THE EMBO JOURNAL

Manuscript EMBO-2013-87344

eIF5B Employs a Novel Domain Release Mechanism to Catalyze Ribosomal Subunit Joining

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Review timeline:

Submission date:	06 November 2013
Editorial Decision:	23 December 2013
Revision received:	06 February 2014
Accepted:	20 February 2014

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

Editor: Anne Nielsen

1st Editorial Decision 23 December 2013

Thank you for submitting your manuscript for consideration by the EMBO Journal and my apologies for the unusually long duration of the review process in this case. Your manuscript has now been seen by three referees whose comments are shown below.

As you will see from the report all three referees express high interest in your study and praise the quality of the structural work presented; however, ref#1 and #3 both raise concerns that the ITC analysis is less solid in substantiating the conclusions made. While ref#3 is consequently rather critical about the overall impact of the study and finds that additional structural insight would be required to fully demonstrate the dissociative switch mechanism, ref#1 finds that the existing data are sufficiently strong to warrant publication but does asks that biochemical data should be included, if possible, to provide further experimental support for the proposed mechanism.

In light of the overall positive recommendations from the referees, I would like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version.

Based on the referee comments I would ask you to particularly focus your efforts on:

-> addressing all points raised by ref#1
-> extensively revise the nomenclature, manuscript text and overall data presentation as requested

by refs #1 and #2
-> critically discuss the potential caveats of ITC measurements as pointed out by ref#3.
In addition, you should take care to better incorporate and discuss the existing literature (especially the recent structures of eIF5B on the ribosome) as pointed out by all three referees.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website:
http://emboj.embopress.org/about#Transparent_Process

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORT:

Referee #1:

The paper by Kuhle and Ficner presents six high-resolution crystal structures of eIF5B from two different organisms. Due to the high resolution, the structures provide novel insights into the structure and rearrangements of the switch I and II regions and the P loop upon binding of GTP or GDP. Comparisons between the GTP- and GDP-bound structures lead the authors to a hypothesis concerning the overall nucleotide-dependent domain rearrangement in eIF5B. The suggestion is clearly tentative as most of the structures, except for the apo-form, are truncated versions of eIF5B. The hypothesis is further tested biochemically using ITC which seems to be consistent with the idea that binding of GTP results in the dissociation of domain III from the switch II region of domain I. Finally, the authors dock the structures onto the 70S ribosome and suggest a model for eIF5B conformation on the ribosome. The data are novel and interesting and important for the understanding of translational GTPases. It is particularly timely given the new structures of IF2 from the Steitz/Klaholz labs. In comparison to those structures, the present eIF5B structure has a better resolution in general and particularly at the critical regions (P loop, switches) and has various bound nucleotides. Notably, the authors of the present paper, similarly to the Steitz/Klaholz consortium) do not support the "articulated lever" mechanism of communication between the GTPase and initiator tRNA proposed earlier; however the alternative models differ from each other. In summary, the present paper is timely and will contribute important new results for the discussion of the role of eIB5B/IF2.

Specific comments.

1. The authors use too many terms in quotes, such as 'active' and 'Inactive' forms of GTP, 'dissociative switch mechanism', 'articulated level model', etc. This gives a strong impression that the authors do not believe that the respective forms are really 'active' or 'inactive'. In some cases it is even justified, as the authors correctly point out. I read these quotes as a psychological problem the authors have with the uncertainties/inaccuracies of the different eIF5B forms, e.g. the GTP form becomes 'inactive' on the ribosome, as it has to change the conformation to bind properly. My suggestion is to avoid this problem altogether by giving the exact nature of the conformation (e.g. GTP-bound form) without qualifying it as 'active' or 'inactive'. In the case of EF-Tu, the GTP-bound form is really active and the GDP-fom is really inactive, so the quotes can be omitted. In the case of EF-G, the issue is as uncertain as with eIF5B (because there are so many different potential conformations involved) and I would not use the active-inactive nomenclature either.
2. The term 'dissociative switch' is rather misleading because it sounds too close to the 'dissociative mechanism' for GTP hydrolysis, which is another actively discussed issue for the GTPases in general. The authors should find a better, more precise term with less overlap with the terms already

in use.
3. The 'dissociative switch' mechanism cries for biochemical validation. The best way would be to identify the mutants which may affect the interactions between switch 2 and domain 3 and test their effect on the activity of eIF5B. While I understand that this may be beyond the scope of the paper, at minimum the author should prepare a Table of the potentially involved residues and compare them with those available in the literature. There is some discussion in this direction, but the text is insufficiently focused and not illustrated.
4. P. 13, 'To investigate the effect of Mg...' I am surprised that nucleotides bind at all to a GTP binding protein in the absence of Mg2+. Even more confusing, the affinity of eIF5B to GDP appears to INCREASE in the absence of Mg2+ (Table II). This does not agree with what is known for other GTP-binding proteins, because Mg2+ is expected to stabilize the changes on GTP/GDP upon binding to the protein. What is the explanation for this effect? What is the residual Mg2+ in the buffer?
5. Abstract: 'Effector' domain III....activated eIF5B'. What is the evidence that domain III is an 'effector domain'? 'Activated' reads like 'GTPase activated' in this context, which is not what the authors want to say. 'Activated' is not the same as the 'GTP-bound form', and there is further evidence in the paper that the notion 'active' is a misnomer.
6. Figure 2: Identify the P loop in the GTP-bound structure
7. Figure 3: the structure of EF-G in the GDP form is lacking most of domain 3. It is not clear to me how this incomplete structure can be compared with the rest.
8. Fig 5E: indicate error bars and describe the significance of all measurements. How many independent measurements were done, including biological and technical replicates? What is the evidence that the deviation from the linear behavior at low T{degree sign} is not caused by precipitation, aggregation, misfolding or any other type of protein inactivation?
9. Table 1, column 3, construct purified: is it a misprint, should probably read eIF5B870C)?
10. P. 3, 'EF-Tu, eIF5B, IF2...' For the sake of completeness, the authors may want to mention RF3 and SelB.
11. P. 11, 'At the center of the unresolved issues...' Besides the fact that the whole sentence is awkward, there are several structures of IF2 and eIF5B in different forms. This should be mentioned here, rather than ignoring the previous work.
12. P. 16. 'pointing...towards the beak of the 30S subunit and the so-called GAC". The reader will most probably not know what the ribosome 'beak" is and will be confused by the term "GTPase activating center' which in reality is not activating GTP hydrolysis. Simply name the respective elements of the ribosome, i.e. helix nn of 16S or 23S rRNA or protein yyy.
13. P. 17: 'In contrast to previous assumptions (Roll-Mecak)... Those authors based their suggestion on the comparisons of crystal structures, exactly as the authors of the present paper do. The difference should be explained and the previous work should be taken with a bit more care, even if the authors disagree with the interpretation.
14. P. 18: 'only in this more compact GTP conformation... finishing with the citation to Nissen et al. While Nissen et al. provided the crystal structure, the fact that only the GTP-bound form of EF-Tu binds aminoacyl-tRNA has been known decades before the crystal structure. A credit should be given to the primary work.

