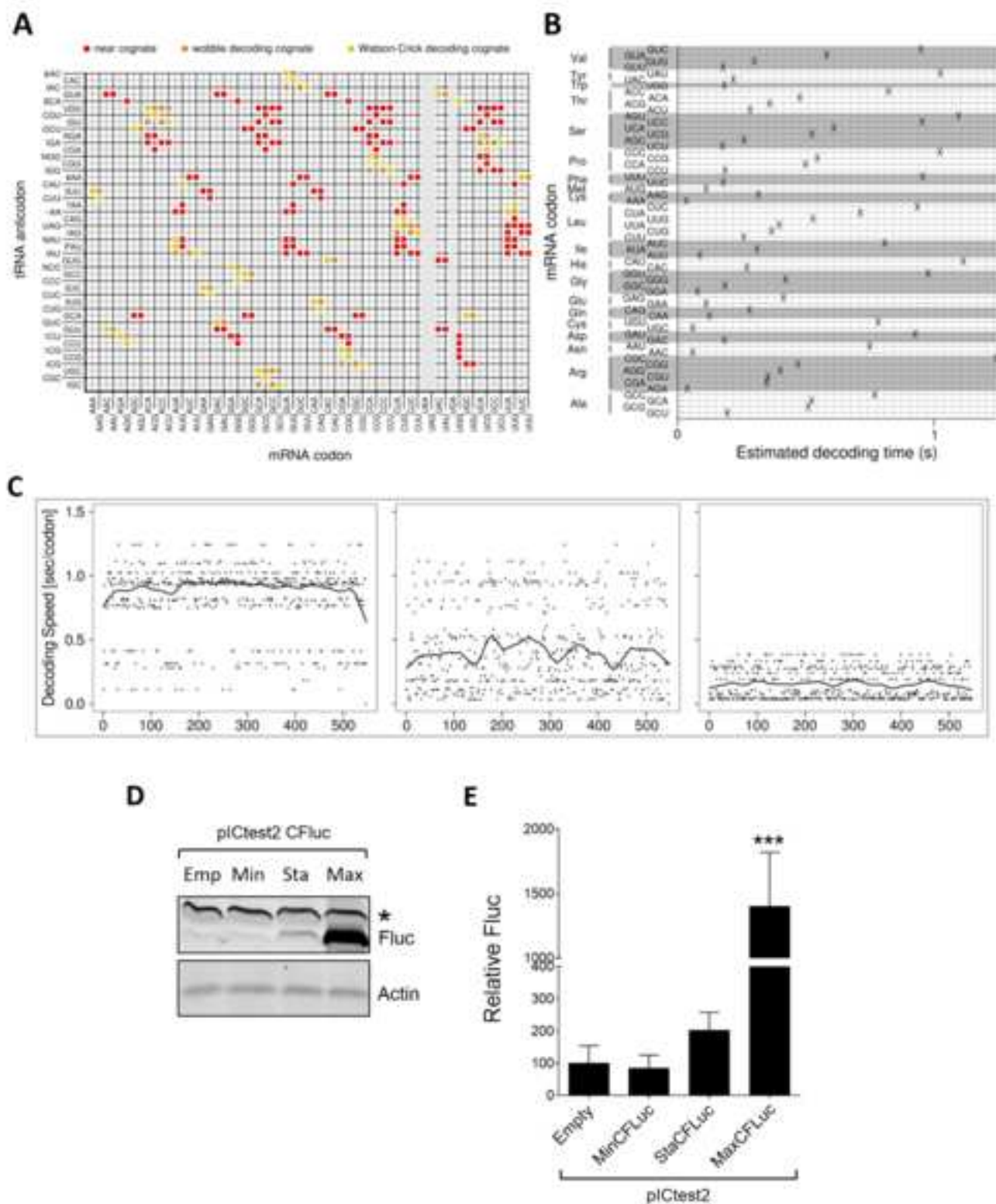


Figure S2, related to **Figure 1A, B** and **C**. Schematic presentations of the human decoding system and estimated decoding times and western blot analysis of Min/Sta/MaxCFLuc expression in HEK293 cells. **A)** The human decoding system. **B)** Estimated decoding times in the human decoding system. **C)** Speed comparison of the three luciferase sequences. For **A-C**, see Supplementary Materials & Methods for details. **D)** HEK293 cells were transfected with empty piCtest2 or with piCtest2 vector expressing Min/Sta/MaxCFLuc constructs. 48 h later, cells were lysed and Fluc protein expression levels were analysed by SDS-PAGE/WB. Actin was used as a 'loading control'. *: non-specific band. **E)** Quantification of **C**. ***, $P < 0.001$, as determined by two-way ANOVA followed by Dunnett's test. $n = 4$.

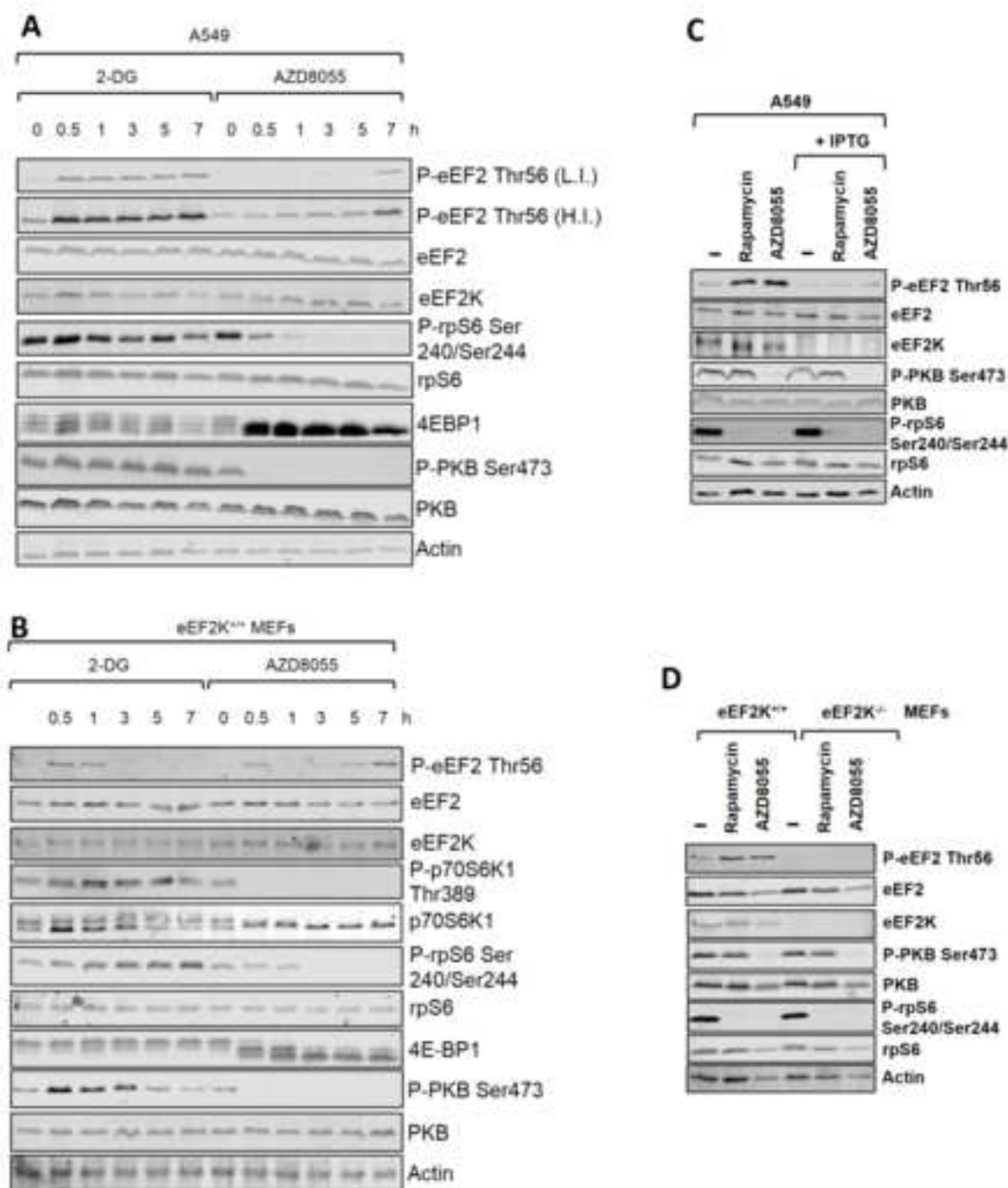


Figure S3, related to Figure 2A and B. Cell and time-dependent stimulation of eEF2 phosphorylation under 2-DG or AZD8055 treatment. A) A549 cells or eEF2^{+/+} **B)** MEFs were either pre-cultured in low glucose (5.5 mM) DMEM for 1 h, before treatment with 10 μ M 2-DG; or cultured in high glucose (11.1 mM) DMEM in the presence of 1 μ M AZD8055 for the indicated periods of time. **C)** A549 cells were cultured in growth medium and then either mock-treated with vehicle (DMSO), or treated with rapamycin (200 nM) or AZD8055 (1 μ M) for 16 h. **D)** eEF2^{+/+} and eEF2^{-/-} MEFs were incubated with vehicle (DMSO), rapamycin (200 nM) or AZD8055 (1 μ M) for 16 h. Cell lysates were analysed by SDS-PAGE/Western blotting. L. I. = light intensity; H. I. = high intensity.

