nature portfolio

# Peer Review File

# The mechanism of mRNA cap recognition

Corresponding Author: Professor Ruben Gonzalez Jr

**This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version**

**Redactions – unpublished data**
Parts of this Peer Review File have been redacted as indicated to maintain the confidentiality of unpublished data.

.

Version 0:

Reviewer comments:

Referee #1

(Remarks to the Author)
Gentry et al. report a novel method to directly and quantitatively observe mRNA activation by the trimeric translation initiation factor eIF4F, in real time and at the single-molecule level. The authors present thought-provoking findings that have important implications for a central mechanistic paradigm in protein synthesis and its regulation. In particular, the authors demonstrate convincingly that the core eIF4F subunit, eIF4G, intrinsically binds mRNA rapidly and distributively along its length, with high longevity on the timescale of translation initiation. The authors show that the dynamics of this interaction are independent of the mRNA cap. Thus, the results challenge the conventional view that the mRNA 5' cap is the initial contact between mRNA and eIF4F. Furthermore, the data place the eIF4F helicase subunit, eIF4A, as playing a central role in driving specific recognition of the mRNA cap, by disassembling eIF4EG•mRNA complexes that do not engage the cap. This contrasts with the expectation that the principal role of eIF4A is to resolve secondary structures near the mRNA 5' end. Taken together, the findings highlight a need to rethink textbook models for the mechanism of initiation of eukaryotic protein synthesis.

The manuscript and its findings are conceptually and technologically of broad interest within and beyond the field of translation initiation. The experimental approach is valid and highly appropriate for studying mRNA activation, and has produced high-quality data which are presented clearly throughout. The authors include appropriate statistical analyses of the data and comparisons between conditions.

The conclusions are overall robust, valid, and reliable, and the experiments are well-controlled and very carefully and appropriately analyzed and interpreted to ensure this. However, a number of aspects could benefit from additional data to strengthen the conclusions, in my view:

1. The study examines only a single mRNA, RPL41A. eIF4G shows widely-varying binding to different mRNA sequences, both in vivo and in vitro (Costello et al., Genome Biol. 16(1), 10; Zinshteyn et al., RNA 23(9), 1365). The behavior observed in the present study will be most relevant for mRNAs where eIF4G non-specifically binds internal sequences rapidly and tightly (and faster than the eIF4E–cap interaction). In a similar vein, removal of the cap from the full-length yeast NCE102 mRNA practically abolished eIF4EG–mRNA 5'-end interaction detected in a single-molecule FRET assay for cap recognition (Çetin and O'Leary, Nucleic Acids Res. 50(14), 8240), raising the possibility that different eIF4EG interaction dynamics may be observed with different mRNAs. Thus, including data obtained with additional mRNAs in this assay system, such as for those with known differential eIF4G/eIF4F binding, would further strengthen the generality of the conclusions.

2. The authors convincingly show that addition of eIF4A to eIF4EG does not alter the pre-steady-state rate and cap-dependence of eIF4EG–mRNA association. In these experiments, eIF4EG is co-delivered to mRNA with eIF4A (based on my reading of the description from lines 691-694 of the compiled PDF). However, this does not necessarily recapitulate the situation for translation initiation in vivo, where the large cellular excess of eIF4A could mean that "free" (i.e., not eIF4F-bound) eIF4A molecules are already associated with mRNA as the mRNA is intercepted by eIF4EG (or eIF4F). Pre-bound free eIF4A could sterically block eIF4G–mRNA interaction, inhibiting eIF4EG binding to internal mRNA sites. In the cellular context, similar arguments could be made for ribosomes and other RNA-binding proteins, whose occupancy on internal mRNA sites would compete with eIF4G binding. Should that be the case, the cap structure could again become a kinetically-competitive point of initial attachment for eIF4F to the mRNA. Thus, in my view, data should be included from experiments where mRNA is pre-incubated with eIF4A(±ATP), and/or an RNA-binding protein that is not a translation factor, prior to eIF4EG delivery. Similarly, including the steady-state mRNA association rates for eIF4EG+A (not reported in the present

Extended Data Figure 6) could strengthen the conclusions. Alternatively, including data that show the eIF4A-mRNA interaction has equilibrated on the timescale of eIF4G-mRNA association would address this point.

3. A key conclusion is that eIF4A acts as a “strippase” to disassemble non-productive eIF4EG•mRNA complexes, and this is an important and intriguing result. However, the mechanism of this disassembly is not entirely clear. On one hand, the ATP-dependence could point to an input of energy needed to effect eIF4G dissociation. Alternatively, it could reflect the fact that eIF4A-RNA interaction is ATP-dependent, particularly in the case of single-stranded RNA (Rajagopal et al., J. Biol. Chem. 287(24), 20301): from this point of view, with ATP present eIF4A can more effectively compete with eIF4G for binding to mRNA, leading to displacement of eIF4G (“trans-facilitated dissociation”, as it were). These possibilities could be differentiated by substitution of ATP with a non- or slowly-hydrolyzable ATP analog; if only binding is required, the effect should still be observed with the analog.

4. The authors favor an allosteric mechanism, linked to eIF4E-induced eIF4G conformational changes, for why eIF4A-mediated eIF4EG•mRNA dissociation fails at the cap. However, the nature of this allosteric mechanism is also not clear. An alternative explanation would be that eIF4A cannot effectively remove the cap-bound eIF4F because the eIF4E–cap attachment lifetime (4-5 s; Fig. 2a and supplementary table) is sufficiently longer than the time required for eIF4G–mRNA rebinding after transient displacement by eIF4A through a non-allosteric mechanism. The FRET fluctuations attributed to eIF4G “hopping” (Extended Data Fig. 4) would appear consistent with fast eIF4G–mRNA rebinding on this timescale. These possibilities could be differentiated with eIF4G mutants impaired in RNA binding (please see #6, below).

5. While the FRET data clearly indicate similar eIF4G–mRNA binding kinetics for the +14-labeled and +92-labeled mRNAs, there is an assumption that these reflect different eIF4G–mRNA binding sites. While this is in principle a reasonable assumption, in the complex structural landscape of a full-length mRNA, it is possible that the +92 label may be close in space to the eIF4G FRET acceptor when eIF4G is bound near the cap, even if the label is remote in terms of the sequence. Since the model implies that non-specific eIF4G–mRNA binding should be relatively insensitive to RNA labeling position, including data from a second, more cap-distal labeling position could bolster the conclusions.

6. There are a number of eIF4F subunit variants that could be used to strengthen the conclusions. These include mutations to individual or multiple eIF4G RNA-binding domains (Berset et al., RNA 9(7), 871), and mutations in eIF4A that impair eIF4G–eIF4A interaction (Oberer et al., Genes Dev. 19(18), 2212; Schütz et al., Proc. Natl. Acad. Sci. U.S.A. 105(28), 9564). Increasing impairment of eIF4G–RNA binding by serial mutation of the eIF4G–RNA binding domains should reduce and eventually abolish the concentration effects on eIF4G•RNA dissociation, providing additional evidence for facilitated dissociation. Impairment of the eIF4A–eIF4G interaction would allow more unambiguous distinction between allosteric and direct-competition models for eIF4A displacement of eIF4G.

7. The authors show that the eIF4G–mRNA dissociation rates are increased at elevated eIF4(E)G concentration, and this underpins the proposal of facilitated dissociation. However, they do not show a plot to visualize the nature of the concentration dependence, instead showing only representative traces at two concentration points. If I am understanding correctly, the expectation would be that the off-rate should increase linearly with eIF4G concentration and then potentially saturate (depending on the accessible eIF4G concentration range in the experiment). A rate-concentration plot of this kind, with inclusion of additional concentration points as needed (most or all of the data already appear to be in the supplementary table), would emphasize the conclusions around facilitated dissociation.

8. Minor: Yeast and human eIF4F have different molecular architectures and biochemical activities, especially in terms of RNA binding and eIF4A activity. Thus, the full applicability to human eIF4F of the present model, developed with yeast, is not necessarily guaranteed. Addressing this experimentally is very clearly well beyond the scope of the present study, but in my view these differences and their implications should be more explicitly pointed out.

Referee #2

(Remarks to the Author)
Models of eukaryotic translation initiation define an early event in which the eukaryotic initiation factor (eIF) 4E binds the 5’-cap of the mRNA and recruits the rest of the eIF4F complex (eIF4G and eIF4A), thereby activating the mRNA. In this model, eIF4G serves as a scaffold for recruitment of factors (including the 43S pre-initiation complex (PIC)) and eIF4A serves as a helicase, unwinding the mRNA 5’UTR to facilitate 43S PIC scanning. Importantly, pieces of evidence have challenged this paradigm but an alternative model which comprehensively accounts for the data has not emerged.

In this manuscript, Gentry et al. develop a single-molecule fluorescence resonance energy transfer (smFRET) assay from S. cerevisiae components that allows them to biochemically test key aspects of this model under steady state and pre-steady state regimes. The authors convincingly validate this assay and use it to make several key observations: 1) eIF4G associates tightly along the length of an mRNA in an eIF4E- and cap-independent manner; 2) eIF4G undergoes concentration-dependent dissociation kinetics, suggestive of multivalent interactions with its mRNA substrate; 3) eIF4E alone does not initially help localize eIF4G to the cap; 4) eIF4A destabilizes eIF4E/G on mRNA to provide a “strippase” function; and 5) eIF4A-mediated destabilization is only inhibited in the context of eIF4E, ATP, and an mRNA cap. Together, these observations lead the authors to propose a new model. In this model, the eIF4F complex (driven by eIF4G) binds stochastically along the length of the mRNA, eIF4A efficiently strips eIF4F from non-productive locations, and only in the context of eIF4E recognition of 5’-cap is the eIF4A stripping function inhibited. This constitutes the activated state of the mRNA, ready for 43S PIC recruitment by eIF4G. These biochemical observations, if validated in vivo and evolutionarily conserved, would fundamentally change our understanding of how protein synthesis is initiated in eukaryotic organisms.

Despite the potential importance of this work, the impact of the claims is limited by the fact that all key conclusions derive from a single assay. The work would be enhanced by an attempt at cross-validation using alternative methods or in vivo systems. As an initial experiment, the authors already have an assay for PIC recruitment (Fig. S1B). It would be nice to show that eIF4A is required for 43S PIC recruitment, as predicted by their model (with acknowledgement that a similar result has been shown using human components; Sokabe et al., 2017). An even more exciting prediction in vivo would be that eIF4A depletion or inhibition would lead to eIF4G being stuck along the length of mRNA (or at least coating the 5' UTR) genome wide rather than enriched proximal to the cap. This could be tested by a CLIP experiment. Additionally, it would be nice to validate the model using mutant components. An eIF4E cap-binding mutant should no longer be able to recognize the 5'-cap of the mRNA and should therefore not prevent eIF4A "strippase" activity. The authors could do more with testing different ATP analogs or eIF4A mutants in Figure S7B to determine whether "strippase" activity requires ATP hydrolysis. Finally, the authors need some quantitation for Figures S2 and S4. Single traces are not sufficient evidence for their eIF4G facilitated dissociation and hopping models.

The discussion of the final model for mRNA activation (lines 417-433) was clear. However, beyond these initial steps of "mRNA activation" that the authors explore in this manuscript, subsequent discussion points are considerably more speculative, and this should be made clearer in the text. Further, there are several discussion points that would be best to address in a more systematic manner, either with experiments or with text:
1. The authors speculate that 43S PICs should stimulate eIF4A strippase activity (lines 425-427). This idea could be tested by supplying 43S PICs in their smFRET assay.
2. The authors mention that stimulation of "strippase" activity by 43S PICs would allow eIF4F exchange for the 43S PIC at each initiation event (lines 427-428). This does not seem compatible with the model of cap-tethered scanning which is gaining traction (Bohlen et al., 2020). Please clarify or reconcile.
3. How does this work reconcile with recent structures suggesting that two copies of eIF4A are found associated with 43S PICs (Brito Querido et al., 2022)?
4. How does this work reconcile with literature demonstrating that eIF4A's helicase functon(in addition to its "strippase function defined here) can be stimulated by eIF4E (Feoktistova et al., 2013)? And that eIF4E promotes eIF4F-mRNA binding in a cap-independent manner (Izodoro et al., 2022)? And that ATP promotes eIF4F-mRNA binding in a cap-independent manner (Izodoro et al., 2022)?
5. The authors suggest that their work explains the phenomenon of translation bursting (lines 432-433). However, it is unclear how this relates. Please clarify or reconcile.
6. How does this work reconcile with in vivo work using eIF4A inhibitors (i.e. silvestrol, hippuristinol, RocA)?