Referee #2:

The translation initiation factor IF2/aIF5B/eIF5B is a universally conserved GTPase that promotes ribosomal subunit joining. Its mechanism of action is incompletely understood, particularly with respect to the influence of its nucleotide binding state/GTP hydrolysis on its function. Here, Kuhle and Ficner report X-ray crystallographic data that lead to an interesting alternative to an earlier "articulated lever" model that was based on the crystal structure of Methanobacterium thermoautotrophicum aIF5B (Roll-Mecak et al., 2000) and that suggested that conformational differences in the switch regions in aIF5B's G-domain induced by nucleotide binding are minor but are amplified to cause a rigid pendulum-like movement in domain IV.
In this interesting and clearly presented manuscript, Kuhle and Ficner report that they have determined the crystal structure of Chaetomium thermophilum eIF5B (consisting of the G domain with domains II - IV) in apo form, and the crystal structures of various domains of C. thermophilum and Saccharomyces cerevisiae eIF5B in apo-, GDP-bound and GTP-bound forms at resolutions from

1.83 to 3.02 Angstroms. These individual domains have a similar topology and arrangement to domains in the previously reported full-length M. thermoautotrophicum aIF5B, but domain III is substantially displaced between the three structures, and the position of domain IV in them is variable. Importantly, the switch 1 and switch 2 regions undergo substantial conformational changes on binding of GTP, so that direct interactions with the gamma-phosphate of GTP are established. Binding of GTP also leads to rotation of domain II so that it forms a new interaction with the G domain, and likely to dissociation of domain III from the G domain (and consequent movement of the linked domain IV). These changes are mostly reversed following GTP hydrolysis, although switch 2 and domain III remain somewhat displaced in the GDP-bound state relative to their "apo" positions. These observations are supported by the authors' isothermal calorimetry data, and together suggest that eIF5B switches between inactive apo/GDP-bound and active GTP-bound states. These observations constitute a significant advance, and are in direct contrast to observations concerning M. thermoautotrophicum aIF5B, thus contradicting the 'articulated lever' model. Kuhle and Ficner term their new model the "dissociative switch mechanism" because of the dissociation of domain III of eIF5B and the consequent movement of domain IV that are induced by binding of GTP. The authors correctly note some of the data that have previously caused the "articulated lever" model to be questioned. Various alternative models have been proposed to accommodate these data, such as the "conformational switching" model (e.g. Hauryliuk et al., (2008) Biophys J. 95: 1704-1715; Pavlov et al. (2011) EMBO J. 30: 289-301), in which binding of GTP is not sufficient for activation of eIF5B/IF2, and in which an equilibrium between inactive and active GTP-bound forms is shifted towards the latter on binding to an auxiliary cofactor, such as the ribosome. It would therefore be appropriate if the authors related their findings to this and other models for eIf5B's activity, rather than restricting their comparisons to the Roll-Mecak model.
Importantly, immediately before submission of the present manuscript, a cryoEM study by V. Ramakrishnan and colleagues published online and in print (Fernandez et al., Science 342, 1240585 (2013)) reported a similar coordinated reorientation of domains III and IV relative to the G domain and domain II as well as interactions of domain IV with P-site initiator tRNA for ribosome-associated yeast eIF5B bound to a non-hydrolyzable GTP analogue. The present study is at a significantly higher resolution than this work, and thus affords more detailed insights. Nevertheless, the text should be revised such that the description of the modeling of eIF5B's interaction with the ribosome (pp15-16 in the present manuscript) and discussions of the shifting of domain III (pp17-19) and of the dependence of eIF5B-mediated subunit joining on the presence of initiator tRNA in the P site of the 40S subunit (pp20-21) take these other data into account.

Referee #3:

The paper by Kuhle and Ficner describes six different structures of the initiation factor eIF5B from C.thermophilus and S.cerevisiae. Previously only the structure of the apo form of an archeal homologue was known. Here they describe the apo form of a construct comprising domains I-IV, the GDP-Bound form of domains I-III and the GTP analogue form of domains I-II. While the apo and GDP-bound structures are very similar and similar to previous structures, the GTP-bound structure shows very convincingly that the proteins goes through very large conformational changes, in particular of switch I and II, to induce canonical interaction between the phosphates and Mg. They also show that the GTP-bound structure is very similar to that of EF-Tu and can convincingly demonstrate that the binding site of the G domain (domain I) and the mechanism of GTP hydrolysis on the ribosome should be very similar between the translation factors.
So far, so good
The authors also present very detailed ITC measurements for the binding of various nucleotides in the absence of presence of Mg, and deduce from the hat capacity some very wild speculations about the interface between domains. It should be pointed out that even small changes in proteins can cause large changes in DH and DS and heat capacities, which people have a hard time to interpret..

The authors go on to propose a what they call a dissociative switch mechanism that is based on the assumption that domain III is released from its interaction with domain I and II. However there is no structure that would back up this proposal and the evidence from ITC et al is at best questionable. For a full understanding of the role of eIF5B in joining the ribosomal subunits, the location of domain IV would also be very helpful but it is also not seen in the GTP-bound structure.

In summary I think there are some nice results but the story is definitively heavily oversold.

Some specific comments:

It would be easier if Figures 2 A,B,C would show the switches in the same colour, in a background of different colours for each structure
some Figures are way too busy, ie Fig. 2D, 3A,
Fig. 5 is fairly useless, we know how an ITC curve looks like and I trust that they are correctly interpreted, as shown in Table 2
The paper needs some language correction, ie evolutionary conservatism (p3) is probably meant to mean conservation ect

1st Revision - authors' response 06 February 2014

Referee #1:

1. The authors use too many terms in quotes, such as 'active' and 'Inactive' forms of GTP, 'dissociative switch mechanism', 'articulated level model', etc. This gives a strong impression that the authors do not believe that the respective forms are really 'active' or 'inactive'. In some cases it is even justified, as the authors correctly point out. I read these quotes as a psychological problem the authors have with the uncertainties/inaccuracies of the different eIF5B forms, e.g. the GTP form becomes 'inactive' on the ribosome, as it has to change the conformation to bind properly.

Reply:

We originally used these quotes to identify 'active' and 'inactive' forms, 'dissociative switch mechanism', 'articulated level model', etc. as definitions of words or phrases in specific contexts. However, we removed them in most cases in order to avoid the impression of uncertainties concerning the different functional activation states of eIF5B.

My suggestion is to avoid this problem altogether by giving the exact nature of the conformation (e.g. GTP-bound form) without qualifying it as 'active' or 'inactive'. In the case of EF-Tu, the GTP-bound form is really active and the GDP-fom is really inactive, so the quotes can be omitted. In the case of EF-G, the issue is as uncertain as with eIF5B (because there are so many different potential conformations involved) and I would not use the active-inactive nomenclature either.

Reply:

We agree with referee #1 that the nomenclature is problematic in our case. Originally we followed two of the most frequently cited reviews on GTPase function, where the GDP-bound state is either termed ‘inactive’ or ‘off-state’ by Bourne et al (Nature, 1991) and Vetter & Wittinghofer (Science, 2001) while the GTP-bound state is termed ‘active’ or ‘on-state’. In both cases the GTP-bound form (active/on-state) is *structurally* defined as the conformation of the G domain in which the invariant Thr of switch 1 and Gly of switch 2 form direct contacts to the GTP-γ-phosphate and it is *functionally* defined as the state that allows high affinity and productive binding to the effector molecules of the G protein. Thus, in both cases GTP binding and the following conformational changes of the G domain are considered as an activation process of the G protein that precedes and is distinct from the activation for GTP hydrolysis (GTPase activation) itself.