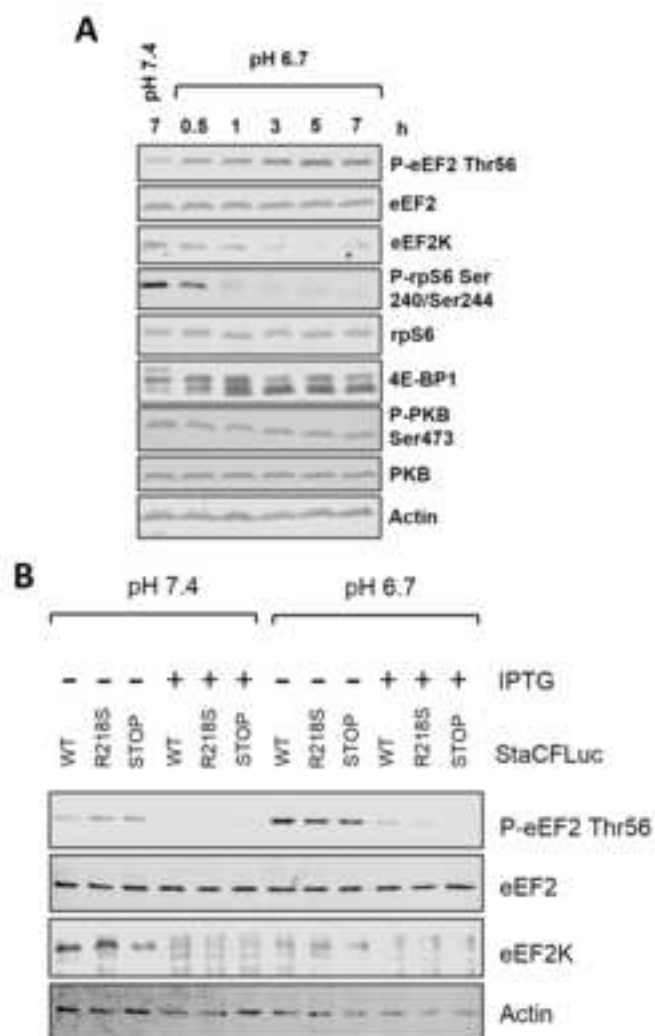


Figure S4, related to Figure 2C. Western blot analysis of A549 cells cultured under acidotic stress. A549 cells were treated with IPTG (1 μ M) for 5 days to induce the expression of shRNA against *EEF2K*. Cells were then either untransfected (**A**) or transfected with plasmids encoding (**B**) StaCFLuc or WT, R218S or STOP plasmids for 24 h, and then cultured in medium buffered at pH 7.4 or 6.7 for the indicated periods of time (in **A**), or 16 h (in **B**). Cells were then lysed and subjected to Western blot analysis with indicated antibodies.

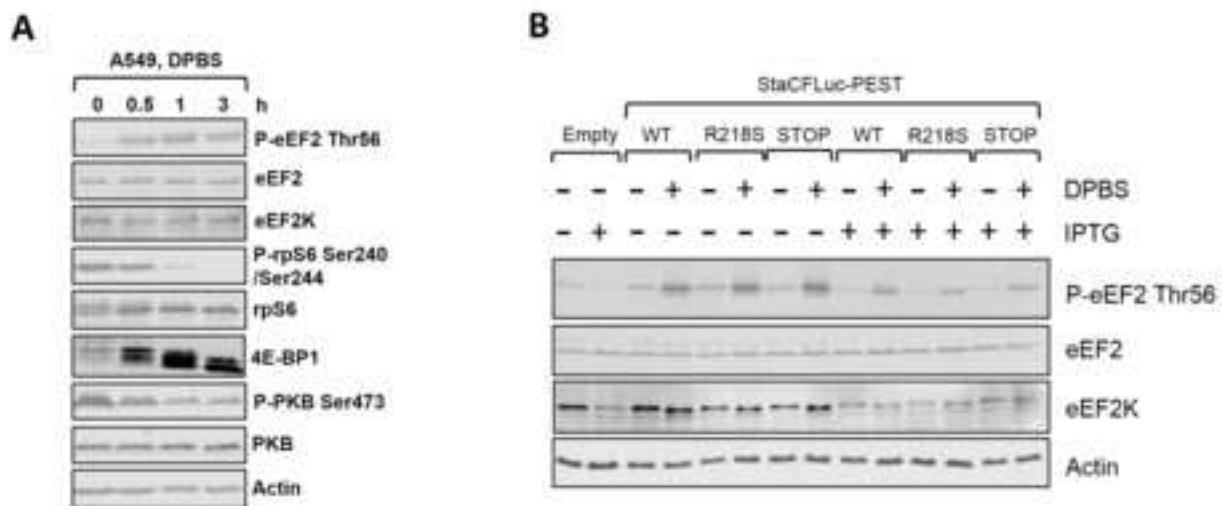


Figure S5, related to Figure 2D. Western blot analysis of A549 cells cultured under nutritional stress. A) A549 cells cultured in DPBS for up to 3 h before lysed and subjected to Western blot analysis. **B)** A549 cells were treated with IPTG (1 μ M) for 5 days to induce the expression of shRNA against *EEF2K*. Cells were then transfected with plasmids encoding StaCFLuc WT, R218S or STOP, and then cultured in DPBS for 3 h, followed by lysis and SDS-PAGE/Western blotting against indicated phosphorylated (P-) or total proteins.

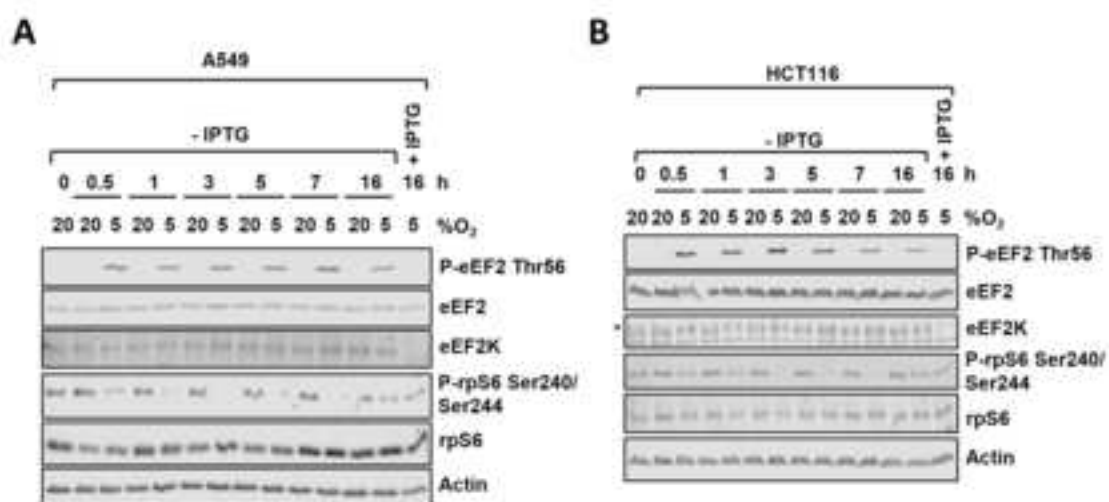


Figure S6, related to Figure 2E and F. Western blot analysis of A549 and HCT116 cells cultured under hypoxia. A549 (A) or HCT116 (B) cells were treated with IPTG (1 μ M) for 5 days to induce the expression of shRNA against *EEF2K*. Cells were then cultured in either 20% or 5% O₂ for the indicated periods of time, followed by lysis and Western blot analysis.