Finally, the title is too broad and should be revised to something that more specifically identifies the important features of the authors' work and does not over-reach. Overall, this work is exciting and could substantially change our understanding of translation initiation in eukaryotic cells.

References:
1. Sokabe M, Fraser CS. A helicase-independent activity of eIF4A in promoting mRNA recruitment to the human ribosome. Proc Natl Acad Sci U S A. 2017 Jun 13;114(24):6304-6309.
2. Bohlen J, Fenzl K, Kramer G, Bukau B, Teleman AA. Selective 40S Footprinting Reveals Cap-Tethered Ribosome Scanning in Human Cells. Mol Cell. 2020 Aug 20;79(4):561-574.e5.
3. Jailson Brito Querido, Masaaki Sokabe, Irene Díaz-López, Yuliya Gordiyenko, Christopher S. Fraser, V. Ramakrishnan. The structure of a human translation initiation complex reveals two independent roles for the helicase eIF4A bioRxiv 2022.12.07.519490
4. Feoktistova K, Tuvshintogs E, Do A, Fraser CS. Human eIF4E promotes mRNA restructuring by stimulating eIF4A helicase activity. Proc Natl Acad Sci U S A. 2013 Aug 13;110(33):13339-44.
5. Izidoro MS, Sokabe M, Villa N, Merrick WC, Fraser CS. Human eukaryotic initiation factor 4E (eIF4E) and the nucleotide-bound state of eIF4A regulate eIF4F binding to RNA. J Biol Chem. 2022 Oct;298(10):102368.

Referee #3

(Remarks to the Author)
Gentry et al used single-molecule FRET microscopy to study how subunits of eIF4F complex bind to the 5' cap of mRNAs during translation initiation. This step of translation initiation is regulated by mTOR pathway and implicated in translation dysregulation in many human diseases. The authors demonstrate that the eIF4E•eIF4G complex initially binds in a cap-independent manner all along mRNA and then moves via one-dimensional diffusion (sliding and "hopping") until it finds the 5' cap. The authors also show that another subunit of eIF4F complex, RNA helicase eIF4A, displaces non-specifically bound eIF4E•eIF4G and thus accelerate eIF4E•eIF4G 1-D "search" for the 5' cap.

The authors performed very challenging, state-of-the-art single-molecule measurements. Very impressively, they overcome several technical hurdles such as purifying full-length eIF4G and fluorescent labeling of mRNA at internal positions (rather than RNA termini). Their data analysis is meticulous. The manuscript is well written. Their observation of eIF4G diffusion along mRNA is intriguing and has important mechanistic implications for translation initiation. While 1-D search for specific binding sites was previously demonstrated for a few DNA binding proteins, 1-D diffusion along mRNA is less well understood. To my knowledge, previous single-molecule demonstrations of 1-D diffusion along RNA were mostly limited to Ago-miRNA complex (Chandradoss et al, Cell 2015; Cui and Joo RNA Biol 2019 and references therein). Hence, authors'

findings extend beyond the field of protein synthesis and are of substantial interest for the broad readership of Nature. As outlined below, I think that some authors' conclusions might need be toned down. Furthermore, the authors might consider performing some additional experiments. Nevertheless, I believe that after appropriate revisions, this manuscript will be suitable for publication in Nature.

My main concerns are related to the authors' conclusion that "the primary function of eIF4A during mRNA activation is that of an ATP-dependent "strippase". The authors used concentrations of eIF4A that were 3 orders of magnitude higher than concentrations of eIF4E and eIF4G in their experiments. It is not clear whether "strippase" activity belongs to eIF4A bound to eIF4E•eIF4G complex or additional copies of eIF4A bound throughout the mRNA. This issue is not easily addressable by experiments. To track eIF4A binding, eIF4A could be labeled with a spectrally distinct fluoropohore.
However, yeast eIF4A binds to mRNA and eIF4E•eIF4G•mRNA with relatively low affinities (KD of 200 nM and 30 nM, respectively, Mitchell et al Mol Cell 2010) that will greatly complicate tracking eIF4A binding using TIRF microscopy. Nevertheless, the authors should discuss the possibility that eIF4A might have different functions depending on whether it is bound to mRNA alone or as a subunit of eIF4F complex.

Displacing proteins from RNA is thought to be one of the prominent biochemical activities of DEAD box helicases (e.g. please see review articles by Eckhard Jankowsky and references therein). This raises a question whether the "strippase" activity is a unique property of eIF4A or other DEAD box helicases can similarly destabilize non-specifically bound eIF4E•eIF4G and thus promote its binding to the 5' cap. The authors could address this question experimentally replacing eIF4A with Ded1 and other DEAD box helicases in their assays.

Other concerns:

1) 1-D diffusion/hopping of eIF4E•eIF4G along mRNA seems to be one of the key findings of this work. However, the authors mention this in passing and refer the reader to a supplemental figure for more information. The authors could elaborate on their observations supporting 1-D diffusion of eIF4E•eIF4G in the main text (e.g. presence of smFRET traces showing fluctuations between high and low FRET values without reaching 0 FRET as evidence of sliding; repetitive re-binding of eIF4E•eIF4G after unbound proteins were removed from the slide as evidence of "hopping").

2) The authors might want to explicitly state that eIF4E•eIF4G binds non-specifically and "hops" along mRNA in both absence and presence of eIF4A. Otherwise, in the light of "strippase" activity of eIF4A, the reader might erroneously conclude that eIF4E•eIF4G non-specific binding and "hopping" along mRNA observed in the absence of eIF4A are not biologically relevant.

3) The authors might want to discuss what the fast and slow phases of bi-phasic kinetics likely correspond to and justify their use of the weighted average rate constant, kav.

4) In the supplementary Excel file, the authors do not report equilibrium association (on) rates for most of their experiments. How similar/different were those rates from pre-steady state on rates? The difference between pre-steady state and equilibrium association (on) rates might provide another evidence for 1-D diffusion of eIF4E•eIF4G along mRNA.

5) The title of the manuscript "The mechanism of mRNA activation" seems a bit vague and slightly misleading. Translational activation of mRNA implies some form of regulation. The authors have not yet investigated the effects of different mRNA sequences and secondary structures on eIF4F binding in their single-molecule assays. This would likely be beyond the scope of the current manuscript. However, in the absence of such studies, the authors might want to choose a title that describes their findings more precisely.

6) Authors' nomenclature for mRNA constructs used in their work is a bit confusing because the "capped" and "internal" mRNAs are both capped.

Version 1:

Reviewer comments:

Referee #1

(Remarks to the Author)
The authors have thoroughly addressed my comments from the first round of review. The key results remain essentially unchanged from the initial manuscript. However, the new data generally enhance the robustness and reliability of the conclusions, particularly around mechanistic aspects of the facilitated dissociation and eIF4A's role in cap recognition. The additions and modifications are significant, and their description in the text is clear. The methodology and data analysis/presentation, including the statistical aspects, are appropriate for both the initial and newly-added components of the study. I agree with the authors' preference to not include the new NCE102 data in the revised manuscript. Overall, the study provides an original, significant, thought-provoking and timely re-assessment of eIF4F function and dynamics. It will likely be of substantial interest for the translation field and for the broader readership of Nature.

Referee #2

(Remarks to the Author)

This is an impressive body of work which changes our understanding of translation initiation mechanisms in eukaryotic cells. The authors have mostly addressed our comments from the first round of Review. Important additions include experiments demonstrating that eIF4A is required for PIC recruitment and that eIF4A strippase activity does not require ATP hydrolysis. As requested, the authors also attempted to test whether the inhibition of eIF4A strippase activity is lost with an eIF4E binding mutant that no longer binds the cap. However, we note that they did not appear to include eIF4B in this assay (Ext. Data Fig. 7a) and we note that eIF4B is required for inhibition of eIF4A strippase activity based on the koff constants in Ext. Data Table 2. It is unclear why eIF4B was not included (unless the figure was simply mis-labeled) and this suggests that the authors have not addressed whether an eIF4E mutant which can no longer bind the cap is able to inhibit eIF4A strippase activity. In general, a discussion of the dependence of eIF4B for the cap-dependent, eIF4E/G-dependent inhibition of eIF4A strippase activity is largely lacking and seems to be important for understanding this regulation. We also note that because eIF4G binds along the length of mRNAs with high affinity in the absence of eIF4A, the loss of PIC recruitment when eIF4A is omitted (Ext. Data Fig. 8d) suggests additional layers of regulation which are interesting but certainly outside the scope of this manuscript.

We maintain that the title should be revised. In our view, the title devalues both the large body of groundwork upon which their study is based, as well as future mechanistic insights which will expand their work. We suggest that they title their manuscript one of the alternative titles that they offer. Considering all the above, I believe their body of work offers an extraordinary conceptual advance and is essentially suitable for publication.

Referee #3

(Remarks to the Author)
The authors have comprehensively addressed all issues raised in previous reviews. I believe that this manuscript should be accepted for publication in its current form.

Version 2:

Reviewer comments:

Referee #2

(Remarks to the Author)
The authors have completely addressed my concerns. Regarding the authors' question of which version of Extended Data 2 to use, either seems suitable and should be up to the authors' discretion. I believe this manuscript should be accepted in its current form.

We would like to thank the editor for organizing the review process and the reviewers for their careful reading of our manuscript. We are particularly grateful for the reviewers' insightful comments, which have allowed us to revise our work significantly improving its quality and strengthening its conclusions. As detailed below in our point-by-point responses to the reviewers' concerns, we have generated 3 new molecular constructs, collected 30,640 new $E_{FRET}$ *vs.* time trajectories over 32 new smFRET experiments conducted in 66 flowcells, executed 10 new electrophoretic mobility shift assays, completed 1 new fluorescence anisotropy titration, carried out additional analyses of all the data, and made significant revisions to the text and figures. All told, we have nearly doubled the data content of the original study. We hope these additions and changes have made it more straightforward to follow our results, clarified our interpretations of the data, and focused and strengthened our conclusions. We are pleased with the evolution of our manuscript into its current form, and again thank the reviewers for their feedback. We sincerely hope that the editor and reviewers find our revised manuscript to now be acceptable for publication in *Nature.*

**REVEWER 1**

COMMENT 1.1.: Gentry et al. report a novel method to directly and quantitatively observe mRNA activation by the trimeric translation initiation factor eIF4F, in real time and at the single-molecule level. The authors present thought-provoking findings that have important implications for a central mechanistic paradigm in protein synthesis and its regulation. In particular, the authors demonstrate convincingly that the core eIF4F subunit, eIF4G, intrinsically binds mRNA rapidly and distributively along its length, with high longevity on the timescale of translation initiation. The authors show that the dynamics of this interaction are independent of the mRNA cap. Thus, the results challenge the conventional view that the mRNA 5' cap is the initial contact between mRNA and eIF4F. Furthermore, the data place the eIF4F helicase subunit, eIF4A, as playing a central role in driving specific recognition of the mRNA cap, by disassembling eIF4EG•mRNA complexes that do not engage the cap. This contrasts with the expectation that the principal role of eIF4A is to resolve secondary structures near the mRNA 5' end. Taken together, the findings highlight a need to rethink textbook models for the mechanism of initiation of eukaryotic protein synthesis.

The manuscript and its findings are conceptually and technologically of broad interest within and beyond the field of translation initiation. The experimental approach is valid and highly appropriate for studying mRNA activation, and has produced high-quality data which are presented clearly throughout. The authors include appropriate statistical analyses of the data and comparisons between conditions.

The conclusions are overall robust, valid, and reliable, and the experiments are well-controlled and very carefully and appropriately analyzed and interpreted to ensure this. However, a number of aspects could benefit from additional data to strengthen the conclusions, in my view:

RESPONSE1.1.: We thank the reviewer for their very kind words.