Due to the multi-domain architecture of eIF5B and the stepwise activation mechanism that we propose for eIF5B, it is difficult to find a simple and unifying nomenclature that is in agreement with the above definitions but does not result in confusion. We revised our nomenclature, trying to follow the suggestions by referee #1, as follows:

When we refer to the G domain and the reorganization of the switch regions alone, we continue to use the active-inactive nomenclature. The reason is that the terms 'active'/'inactive' for the G domain have a strict and unambiguous structural definition according to Bourne et al and Vetter & Wittinghofer, which is critical to relate our own eIF5B·GTP structure to other Ras-related GTPases. This is important to highlight the difference between the GTP-bound eIF5B G domain that is 'activated' in the classical sense (in our case) and the conformations of the G domains in structures of aIF5B and IF2 that are likewise called 'GTP-bound forms' (Roll-Mecak et al, Cell (2000); Simonetti et al, Acta Cryst. Section D (2013)) but are not activated in the classical sense as defined by Bourne et al and Vetter & Wittinghofer.

As suggested by referee #1 we replaced the term 'active' for GTP-bound eIF5B. Instead we now use either 'GTP-bound state' of eIF5B or simply 'eIF5B·GTP'. We avoided the term 'GTP-bound form' as it would give the wrong impression that we assume *one defined* conformation which GTP-bound eIF5B adopts in solution prior to its interactions to the ribosome. However, a critical point of the domain release mechanism is that exactly this is not the case for eIF5B·GTP in solution.

For the defined conformation of eIF5B·GTP that is stabilized only in the context of the 48S pre-IC, we use the term 'subunit joining competent conformation'.

Finally, for the *process* that covers the steps from GTP-binding to the resulting GTP-induced conformational changes in G domain and the overall domain architecture of eIF5B to allow high affinity and productive interactions with its ribosomal effector complexes, we continue to use the term 'activation process', in agreement with the functional definition by Bourne et al (Nature, 1991) and Vetter & Wittinghofer (Science, 2001) for the activation of G proteins in general.

2. The term 'dissociative switch' is rather misleading because it sounds too close to the 'dissociative mechanism' for GTP hydrolysis, which is another actively discussed issue for the GTPases in general. The authors should find a better, more precise term with less overlap with the terms already in use.

Reply:

The term was replaced by 'domain release mechanism'.

3. The 'dissociative switch' mechanism cries for biochemical validation. The best way would be to identify the mutants which may affect the interactions between switch 2 and domain 3 and test their effect on the activity of eIF5B. While I understand that this may be beyond the scope of the paper, at minimum the author should prepare a Table of the potentially involved residues and compare them with those available in the literature. There is some discussion in this direction, but the text is insufficiently focused and not illustrated.

Reply:

Independent biochemical as well as structural support for the domain release mechanism is provided by several previous studies, including the newly published study on the cryo-EM structures of 80S-bound eIF5B·GDPCP (Fernandez et al, Science (2013)) (see also response to comments of referees #2 & #3). Moreover, there are several biochemical studies on the influence of mutations in residues that we predict to be involved in the domain release mechanism (e.g. Thr439, Gly479 and Asp740) that as well provide evidence in favour of the proposed model for eIF5B activation (e.g. Shin et al, Mol. Cell. Biol. (2007); Shin et al, Cell (2002)).

In order to provide a more comprehensive and focused comparison of the available mutational and biochemical studies on eIF5B activity and their interpretation in the context of our model, we included Table S1 that lists mutations of residues involved in the conformational switch mechanism of eIF5B. We also included Figure S7B/C for a better illustration. In order to provide a clearer picture of the proposed mechanism and for better understandability, we rewrote the section "eIF5B

combines the classical GTP operated switch mechanism in the G domain with a novel mechanism of activation for a trGTPase" (pages 15-17), which includes the discussion of previous biochemical data (last paragraph) and now reads as follows:

"TrGTPases such as eIF5B and EF-Tu are multidomain proteins which consist of a universally conserved structural core composed of the G domain and domain II that is supplemented with additional functional domains related to the respective role of the GTPase during translation. The activation of trGTPases by GTP is therefore not merely restricted to the G domain but involves a reorganization of the overall domain arrangement, induced by a modulation of the interactions between G domain and the downstream functional domains.

This principle was first established for the elongation factor EF-Tu. Here, the GTP-induced transition from the inactive apo form to the GTP-bound state depends on the rearrangement of the switch regions in the G domain that follows the canonical switch mechanism of Ras-like GTPases (Berchtold et al, 1993). As a consequence, domain II, which is separated from the G domain in inactive EF-Tu, stably associates with the reorganized G domain involving the newly formed surface of the activated switch 2 (Berchtold et al, 1993) (Fig. 3C). Only in this more compact GTP-conformation EF-Tu is able to form a stable ternary complex with aminoacyl-tRNA – involving also the reorganized switch 1 – for delivery of the latter to the ribosome (Abrahamson et al, 1985; Delaria et al, 1991; Louie & Jurnak, 1985; Nissen et al, 1995; Romero et al, 1985). Thus, the *presence* of the defined *active* conformation of switch 1 and 2 functions as the critical signal and thereby is a necessity for the overall activation of EF-Tu (Fig. 3C).

The same basic principle of activation also applies to eIF5B: the G domain follows the classical molecular switch mechanism (Fig. 2) and the signal of G domain activation is propagated into domains II-IV through the reorganization of the switch regions. However, the mechanism by which this signal transduction is achieved appears to be different from that in EF-Tu and so far unprecedented in trGTPases: in eIF5B the GTP-induced *absence* of the *inactive* conformation of switch 2 and the resulting release of domain III seems to be the decisive signal that renders eIF5B·GTP activated for productive interactions with the ribosome (Fig. 3). This does not imply that the GTP-bound conformation of the G domain is irrelevant for eIF5B function but is most likely required for tight interactions of the G domain with the large ribosomal subunit and GTPase activity (Fig. 4) as well as to prevent the reassociation of domain III before GTP hydrolysis. In turn this means that the defined GTP-conformation is not as critical for productive interactions between eIF5B and its effector molecules as it is for EF-Tu.