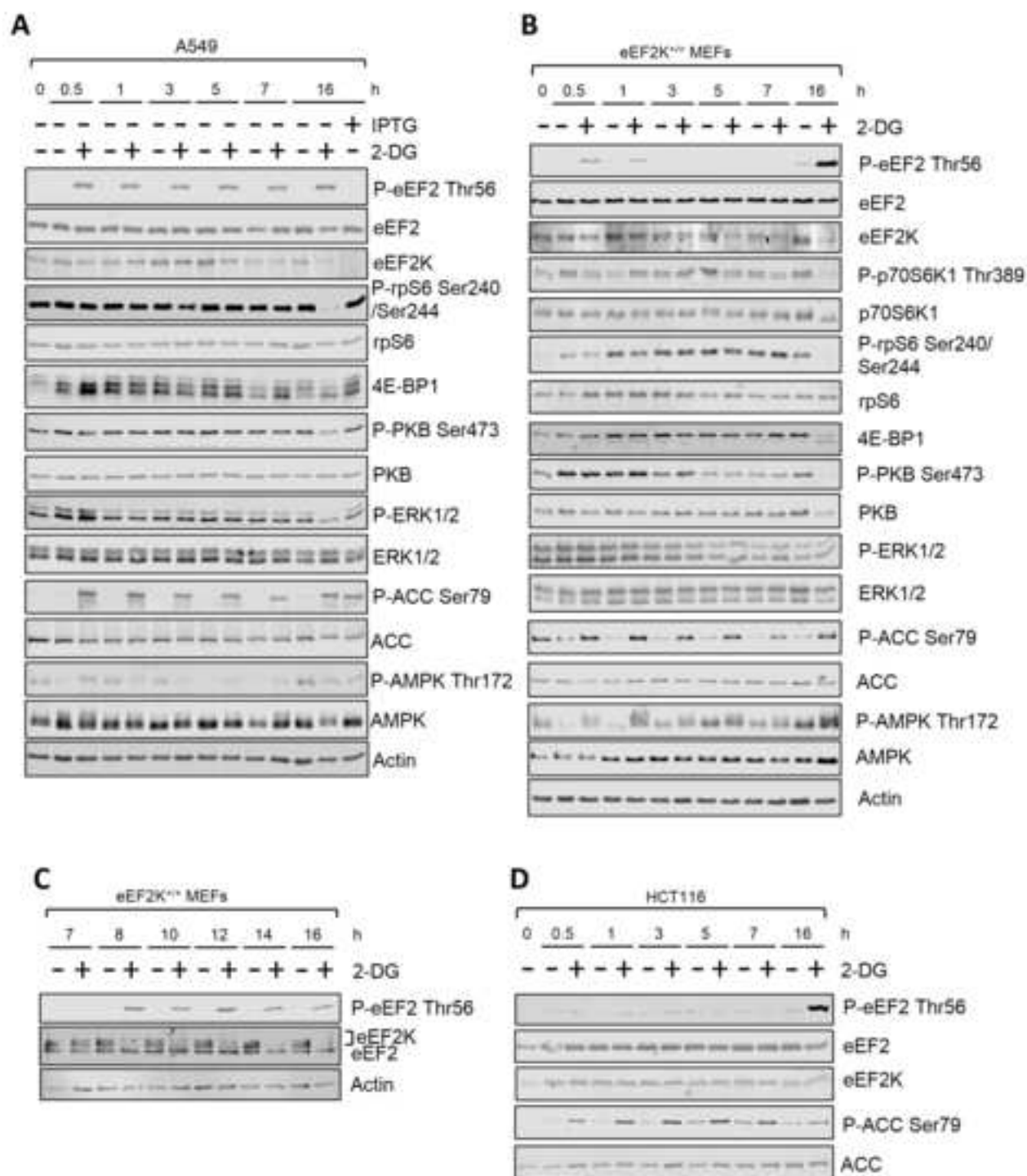


Figure S7, related to Figure 2. The effect of 2-DG on upstream and downstream mTORC signalling pathways in MEFs and HCT116 cells. A) and B, C) eEF2^{+/+} MEFs or D) HCT116 cells were pre-incubated in low glucose (5.5 mM) DMEM for 1 h, and then treated with 2-DG (10 mM) for the indicated periods of time, followed by lysis and Western blot analysis.

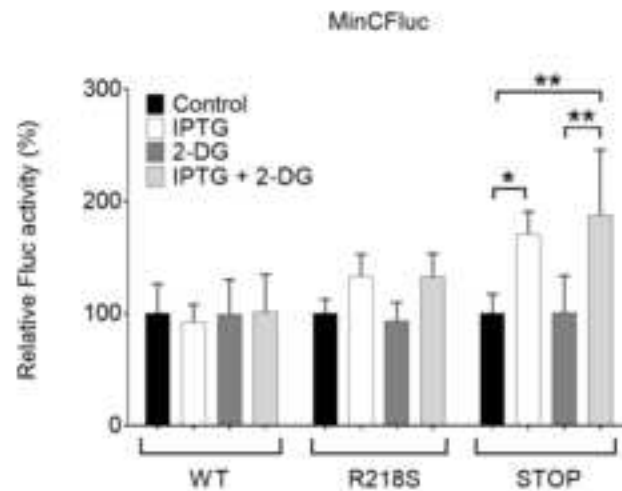
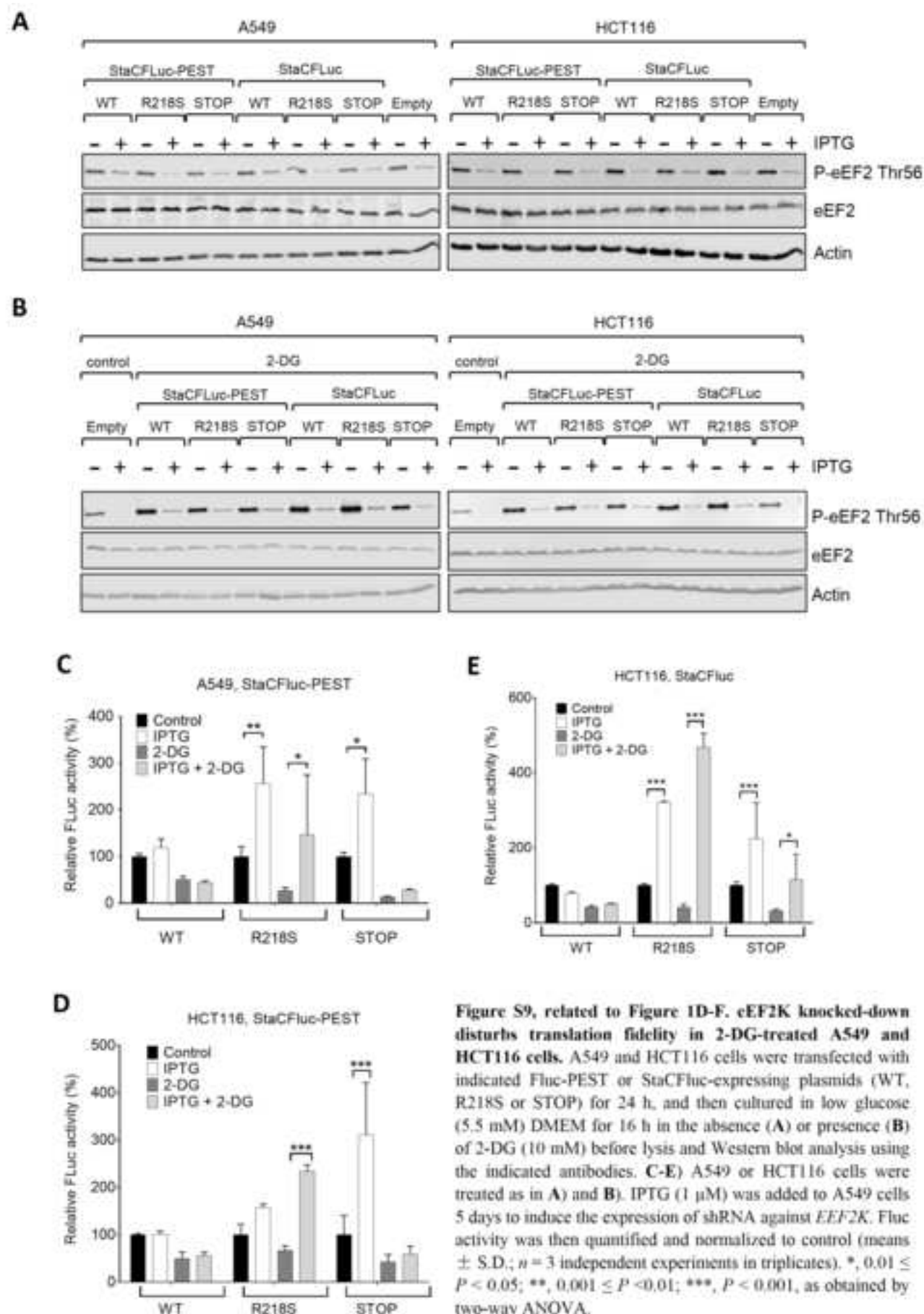


Figure S8, related to Figure 1E and F. The effect of eEF2K on translation fidelity of MinCFluc. IPTG (1 μ M) was added to A549 cells 5 days before the experiment to induce expression of an shRNA against *EEF2K*. A549 cells were transfected with MinCFluc WT, R218S or STOP constructs for 24 h, cells were then cultured in DMEM containing low glucose (5.5 mM) for 1 h, before treatment with 2-DG (10 mM) for 16 h. Fluc activity was quantified using luciferase assay and normalized to mock-treated control. Results are given as means \pm S.D.; $n = 3$ independent experiments each performed in triplicate.