COMMENT 1.2.: The study examines only a single mRNA, RPL41A. eIF4G shows widely-varying binding to different mRNA sequences, both in vivo and in vitro (Costello et al., Genome Biol. 16(1), 10; Zinshteyn et al., RNA 23(9), 1365). The behavior observed in the present study will be most relevant for mRNAs where eIF4G non-specifically binds internal sequences rapidly and tightly (and faster than the eIF4E–cap interaction). In a similar vein, removal of the cap from the full-length yeast NCE102 mRNA practically abolished eIF4EG–mRNA 5'-end interaction detected in a single-molecule FRET assay for cap recognition (Çetin and O'Leary, Nucleic Acids Res. 50(14), 8240), raising the possibility that different eIF4EG interaction dynamics may be observed with different mRNAs. Thus, including data obtained with additional mRNAs in this assay system, such as for those with known differential eIF4G/eIF4F binding, would further strengthen the generality of the conclusions.

RESPONSE 1.2.: We think this is a really interesting point and would like to thank the reviewer for raising it. While we agree with the reviewer that further information could be gained from studying additional mRNA sequences, we believe these studies to be outside the scope of the present work. Instead, the present work is focused on dissecting the association of eIF4G:E in many contexts to the full body of one mRNA, effectively sampling multiple possible different eIF4G binding sequences/locations. Nonetheless, in response to this comment, we have expanded this focus to encompass several new constructs (denoted by an asterisk) and now report rates for, Cy5-eIF4G:E binding to: (*i*) capped rpl41a with a cap-proximal fluorophore; (*ii*) uncapped rpl41a with a cap-proximal fluorophore; (*iii*) capped rpl41a with a cap-distal fluorophore at position A93; (*iv**) uncapped rpl41a with a cap-distal fluorophore at position A211; (*v**) the capped, but isolated 24-nucleotide 5' UTR of rpl41a

encompassing the fluorophore label at A14; and (*vi**) an uncapped, 47-nucleotide internal fragment of rpl41a encompassing the fluorophore label at A93.

One limitation in our ability to study other mRNAs is the efficiency with which the ribozyme-based approach labels mRNAs and the poor scaling of this ribozyme reaction to longer mRNA constructs. Nonetheless, we fully appreciate and agree with the reviewer's point that different mRNA sequences or structures may possess unique features that modulate the attachment and/or activity of eIF4F. Consequently, we have edited the final paragraph of the discussion section to highlight that we cannot exclude the possibility that in some contexts eIF4E association with the cap may be the primary driver of the interaction of eIF4F with the mRNA and instead argue that the focus on cap-proximal sequences which regulate eIF4F needs to be supplemented with studies that seriously consider the interaction of eIF4G with the body of the mRNA as well (the full paragraph is also found in RESPONSE 1.8., below). Given the generality of cap-dependent recruitment and the numerous studies detailing the cap-independent association of eIF4G with mRNA[1-8], we would expect these regulatory sequences to be fairly sparse. Although our model's description of attachment to the cap is important, an equally important feature of our model is what happens to eIF4G if it does not bind near the cap or if eIF4E is not present due to decreased expression or sequestration by a 4E-binding protein or similar. In these cases, eIF4A will rapidly strip eIF4G from the mRNA. Because the sequence of the open reading frame of an mRNA is constrained by the peptide sequence it encodes, we envision binding of eIF4G to the body of the mRNA, and subsequent release by eIF4A, to be unavoidable. To this point, in Supplementary Figure 3C of Çetin and O'Leary ((2022) *Nucleic Acids Res*. **50**, 8240) the authors show an electrophoretic mobility shift assay of eIF4G binding to NCE102 mRNA in the absence of eIF4E. Provided their conclusion that eIF4G:E does not effectively associate with the 5' UTR of NCE102 in the absence of a cap, this would require eIF4G:E to bind along the body of the mRNA, where our model would then predict it gets cleared by eIF4A—and thus our study of rpl41a still provides general insight into these special mRNA cases.

[This has been redacted.]

COMMENT 1.3.: The authors convincingly show that addition of eIF4A to eIF4EG does not alter the pre-steady-state rate and cap-dependence of eIF4EG–mRNA association. In these experiments, eIF4EG is co-delivered to mRNA with eIF4A (based on my reading of the description from lines 691-694 of the compiled PDF). However, this does not necessarily recapitulate the situation for translation initiation in vivo, where the large cellular excess of eIF4A could mean that "free" (i.e., not eIF4F-bound) eIF4A molecules are already associated with mRNA as the mRNA is intercepted by eIF4EG (or eIF4F). Pre-bound free eIF4A could sterically block eIF4G–mRNA interaction, inhibiting eIF4EG binding to internal mRNA sites. In the cellular context, similar arguments could be made for ribosomes and other RNA-binding proteins, whose occupancy on internal mRNA sites would compete with eIF4G binding. Should that be the case, the cap structure could again become a kinetically-competitive point of initial attachment for eIF4F to the mRNA. Thus, in my view, data should be included from experiments where mRNA is pre-incubated with eIF4A(±ATP), and/or an RNA-binding protein that is not a translation factor, prior to eIF4EG delivery. Similarly, including the steady-state mRNA association rates for eIF4EG+A (not reported in the present Extended Data Figure 6) could strengthen the conclusions. Alternatively, including data that show the eIF4A-mRNA interaction has equilibrated on the timescale of eIF4G-mRNA association would address this point.

RESPONSE 1.3.: Following the reviewer's suggestion, we have added the steady-state mRNA association rates for eIF4G:E in the presence of eIF4A to **Extended Data Figure 6**. Moreover, we performed the suggested experiments wherein we allowed eIF4A to associate with the mRNA for 5 minutes before injecting the eIF4F complex into the flowcell. The pre-steady-state eIF4G:E association rates obtained from these experiments were only modestly diminished (< 2-fold). The steady-state association and dissociation kinetics were largely unchanged and are now also reported in **Extended Data Figure 6**. It is true that the rates we report in this body of work reflect the preferences of eIF4G to associate with unbound locations on the mRNA and we do not disagree with the reviewer that steric interference may exclude eIF4G from sampling some binding locations *in vivo* and that this may constrain the diffusional landscape, but we find it unlikely that the only available location for eIF4G to bind would be at the productive, cap-proximal location or that no locations outside of the productive, cap-proximal location are free such that only the cap-interaction becomes relevant for association of eIF4G with mRNA.

COMMENT 1.4.: A key conclusion is that eIF4A acts as a "strippase" to disassemble non-productive eIF4EG•mRNA complexes, and this is an important and intriguing result. However, the mechanism of this disassembly is not entirely clear. On one hand, the ATP-dependence could point to an input of energy needed to effect eIF4G dissociation. Alternatively, it could reflect the fact that eIF4A-RNA interaction is ATP-dependent, particularly in the case of single-stranded RNA (Rajagopal et al., J. Biol. Chem. 287(24), 20301): from this point of view, with ATP present eIF4A can more effectively compete with eIF4G for binding to mRNA, leading to displacement of eIF4G ("trans-facilitated dissociation", as it were). These possibilities could be differentiated by substitution of ATP with a non- or slowly-hydrolyzable ATP analog; if only binding is required, the effect should still be observed with the analog.

RESPONSE 1.4.: Following the reviewer's suggestion, along with the suggestion of the other reviewers, we tested the strippase activity of eIF4A in the presence of both slowly hydrolyzing- and non-hydrolyzing analogs of ATP. Both analogs stimulated the strippase activity of eIF4A. This result is highly satisfying in that it agrees with the demonstration that a single ATP hydrolysis event is sufficient to drive mRNA loading[9,10]. If stripping was contingent on ATP hydrolysis, one would instead expect diffusion across the mRNA to require multiple ATP turnover events to reach the cap. In addition, our rates of stripping (~0.2 $s^{-1}$) are faster than the reported rates of ATP hydrolysis by eIF4A in the presence of eIF4G:E (~0.05 $s^{-1}$ )[10]. Based on these results and observations, we do not think eIF4G is displaced *via* the proposed 'trans-facilitated dissociation' events.

To demonstrate that eIF4A strips eIF4G by directly binding to it rather than to mRNA, we titrated eIF4A in the single-molecule, single-turnover stripping assay and compared it to the equilibrium dissociation constant ($K_d$) of eIF4A to the first 51 nucleotides of the rpl41a mRNA as measured by fluorescence anisotropy **(Extended Data Figure 8a-c).** In these experiments, we observed potent, half-maximal, strippase activity between 100-200 nM eIF4A, concentrations of eIF4A that are 10-20-fold lower than those used in our standard strippase assay. These concentrations of eIF4A are also roughly at the $K_d$ of eIF4A binding to eIF4G and the half-maximal concentration ($K_{1/2}$) of eIF4A activity in *in vitro* mRNA recruitment assays[7]. In contrast, our measured $K_D$ for eIF4A binding to the mRNA was between 5-15 μM (within one standard deviation). We think these data strongly indicate that stripping is a product of direct interaction between eIF4A and eIF4G rather than between eIF4A and the mRNA, and that the dependence of the strippase activity of eIF4A on ATP binding may indicate that eIF4G-mRNA preferentially interacts with the ATP-bound conformation of eIF4A.

COMMENT 1.5.: The authors favor an allosteric mechanism, linked to eIF4E-induced eIF4G conformational changes, for why eIF4A-mediated eIF4EG•mRNA dissociation fails at the cap. However, the nature of this allosteric mechanism is also not clear. An alternative explanation would be that eIF4A cannot effectively remove the cap-bound eIF4F because the eIF4E–cap attachment lifetime (4-5 s; Fig. 2a and supplementary table) is sufficiently longer than the time required for eIF4G–mRNA rebinding after transient displacement by eIF4A through a non-allosteric mechanism. The FRET fluctuations attributed to eIF4G "hopping" (Extended Data Fig. 4) would appear consistent with fast eIF4G–mRNA rebinding on this timescale. These possibilities could be differentiated with eIF4G mutants impaired in RNA binding (please see #6, below).

RESPONSE 1.5.: Without contributions from eIF4G towards mRNA binding, the lifetime of the eIF4E interaction with the cap is roughly 500 ms **(Figure 2c)**. The 4-5 s lifetime cited above and in **Figure 2a** is unchanged whether or not a cap is present (**Figure 2c**, **Extended Data Table 1**) and therefore likely reflects that FRET between the mRNA and Cy5-eIF4E in the presence of eIF4G reports on binding of eIF4G within the eIF4G:E complex to mRNA rather than the association of Cy5-eIF4E with the cap. We think this concern is also addressed by additional analyses we have now provided with respect to hopping **(Extended Data Figure 4).** If the fluctuations we interpret as hopping events instead reported on a configurational rearrangement of cap-bound eIF4G:E that alters its distance to the Cy3 fluorophore on the mRNA, we would expect the number of FRET recovery events after apparent dissociation of eIF4G:E to depend only on the presence of the cap and not on the mRNA construct tested. In contrast with this expectation, we observe 5-fold more hopping events (25% *vs.* 5%) in photobleaching-limited, continuous acquisition movies on the capped or uncapped full-length rpl41a constructs *vs.* the capped 24-nucleotide rpl41A construct, which is too short for hopping to occur **(Extended Data Figure 4)**. Thus, we interpret the recovery events as hopping.

COMMENT 1.6.: While the FRET data clearly indicate similar eIF4G–mRNA binding kinetics for the +14-labeled and +92-labeled mRNAs, there is an assumption that these reflect different eIF4G–mRNA binding sites. While this is in principle a reasonable assumption, in the complex structural landscape of a full-length mRNA, it is possible that the +92 label may be close in space to the eIF4G FRET acceptor when eIF4G is bound near the cap, even if the label is remote in terms of the sequence. Since the model implies that non-specific eIF4G–mRNA binding should be relatively insensitive to RNA labeling position, including data from a second, more cap-distal labeling position could bolster the conclusions.