This scenario is in line with earlier biochemical studies that identified mutations in the G domain and domain III that are able to partially activate eIF5B by destabilizing the interactions between domain III and switch 2 in inactive eIF5B. The mutation of Gly479 in switch 2 to Ala was found to reduce GTP binding and to impair subunit joining and ribosome dependent GTP hydrolysis (Shin et al, 2007). Our structural analysis shows that this Gly residue undergoes a peptide flip of ~160° during the transition of switch 2 from its inactive to the active state (Fig. 4B), a conformational change that is energetically not allowed for any other residue. G479A would thus stabilize the inactive switch 2 preventing the formation of the GTPase centre and the release of domain III, ultimately causing the inability of the mutant to promote subunit joining and to hydrolyse GTP. A444V and D740R were identified as two independent intragenic suppressor mutants for G479A that restore nucleotide binding, GTP hydrolysis and subunit joining activities in eIF5B (Shin et al, 2007). Interestingly, Asp740 is located in domain III ~30 Å apart from the nucleotide binding pocket and forms a direct salt bridge to the conserved Arg489 of the *inactive* switch 2 which moves ~15 Å upon GTP binding (Fig. 3B and S7B/C). Consequently, D740R would result in a steric and electrostatic repulsion of the inactive switch 2 and thereby a destabilization of the domain III-switch 2 contact in apo eIF5B. A444V is located at the N-terminus of strand β3 close to Asp476 in the G3 motif and most likely causes the constitutive reduction of the energy barrier that has to be overcome by GDP and GTP to move Asp476 into a GTP-like position thereby facilitating the distortion of the interactions between switch 2 and domain III (Shin et al, 2007) (Fig. S7C). Particularly interesting is that A444V does not only restore GTP dependency in eIF5B but even allows GDP to activate eIF5B for stable interactions with the ribosome (Shin et al, 2007). This demonstrates that the full GTP-conformation in the G domain is not an absolute requirement for stable interactions between eIF5B and the ribosome. Instead, it seems to be critical that the suppressor mutations overcome the increased energy barrier introduced by G479A by destabilizing the inactive conformation and thereby the contact between switch 2 and domain III either directly in the case of D740R or indirectly in the case of A444V (see also Table S1)".

4. P. 13, 'To investigate the effect of Mg...' I am surprised that nucleotides bind at all to a GTP binding protein in the absence of Mg2+. Even more confusing, the affinity of eIF5B to GDP appears to INCREASE in the absence of Mg2+ (Table II). This does not agree with what is known for other GTP-binding proteins, because Mg2+ is expected to stabilize the changes on GTP/GDP upon binding to the protein. What is the explanation for this effect? What is the residual Mg2+ in the buffer?

Reply:

The observation reported here for eIF5B is not unprecedented among GTPases. Although it is true that Mg2+ in most known cases stabilizes the binding of nucleotides (GTP and GDP) to GTP binding proteins, there are a number of examples known where this is not the case. The most prominent examples are the Gα subunits of some heterotrimeric G proteins, for which the complete independency of GDP-binding from Mg2+ has been reported (Higashijima et al, J. Biol. Chem. (1987)) and for which structures have been solved in the GDP-bound form that do not contain Mg2+ (reviewed in: Wittinghofer, The functioning of molecular switch in three dimension. In GTPases, A. Hall ed, Oxford University Press (2000)). More recently, it was shown for the small Ras-related GTPase Arl3 by structural and biochemical analysis that it also does not require Mg2+ for GDP binding (Hillig et al, Structure (2000). Finally, more directly related to the case of eIF5B is the example of the eukaryotic release factor 3 (eRF3). A number of studies (e.g. from the labs of M. Rodnina, T. Pestova or L. Kisselev) have demonstrated that this translational GTPase does not only bind GDP in the absence of Mg2+ but that the affinity of eRF3 to the nucleotide INCREASES with decreasing Mg2+ concentrations, resulting in the highest affinity in the complete absence of Mg2+ (Pisareva et al, J. Biol. Chem. (2006); Mitkevich et al, Nucleic Acids Res. (2006); Kong et al, Mol. Cell, (2004)). It was therefore concluded that Mg2+ might even accelerate GDP release and exchange for GTP (in all the above examples, the independency from Mg2+ was only observed for GDP but not for GTP binding).

A similar but significantly less severe effect was observed in our own ITC experiments for the related GTPase eIF5B. Like in the case of eRF3, where it is not known in molecular detail why Mg2+ reduces the affinity for GDP, the origin for the effect in eIF5B is difficult to identify. However, it becomes apparent from our crystal structures of GDP-bound eIF5B that the Mg2+ ion induces conformational strain on switch 2 and the adjacent regions in the G domain as well as in domain II and III that might thereby destabilize GDP binding.

We also would like to mention that we could confirm the observation that Mg2+ is not required for GDP binding by fluorescence experiments, measuring the association of mant-labeled GDP and GTP to nucleotide-free eIF5B. We observed that mantGDP binding results in an increase of fluorescence upon binding to eIF5B (excitation at 355 nm; emission at 440 nm at 25 °C) in the presence of 3 mM Mg2+ as well as in the absence of Mg2+ (residual Mg2+ was removed by the addition of 1 mM EDTA). In contrast, mantGTP only induced an increase in fluorescence (indicative for binding) in the presence of Mg2+, while the repeated addition of EDTA successively reduced this signal. These results support the assumption from the ITC experiments that Mg2+ is not required for GDP binding and that the observed interactions do not depend on the presence of residual Mg2+ in the buffer. Nonetheless, Mg2+ seems to be necessary for efficient interaction with the triphosphate form of the nucleotide, which is typical for GTP binding proteins. It should also be noted that the structure of the archeal aIF5B·GDP does not contain a Mg2+ ion bound to the GDP molecule (which should be well defined at a resolution of 2.0 Å), despite the fact that the crystallization condition – as the authors of the study explicitly point out – contains 20 mM $MgCl_2$ (Roll-Mecak et al, Cell (2000)).

Originally, we included a detailed discussion of this observation similar to that presented here. However, though interesting for the question how nucleotide exchange might be achieved on eIF5B, it is not directly relevant to the main argument and the conclusions of this manuscript concerning the activation mechanism and function of eIF5B during ribosomal subunit joining. Due to the limited space and the potential distraction from the main points of the argument, we decided to exclude the additional results and this discussion from the manuscript.

5a. Abstract: 'Effector' domain III....activated eIF5B'. What is the evidence that domain III is an 'effector domain'?

Reply:

We used the term ‘effector domain’ in the same sense as used by Eiler et al. (PNAS, (2013)). According to this definition, an ‘effector domain’ is analogous to the effector molecules of small Ras-like GTPases and thus forms stable and functionally relevant contacts to the switch regions of the G domain in the course of the nucleotide cycle. However, we now realized that the term ‘effector domain’ is occasionally used synonymously to ‘effector region’ or ‘effector loop’ for the switch 1 region in the G domain itself. In order to avoid this confusion (which might be the reason for the above question by referee #1) we removed the term ‘effector domain’, except when referring directly to the work by Eiler et al (PNAS, 2013).

5b. 'Activated' reads like 'GTPase activated' in this context, which is not what the authors want to say. 'Activated' is not the same as the 'GTP-bound form', and there is further evidence in the paper that the notion 'active' is a misnomer.

Reply:

We replaced the term ‘activated’ with ‘GTP-bound’ eIF5B.

6. Figure 2: Identify the P loop in the GTP-bound structure

Reply:

Done

7. Figure 3: the structure of EF-G in the GDP form is lacking most of domain 3. It is not clear to me how this incomplete structure can be compared with the rest.

Reply:

We agree with the referee that the used structure of EF-G lacks most of domain 3, which might result in confusion or misunderstandings. Due to the limited space and the fact that the comparison to EF-G is not essential for our argument, which is instead primarily based on the well studied case of EF-Tu, we decided to leave out the example of EF-G entirely.