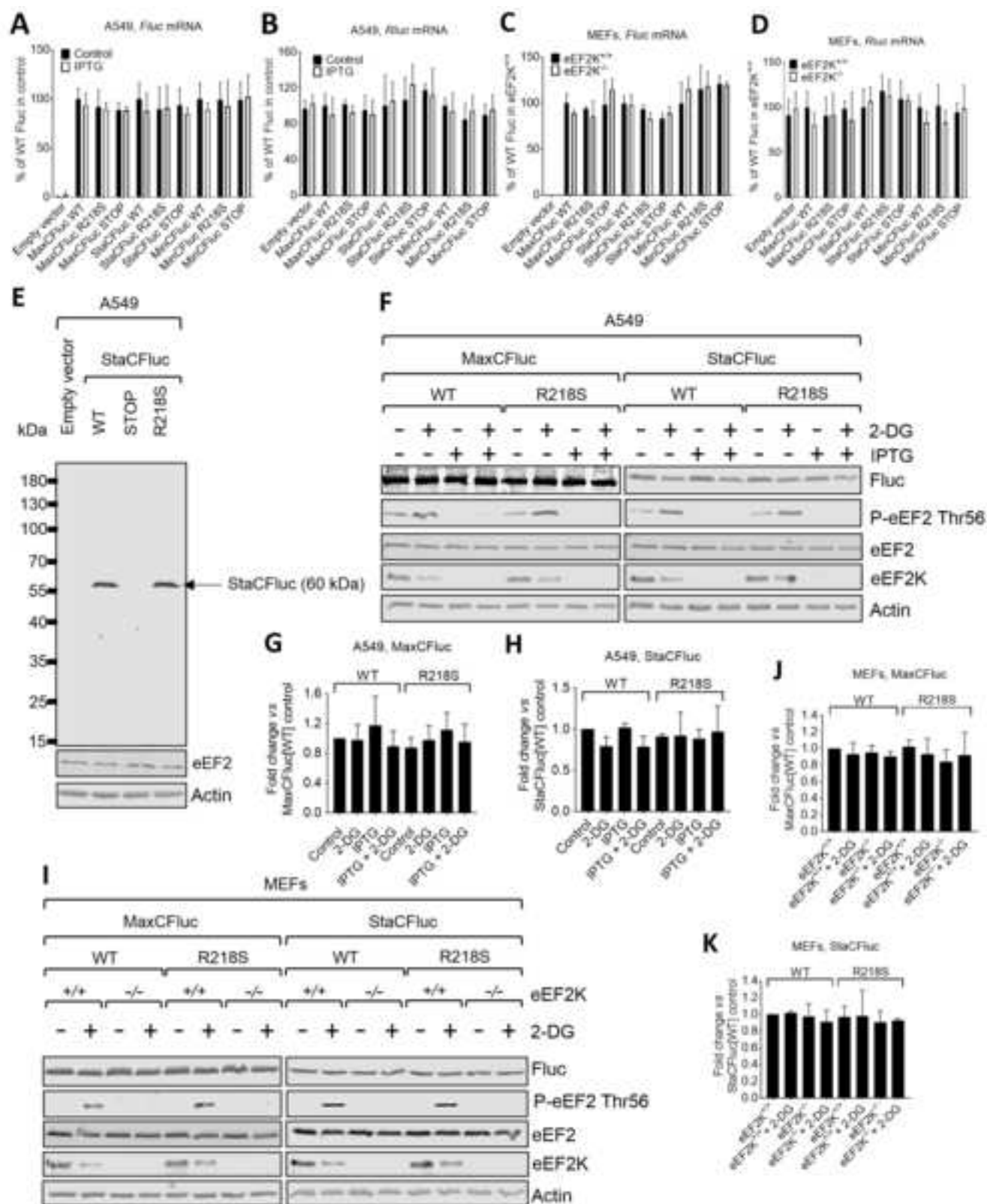


Figure S10, related to Figure 1D-F. eEF2K does not affect the mRNA and protein stability of Fluc[WT] and mutants. **A-D)** A549 cells or MEFs were transfected with indicated Fluc constructs, after 40 h, *Fluc* (in **A** and **C**) and *Rbc* (in **B** and **D**) mRNA levels in A549 (in **A** and **B**) as well as eEF2K^{+/+} and eEF2K^{-/-} MEFs (in **C** and **D**) were measured by qPCR. Human/mouse B2M were used as housekeeping genes. $n = 3$. **E-G)** A549 cells or (**H** and **I**) MEFs were transfected with indicated Fluc constructs; **E)** After 40 h, cells were lysed and immunoblotted for Fluc, eEF2 and actin (as a loading control). Note that StaCF_{Fluc}[STOP] was undetectable using our Fluc antibody. **F** and **I)** 24 h after transfection, cells were incubated in low glucose (5.5 mM) medium for 1 h, before the addition of 2-DG (10 μ M). 16 h later, cells were lysed and immunoblotted against indicated phospho- (P-) or total proteins. **G** and **H)** Quantification of Fluc levels in **F**, $n = 3$. **J** and **K)** Quantification of Fluc levels in **I**, $n = 3$.

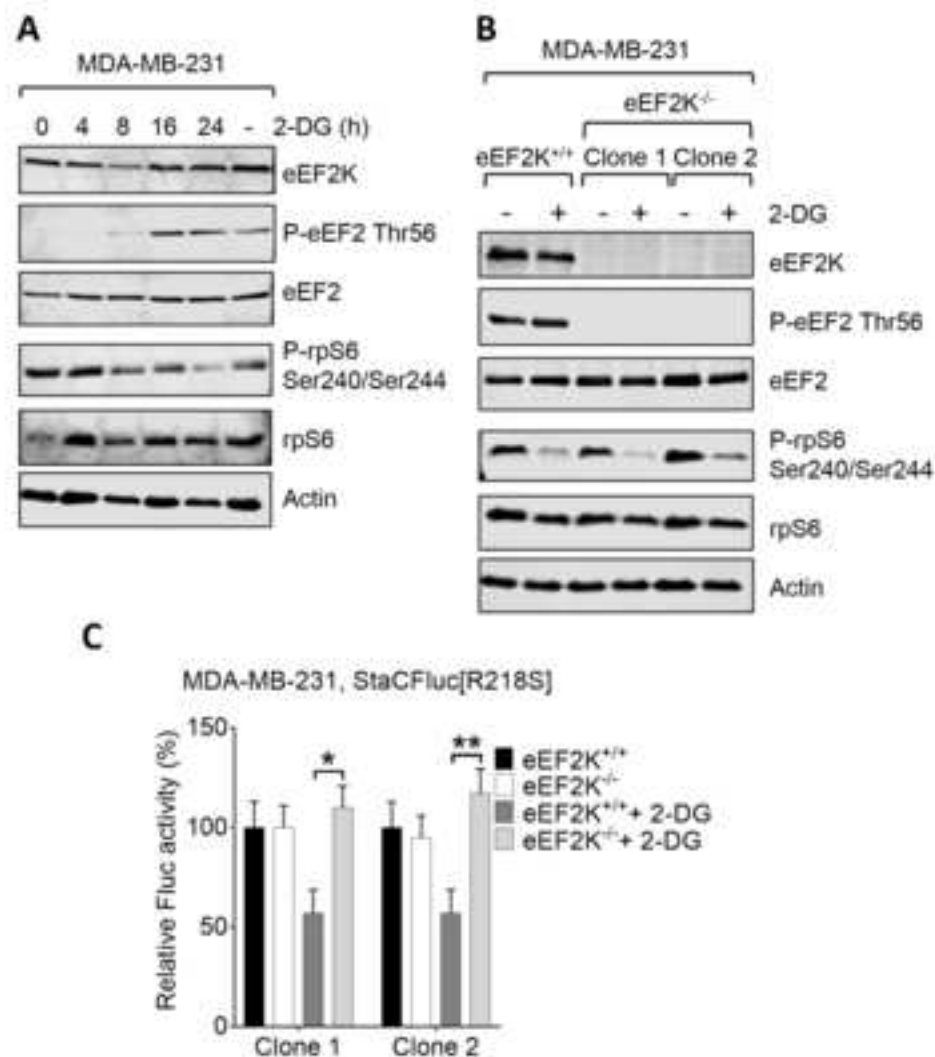


Figure S11, related to Figure 1D-F. CRISPR-Cas9-directed eEF2K knockout in MDA-MB-231 cells possess decreased translation fidelity in MDA-MB-231 cells. **A** and **B**) eEF2K WT and KO MDA-MB231 cells were cultured in low (5.5 mM) glucose DMEM for 1 h, 2-DG (10 mM) was then added and cells were cultured further for the indicated periods of time (in **A**) or for 1 h (in **B**), followed by cell lysis and immunoblotting analysis. **C**) eEF2K WT and KO MDA-MB231 cells were transfected with StaCFluc R218S construct. After 24 h, cells were then cultured in DMEM containing low glucose (5.5 mM) for 1 h, before treatment with 2-DG (10 mM) for 24 h. Fluc activity was quantified by luciferase assay and normalized to mock-treated control. Results are given as means \pm S.D.; $n = 5$ independent experiments each performed in triplicate.

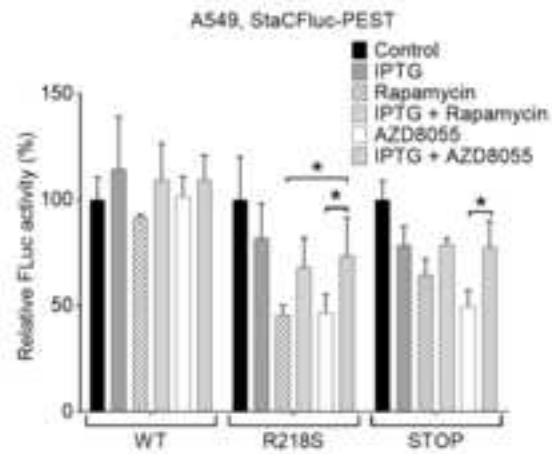


Figure S12, related to Figure 2A. eEF2K knocked-down impairs StaCFluc-PEST translation fidelity in 2-DG-treated A549 cells. IPTG (1 μ M) was added to A549 cells 5 days before the experiment to induce the expression of an shRNA against *EEF2K*. A549 cells were transfected with StaCFluc-PEST WT, R218S or STOP constructs. After 24 h, cells were treated with vehicle (DMSO, as a control), rapamycin (200 nM) or AZD8055 (1 μ M) for 16 h. Fluc activity was then measured and is expressed as means \pm S.D.; $n = 4$ independent experiments conducted in triplicate.