RESPONSE 1.6.: Following the reviewer's suggestion, we generated and repeated our experiments with an additional, more cap-distal, labeling position on rpl41a located at nucleotide A211 in addition to the original position at A93. The ribozyme-based labeling efficiency of this construct was low, which limited the number of $E_{FRET}$ trajectories we could collect, but the eIF4G:E binding kinetics match those of the eIF4G:E binding experiments with the fluorophores at A14 and A93, suggesting that eIF4G:E non-specifically samples the mRNA. To further strengthen the argument that the region around A93 contains a binding site for eIF4G:E that is distinct from the cap-proximal one reported on by the fluorophore on A14, we transcribed a 47-nucleotide, uncapped, RNA fragment encompassing the internal region of rpl41a that contains A93 and performed smFRET experiments with an A93-flurophore-labeled variant of this fragment. Just like it did for the isolated 5' UTR of rpl41a, eIF4G:E exhibited robust, non-specific binding to this isolated internal fragment, demonstrating that eIF4G:E is capable of similarly interacting with both of these regions of the rpl41a mRNA.

To report these new results in our manuscript, we added the following statement to the Results section and added the data described above in **Extended Data Figure 3d and e**:

> *"In order to eliminate the possibility that our cap-proximal and cap-distal fluorophores were reporting on the same, cap-proximal binding location due to mRNA structure, we generated a short, 47 nucleotide, RNA fragment roughly 20 nucleotides upstream and downstream of the cap-distal fluorophore and observed binding between Cy5-eIF4G:E and this isolated fragment (**Extended Data Figure 3d,e**). Consistent with the cap-distal fluorophore reporting on a distinct location, eIF4G:E tightly and rapidly associated with this isolated, uncapped internal fragment similarly to how it bound the cap-distal location in the context of the full-length, capped mRNA. Furthermore, we also tested binding to a second cap-distal fluorophore position on an uncapped mRNA roughly 100 nucleotides downstream of the first location, achieving the same result **(Extended Data Figure 3d,e).**"*

COMMENT 1.7.: There are a number of eIF4F subunit variants that could be used to strengthen the conclusions.

These include mutations to individual or multiple eIF4G RNA-binding domains (Berset et al., RNA 9(7), 871), and mutations in eIF4A that impair eIF4G–eIF4A interaction (Oberer et al., Genes Dev. 19(18), 2212; Schütz et al., Proc. Natl. Acad. Sci. U.S.A. 105(28), 9564). Increasing impairment of eIF4G–RNA binding by serial mutation of the eIF4G–RNA binding domains should reduce and eventually abolish the concentration effects on eIF4G•RNA dissociation, providing additional evidence for facilitated dissociation. Impairment of the eIF4A–eIF4G interaction would allow more unambiguous distinction between allosteric and direct-competition models for eIF4A displacement of eIF4G.

RESPONSE 1.7.: While we agree with the reviewer that if our aim was to dissect the precise molecular basis for the facilitated dissociation of eIF4G it would be necessary to mutate the RNA binding domains of eIF4G, we believe this aim is outside the scope of the current work. Instead, our intent in the current work is simply to emphasize that facilitated dissociation creates the potential for experimental artifacts and misinterpretations. Ultimately, our discovery of facilitated dissociation by eIF4G shaped our decision to use pre-steady-state measurements throughout the work to characterize the binding behavior of various eIF4G-containing complexes. Nonetheless, in response to this comment and Comment 1.8., below, we have performed additional experiments to provide more evidence of facilitated dissociation of eIF4G (detailed in Response 1.8., below) as well as for the role of direct eIF4G-eIF4A interactions in stripping (see Response 1.4., above, for a discussion of eIF4A).

COMMENT 1.8.: The authors show that the eIF4G–mRNA dissociation rates are increased at elevated eIF4(E)G concentration, and this underpins the proposal of facilitated dissociation. However, they do not show a plot to visualize the nature of the concentration dependence, instead showing only representative traces at two concentration points. If I am understanding correctly, the expectation would be that the off-rate should increase linearly with eIF4G concentration and then potentially saturate (depending on the accessible eIF4G concentration range in the experiment). A rate-concentration plot of this kind, with inclusion of additional concentration points as needed (most or all of the data already appear to be in the supplementary table), would emphasize the conclusions around facilitated dissociation.

RESPONSE 1.8.: We thank the reviewer for highlighting the need for additional clarity surrounding facilitated dissociation in this and the previous comments. We have plotted the concentration-dependent steady-state eIF4G:E dissociation rate for the capped, 24-nucleotide construct in **Extended Data Figure 2e.** Moreover, in addition to showing the steady-state eIF4G:E dissociation rates, we performed pre-steady-state experiments where we competed Cy5-eIF4G:E that had been pre-bound to the capped, 24-nucleotide construct off of the mRNA by serially injecting increasing amounts of unlabeled eIF4G:E into the flowcell. Interestingly, the rate of exchange between Cy5-eIF4G:E and unlabeled eIF4G:E in these experiments appears to have two phases—a steep increase in rate at low concentrations and a more moderate increase at higher concentrations **(Extended Data Figure 2g).**

To introduce these results into our manuscript, we added the following paragraph to the Results section:

> *"To confirm this phenomenon, we allowed Cy5-eIF4G:E to associate with the 24-nucleotide 5' UTR of rpl41a, injected unlabeled eIF4G:E into the flowcell at increasing concentrations, and observed the concentration-dependent exchange of the fluorophore-labeled eIF4G:E with unlabeled eIF4G:E (**Extended Data Figure 2g)**. Due to facilitated dissociation, off events measured in steady-state can represent stochastic dissociation, facilitated exchange with another molecule, or conformational fluctuations that happen during those exchange events. This leads to multi-phasic steady-state off rates, which we fit to bi-exponential functions. Throughout this article we report the population-weighted average of the two phases in order to contrast the pre-steady-state stochastic dissociation events from the convoluted steady-state exchange events **(Extended Data Figure 3a)**, but all population weights can be found in **Supplemental Table 1**."*

Moreover, we further confirmed that facilitated dissociation occurs on full-length rpl41a by performing these pre-steady-state exchange experiments with 5 nM and 400 nM unlabeled eIF4G:E using the capped construct **(Extended Data Figure 3c)**.

To motivate our use of pre-steady-state experiments in our manuscript, we edited the following statement in the main text:

> *"Notably, we observe that eIF4G:E facilitates its own dissociation on the full-length constructs as well, convoluting the steady-state data (**Extended Data Figure 3a-c).** Therefore, to measure dissociation rates, we performed pre-steady-state experiments wherein we started with Cy3-mRNA molecules pre-*

*bound to Cy5-eIF4G/G:E, removed all free protein by flushing the flowcell with buffer lacking initiation factors (**Figure 1b, ejection**), and measured the time required for Cy5-eIF4G/G:E dissociation (**Figure 1d**).”*

COMMENT 1.9.: Minor: Yeast and human eIF4F have different molecular architectures and biochemical activities, especially in terms of RNA binding and eIF4A activity. Thus, the full applicability to human eIF4F of the present model, developed with yeast, is not necessarily guaranteed. Addressing this experimentally is very clearly well beyond the scope of the present study, but in my view these differences and their implications should be more explicitly pointed out.

RESPONSE 1.9.: While there are some differences between yeast and mammalian eIF4F, we do not believe these differences impact the findings of our current work. Reports of RNA-binding by mammalian eIF4F vary widely (see Response 2.6 for an expanded discussion on this), but our work is consistent with work using full-length eIF4F. The molecular architectures of the yeast and mammalian proteins appear to be different, but we think this is a consequence of nomenclature. The “RNA binding region” of mammalian eIF4G is composed of two RNA binding domains that sandwich the eIF4A binding domain—these are analogous to RNA2 and RNA3 in yeast[4]. While studies in mammalian systems have identified an eIF3-binding motif in what would be a linker region between the eIF4A and RNA3 binding domains in yeast, it’s clear that this interaction is expected to occur in yeast even though it has not yet been identified. Moreover, if one considers the cryptic RNA binding site in the N-terminus of mammalian eIF4G[11] as an equivalent for RNA1, which precedes the PABP binding domain, then the molecular architecture of the yeast and mammalian proteins are quite similar. In this respect, the mammalian eIF4G just has a C-terminal expansion containing an additional eIF4A binding domain and a kinase binding domain. The functional consequence of possessing motifs that increase the affinity of eIF4G for eIF3 and other kinases simply increases the probability of additional regulation of eIF4F—whether or not that regulation occurs during mRNA activation. The additional eIF4A-binding domain and the enhanced affinity that mammalian eIF4G has for eIF4A would aid the search process of our proposed model by increasing the probability that eIF4A would be present during non-specific mRNA binding. Many of the differences regarding the biochemical activity of eIF4A between these systems have to do with eIF4B-driven stimulation of eIF4A[12]. We have demonstrated that eIF4B does not contribute to the mechanistic steps that are elucidated in our current work. We, and others, expect the core mechanism of eIF4F-driven mRNA recruitment to be conserved across eukaryotes. Notably, human eIF4E is able to compensate for yeast eIF4E in live cells[13,14]. This suggests that human eIF4E has conserved the ability to elicit the same allosteric effects in yeast eIF4G1. Yet, it has simultaneously drifted far enough to be unable to interact with yeast eIF4E-binding proteins, which share a binding interface with eIF4G1[13,14].

*“Much effort has gone into looking for mRNA sequences, or proteins that regulate eIF4F binding at the 5’ end[8,15-17], and although we do not observe a 5’ bias in the system interrogated here, we cannot exclude the possibility that elements positioned near the cap may play critical regulatory roles and sequences with which eIF4G poorly binds likely exist[17,18]. However, our results suggest cells may also regulate the translation efficiency by encoding factors that accelerate the search for the cap, rather than the stability of the resulting ‘activated’ mRNA and that consideration of the entire body of the mRNA, and the RNA-binding proteins contained within, is warranted when considering the ability of an mRNA to recruit eIF4F. It is also possible that extreme cap-proximal RNA sequences, which are known to alter the affinity of eIF4E for the cap in the absence of eIF4G[19], also alter the lifetime of the ‘activated’ state. It is worth emphasizing that the study conducted here employed S. cerevisiae eIF4F. However, given the nature of the similarities and differences between S. cerevisiae and mammalian eIF4F (reviewed in [12]), we expect the fundamental mechanism we have detailed here to generalize to higher-eukaryotes, an expectation that must of course be explicitly tested in mammalian systems. Moreover, although our work here focused on cap-dependent translation, cap-independent translation is often eIF4G- and eIF4A dependent[20,21]. This suggests that the allosteric communication transduced by cap-bound eIF4E through eIF4G, which has been observed in other contexts[3,22], can be induced by other factors during non-canonical translation initiation.”*

**REVEWER 2**

COMMENT 2.1.: Models of eukaryotic translation initiation define an early event in which the eukaryotic initiation factor (eIF) 4E binds the 5’-cap of the mRNA and recruits the rest of the eIF4F complex (eIF4G and eIF4A),

thereby activating the mRNA. In this model, eIF4G serves as a scaffold for recruitment of factors (including the 43S pre-initiation complex (PIC)) and eIF4A serves as a helicase, unwinding the mRNA 5'UTR to facilitate 43S PIC scanning. Importantly, pieces of evidence have challenged this paradigm but an alternative model which comprehensively accounts for the data has not emerged.

In this manuscript, Gentry et al. develop a single-molecule fluorescence resonance energy transfer (smFRET) assay from S. cerevisiae components that allows them to biochemically test key aspects of this model under steady state and pre-steady state regimes. The authors convincingly validate this assay and use it to make several key observations: 1) eIF4G associates tightly along the length of an mRNA in an eIF4E- and cap-independent manner; 2) eIF4G undergoes concentration-dependent dissociation kinetics, suggestive of multivalent interactions with its mRNA substrate; 3) eIF4E alone does not initially help localize eIF4G to the cap; 4) eIF4A destabilizes eIF4E/G on mRNA to provide a "strippase" function; and 5) eIF4A-mediated destabilization is only inhibited in the context of eIF4E, ATP, and an mRNA cap. Together, these observations lead the authors to propose a new model. In this model, the eIF4F complex (driven by eIF4G) binds stochastically along the length of the mRNA, eIF4A efficiently strips eIF4F from non-productive locations, and only in the context of eIF4E recognition of 5'-cap is the eIF4A stripping function inhibited. This constitutes the activated state of the mRNA, ready for 43S PIC recruitment by eIF4G. These biochemical observations, if validated in vivo and evolutionarily conserved, would fundamentally change our understanding of how protein synthesis is initiated in eukaryotic organisms.