Briefly: What we intended to show in this figure was that domain III as the functional domain of EF-G that is contacted by the switch regions in the GTP-bound state (PDB-ID 4BTC) does not form any stable contacts to the switch regions of the G domain when GDP is bound instead, which would be in contrast to the situation in eIF5B. In our opinion this becomes obvious from the 1DAR (PDB-ID) structure as domain III is not missing from the crystallized protein but too flexible to be resolved in the electron density, indicating insufficient stabilization by interactions to the other domains (the fragments of domain III that are resolved show that the domain is not incomplete due to a proteolytic cleavage of the protein). Moreover, we chose 1DAR as it is one of the first two EF-G·GDP structures in the PDB and unlike structures that do contain the complete domain III (e.g. 2BM0, 2BM1, 2J7K) does not contain any point mutations.

8. Fig 5E: indicate error bars and describe the significance of all measurements. How many independent measurements were done, including biological and technical replicates?

Reply:

The error bars were included in the new version of Figure 5. Errors for the changes in heat capacity in Table III were indicated. The number of independent technical replicates had been indicated below Table II; the number of biological replicates is now included in the revised version of Table II:

"All measurements were performed two to four times; for GTP binding to both constructs and for GDP binding in the presence of Mg^{2+} experiments were done with two independent purifications of the respective construct; for GDP binding in the absence of Mg^{2+} the experiments were done with protein from one purification."

What is the evidence that the deviation from the linear behaviour at low T{degree sign} is not caused by precipitation, aggregation, misfolding or any other type of protein inactivation?

Reply:

If the deviation from the linear behaviour at low temperatures was caused by protein inactivation, a deviation from the expected stoichiometry of 1:1 between protein and nucleotide should be observed; however, this was not the case and the stoichiometry stayed constant in the temperature interval from 5 to 30 °C for all nucleotides. Moreover, it would be surprising if only GDP binding and only in the presence of $MgCl_2$ should cause precipitation (which was not apparent from ocular inspection), aggregation or misfolding at low temperatures. The gel filtration runs during purification (which were performed at 4 °C) as well as the structural data indicate that low temperatures do not induce a severe aggregation or misfolding of the protein. As we initially also considered this possibility, we performed analytical gel filtration runs with eIF5B *after* the ITC experiment, which as well did not indicate aggregation or a folding/radius of gyration different from the protein before the experiment.

9. Table 1, column 3, construct purified: is it a misprint, should probably read eIF5B870C)?

Reply:

This is not a misprint. As described in the Supplementary Materials and Methods, we tried to crystallize the construct eIF5B(517C) containing G domain and domains II to IV in the presence of GDP, GDPNP or GTP under the same conditions as for the apo form. However, while no crystals were obtained in the presence of GDP or GDPNP, the presence of GTP resulted in the formation of crystals that contained only the construct eIF5B(870C), corresponding to domains III and IV. Analysis of the crystal packing in the resulting structure unambiguously shows that there is no space left to accommodate the G domain or even domain II. Furthermore, we loaded dissolved crystals onto an SDS-gel, which confirmed that the crystals contained only a fragment of the original protein. It therefore seems that the presence of GTP (but not GDP or GDPNP) resulted in an increased susceptibility of the linker between domain III and domain IV to proteolytic cleavage, which is paralleled by the removal of domain III in the eIF5B·GTP crystals by cleavage in the same region. Thus, the construct purified was eIF5B(517C), the protein in the crystal was eIF5B(870C).

10. P. 3, 'EF-Tu, eIF5B, IF2...' For the sake of completeness, the authors may want to mention RF3 and SelB.

Reply:

Although referee #1 is of course correct that RF3 and SelB (as well as eRF3 and eIF2γ) belong to the set of translational GTPases, they were deliberately left out as we tried to put an emphasis on EF-Tu, EF-G and eIF5B (and its orthologs) as the three *universal/ubiquitous* translational GTPases that are found in *all* extant living organisms to point out the importance of eIF5B in ribosomal translation. This is elaborated in the next sentence of the manuscript: "This suggests that the function of eIF5B and its orthologs was fixed at an early stage of cellular evolution before the onset of speciation, which reflects the importance of subunit joining as the final control step in the initiation pathway." By contrast, the EF-G paralog RF3 is found only in (some) bacteria, the EF-Tu paralog eRF3 is found only in eukarya and a/eIF2γ is found only in eukarya and archea. Finally, although SelB is present in all three domains of life and could therefore be considered universal, it is only found sporadically scattered across the different lineages, demonstrating that it is not as essential for the process of translation and cellular life as the three factors that were mentioned.

11. P. 11, 'At the centre of the unresolved issues...' Besides the fact that the whole sentence is awkward, there are several structures of IF2 and eIF5B in different forms. This should be mentioned here, rather than ignoring the previous work.

Reply:

The sentence was rewritten and now reads as follows:

"For aIF5B as well as IF2, crystal structures have previously been solved in the GDPNP- and GTP-bound state, respectively (Roll-Mecak et al, 2000; Simonetti et al, 2013). However, in both cases the G domain remained in the apo/GDP conformation despite the presence of the γ-phosphate. Thus, the knowledge of eIF5B and IF2 function is limited by the fact that up to now no high resolution structural information is available for their GTP-bound forms that is in agreement with the classical concept of molecular switching. Though of paramount importance to the understanding of eIF5B/IF2 function, it is therefore not known what distinguishes the active from the inactive state of the G domain and how these differences modulate the affinity of the overall eIF5B/IF2 to ribosomal effector complexes or influence the mechanism of ribosome induced GTP hydrolysis."

The central questions for our work that formed the basis for our activation model for eIF5B are: what is going on in the G domain, the functional centre of eIF5B, in the course of the nucleotide cycle, what effects do these changes have on the rest of the protein and how is this switch mechanism related to the classical concept of molecular switching for G proteins? To answer this question, high resolution structural information is needed for the eIF5B/IF2 G domain in its GTP-bound conformation that provides the possibility to explain the evolutionary conservation of specific residues that are essential for eIF5B function and that are essential for the classical conformational switch mechanism in other G proteins, for available mutational, genetic or biochemical data as well as for lower resolution cryo-EM structures. Such a structure was not available before, neither for eIF5B nor for IF2. We hope that we were able to formulate this problem in a clearer way in the revised version of the paragraph.

Concerning the structures of IF2 and eIF5B: There are three previous works that provide high resolution structures of IF2 and aIF5B with and without nucleotides that could give insight into the molecular details of their nucleotide cycles: Archeal aIF5B in apo, GDP- and GDPNP-bound state (Roll-Mecak et al, Cell (2000)); IF2 in the apo state and apo state crystals soaked with GDP or GTP (Simonetti et al, Acta Cryst. D (2013)); IF2 in the apo and GDP-bound state (Eiler et al, PNAS (2013)). We referred to all three studies on pages 3-5 (and later on) with reference to their structural work. The conclusions from the work by Roll-Mecak et al. and Eiler et al. concerning eIF5B and IF2 function were discussed in more detail in the paragraph preceding 'At the centre of the unresolved issues…' as well as in the Discussion part (Pages 17 and 23/24, respectively). We

revised both paragraphs (see also the response to comment #13). The paragraph on page 22 concerning the work by Eiler et al (2013) now reads as follows:

"The recent crystal structure of *T. thermophilus* IF2(3-467) in the apo and GDP-bound state as well seems to argue against the domain release mechanism for IF2 as domain III has no direct contact to either of the switch regions (Eiler et al, 2013). The authors propose that the increased length of helix α8 (the linker between domains II and III) compared to α8 in aIF5B accounts for the inability of domain III to contact switch 2 (Eiler et al, 2013). However, the lengths of helix α8 and the following flexible linker to domain III is actually compatible with a direct contact between the N-terminal half of helix α9 and switch 2 as observed in apo a/eIF5B (Fig. 3A/B). Instead, the crystal packing shows that the position occupied by domain III in a/eIF5B is occupied by symmetry related molecules in the IF2 crystals. Crystallization would therefore be selective for the state in which domain III is released, irrespective of its fraction among the IF2 molecules in solution. As we show by means of ITC and observe in the Sc-eIF5B·GDP structure (Fig. S1E and S7A), domain III in eIF5B has the ability to dissociate from the G domain even in the absence of GTP. This, we suggest, also applies to IF2."