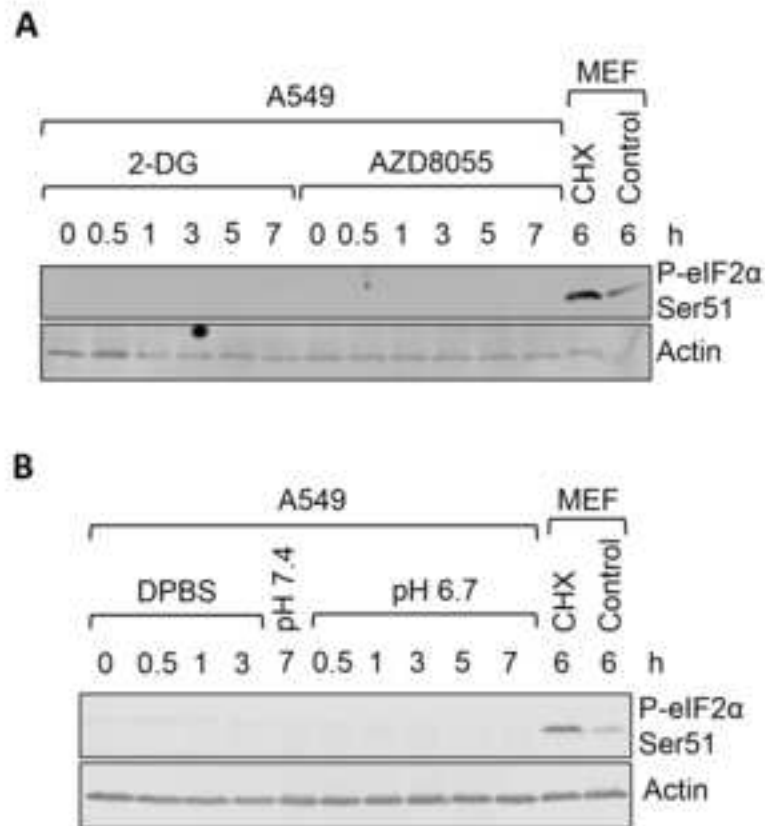


Figure S13, related to Figure 2. Impaired glucose metabolism, mTORC inhibition, acidotic and nutritional stresses are unable to induce eIF2 α phosphorylation. A) and B) A549 cells were cultured in low (5.5 mM) glucose DMEM for 1 h, 2-DG (10 mM) was then added and cells were further cultured for the indicated periods of time. Untreated or cycloheximide (5 μ g/ml, 6 h)-treated MEFs were used as controls. In **A, A549 cells were also treated with AZD8055 (1 μ M); and in **B**, A549 cells were also incubated in medium buffered at pH (7.4 or 6.7), for the indicated period of times. Cells were then lysed, and samples were analysed by immunoblotting with the indicated antibodies.**

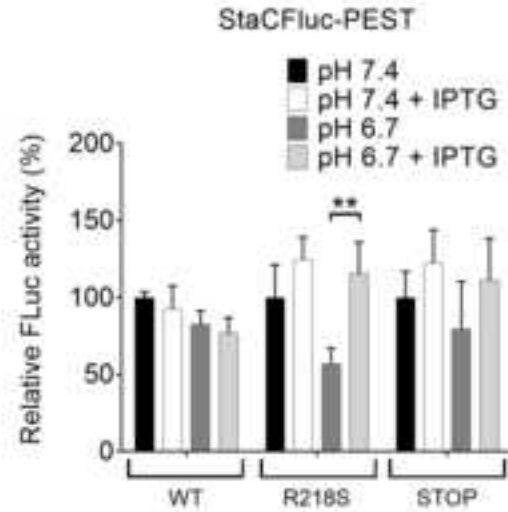


Figure S14, related to Figure 2C-F. The effect of eEF2K knocked-down in StaCFluc-PEST translation fidelity in A549 cells cultured under acidic pH. A549 cells were transfected with StaCFluc-PEST WT, R218S or STOP constructs for 24 h, and were then incubated in pH (7.4 or 6.7) buffered medium for 16 h, before luciferase assay analysis. Fluc activity is expressed as means \pm S.D.; n = 4 independent experiments performed in triplicate.

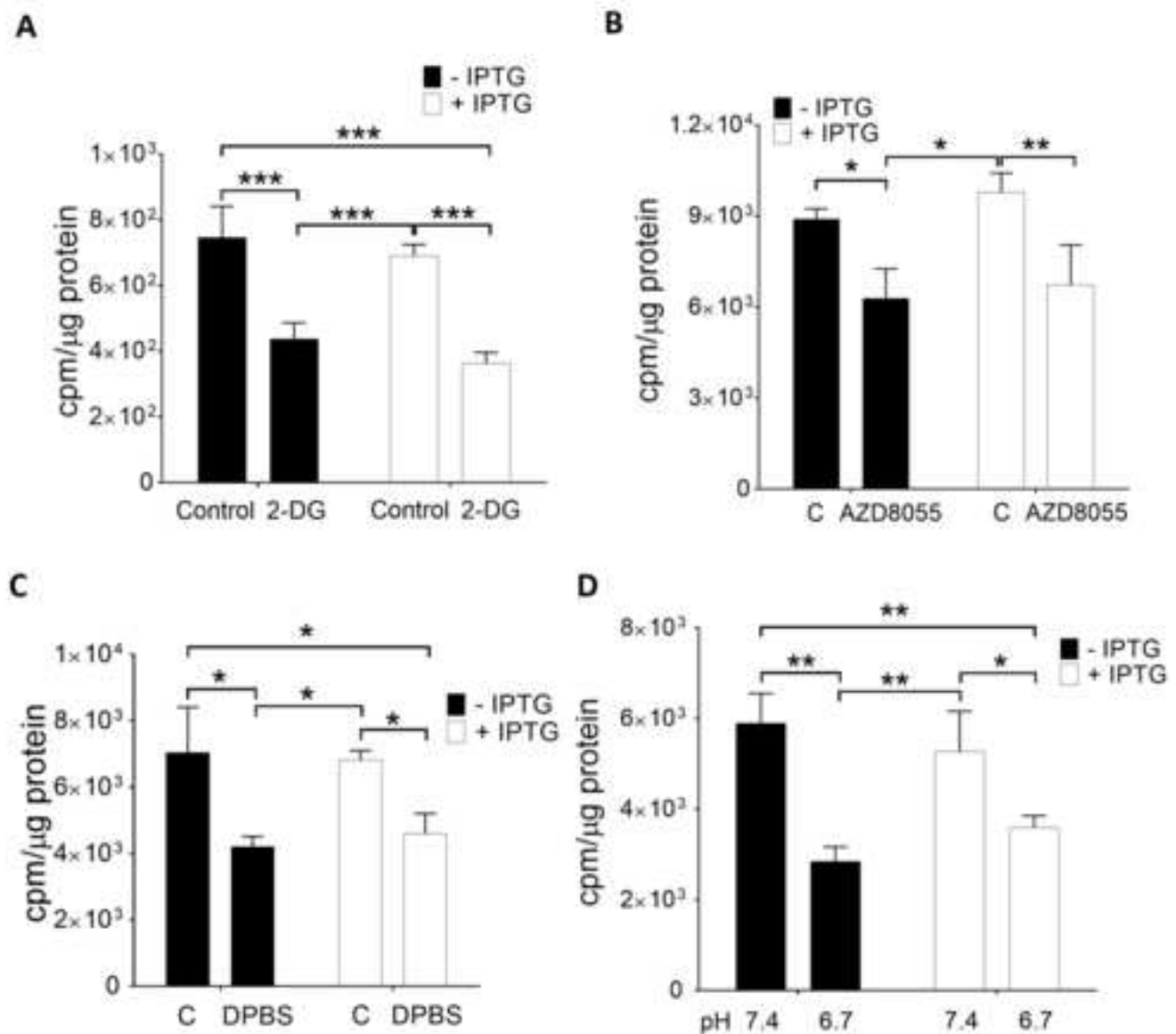


Figure S15, related to Figure 2. eEF2K does not affect global protein synthesis when treated with 2-DG, mTORC inhibitor, or under stresses in A549 cells. For all experiments, IPTG (1 μ M) was added to A549 cells 5 days before experiment to induce the expression of shRNA against *EEF2K*. A549 cells were then: **A)** cultured in Met/Cys-free DMEM supplemented with 5.5 mM glucose for 1 h, before the treatment with 2-DG (10 mM) for 16 h; **B)** cultured in Met/Cys-free DMEM and treated with AZD8055 (1 μ M) for 16 h; **C)** incubated in Met/Cys-free growth medium or DPBS for 3 h; or **D)** incubated in Met/Cys-free pH (7.4 or 6.7) buffered DMEM for 16 h; in the presence of 10 μ Ci [³⁵S]Met-Cys. Rates of protein synthesis were determined, results were presented as counts per minute (cpm) per μ g protein, and expressed as means \pm S.D.; $n = 3$ independent experiments. *, $0.01 \leq P < 0.05$; **, $0.001 \leq P < 0.01$; ***, $P < 0.001$, as obtained by two-way ANOVA.