RESPONSE 2.1.: We thank the reviewer for emphasizing that we have convincingly validated our smFRET assay and for their kind words about the relevance of the work.

COMMENT 2.2.: Despite the potential importance of this work, the impact of the claims is limited by the fact that all key conclusions derive from a single assay. The work would be enhanced by an attempt at cross-validation using alternative methods or in vivo systems. As an initial experiment, the authors already have an assay for PIC recruitment (Fig. S1B). It would be nice to show that eIF4A is required for 43S PIC recruitment, as predicted by their model (with acknowledgement that a similar result has been shown using human components; Sokabe et al., 2017). An even more exciting prediction in vivo would be that eIF4A depletion or inhibition would lead to eIF4G being stuck along the length of mRNA (or at least coating the 5' UTR) genome wide rather than enriched proximal to the cap. This could be tested by a CLIP experiment. Additionally, it would be nice to validate the model using mutant components. An eIF4E cap-binding mutant should no longer be able to recognize the 5'-cap of the mRNA and should therefore not prevent eIF4A "strippase" activity. The authors could do more with testing different ATP analogs or eIF4A mutants in Figure S7B to determine whether "strippase" activity requires ATP hydrolysis. Finally, the authors need some quantitation for Figures S2 and S4. Single traces are not sufficient evidence for their eIF4G facilitated dissociation and hopping models.

RESPONSE 2.2.: We thank the reviewer for these suggestions. In response, we performed several additional mRNA recruitment and smFRET experiments that we hope will satisfy the reviewer's concerns about cross-validation. We began by omitting eIF4A from mRNA recruitment reactions, which, as expected from our model, abolished the ability to generate 48S PICs (**Extended Data Figure 8d)**. We note that this result has also been observed by Lorsch and colleagues[7,10] in the yeast system as well as by Fraser and colleagues (Sokabe et al.) in the human system. Moreover, we performed mRNA recruitment reactions at varying concentrations of eIF4G **(Extended Data Figure 8d)**. This concentration series revealed that eIF4G alone stimulates mRNA recruitment about 10-fold less efficiently than eIF4G:E (e.g., eIF4G at 200 nM leads to similar rates of recruitment to eIF4G:E at 20 nM), which is roughly the degree of stabilization conferred by the cap. To show that this effect was dependent on cap recognition, we purified a mutant eIF4G:E in which one of the two critical tryptophan residues in eIF4E that sandwich the cap is mutated to alanine (eIF4G:$E_{W104A}$), diminishing the ability of this mutant eIF4G:E to bind the cap. As expected from our model, eIF4G:$E_{W104A}$ behaved similarly to eIF4G in the absence of eIF4E in both the mRNA recruitment assay and in our single-molecule stripping assay **(Extended Data Figure 7a)**, wherein eIF4A was able to strip eIF4G:$E_{W104A}$ from the cap. Each of these two results provide independent support for our model, in which eIF4G:E is stripped from non-productive locations by eIF4A unless allosterically inhibited by the eIF4E-cap interaction. As an additional perturbation to cap recognition, we tested whether inclusion of an $m^7$GpppA cap analog in our imaging buffer would allow eIF4G:E to be stripped from the cap or, alternatively, whether it would cause eIF4G:E to behave as if it were continuously at a capped position. To our surprise, eIF4G:E was stripped from the cap in the presence of this cap-analog, illustrating that the cap-analog can compete for cap-binding with eIF4E, but not induce the same allosteric effect that the cap does **(Extended Data Figure 7b)**.

Again following the reviewer's suggestion, we also performed experiments using slowly and non-hydrolyzable ATP analogs to test whether ATP hydrolysis was necessary for stripping (**Extended Data Figure 7c)**. It was not, and a full discussion of this result can be found in Response 1.4, above.

Finally, we performed and analyzed several additional experiments to validate and quantitate facilitated dissociation (please see Response 1.8, above, and **Extended Data Figures 2 and 3).** We also quantified the fraction of $E_{FRET}$ trajectories that exhibited hopping events for the capped and uncapped RNAs across a variety of experimental conditions in **Extended Data Figure 4**. An expanded discussion about hopping requested by Reviewer 3 can be found in Response 3.4, below.

COMMENT 2.3.: The discussion of the final model for mRNA activation (lines 417-433) was clear. However, beyond these initial steps of "mRNA activation" that the authors explore in this manuscript, subsequent discussion points are considerably more speculative, and this should be made clearer in the text. Further, there are several discussion points that would be best to address in a more systematic manner, either with experiments or with text:

The authors speculate that 43S PICs should stimulate eIF4A strippase activity (lines 425-427). This idea could be tested by supplying 43S PICs in their smFRET assay.

RESPONSE 2.3.: Experiments in which we deliver 43S PICs to activated mRNAs are exciting and ongoing in our lab, but are both technically challenging and outside the scope of the current work. To expand on the technical challenges: Although mRNA recruitment has been performed in single-molecule assays[9,23], it is generally performed under conditions in which eIF4F is in a multi-turnover context. Pre-activating the mRNA and subsequently depleting eIF4F makes one reliant on efficient recruitment happening substantially faster than the eIF4A-assisted dissociation of eIF4G:E from the cap. Due to the noise inherent to single-molecule assays, generating an effect large enough to confidently report would require mRNA recruitment to proceed in less than 30 seconds. Nonetheless, in order to articulate the significance of our model, it seems necessary to hypothesize on the step immediately downstream from the one that we have just mechanistically interrogated. To emphasize the speculative nature of such hypothesizing, we have clearly started the sentence in the Discussion section where this is discussed with the term "We speculate…".

COMMENT 2.4.: The authors mention that stimulation of "strippase" activity by 43S PICs would allow eIF4F exchange for the 43S PIC at each initiation event (lines 427-428). This does not seem compatible with the model of cap-tethered scanning which is gaining traction (Bohlen et al., 2020). Please clarify or reconcile.

RESPONSE 2.4.: We do not think our exchange model is mutually exclusive with a cap-tethered scanning model. Specifically, because the cap-tethered scanning model presented in Bohlen et al. invokes a ribosome-bound eIF4F, the exchange predict by our exchange model could simply involve binding of eIF4F to the ribosome during loading followed by reassociation with the cap. Moreover, Bohlen et al. found a cell line in which they inferred scanning was not cap-tethered. Given that the cap-tethered scanning activity, at least in this cell line, can be turned off, then cap tethering is likely not an intrinsic property of the factors themselves, and may be assisted by additional protein factors that we would lack in our *in vitro* system. Additionally, although the 48S PIC structure described by Brito Querido et al., 2022 (see Comment 2.5,below) contains density for 48S PIC-bound eIF4F, neither the cap nor eIF4E exhibited clear density, a result that contradicts the expectations of a stable cap-tethered structure. Importantly, we do not expect the footprint of eIF4G on the mRNA to remain the same during the loading event because, on mRNAs with short 5' UTRs such as rpl41a, eIF4G would occlude the start codon and block initiation. To emphasize the speculative nature of this hypothesis in the Discussion section of our manuscript, we have removed the word "likely", overall softened our language, and clarified the text in our manuscript to read

> *"In the next steps of the mechanism, we speculate that 43S PICs can contact eIF4G:E:A on the activated mRNA and reactivate the strippase modality of eIF4A, since PICs are known to stimulate ATP hydrolysis by eIF4A[10], and while ATP hydrolysis is not required for stripping from uncapped mRNA positions, a single ATP hydrolysis event is both necessary and sufficient to drive mRNA loading[9,10]. This reactivation could then facilitate either a configurational rearrangement or complete exchange of eIF4F with the 43S PIC, which then loads onto the mRNA. This exchange could possibly lead to the direct association of eIF4G with the 48S PIC, an event that has been inferred from in vivo experiments suggesting that a ribosome-*

*bound eIF4F holds onto the cap during scanning[24] and recent structures that observe eIF4F on the exit channel side of the ribosome[25].”*

COMMENT 2.5.: How does this work reconcile with recent structures suggesting that two copies of eIF4A are found associated with 43S PICs (Brito Querido et al., 2022)?

RESPONSE 2.5.: Because the structures reported in Brito Querido et al., 2022 are of 48S PICs that are formed several steps downstream of the mRNA activation process we have studied here (i.e., after loading, scanning, and start-codon recognition), we think it would be inappropriate for us to comment on the steps leading up to formation of these structures.

COMMENT 2.6.: How does this work reconcile with literature demonstrating that eIF4A’s helicase functon(in addition to its “strippase function defined here) can be stimulated by eIF4E (Feoktistova et al., 2013)? And that eIF4E promotes eIF4F-mRNA binding in a cap-independent manner (Izodoro et al., 2022)? And that ATP promotes eIF4F-mRNA binding in a cap-independent manner (Izodoro et al., 2022)?

RESPONSE 2.6.: The stimulation of helicase activity reported in Feokistova et al. is both small (~3-fold) and, in any event, not present in yeast[6]. Moreover, eIF4A is required even on completely unstructured mRNAs, and therefore, at the minimum posseses an essential, helicase-independent activity in addition to whatever role it has as a helicase. We have clarified our Discussion section as shown below:

*“Although the helicase activity of eIF4A is modestly stimulated by eIF4G:E, it is still a poor RNA helicase[3,10,15]. Moreover, because eIF4A is essential for mRNA recruitment even on unstructured mRNAs[10,26,27], which likely do not require a helicase, we speculate that, in addition to its helicase activity, a primary function of eIF4A as part of eIF4F during mRNA activation is that of an ATP-dependent “strippase”.”*

Moreover, the increase in helicase activity could be an indirect consequence of a change in the conformational landscape of eIF4A more generally, and demonstrates communication through eIF4G between eIF4A and eIF4E. We reference the allosteric communication, which we think this example exemplifies, in the concluding sentence of the discussion:

*“This suggests that the allosteric communication transduced by cap-bound eIF4E through eIF4G, which has been observed in other contexts[3,22], can be induced by other factors during non-canonical translation initiation”*

While the concluding model we propose in this article conflicts with the model proposed in Izodoro et al., we do not think the bodies of work are easily comparable. The experiments in Izodoro et al. are performed with truncations of eIF4G and use model mRNAs rather than native sequences: these experimental constraints could easily affect the results. It is entirely possible that allosteric communication to other parts of eIF4G1, like what they observe with the eIF4E binding domain, alter the RNA binding preferences of the complex. Moreover, while facilitated dissociation has not been demonstrated for human eIF4G1, it could easily complicate the interpretation of the anisotropy results they obtained. Additionally, Izodoro et al. lacks many of the experiments required for direct comparison with our work here: no experiments were performed in the absence of eIF4A nor was the effect of the cap tested. Finally, the affinities reported in Izodoro et al. differ greatly from other measurements working in higher eukaryotes. Our own work in collaboration with Dixie Goss reports affinities of truncated eIF4G to native mRNA UTRs that are an order of magnitude tighter than those reported in Izodoro et al. (albeit in the absence of eIF4A)[4]. Longer constructs of human eIF4G have been demonstrated to tightly bind β-globin mRNA at low nM concentrations[28]. The paper most comparable to the work we detail here is Kaye et al. which uses full-length rabbit eIF4G and tests the affinity of eIF4F to a variety of mRNA constructs as a function of ATP and the cap[5]. Our results are entirely consistent with their experiments, which show that eIF4F’s association to mRNA is high-affinity (irrespective of ATP), non-specific, and not controlled by the cap. We think the variation in the results between these studies likely reflect the technical limitations and complexity imposed by working in the human system and avoiding these technical challenges was a strong motivating factor in our decision to work in the more tractable, but still conserved, *Saccharomyces cerevisiae* model system. Based on these other literature reports, it is our expectation that the mechanism we arrive at in our work will generalize to the mammalian system, but experiments are obviously needed to verify that. As included in our response to comment 1.9, above,

we have added a discussion of the yeast vs mammalian system to the end of the Discussion section of our manuscript.