The work by Simonetti et al. (Acta Cryst. D (2013)) on the IF2·GTP structure is very problematic. This structure was obtained by *soaking* GTP into crystals that had been obtained with the apo form of the protein. An analysis of the crystal packing shows that switch 1 and switch 2 form extensive contacts to symmetry related molecules, which make the conformational changes that would allow the conserved switch regions to contact the GTP molecule and adopt the canonical conformation observed in EF-Tu, Ras or eIF5B physically impossible. Consequently, the G domain remains virtually unchanged upon GTP binding without any direct contacts formed between the switch regions and the GTP molecule. It is therefore in our opinion impossible to deduce representative conformational changes for the IF2 G domain upon GTP binding or its GTP-bound conformation based on this structure. As we do think that these structures are important for our own work in the respect that they demonstrate the structural homology between eIF5B and IF2, we cited Simonetti et al. (Acta Cryst. D (2013)) in the context of the domain architecture of eIF5B and IF2 (Pages 3 and 7). We introduced new citations in the discussion, where we originally quoted the structures themselves. Moreover, we incorporated a discussion of this IF2·GTP structure in the last section of the discussion part:

"The assumption that the domain release mechanism also applies to IF2 seems to be contradicted by two recent structural studies (Eiler et al, 2013; Simonetti et al, 2013). The only so far available high resolution structure of GTP-bound IF2 appears to indicate that its G domain does not follow the classical switch mechanism, as switch 1 remains virtually unchanged upon GTP binding and switch 2 undergoes only a small local rearrangement without forming a contact to the γ-phosphate (Simonetti et al, 2013). However, it is important to note that this IF2·GTP structure was obtained by soaking GTP into crystals of apo IF2, in which both switch regions are fixed by extensive contacts to symmetry related molecules. A reorganization of the G domain that would allow the G2 and G3 motifs to contact the GTP molecule in the classical way is thus most likely prevented by crystal contacts and not due to a non-classical behaviour of IF2."

12. P. 16. 'pointing...towards the beak of the 30S subunit and the so-called GAC". The reader will most probably not know what the ribosome 'beak" is and will be confused by the term "GTPase activating centre' which in reality is not activating GTP hydrolysis. Simply name the respective elements of the ribosome, i.e. helix nn of 16S or 23S rRNA or protein yyy.

Reply:

As the model of eIF5B·GTP has become obsolete with the now available cryo-EM structure of eIF5B on the 80S ribosome, we decided to remove our detailed modelling procedure and instead refer to the work by Fernandez et al. (Science, 2013). Please also see the response to comments by referee #2.

13. P. 17: 'In contrast to previous assumptions (Roll-Mecak)... Those authors based their suggestion on the comparisons of crystal structures, exactly as the authors of the present paper do. The difference should be explained and the previous work should be taken with a bit more care, even if the authors disagree with the interpretation.

Reply:

We tried to give a description of the articulated lever model proposed by Roll-Mecak et al (2000) two times: In the introduction (page 5):

"The non-classical 'articulated lever model' for eIF5B/IF2 function, which is based on crystal structures of aIF5B, assumes that a GTP induced ~2 Å shift in switch 2 is amplified into a ~5 Å movement of domain IV (Roll-Mecak et al, 2000). According to this model, neither switch 1 nor switch 2 undergo a conformational change or form direct contacts to the γ-phosphate…".

And at the end of the quoted paragraph of the Discussion (page 17): "This 'dissociative switch mechanism' stands in stark contrast to the previously proposed 'articulated lever model' for eIF5B/IF2 function, where GTP-induced conformational changes are limited to a moderate ~2 Å shift in switch 2 of the G domain, resulting in a rigid ~5 Å movement of domain IV (Roll-Mecak et al, 2000) (Fig. S2)".

In order to make the differences between our model and the articulated lever model clearer, we changed the first part to (page 5):

"The 'articulated lever' model for eIF5B/IF2 function, which is based on crystal structures of aIF5B, assumes that a GTP induced ~2 Å shift in switch 2 is amplified by an *en bloc* rearrangement of domains II to IV into a ~5 Å movement of domain IV (Roll-Mecak et al, 2000). According to this model, neither switch 1 nor switch 2 undergo the conformational changes or form the direct contacts to the γ-phosphate that are typical for the classical molecular switch."

The discussion part on page 17 (now page 15) was changed to:

"Moreover, the domain release mechanism is in stark contrast to the previously proposed non-classical articulated lever model for eIF5B/IF2 function, in which the GTP-induced conformational changes in the G domain are limited to a ~2 Å shift in switch 2. This causes a rigid body movement of domains III and IV and a displacement of the latter by ~5 Å as ultimate result of eIF5B activation (Roll-Mecak et al, 2000; Roll-Mecak et al, 2001). In contrast to the release mechanism, this involves neither a conformational change in switch 1 or switch 2 to form the canonical catalytic GTPase centre nor requires the loss or the formation of contacts between G domain and domains II and III at any stage of the activation process (Fig. S8). This model therefore does not explain why switch 1 and switch 2 are universally conserved and why the mutagenesis of conserved residues in both motifs results in severe functional defects in eIF5B (Lee et al, 2002; Shin & Dever, 2007; Shin et al, 2002)."

To illustrate this difference, we included additional images in Figure S8.

14. P. 18: 'only in this more compact GTP conformation... finishing with the citation to Nissen et al. While Nissen et al. provided the crystal structure, the fact that only the GTP-bound form of EF-Tu binds aminoacyl-tRNA has been known decades before the crystal structure. A credit should be given to the primary work.

Reply:

Primary work was incorporated: Abrahamson et al, 1985; Delaria et al, 1991; Louie & Jurnak, 1985; Romero et al, 1985)

Referee #2:

The authors correctly note some of the data that have previously caused the "articulated lever" model to be questioned. Various alternative models have been proposed to accommodate these data, such as the "conformational switching" model (e.g. Hauryliuk et al., (2008) Biophys J. 95: 1704-1715; Pavlov et al. (2011) EMBO J. 30: 289-301), in which binding of GTP is not sufficient for activation of eIF5B/IF2, and in which an equilibrium between inactive and active GTP-bound forms is shifted towards the latter on binding to an auxiliary cofactor, such as the ribosome. It would therefore be appropriate if the authors related their findings to this and other models for eIf5B's activity, rather than restricting their comparisons to the Roll-Mecak model.

Reply:

The alternative models have been included into the introduction as well as in the discussion of eIF5B and IF2 function.