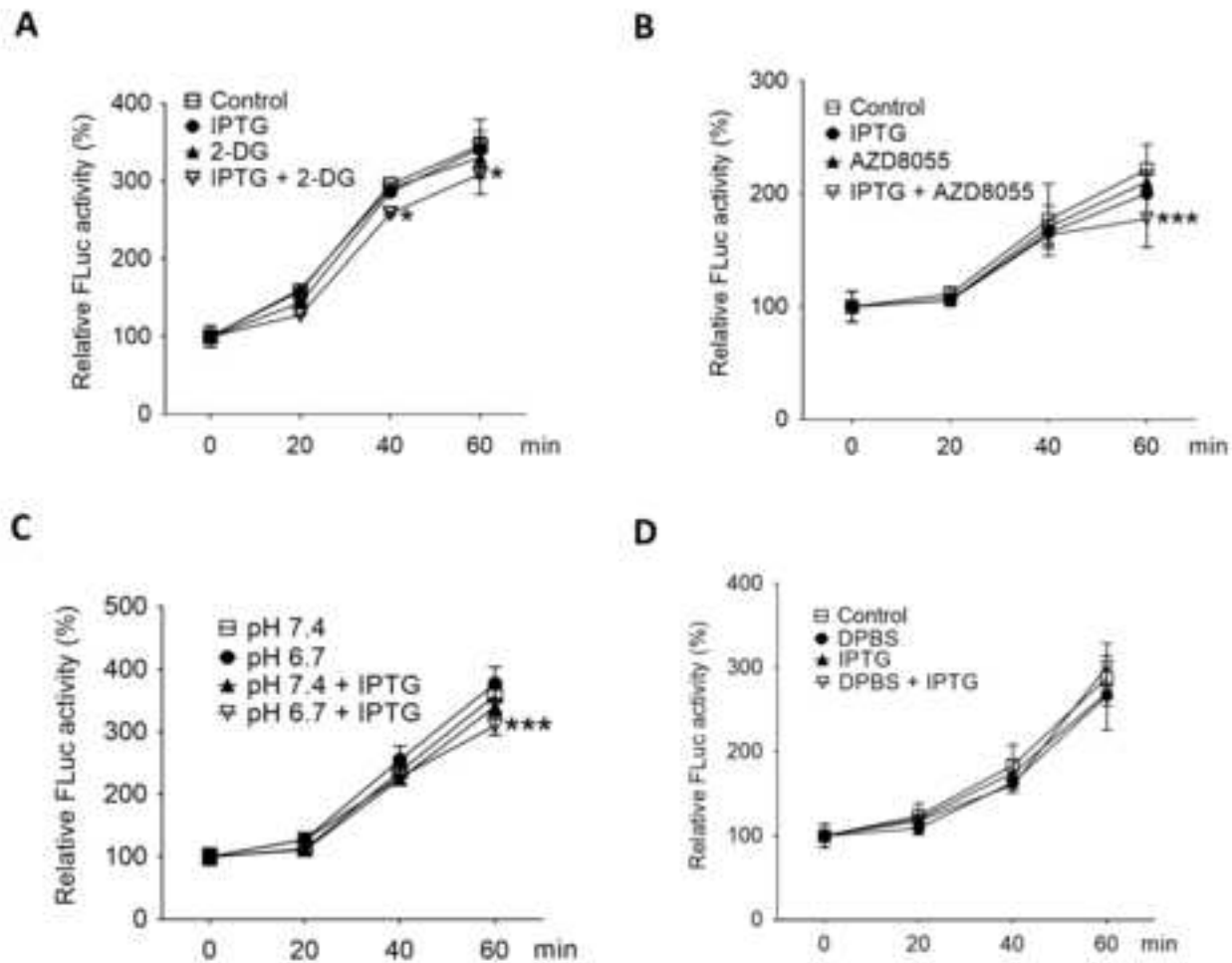


Figure S16, related to Figure 2. The role of cEF2K in the maintenance of chaperon activity. For all experiments, IPTG (1 μ M) was added to A549 cells 5 days before experiment to induce the expression of shRNA against I. **A)** A549 cells were pre-incubated in low glucose (5.5 mM) DMEM for 1 h before the addition of 2-DG (10 mM) for 16 h. **B)** A549 cells were treated with either vehicle (DMSO) as a control or AZD8055 (1 μ M) for 16 h. **C)** A549 cells were cultured in DMEM buffered at pH 7.4 or 6.7 for 16 h. **D)** A549 cells were cultured in growth medium or DPBS for 3 h. Cells were then lysed and lysates were incubated with previously heat-denatured Fluc at room temperature. Fluc refolding was assessed by measuring Fluc activity at the indicated time points. Data are shown as percentage of control (means \pm S.D.; $n = 3$ independent experiments in triplicates). *, $0.01 \leq P < 0.05$; ***, $P < 0.001$ compared to non-treated controls, obtained by two-way ANOVA.

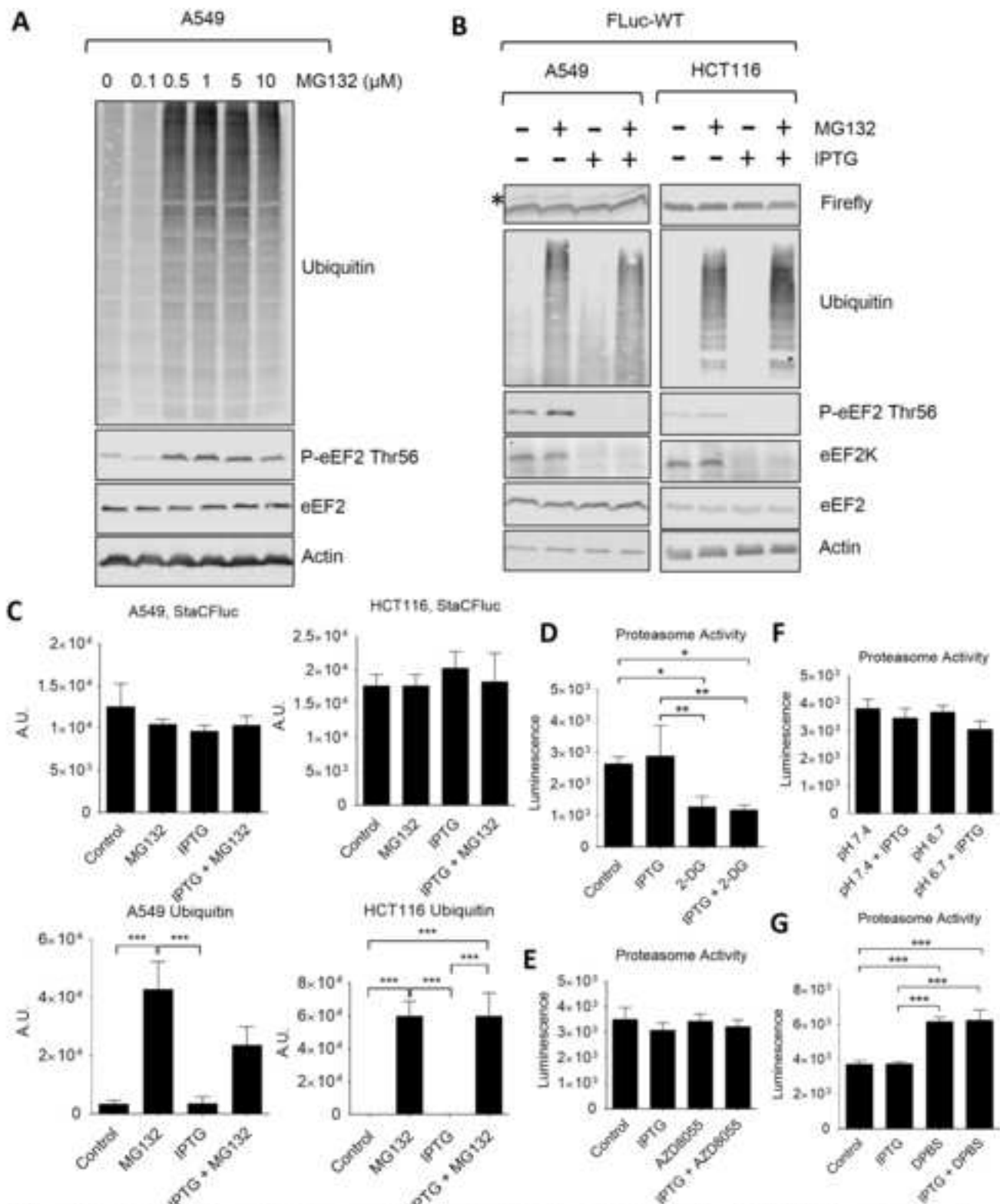


Figure S17, related to Figure 2. Proteasome activity is not affected by eEF2K activation. **A**) A549 cells were treated with the indicated concentrations of MG132 for 16 h. **B**) A549 and HCT116 cells were transfected with WT StaCFluc, and then treated with 10 μM MG132 for 16 h. *: non-specific band. For **A** and **B**, cells were then lysed and proteins were immunoblotted with indicated antibodies. **C**) Densitometric quantifications (of Fluc and ubiquitin) of **B**. **D**) A549 cells were cultured in low glucose (5.5 mM) medium for 1 h, followed by the addition of 2-DG (10 mM) for a period of 16 h. Results are shown in arbitrary units (A.U.) and expressed as means \pm S.D. ($n = 6$ (A549) or 3 (HCT116) independent experiments). **E**) A549 cells were cultured with vehicle (DMSO) or AZD8055 (1 μM) for 16 h. **F**) A549 cells were incubated in DMEM buffered at pH (7.4 or 6.7) for 16 h. **G**) A549 cells were cultured in growth medium or DPBS for 3 h. For **B-G**, cells were treated with IPTG (1 μM) for 5 days to induce the expression of shRNA against *EEF2K*. In **D-G**, proteasome activity was measured by the Proteasome-GloTM chymotrypsin-like cell based assay system at the end of the incubation period. *, $0.01 \leq P < 0.05$; **, $0.001 \leq P < 0.01$; ***, $P < 0.001$, as obtained by one-way ANOVA.