COMMENT 2.7.: The authors suggest that their work explains the phenomenon of translation bursting (lines 432-433). However, it is unclear how this relates. Please clarify or reconcile.

RESPONSE 2.7.: Following the reviewer's advice, we have clarified our statement to read (the change is underlined):

> *"Notably, the stochastic sampling of the mRNA by eIF4F that we observe explains early reports that 4-5 rounds of eIF4F activity were required to facilitate recruitment[12,29] and recent in vivo reports that translation levels occur in oscillating bursts which can be modulated by eIF4F, <u>whereby the ability to eIF4F to diffuse along the mRNA could lead it to retained on the mRNA for multiple initiation cycles[30]</u>."*

COMMENT 2.8.: How does this work reconcile with in vivo work using eIF4A inhibitors (i.e. silvestrol, hippuristinol, RocA)?

RESPONSE 2.8.: Because eIF4A exhibits a number of eIF4F-independent functions it is difficult to speculate about the phenotypic consequences of eIF4A inhibitors. However, we do note that because hippuristinol prevents the association of eIF4F with mRNA that is inherent to some step in all proposed models, the outcome of its inhibitory function is expected to be similar across all models[31]. At first glance, the role of rocaglates such as silvestrol and RocA as inhibitors that induce clamping eIF4A down on poly-purine sequences is consistent with our model of non-specific association of eIF4F with mRNA[32]. Specifically, within the context of our model, we expect that clamping these complexes down would inhibit the search for the cap and, consequently, translation initiation. Nonetheless, this expected result is anticipated to be similar for other models. This is because the inhibitor would be expected to also clamp free eIF4A down to the mRNA, depleting the cellular pool of eIF4A and inhibiting translation initiation in a manner indistinguishable from what we would expect to observe in the context of our model. Given that all models proposed thus far invoke the essential nature of eIF4A, it is not surprising that the mechanisms of action of these inhibitors can be generally reconciled with all models. Because of these generalities, we have opted not to discuss inhibitors in the Discussion section of our manuscript.

COMMENT 2.9.: Finally, the title is too broad and should be revised to something that more specifically identifies the important features of the authors' work and does not over-reach. Overall, this work is exciting and could substantially change our understanding of translation initiation in eukaryotic cells.

RESPONSE 2.9.: We thank the reviewer for their kind words about our work. However, we respectfully disagree with the reviewer that our original title it too broad, overreaches, and does not specifically identify the important features of our work. By precisely defining the molecular functions of the three components of eIF4F and the molecular mechanism through which eIF4F searches for, recognizes, and discriminates the 5' cap, we have revealed the nature of mRNA activation and the identity of the activated mRNA. Consequently, we think our title, 'The mechanism of mRNA activation', is the one that most accurately describes our work. Nonetheless, if revision of our title is required, we would propose 'The mechanism of mRNA cap recognition' or 'eIF4A drives mRNA cap-recognition by eIF4F'. However, we would argue that neither of these titles provides a comprehensive description of what our work has achieved.

**<u>REVEWER 3</u>**

COMMENT 3.1.: Gentry et al used single-molecule FRET microscopy to study how subunits of eIF4F complex bind to the 5' cap of mRNAs during translation initiation. This step of translation initiation is regulated by mTOR pathway and implicated in translation dysregulation in many human diseases. The authors demonstrate that the eIF4E•eIF4G complex initially binds in a cap-independent manner all along mRNA and then moves via one-dimensional diffusion (sliding and "hopping") until it finds the 5' cap. The authors also show that another subunit of eIF4F complex, RNA helicase eIF4A, displaces non-specifically bound eIF4E•eIF4G and thus accelerate eIF4E•eIF4G 1-D "search" for the 5' cap.

The authors performed very challenging, state-of-the-art single-molecule measurements. Very impressively, they overcome several technical hurdles such as purifying full-length eIF4G and fluorescent labeling of mRNA at internal positions (rather than RNA termini). Their data analysis is meticulous. The manuscript is well written.

Their observation of eIF4G diffusion along mRNA is intriguing and has important mechanistic implications for translation initiation. While 1-D search for specific binding sites was previously demonstrated for a few DNA binding proteins, 1-D diffusion along mRNA is less well understood. To my knowledge, previous single-molecule demonstrations of 1-D diffusion along RNA were mostly limited to Ago-miRNA complex (Chandradoss et al, Cell 2015; Cui and Joo RNA Biol 2019 and references therein). Hence, authors' findings extend beyond the field of protein synthesis and are of substantial interest for the broad readership of Nature. As outlined below, I think that some authors' conclusions might need be toned down. Furthermore, the authors might consider performing some additional experiments. Nevertheless, I believe that after appropriate revisions, this manuscript will be suitable for publication in Nature.

RESPONSE 3.1.: We thank the reviewer for their kind comments and for highlighting previous studies showing that Ago-miRNA complexes diffuse along mRNA.

COMMENT 3.2.: My main concerns are related to the authors' conclusion that "the primary function of eIF4A during mRNA activation is that of an ATP-dependent "strippase". The authors used concentrations of eIF4A that were 3 orders of magnitude higher than concentrations of eIF4E and eIF4G in their experiments. It is not clear whether "strippase" activity belongs to eIF4A bound to eIF4E•eIF4G complex or additional copies of eIF4A bound throughout the mRNA. This issue is not easily addressable by experiments. To track eIF4A binding, eIF4A could be labeled with a spectrally distinct fluoropohore. However, yeast eIF4A binds to mRNA and eIF4E•eIF4G•mRNA with relatively low affinities (KD of 200 nM and 30 nM, respectively, Mitchell et al Mol Cell 2010) that will greatly complicate tracking eIF4A binding using TIRF microscopy. Nevertheless, the authors should discuss the possibility that eIF4A might have different functions depending on whether it is bound to mRNA alone or as a subunit of eIF4F complex.

RESPONSE 3.2.: We appreciate the concerns the reviewer has brought up here. We have amended our statement to include the underlined text: *"a primary function of eIF4A within the eIF4F complex during mRNA activation is that of an ATP-dependent "strippase".* Moreover, we have substantially edited that paragraph, describing our speculation around eIF4A, to include the concerns in Response 3.3 and included a statement about how free eIF4A may be participating with other components in the system (see Response 3.3, below).

We agree with the reviewer that fluorophore labeling of eIF4A and working with fluorophore-labeled eIF4A at the required concentrations is not currently feasible with TIRF microscopy. Beyond this, we note that the $K_D$s the reviewer has referenced from Mitchell et al. correspond to the affinity of eIF4A for eIF4G or eIF4G:E and not for RNA. The same lab subsequently published an ~2 μM affinity of eIF4A for a longer, but similarly degenerate, RNA relative to that used in Mitchell et al.[8] Moreover, eIF4A is consistently reported to exhibit a $K_D$ for RNA in the micromolar range[8,33,34], and we have now performed and added anisotropy measurements to our manuscript demonstrating an ~5-15 μM $K_D$ (within 1 standard deviation) of eIF4A for the first 51 nucleotides of rpl41a **(Extended Data Figure 8c)**. In contrast, the affinity of eIF4A for eIF4G is in the high tens to low hundreds of nM. We therefore sought to distinguish whether stripping occurred due to the direct interaction of eIF4A with eIF4G or with RNA by titrating the concentration of eIF4A in our eIF4G:E stripping assay (see Response 1.4, above, and **Extended Data Figure 8a-c**). The results of these experiments demonstrate that the half-maximal stripping rates of eIF4A occur at eIF4A concentrations of 100-200 nM, a concentration range that roughly matches the affinity of eIF4A for eIF4G and that is more than an order of magnitude lower than the affinity of eIF4A for RNA.

COMMENT 3.3.: Displacing proteins from RNA is thought to be one of the prominent biochemical activities of DEAD box helicases (e.g. please see review articles by Eckhard Jankowsky and references therein). This raises a question whether the "strippase" activity is a unique property of eIF4A or other DEAD box helicases can similarly destabilize non-specifically bound eIF4E•eIF4G and thus promote its binding to the 5' cap. The authors could address this question experimentally replacing eIF4A with Ded1 and other DEAD box helicases in their assays.

RESPONSE 3.3.: We thank the reviewer for his comment and have added the following passage to the appropriate paragraph in our Discussion:

> *"Because other DEAD-box proteins have been shown to displace proteins from RNA[35], we cannot exclude the possibility these proteins (e.g. ded1, dbp1[26,27,36,37]) share activities that are redundant with those of the essential eIF4F subunit, eIF4A."*

COMMENT 3.4.: 1-D diffusion/hopping of eIF4E•eIF4G along mRNA seems to be one of the key findings of this

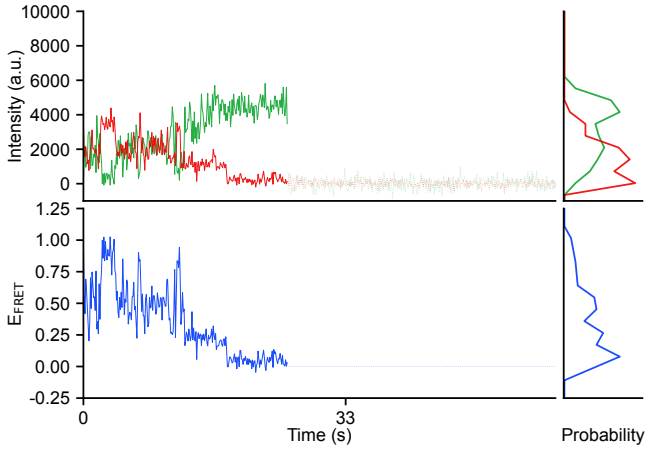
work. However, the authors mention this in passing and refer the reader to a supplemental figure for more information. The authors could elaborate on their observations supporting 1-D diffusion of eIF4E•eIF4G in the main text (e.g. presence of smFRET traces showing fluctuations between high and low FRET values without reaching 0 FRET as evidence of sliding; repetitive re-binding of eIF4E•eIF4G after unbound proteins were removed from the slide as evidence of "hopping").

RESPONSE 3.4.: The reviewer is correct that hopping is a key finding of this work and we agree that not enough time was devoted to it in the main text. Consequently, we have expanded our discussion of hopping in the Results section by adding the following statements to the text:

> *"These dissociation rates may also not necessarily reflect true dissociation rates from the mRNA molecule because facilitated dissociation may allow for intra-mRNA 'hopping'[38], whereby a microdissociated RNA binding domain of eIF4G may bind to another segment on the same RNA and facilitate the movement of eIF4G along the mRNA (**Extended Data Figure 4**). These hopping events manifest as a recovery of $E_{FRET}$ after dissociation of eIF4G from the fluorophore-proximal location. On the capped, 24-nucleotide UTR of rpl41a that contains only a single eIF4G:E binding site, recovery of $E_{FRET}$ only occurs ~5% of the time **(Extended Data Figure 4)**, which may be due to conformational fluctuations of eIF4G:E or the mRNA or to photoblinking of the Cy5 fluorophore. In contrast, on full-length rpl41a, hopping events can be observed in ~25 % of our trajectories under constant illumination. In conditions where we preserve the fluorophore by shuttering the laser, this increases to ~45 %, although it also allows for the possibility of transient events being missed during the time the laser is shuttered off. In both cases, detection of a bone fide hopping event requires two observations: dissociation of eIF4G from the fluorophore-proximal location and recovery of $E_{FRET}$ when the same eIF4G, or another eIF4G bound elsewhere on the mRNA, hops back to a fluorophore-proximal position. The stringency of these requirements, combined with the short lifetimes of our fluorophores likely lead to a systematic under-representation of how often hopping events occur."*

Notably, our smFRET signal is able to detect the phenomenon of hopping, but is an imperfect signal to study it outside of a phenomenological context. This is largely because of the stringent detection requirements described above. Despite this, we have attempted to more directly investigate hopping by tethering eIF4G, rather than the mRNA, to the surface of our microfluidic flowcells. Such an experimental scheme would ensure that only a single eIF4G is present on the mRNA and, using this surface-tethered eIF4G, we intended to combine FRET and fluorescence co-localization signals using an alternating laser excitation (ALEX) variant of our standard TIRF microscopy experiments. Such an approach would allow us to unambiguously distinguish between dissociation events and hopping events. Unfortunately, however, all of our attempts to generate eIF4G constructs capable of surface-tethering to date have yielded biochemically inactive eIF4G molecules.