Page 5:

"However, low resolution cryo-EM reconstructions of bacterial and eukaryal 70S/80S ICs revealed conformations of IF2 and eIF5B that are incompatible with the articulated lever model (Allen et al, 2005; Fernandez et al, 2013). In order to reconcile the contradictory experimental data with the classical concept of molecular switching it was suggested that eIF5B and IF2 follow a mechanism of 'cofactor dependent conformational switching', in which GTP binding alone is insufficient to activate eIF5B/IF2 but requires the ribosome as a cofactor that shifts the equilibrium between an inactive and an active GTP-bound form toward the latter (Hauryliuk et al, 2008; Pavlov et al, 2011). More recently…"

Page 14:

"This mechanism for the activation of eIF5B in solution contradicts earlier assumptions that GTP alone is insufficient to induce the conformational switch in free eIF5B in the absence of the ribosome as auxiliary cofactor, which forms the basis for the hypothesis that eIF5B follows a mechanism of conditional switching (Hauryliuk et al, 2008)."

Page 18:

"Consequently, for the domain release model a distinction has to be drawn between the 'activated state' of eIF5B·GTP in solution and its 'subunit joining competent conformation' on the 48S pre-IC, which is only one of the many possible conformations accessible to the free eIF5B·GTP, and which requires the reduction of conformational freedom of domains III and IV and their stabilization in the correct orientation. This proposed dependency of eIF5B·GTP on the ribosomal effector complex resembles the hypothesis of conditional switching for eIF5B (Hauryliuk et al, 2008). However, the critical conceptual difference is that eIF5B does not require the ribosome as cofactor to induce the GTP-dependent conformational switch, but instead depends on the ribosomal effector complex to stabilize the sole conformation of GTP-bound eIF5B (among the many possible) capable to promote the association of the 60S subunit. The recent cryo-EM structures"

Page 22:

"As for eIF5B, the domain release model can explain why IF2 requires GTP for efficient interactions with ribosomal complexes and the fMet-tRNA^{fMet} in the context of the 30S pre-IC (Antoun et al, 2003; Antoun et al, 2004), why IF2·GTP is unable to promote subunit docking in the absence of the initiator-tRNA (Antoun et al, 2006) and finally, why even GDP is able to partially activate IF2 for ribosome binding and subunit joining in *in vitro* studies despite its inability to stabilize the GTP-conformation of the G domain. With the critical conceptual difference that in the domain release mechanism the GTP-dependent conformational switch in IF2 precedes and is therefore not a consequence of its interaction with the ribosome, this hypothesis of a common mechanism for eIF5B and IF2 is in agreement with previous proposals of a stepwise activation mechanism for IF2 by GTP and the 30S·fMet-tRNA^{fMet} complex made on the basis of biochemical experiments (Antoun et al, 2003; Pavlov et al, 2011)."

Importantly, immediately before submission of the present manuscript, a cryoEM study by V. Ramakrishnan and colleagues published online and in print (Fernandez et al., Science 342, 1240585 (2013)) reported a similar coordinated reorientation of domains III and IV relative to the G domain and domain II as well as interactions of domain IV with P-site initiator tRNA for ribosome-associated yeast eIF5B bound to a non-hydrolyzable GTP analogue. The present study is at a significantly higher resolution than this work, and thus affords more detailed insights. Nevertheless, the text should be revised such that the description of the modelling of eIF5B's interaction with the ribosome (pp15-16 in the present manuscript) and discussions of the shifting of domain III (pp17-19) and of the dependence of eIF5B-mediated subunit joining on the presence of initiator tRNA in the P site of the 40S subunit (pp20-21) take these other data into account.

Reply:

We included a discussion of the study by Fernandez et al. in the revised version of the manuscript (see below). Here, we would like to take the opportunity to point out that the modelling of eIF5B on the ribosome (pages 15-16 and Figure 4 in the first version of the manuscript) was done without the knowledge of any details from the new cryo-EM structure by Fernandez et al. (online publication of the paper was on 7th November, one day after the submission of this manuscript; the structures were released on the 20th).

Our modelling of domains III and IV becomes obsolete with this new structure. Our primary intention was to visualize the necessity to reorganize domains III and IV relative to domains I and II to allow interactions with the tRNA and that this would require the loss of all interactions between domain III and domains I and II that are found in the apo form of eIF5B by the 'domain release mechanism' (formerly 'dissociative switch'). This is exactly what becomes directly apparent from the new cryo-EM structures by Fernandez et al. Concerning the modelling of domains I and II of eIF5B·GTP onto the ribosome we refer to the cryo-EM structures in Figures 3, 4 and S5 and in the discussion of the putative GTPase mechanism (page 19):

"Our own structural investigations show that the catalytic centres in eIF5B·GTP and free EF-Tu·GDPNP exhibit nearly identical positions for residues implicated in ribosome binding and GTP hydrolysis (Fig. 4). In line with the recent cryo-EM model of ribosome bound eIF5B (Fernandez et al, 2013), this suggests that domains I and II of eIF5B bind the 80S ribosome in the same way as the bacterial elongation factor (Fig. 4 and S5A/B)."

Furthermore, their structures of eIF5B(domains I-IV) and eIF5B(domains I and II) on the 80S ribosome provide direct evidence for our proposed domain release mechanism that predicts that domains III and IV are flexible and therefore disordered when the critical contact to the tRNA is lost (in the case of Fernandez et al due to deacylation of the tRNA). We included these observations in the revised version of the introduction and discussion section (page 18):

"However, the critical conceptual difference is that eIF5B does not require the ribosome as cofactor to induce the GTP-dependent conformational switch, but instead depends on the ribosomal effector complex to stabilize the sole conformation of GTP-bound eIF5B (among the many possible) capable to promote the association of the 60S subunit. The recent cryo-EM structures of eIF5B on the 80S ribosome demonstrate that this stability is primarily provided by the methionylated 3'-CCA end of Met-tRNA$_i^{Met}$ (Fernandez et al, 2013). In agreement with the domain release mechanism, the cryo-EM structures show that the activation of the G domain by GDPCP is sufficient to induce the release of domain III from switch 2 and domain II, but insufficient to stabilize domains III and IV in a defined conformation, as they remain disordered in the absence of amino-acylated tRNA. Only through the interactions between domain IV and the amino-acylated tRNA, domain III and IV become stabilized in their subunit joining competent conformation in which domain III is released from all its contacts to the G domain and domain II that are also found in the apo state of eIF5B and is reoriented relative to domain I and II by 65° (Fig. 3D and S5C) (Fernandez et al, 2013)."

Referee #3:

The paper by Kuhle and Ficner describes six different structures of the initiation factor eIF5B from C.thermophilus and S.cerevisiae. Previously only the structure of the apo form of an archeal homologue was known. Here they describe the apo form of a construct comprising domains I-IV, the GDP-Bound form of domains I-III and the GTP analogue form of domains I-II.

Reply:

We would like to emphasize that it is not a GTP analog but true GTP that was used to obtain this structure. This is important as it might explain why the aIF5B·GDPNP structure (Roll-Mecak et al, Cell (2000)) in contrast to eIF5B·GTP remained in a conformation nearly identical to that of the apo and GDP-bound form, similar to the observations for other translational GTPases (e.g. EF-G and eRF3) bound to GDPNP.