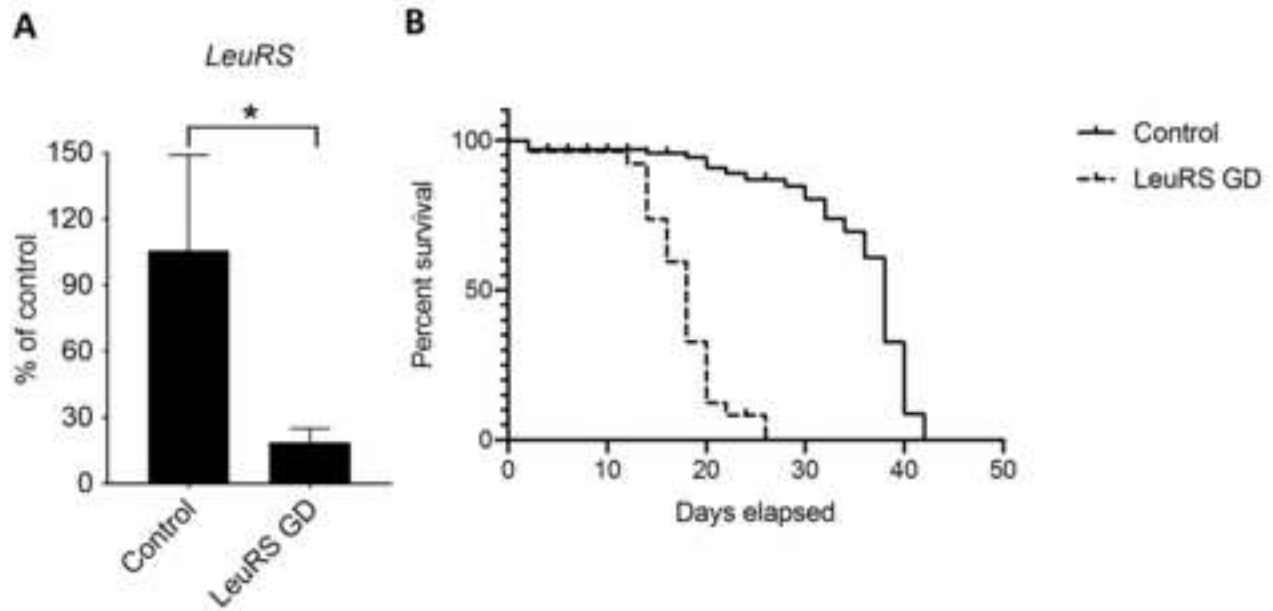


Figure S18, related to Figure 6A. *LeuRS* knocked down in *D. melanogaster* reduces lifespan. A) qPCR analysis of *Drosophila LeuRS* (CG33123) mRNA levels in control (*Actin-GAL4⁺; tub-GAL80^{+/+}*) and *LeuRS* ubiquitous knockdown (*Actin-GAL4⁺; tub-GAL80^{+/+}/UAS-LeuRS GD*) flies after 2 days at 29°C. **B)** Survival curve comparison for *LeuRS* ubiquitous knockdown during adult stages compared to control. Percent survival of flies is graphed against survival time (in days). Two independent experiments were performed: Experiment 1: control: $n = 125$, median survival: 32 days; *LeuRS GD*: $n = 64$, median survival: 18 days; Experimental 2: control: $n = 94$, median survival: 38 days; *LeuRS GD*: $n = 54$, median survival: 18 days, $P < 0.001$.

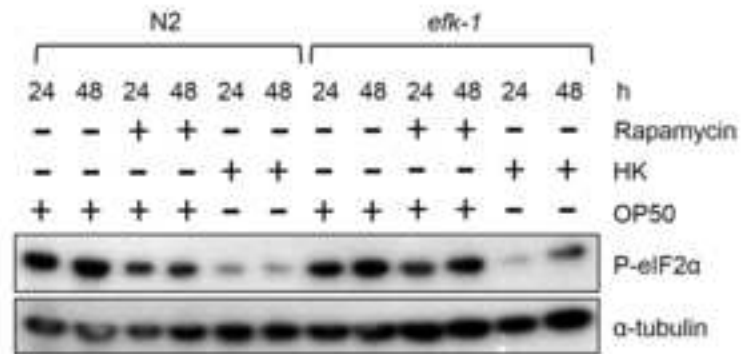


Figure S19, related to Figure 6B and D. *efk-1* knocked down in *C. elegans* does not alter eIF2 α phosphorylation.

C. elegans worms were synchronized to L1 stage, *efk-1* gene knockdown was introduced by feeding using HT115(DE3) host bacteria transformed with the RNAi. After the indicated periods of times, L4 worms (30 worms/sample) were collected and subjected to SDS-PAGE/immunoblotting against P-eIF2 α , as well as α -tubulin as a loading control.

Supplementary figure legends

Figure S1, related to Figure 1A, B and C. Maps of reporter plasmids highlighting functional features. A) pICtest2 empty; B) pICtest2 MinCFluc; C) pICtest2 StaCFluc; D) pICtest2 MaxCFluc; E) pICtest2 Fluc-PEST.

Figure S2, related to Figure 1A, B and C. Schematic presentations of the human decoding system and estimated decoding times and western blot analysis of Min/Sta/MaxCFluc expression in HEK293 cells. A) The human decoding system. B) Estimated decoding times in the human decoding system. C) Speed comparison of the three luciferase sequences. For A-C, see Supplementary Materials & Methods for details. D) HEK293 cells were transfected with empty pICtest2 or with pICtest2 vector expressing Min/Sta/MaxCFluc constructs. 48 h later, cells were lysed and Fluc protein expression levels were analysed by SDS-PAGE/WB. Actin was used as a 'loading control'. *: non-specific band. E) Quantification of C. ***, $P < 0.001$, as determined by two-way ANOVA followed by Dunnett's test. $n = 4$.

Figure S3, related to Figure 2A and B. Cell and time-dependent stimulation of eEF2 phosphorylation under 2-DG or AZD8055 treatment. A) A549 cells or eEF2K^{+/+} B) MEFs were either pre-cultured in low glucose (5.5 mM) DMEM for 1 h, before treatment with 10 μ M 2-DG; or cultured in high glucose (11.1 mM) DMEM in the presence of 1 μ M AZD8055 for the indicated periods of time. C) A549 cells were cultured in growth medium and then either mock-treated with vehicle (DMSO), or treated with rapamycin (200 nM) or AZD8055 (1 μ M) for 16 h. D) eEF2K^{+/+} and eEF2K^{-/-} MEFs were incubated with vehicle (DMSO), rapamycin (200 nM) or AZD8055 (1 μ M) for 16 h. Cell lysates were analysed by SDS-PAGE/Western blotting. L. I. = light intensity; H. I. = high intensity.

Figure S4, related to Figure 2C. Western blot analysis of A549 cells cultured under acidotic stress. A549 cells were treated with IPTG (1 μ M) for 5 days to induce the expression of shRNA against *EEF2K*. Cells were then either untransfected (A) or transfected with plasmids encoding (B) StaCFluc or WT, R218S or STOP plasmids for 24 h, and then cultured in medium buffered at pH 7.4 or 6.7 for the indicated periods of time (in A), or 16 h (in B). Cells were then lysed and subjected to Western blot analysis with indicated antibodies.

Figure S5, related to Figure 2D. Western blot analysis of A549 cells cultured under nutritional stress. A) A549 cells cultured in DPBS for up to 3 h before lysed and subjected to Western blot analysis. B) A549 cells were treated with IPTG (1 μ M) for 5 days to induce the expression of shRNA against *EEF2K*. Cells were then transfected with plasmids encoding StaCFluc WT, R218S or STOP, and then cultured in DPBS for 3 h, followed by lysis and SDS-PAGE/Western blotting against indicated phosphorylated (P-) or total proteins.

Figure S6, related to Figure 2E and F. Western blot analysis of A549 and HCT116 cells cultured under hypoxia. A549 (A) or HCT116 (B) cells were treated with IPTG (1 μ M) for 5 days to induce the expression of shRNA against *EEF2K*. Cells were then cultured in either 20% or 5% O₂ for the indicated periods of time, followed by lysis and Western blot analysis.