We do see some examples of trajectories that could contain 'sliding' events (see example trajectory below) but they are so infrequent that we are hesitant to interpret them as such given the possibility that they might reflect rare photophysical artifacts rather than *bone fide* sliding events.



COMMENT 3.5.: The authors might want to explicitly state that eIF4E•eIF4G binds non-specifically and "hops" along mRNA in both absence and presence of eIF4A. Otherwise, in the light of "strippase" activity of eIF4A, the reader might erroneously conclude that eIF4E•eIF4G non-specific binding and "hopping" along mRNA observed in the absence of eIF4A are not biologically relevant.

RESPONSE 3.5.: We thank the reviewer for catching this important oversight. We have added the explicit statement (underlined) to our discussion section.

> *"Collectively, our results allow us to propose a new model for mRNA activation (**Figure 4**). In the first step, eIF4G:E stochastically binds to the mRNA. In the next step, eIF4A attempts to strip eIF4G:E from the mRNA, bifurcating the cycle. If eIF4G:E is bound in a cap-distal, non-productive location, eIF4A is able to eject eIF4G:E from its current binding location by either facilitating hopping to a new location, which we observe both in the presence and absence of eIF4A (**Extended Data Figure 4**), on the mRNA or releasing eIF4G:E from the mRNA entirely"*

COMMENT 3.6.: The authors might want to discuss what the fast and slow phases of bi-phasic kinetics likely correspond to and justify their use of the weighted average rate constant, kav.

RESPONSE 3.6.: We thank the reviewer for highlighting that this is missing from the main text. Generally, we believe that one phase in our biphasic kinetics corresponds to a slow, photobleaching-limited stochastic dissociation event, and that the other phase reflects either a steady-state exchange or conformational fluctuation (if exchange is attempted but not completed). Although interesting in its own right, for the purpose of our investigation here we largely treat exchange as a complicating factor which convolutes our ability to analyze our experimental data. While we have further expanded the experiments demonstrating the extent of facilitated dissociation in our steady-state experiments (see Response 1.8, above, and the amended **Extended Figures 2 and 3**), we largely do this to motivate our focus on the pre-steady-state rates. To address this in the main text of our manuscript, we have added the paragraph detailed in Response 1.8, above, to the Results section.

COMMENT 3.7.: In the supplementary Excel file, the authors do not report equilibrium association (on) rates for most of their experiments. How similar/different were those rates from pre-steady state on rates? The difference between pre-steady state and equilibrium association (on) rates might provide another evidence for 1-D diffusion of eIF4E•eIF4G along mRNA.

RESPONSE 3.7.: We thank the reviewer for catching this oversight. We have updated the Supplementary Excel file to contain the steady-state association rates for all of our injection experiments. Generally speaking, the equilibrium association rates either match the pre-steady-state association rate or show a modest (< 2-fold) decrease—a small enough change that we hesitate to draw conclusions from it.

COMMENT 3.8.: The title of the manuscript "The mechanism of mRNA activation" seems a bit vague and slightly misleading. Translational activation of mRNA implies some form of regulation. The authors have not yet investigated the effects of different mRNA sequences and secondary structures on eIF4F binding in their single-molecule assays. This would likely be beyond the scope of the current manuscript. However, in the absence of such studies, the authors might want to choose a title that describes their findings more precisely.

RESPONSE 3.8.: Reviewer 2 expressed a similar concern. Please see our response to Comment 2.9, above.

COMMENT 3.9.: Authors' nomenclature for mRNA constructs used in their work is a bit confusing because the "capped" and "internal" mRNAs are both capped.

RESPONSE 3.9.: Following the reviewer's suggestion, we have altered the name of the constructs to be "Capped" "Uncapped" and "Cap-distal" to highlight that the internal position is on a capped mRNA.

**References**


1 Blanchard, S. C., Gonzalez, R. L., Kim, H. D., Chu, S. & Puglisi, J. D. tRNA selection and kinetic proofreading in translation. *Nat Struct Mol Biol* **11**, 1008-1014, doi:10.1038/nsmb831 (2004).

2 Andreou, A. Z. & Klostermeier, D. eIF4B and eIF4G jointly stimulate eIF4A ATPase and unwinding activities by modulation of the eIF4A conformational cycle. *J Mol Biol* **426**, 51-61, doi:10.1016/j.jmb.2013.09.027 (2014).

3 Feoktistova, K., Tuvshintogs, E., Do, A. & Fraser, C. S. Human eIF4E promotes mRNA restructuring by stimulating eIF4A helicase activity. *Proc Natl Acad Sci U S A* **110**, 13339-13344, doi:10.1073/pnas.1303781110 (2013).

4 Haizel, S. A., Bhardwaj, U., Gonzalez, R. L., Jr., Mitra, S. & Goss, D. J. 5'-UTR recruitment of the translation initiation factor eIF4GI or DAP5 drives cap-independent translation of a subset of human mRNAs. *J Biol Chem* **295**, 11693-11706, doi:10.1074/jbc.RA120.013678 (2020).

5 Kaye, N. M., Emmett, K. J., Merrick, W. C. & Jankowsky, E. Intrinsic RNA binding by the eukaryotic initiation factor 4F depends on a minimal RNA length but not on the m7G cap. *J Biol Chem* **284**, 17742-17750, doi:10.1074/jbc.M109.009001 (2009).

6 Krause, L., Willing, F., Andreou, A. Z. & Klostermeier, D. The domains of yeast eIF4G, eIF4E and the cap fine-tune eIF4A activities through an intricate network of stimulatory and inhibitory effects. *Nucleic Acids Res* **50**, 6497-6510, doi:10.1093/nar/gkac437 (2022).

7 Mitchell, S. F. *et al.* The 5'-7-methylguanosine cap on eukaryotic mRNAs serves both to stimulate canonical translation initiation and to block an alternative pathway. *Mol Cell* **39**, 950-962, doi:10.1016/j.molcel.2010.08.021 (2010).

8 Rajagopal, V., Park, E. H., Hinnebusch, A. G. & Lorsch, J. R. Specific domains in yeast translation initiation factor eIF4G strongly bias RNA unwinding activity of the eIF4F complex toward duplexes with 5'-overhangs. *J Biol Chem* **287**, 20301-20312, doi:10.1074/jbc.M112.347278 (2012).

9 Wang, J. *et al.* Rapid 40S scanning and its regulation by mRNA structure during eukaryotic translation initiation. *Cell* **185**, 4474-4487 e4417, doi:10.1016/j.cell.2022.10.005 (2022).

10 Yourik, P. *et al.* Yeast eIF4A enhances recruitment of mRNAs regardless of their structural complexity. *Elife* **6**, doi:10.7554/eLife.31476 (2017).

11 Pequeno, A. Bridging the Gap: Insights into the eIF4E-eIF4G1-PABP1-mRNA complex. *UC San Diego Electronic Theses and Dissertations* (2018).

12 Merrick, W. C. eIF4F: a retrospective. *J Biol Chem* **290**, 24091-24099, doi:10.1074/jbc.R115.675280 (2015).

13 Altmann, M., Muller, P. P., Pelletier, J., Sonenberg, N. & Trachsel, H. A mammalian translation initiation factor can substitute for its yeast homologue in vivo. *J Biol Chem* **264**, 12145-12147 (1989).

14 Hughes, J. M. *et al.* Translational repression by human 4E-BP1 in yeast specifically requires human eIF4E as target. *J Biol Chem* **274**, 3261-3264, doi:10.1074/jbc.274.6.3261 (1999).

15 Rajyaguru, P., She, M. & Parker, R. Scd6 targets eIF4G to repress translation: RGG motif proteins as a class of eIF4G-binding proteins. *Mol Cell* **45**, 244-254, doi:10.1016/j.molcel.2011.11.026 (2012).

16 Otero, L. J., Ashe, M. P. & Sachs, A. B. The yeast poly(A)-binding protein Pab1p stimulates in vitro poly(A)-dependent and cap-dependent translation by distinct mechanisms. *EMBO J* **18**, 3153-3163, doi:10.1093/emboj/18.11.3153 (1999).

17 Clarkson, B. K., Gilbert, W. V. & Doudna, J. A. Functional overlap between eIF4G isoforms in Saccharomyces cerevisiae. *PLoS One* **5**, e9114, doi:10.1371/journal.pone.0009114 (2010).

18 Cetin, B. & O'Leary, S. E. mRNA- and factor-driven dynamic variability controls eIF4F-cap recognition for translation initiation. *Nucleic Acids Res* **50**, 8240-8261, doi:10.1093/nar/gkac631 (2022).

19 Tamarkin-Ben-Harush, A., Vasseur, J. J., Debart, F., Ulitsky, I. & Dikstein, R. Cap-proximal nucleotides via differential eIF4E binding and alternative promoter usage mediate translational response to energy stress. *Elife* **6**, doi:10.7554/eLife.21907 (2017).

20 Marcotrigiano, J. *et al.* A conserved HEAT domain within eIF4G directs assembly of the translation initiation machinery. *Mol Cell* **7**, 193-203, doi:10.1016/s1097-2765(01)00167-8 (2001).

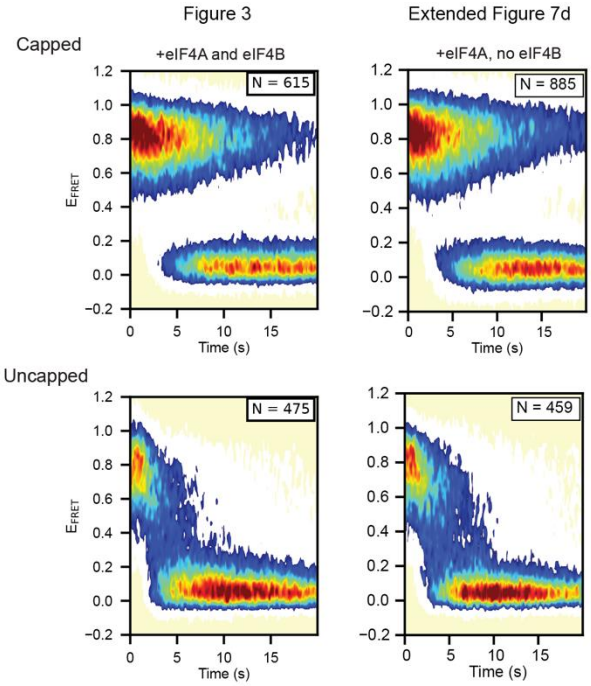
21 Martinez-Salas, E., Francisco-Velilla, R., Fernandez-Chamorro, J. & Embarek, A. M. Insights into Structural and Mechanistic Features of Viral IRES Elements. *Front Microbiol* **8**, 2629, doi:10.3389/fmicb.2017.02629 (2017).