Figure S7, related to Figure 2. The effect of 2-DG on upstream and downstream mTORC signalling pathways in MEFs and HCT116 cells. A) and B, C) eEF2K^{+/+} MEFs or D) HCT116 cells were pre-incubated in low glucose (5.5 mM) DMEM for 1 h, and then treated with 2-DG (10 mM) for the indicated periods of time, followed by lysis and Western blot analysis.

Figure S8, related to Figure 1E and F. The effect of eEF2K on translation fidelity of MinCFluc. IPTG (1 μ M) was added to A549 cells 5 days before the experiment to induce expression of an shRNA against *EEF2K*. A549 cells were transfected with MinCFluc WT, R218S or STOP constructs for 24 h, cells were then cultured in DMEM containing low glucose (5.5 mM) for 1 h, before treatment with 2-DG (10 mM) for 16 h. Fluc activity was quantified using luciferase assay and normalized to mock-treated control. Results are given as means \pm S.D.; $n = 3$ independent experiments each performed in triplicate.

Figure S9, related to Figure 1D-F. eEF2K knocked-down disturbs translation fidelity in 2-DG-treated A549 and HCT116 cells. A549 and HCT116 cells were transfected with indicated Fluc-PEST or StaCFluc-expressing plasmids (WT, R218S or STOP) for 24 h, and then cultured in low glucose (5.5 mM) DMEM for 16 h in the absence (A) or presence (B) of 2-DG (10 mM) before lysis and Western blot analysis using the indicated antibodies. C-E) A549 or HCT116 cells were treated as in A) and B). IPTG (1 μ M) was added to A549 cells 5 days to induce the expression of shRNA against *EEF2K*. Fluc activity was then quantified and normalized to control (means \pm S.D.; $n = 3$ independent experiments in triplicates). *, $0.01 \leq P < 0.05$; **, $0.001 \leq P < 0.01$; ***, $P < 0.001$, as obtained by two-way ANOVA.

Figure S10, related to Figure 1D-F. eEF2K does not affect the mRNA and protein stability of Fluc[WT] and mutants. A-D) A549 cells or MEFs were transfected with indicated Fluc constructs, after 40 h, *Fluc* (in A and C) and *Rluc* (in B and D) mRNA levels in A549 (in A and B) as well as eEF2K^{+/+} and eEF2K^{-/-} MEFs (in C and D) were measured by qPCR. Human/mouse B2M were used as housekeeping mRNAs. $n = 3$. A549 cells (E-G) or MEFs (H and I) were transfected with indicated Fluc constructs; E) After 40 h, cells were lysed and immunoblotted for Fluc, eEF2 and actin (as a loading control). Note that StaCFluc[STOP] was undetectable using our Fluc antibody. F and I) 24 h after transfection, cells were incubated in low glucose (5.5 mM) medium for 1 h, before the addition of 2-DG (10 μ M). 16 h later, cells were lysed and subjected to immunoblot analysis for the indicated phospho- (P-) or total proteins. G and H) Quantification of Fluc levels in F. $n = 3$. J and K) Quantification of Fluc levels in I. $n = 3$.

Figure S11, related to Figure 1D-F. CRISPR-Cas9-directed eEF2K knockout in MDA-MB-231 cells possess decreased translation fidelity in MDA-MB-231 cells. A and B) eEF2K WT and KO MDA-MB231 cells were cultured in low (5.5 mM) glucose DMEM for 1 h. 2-DG (10 mM) was then added and cells were cultured further for the indicated periods of time (in A) or for 1 h (in B), followed by cell lysis and immunoblotting analysis. C) eEF2K WT and KO MDA-MB231 cells were transfected with StaCFluc R218S construct. After 24 h, cells were then cultured in DMEM containing low glucose (5.5 mM) for 1 h, before treatment with 2-DG (10 mM) for 24 h. Fluc activity was quantified by luciferase assay and normalized to mock-treated control. Results are given as means \pm S.D.; $n = 5$ independent experiments each performed in triplicate.

Figure S12, related to Figure 2A. eEF2K knocked-down impairs StaCFluc-PEST translation fidelity in 2-DG-treated A549 cells. IPTG (1 μ M) was added to A549 cells 5 days before the experiment to induce the expression of an shRNA against *EEF2K*. A549 cells were transfected with StaCFluc-PEST WT, R218S or STOP constructs. After 24 h, cells were treated with vehicle (DMSO, as a control), rapamycin (200 nM) or AZD8055 (1 μ M) for 16 h. Fluc activity was then measured and is expressed as means \pm S.D.; $n = 4$ independent experiments conducted in triplicate.

Figure S13, related to Figure 2. Impaired glucose metabolism, mTORC inhibition, acidotic and nutritional stresses are unable to induce eIF2 α phosphorylation. **A)** and **B)** A549 cells were cultured in low (5.5 mM) glucose DMEM for 1 h, 2-DG (10 mM) was then added and cells were further cultured for the indicated periods of time. Untreated or cycloheximide (5 μ g/ml, 6 h)-treated MEFs were used as controls. In **A**, A549 cells were also treated with AZD8055 (1 μ M); and in **B**, A549 cells were also incubated in medium buffered at pH (7.4 or 6.7), for the indicated period of times. Cells were then lysed, and samples were analysed by immunoblotting with the indicated antibodies.

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Figure S16, related to Figure 2. The role of eEF2K in the maintenance of chaperon activity. For all experiments, IPTG (1 μ M) was added to A549 cells 5 days before experiment to induce the expression of shRNA against I. **A)** A549 cells were pre-incubated in low glucose (5.5 mM) DMEM for 1 h before the addition of 2-DG (10 mM) for 16 h. **B)** A549 cells were treated with either vehicle (DMSO) as a control or AZD8055 (1 μ M) for 16 h. **C)** A549 cells were cultured in DMEM buffered at pH 7.4 or 6.7 for 16 h. **D)** A549 cells were cultured in growth medium or DPBS for 3 h. Cells were then lysed and lysates were incubated with previously heat-denatured Fluc at room temperature. Fluc refolding was assessed by measuring Fluc activity at the indicated time points. Data are shown as percentage of control (means \pm S.D.; $n = 3$ independent experiments in triplicates). *, $0.01 \leq P < 0.05$; ***, $P < 0.001$ compared to non-treated controls, obtained by two-way ANOVA.

Figure S17, related to Figure 2. Proteasome activity is not affected by eEF2K activation. **A)** A549 cells were treated with the indicated concentrations of MG132 for 16 h. **B)** A549 and HCT116 cells were transfected with WT StaCFluc, and then treated with 10 μ M MG132 for 16 h. *: non-specific band. For **A** and **B**, cells were then lysed and proteins were immunoblotted with indicated antibodies. **C)** Densitometric quantifications (of Fluc and ubiquitin) of **B**. **D)** A549 cells were cultured in low glucose (5.5 mM) medium for 1 h, followed by the addition of 2-DG (10 mM) for a period of 16 h. Results are shown in arbitrary units (A.U.) and expressed as means \pm S.D. ($n = 6$ (A549) or 3 (HCT116) independent experiments). **E)** A549 cells were cultured with vehicle (DMSO) or AZD8055 (1 μ M) for 16 h. **F)** A549 cells were incubated in DMEM buffered at pH (7.4 or 6.7) for 16 h. **G)** A549 cells were cultured in growth medium or

DPBS for 3 h. For **B-G**, cells were treated with IPTG (1 μ M) for 5 days to induce the expression of shRNA against *EEF2K*. In **D-G**, proteasome activity was measured by the Proteasome-Glo™ chymotrypsin-like cell based assay system at the end of the incubation period. *, $0.01 \leq P < 0.05$; **, $0.001 \leq P < 0.01$; ***, $P < 0.001$, as obtained by one-way ANOVA.

Figure S18, related to Figure 6A. LeuRS knock-down in *D. melanogaster* reduces lifespan.

A) qPCR analysis of *Drosophila* *LeuRS* (CG33123) mRNA levels in control (*Actin-GAL4/+; tub-GAL80^{ts}/+*) flies and flies with ubiquitous knockdown of *LeuRS* (*Actin-GAL4/+; tub-GAL80^{ts}/UAS-LeuRS GD*) after 2 days at 29°C. **B)** Survival curve comparison for *LeuRS* ubiquitous knockdown during adult stages compared to control. Percent survival of flies is graphed against survival time (in days). Two independent experiments were performed: Experiment 1: control: $n = 125$, median survival: 32 days; *LeuRS GD*: $n = 64$, median survival: 18 days; Experimental 2: control: $n = 94$, median survival: 38 days; *LeuRS GD*: $n = 54$, median survival: 18 days. $P < 0.001$.

Figure S19, related to Figure 6B and D. *efk-1* knock-down in *C. elegans* does not alter eIF2 α phosphorylation.

C. elegans worms were synchronized to L1 stage, *efk-1* mRNA knockdown was introduced by feeding using HT115(DE3) host bacteria transformed with the RNAi. After the indicated periods of times, L4 worms (30 worms/sample) were collected and subjected to SDS-PAGE/immunoblotting against P-eIF2 α , as well as α -tubulin as a loading control.