22 Hershey, P. E. *et al.* The Cap-binding protein eIF4E promotes folding of a functional domain of yeast translation initiation factor eIF4G1. *J Biol Chem* **274**, 21297-21304, doi:10.1074/jbc.274.30.21297 (1999).
23 Hong, H. J., Zhang, A. L., Conn, A. B., Blaha, G. & O'Leary, S. E. Single-Molecule Tracking Reveals Dynamic Regulation of Ribosomal Scanning. *bioRxiv*, 2023.2009.2004.555162, doi:10.1101/2023.09.04.555162 (2023).
24 Bohlen, J., Fenzl, K., Kramer, G., Bukau, B. & Teleman, A. A. Selective 40S Footprinting Reveals Cap-Tethered Ribosome Scanning in Human Cells. *Mol Cell* **79**, 561-574 e565, doi:10.1016/j.molcel.2020.06.005 (2020).
25 Brito Querido, J. *et al.* The structure of a human translation initiation complex reveals two independent roles for the helicase eIF4A. *Nat Struct Mol Biol* **31**, 455-464, doi:10.1038/s41594-023-01196-0 (2024).
26 Sen, N. D., Zhou, F., Ingolia, N. T. & Hinnebusch, A. G. Genome-wide analysis of translational efficiency reveals distinct but overlapping functions of yeast DEAD-box RNA helicases Ded1 and eIF4A. *Genome Res* **25**, 1196-1205, doi:10.1101/gr.191601.115 (2015).
27 Gupta, N., Lorsch, J. R. & Hinnebusch, A. G. Yeast Ded1 promotes 48S translation pre-initiation complex assembly in an mRNA-specific and eIF4F-dependent manner. *Elife* **7**, doi:10.7554/eLife.38892 (2018).
28 Yanagiya, A. *et al.* Requirement of RNA binding of mammalian eukaryotic translation initiation factor 4GI (eIF4GI) for efficient interaction of eIF4E with the mRNA cap. *Mol Cell Biol* **29**, 1661-1669, doi:10.1128/MCB.01187-08 (2009).
29 Pause, A., Methot, N., Svitkin, Y., Merrick, W. C. & Sonenberg, N. Dominant negative mutants of mammalian translation initiation factor eIF-4A define a critical role for eIF-4F in cap-dependent and cap-independent initiation of translation. *EMBO J* **13**, 1205-1215, doi:10.1002/j.1460-2075.1994.tb06370.x (1994).
30 Livingston, N. M. *et al.* Bursting translation on single mRNAs in live cells. *Mol Cell* **83**, 2276-2289 e2211, doi:10.1016/j.molcel.2023.05.019 (2023).
31 Cencic, R. & Pelletier, J. Hippuristanol - A potent steroid inhibitor of eukaryotic initiation factor 4A. *Translation (Austin)* **4**, e1137381, doi:10.1080/21690731.2015.1137381 (2016).
32 Chu, J. *et al.* Rocaglates Induce Gain-of-Function Alterations to eIF4A and eIF4F. *Cell Rep* **30**, 2481-2488 e2485, doi:10.1016/j.celrep.2020.02.002 (2020).
33 Izidoro, M. S., Sokabe, M., Villa, N., Merrick, W. C. & Fraser, C. S. Human eukaryotic initiation factor 4E (eIF4E) and the nucleotide-bound state of eIF4A regulate eIF4F binding to RNA. *J Biol Chem* **298**, 102368, doi:10.1016/j.jbc.2022.102368 (2022).
34 Lorsch, J. R. & Herschlag, D. The DEAD box protein eIF4A. 1. A minimal kinetic and thermodynamic framework reveals coupled binding of RNA and nucleotide. *Biochemistry* **37**, 2180-2193, doi:10.1021/bi972430g (1998).
35 Linder, P. & Jankowsky, E. From unwinding to clamping - the DEAD box RNA helicase family. *Nat Rev Mol Cell Biol* **12**, 505-516, doi:10.1038/nrm3154 (2011).
36 Sen, N. D. *et al.* Functional interplay between DEAD-box RNA helicases Ded1 and Dbp1 in preinitiation complex attachment and scanning on structured mRNAs in vivo. *Nucleic Acids Res* **47**, 8785-8806, doi:10.1093/nar/gkz595 (2019).
37 Sharma, D. & Jankowsky, E. The Ded1/DDX3 subfamily of DEAD-box RNA helicases. *Crit Rev Biochem Mol Biol* **49**, 343-360, doi:10.3109/10409238.2014.931339 (2014).
38 Kosar, Z., Attar, A. G. & Erbas, A. Facilitated dissociation of nucleoid-associated proteins from DNA in the bacterial confinement. *Biophys J* **121**, 1119-1133, doi:10.1016/j.bpj.2022.03.002 (2022).

COMMENT 1: This is an impressive body of work which changes our understanding of translation initiation mechanisms in eukaryotic cells. The authors have mostly addressed our comments from the first round of Review. Important additions include experiments demonstrating that eIF4A is required for PIC recruitment and that eIF4A strippase activity does not require ATP hydrolysis. As requested, the authors also attempted to test whether the inhibition of eIF4A strippase activity is lost with an eIF4E binding mutant that no longer binds the cap. However, we note that they did not appear to include eIF4B in this assay (Ext. Data Fig. 7a) and we note that eIF4B is required for inhibition of eIF4A strippase activity based on the koff constants in Ext. Data Table 2. It is unclear why eIF4B was not included (unless the figure was simply mis-labeled) and this suggests that the authors have not addressed whether an eIF4E mutant which can no longer bind the cap is able to inhibit eIF4A strippase activity. In general, a discussion of the dependence of eIF4B for the cap-dependent, eIF4E/G-dependent inhibition of eIF4A strippase activity is largely lacking and seems to be important for understanding this regulation. We also note that because eIF4G binds along the length of mRNAs with high affinity in the absence of eIF4A, the loss of PIC recruitment when eIF4A is omitted (Ext. Data Fig. 8d) suggests additional layers of regulation which are interesting but certainly outside the scope of this manuscript.

RESPONSE 1: We thank the reviewer for their kind words. With regards to this last point, as discussed further below, we believe the way we summarized the data in Extended Data Table 2 has led to some confusion. Specifically, the presence of eIF4B is not required to stabilize eIF4G:E+A at the cap. Therefore, our use of only eIF4A, instead of eIF4A+B, when assessing the ability of the $eIF4E_{W104A}$ mutant to inhibit stripping from the cap is valid.

The dispensable nature of eIF4B in our assay is most clearly visible upon comparing surface contour plots taken from Figure 3 and Extended Data Figure 7d. For the reviewer's convenience, we demonstrate this comparison here by placing the two pairs of 2D histograms next to each other. The comparison illustrates the stripping activity of eIF4A in the capped *vs.* uncapped mRNA cases (top and bottom panels, respectively) as a function of eIF4B (left- and right-side panels, respectively).




As can be seen, eIF4G:E remains bound to the capped position until photobleaching in the presence or absence of eIF4B. Moreover, it is removed from uncapped positions in both cases. When summarizing this result in Extended Data Table 2, we had initially not performed the eIF4B drop-out experiment (*i.e.*, the –eIF4B condition) under the laser shuttering condition and we therefore could only report the bounded $k_{off}$, which was limited by photobleaching. This bound was denoted by the superscript 'c' in the initial table entry and is the reason why we bounded the $K_D$. In hindsight, we likely did not emphasize this superscript and

corresponding footnote adequately, for example by including a "<" sign to the $k_{off}$. To address this, we have generated two alternative revisions to Extended Data Table 2 that will fully resolve any confusion. In Version 1, we more clearly denote that $k_{off}$ is bounded by using a "<" sign rather than a specific value that can be compared to the +eIF4B condition. Alternatively, in Version 2, because we have now performed the corresponding shuttering experiment, we can now report the unbounded $k_{off}$ for the –eIF4B condition for the capped construct. This version of the table reveals that eIF4B does not have an effect. Both versions of this table can be found below, and we would be happy to include either in a revised version of the manuscript. Changes made in each version relative to the initial table are highlighted in red.

Version 1

**Summary of rate constants**

| | $k_{on}$ ($uM^{-1}$) | $k_{off}$ ($s^{-1}$) | $K_D$ (pM) |
|---|---|---|---|
| Capped | | | |
| eIF4G | 237 ± 40[a] | 0.0074 ± 0.0003[b] | 31 ± 5 |
| eIF4G:E | 316 ± 5[a] | 0.0050 ± 0.0004[b] | 16 ± 1 |
| eIF4G:E+A | 247 ± 35[a] | < 0.0939 ± 0.0042[a,c] | < 380 ± 57[d] |
| eIF4G:E+A+B | 315 ± 81[a] | 0.0144 ± 0.0009[b] | 46 ± 12 |
| eIF4G+A | 194 ± 28[a] | 0.1293 ± 0.0073[a] | 664 ± 103 |
| eIF4G+A+B | 264 ± 14[a] | 0.2436 ± 0.0043[a] | 922 ± 52 |
| Uncapped | | | |
| eIF4G | 149 ± 7[a] | 0.0069 ± 0.0002[b] | 46 ± 3 |
| eIF4G:E | 334 ± 5[a] | 0.0057 ± 0.0001[b] | 17 ± 1 |
| eIF4G:E+A | 330 ± 3[a] | 0.1823 ± 0.0293[a] | 553 ± 89 |
| eIF4G:E+A+B | 309 ± 87[a] | 0.2399 ± 0.0691[a] | 776 ± 312 |
| Cap-distal | | | |
| eIF4G | 277 ± 33[a] | 0.0066 ± 0.0012[b] | 24 ± 5 |
| eIF4G:E | 256 ± 39[a] | 0.0057 ± 0.0016[b] | 22 ± 7 |
| eIF4G:E+A | 279 ± 17[a] | 0.1949 ± 0.0241[a] | 698 ± 96 |
| eIF4G:E+A+B | 297 ± 10[a] | 0.2408 ± 0.0105[a] | 812 ± 45 |

[a]Rates calculated from movies with framerate of 10 frames per second (fps)
[b]Rates calculated from movies with framerate of 1/3 fps
[c]Photobleaching limited
[d]$K_D$ is the upperbound due to photobleaching limited off rate

Version 2

**Summary of rate constants**

| | $k_{on}$ ($uM^{-1}$) | $k_{off}$ ($s^{-1}$) | $K_D$ (pM) |
|---|---|---|---|
| Capped | | | |
| eIF4G | 237 ± 40[a] | 0.0074 ± 0.0003[b] | 31 ± 5 |
| eIF4G:E | 316 ± 5[a] | 0.0050 ± 0.0004[b] | 16 ± 1 |
| eIF4G:E+A | 247 ± 35[a] | 0.0146 ± 0.0046[b] | 45 ± 16 |
| eIF4G:E+A+B | 315 ± 81[a] | 0.0144 ± 0.0009[b] | 46 ± 12 |
| eIF4G+A | 194 ± 28[a] | 0.1293 ± 0.0073[a] | 664 ± 103 |
| eIF4G+A+B | 264 ± 14[a] | 0.2436 ± 0.0043[a] | 922 ± 52 |
| Uncapped | | | |
| eIF4G | 149 ± 7[a] | 0.0069 ± 0.0002[b] | 46 ± 3 |
| eIF4G:E | 334 ± 5[a] | 0.0057 ± 0.0001[b] | 17 ± 1 |
| eIF4G:E+A | 330 ± 3[a] | 0.1823 ± 0.0293[a] | 553 ± 89 |
| eIF4G:E+A+B | 309 ± 87[a] | 0.2399 ± 0.0691[a] | 776 ± 312 |
| Cap-distal | | | |
| eIF4G | 277 ± 33[a] | 0.0066 ± 0.0012[b] | 24 ± 5 |
| eIF4G:E | 256 ± 39[a] | 0.0057 ± 0.0016[b] | 22 ± 7 |
| eIF4G:E+A | 279 ± 17[a] | 0.1949 ± 0.0241[a] | 698 ± 96 |
| eIF4G:E+A+B | 297 ± 10[a] | 0.2408 ± 0.0105[a] | 812 ± 45 |

[a]Rates calculated from movies with framerate of 10 frames per second (fps)
[b]Rates calculated from movies with framerate of 1/3 fps
[c]Photobleaching limited
[d]$K_D$ is the upperbound due to photobleaching limited off rate

Regardless of this lack of dependence on eIF4B, we agree with the reviewer that the discussion of eIF4B is incredibly limited in the manuscript. It's largely relegated to a single sentence on Line 403 denoting that eIF4B does not have an effect in our assay. We've therefore added the following text to the Discussion (at Line 450) stating:

> *"To our surprise, eIF4B does not appear to contribute to the mechanistic steps of cap-recognition that we observe in this study. However, this finding is consistent with in vivo and biochemical results showing that eIF4B directly interacts with the ribosome, suggesting it may play a role in downstream steps".*

COMMENT 2: We maintain that the title should be revised. In our view, the title devalues both the large body of groundwork upon which their study is based, as well as future mechanistic insights which will expand their work. We suggest that they title their manuscript one of the alternative titles that they offer. Considering all the above, I believe their body of work offers an extraordinary conceptual advance and is essentially suitable for publication.

RESPONSE 2: We understand the reviewer's concerns, but still feel that the title 'The mechanism of mRNA activation' is appropriate for our work.