While the apo and GDP-bound structures are very similar and similar to previous structures, the GTP-bound structure shows very convincingly that the proteins goes through very large conformational changes, in particular of switch I and II, to induce canonical interaction between the phosphates and Mg. They also show that the GTP-bound structure is very similar to that of EF-Tu and can convincingly demonstrate that the binding site of the G domain (domain I) and the mechanism of GTP hydrolysis on the ribosome should be very similar between the translation factors.

So far, so good

The authors also present very detailed ITC measurements for the binding of various nucleotides in the absence of presence of Mg, and deduce from the hat capacity some very wild speculations about the interface between domains. It should be pointed out that even small changes in proteins can cause large changes in DH and DS and heat capacities, which people have a hard time to interpret.

Reply:

As far as we understand, referee #3 has no particular objection to our ITC measurements but in general is sceptical about the used method to correlate ITC derived changes in heat capacity with conformational changes in protein-ligand complexes. However, exactly this method is generally accepted and frequently used to estimate conformational changes in proteins upon ligand binding or the amount of surface area buried or exposed in protein-protein interactions (Connelly and Thomson, 1992; Murphy and Freire, 1992; Perozzo et al., 2004), including studies from the labs of M. Rodnina or M. Ehrenberg on guanine nucleotide binding to various translational GTPases (Hauryliuk et al, PNAS (2008); Hauryliuk et al, JMB (2009); Kononenko et al, Nucleic Acids Res., (2010); Paleskava et al, J. Biol. Chem. (2012)). Like the authors of these studies, we estimate the amount of surface areas buried upon GDP/GTP binding from the change in heat capacity.

As we did point out in the Supplementary Experimental Procedures, this correlation can be problematic, particularly in cases, where the nature of the surfaces involved in the conformational changes is not known: "Since binding of guanine nucleotides can be expected to involve both, apolar and polar residues, a treatment of the obtained ΔC_p values solely based on nonpolar contributions according to Connelly et al. (1992) would be insufficient. At the same time, the differential treatment by calculating the individual contributions of apolar and polar groups to the total surface area often proved to be inaccurate, especially in cases where the interactions did not conform to a rigid-body binding model (Faergeman et al., 1996; Frisch et al., 1997; Holdgate et al., 1997; Schrift et al., 2006). We therefore use…" In our case, the surfaces that are involved in the observed or assumed conformational changes in eIF5B are known from our structures. In order to account for the inaccuracies associated with the estimations, we avoided to give the number of residues that might be involved in the conformational changes (as done e.g. in Hauryliuk et al, PNAS (2008) and Paleskava et al, J. Biol. Chem. (2012)) but gave two limiting values for the possible changes in solvent accessible surface areas: "We therefore use two values for the area coefficients to estimate the total surface area upon ligand binding: $\Delta c_{max} = 0.24$ (= $0.7 \cdot \Delta c_{ap} + 0.3$ ·

Δc_p) as the upper limit case, assuming ~70% apolar and ~30% polar groups contributing to the total ΔASA (calculated from the crystal structures of Ct-eIF5B in its apo, GDP and GTP forms using the program AREAIMOL from the CCP4 Program Suite 6.3.0) and $\Delta c_{min} = 0.45$ as the lower limit case in which all involved residues are apolar." Despite the relatively large differences between these two estimates, they provide a very clear tendency that can be directly correlated with the *observed* conformational changes upon GDP or GTP binding in the crystal structures (e.g. GDP binding to eIF5B(517C) or GTP binding to eIF5B(517-858)). This demonstrates that the use of the method to infer conformational changes from the change in heat capacity is in principle valid in our case within the accuracy that we require to deduce the dissociation of an entire domain and the resulting exposure of large amounts of surface area (indicated by a 360% larger ΔC_p for GTP binding in the absence of domain III (-553 cal x mol^{-1} x K^{-1}) compared to the value in its presence (-155 cal x mol^{-1} x K^{-1})).

In order to make the correlation between our ITC results and those obtained from the structures clearer we included a column in Table III that contains the values for the change in surface area upon nucleotide binding, calculated from the structures. We furthermore rewrote the results part for the thermodynamic data as follows:

"To probe the conformational changes in eIF5B upon GTP binding and to test the particular influence of domain III we performed ITC experiments with two different constructs, one comprising domains I-IV (Ct-eIF5B(517C)), the other comprising only domains I and II (Ct-eIF5B(517-858)). In both cases, GTP binding was driven by favourable negative changes in binding enthalpy (ΔH = -9.34 kcal/mol for domains I-IV and -18.8 kcal · mol^{-1} for domains I-II at 30 °C) and opposed by unfavourable entropic contributions (Table II).

For both constructs, ΔH plotted against the temperature results in a straight line with negative slope (Fig. 5A) representing the change in heat capacity (ΔC_p) which can be used as estimate for the change in solvent accessible surface area (ΔASA) upon complex formation (see Materials and Methods). For Ct-eIF5B(517C) a ΔC_p of -155 cal·mol^{-1}·K^{-1} is calculated, corresponding to 344 to 646 $Å^2$ of surface area that become buried upon GTP binding (Table III). In contrast, GTP binding to Ct-eIF5B(517-858), the construct lacking domains III and IV, gives a ΔC_p of -553 cal·mol^{-1}·K^{-1}, corresponding to a GTP-dependent surface burial of 1229 to 2304 $Å^2$, in agreement with the ~1800 $Å^2$ that become buried by switch 1 and the β13-β14 loop in domains I and II according to the crystal structures (Table III). Thus, the presence of domains III and IV contributes to the overall ΔC_p of -155 cal·mol^{-1}·K^{-1} in Ct-eIF5B(517C) · GTP with +398 cal · mol^{-1} · K^{-1} to compensate the contribution of -553 cal·mol^{-1}·K^{-1} by domains I and II alone. This corresponds to a ΔASA of 884 to 1658 $Å^2$ that are *exposed* upon GTP binding simultaneously to the *burial* of ~1800 $Å^2$ (or 1229 to 2304 $Å^2$) in domains I and II. The only reasonable candidates that can account for this compensatory effect are the surface areas buried between domain III and the G domain (~1150 $Å^2$) and domain II (~700 $Å^2$), respectively, in apo eIF5B (Fig. 5B). Thus, these data indicate that domain III is released from most or all its contacts with the G domain and domain II in response to the GTP-induced rearrangement of switch 2.

GDP binding to Ct-eIF5B(517C) was driven by favourable contributions of both, binding enthalpy and entropy (ΔH = -6.65 kcal/mol and TΔS = 0.9 kcal/mol at 30 °C) (Table II). In contrast to GTP binding, the temperature dependency of ΔH was not linear for GDP binding; instead the data between 10 and 30 °C fit better to a second order polynomial function, indicating a strong temperature dependency of ΔC_p (Fig. 5A). This suggests that the amount of contact surface within the formed complex changes over the used temperature range. At higher temperatures (30 °C) GDP binding results in a ΔC_p of -140 cal·mol^{-1}·K^{-1}, corresponding to a surface burial of 311 to 583 $Å^2$ (Table III), which agrees well with a ΔASA of ~400 $Å^2$ for GDP binding according to the crystal structures. However, the negative value for ΔC_p decreases with lower temperatures. Below 10 °C the second order polynomial behaviour of ΔH breaks down and ΔC_p changes sign, indicating a net *exposure* of ASA upon GDP binding. Here, three observations based on the eIF5B · GDP structures are of particular interest: i) GDP/Mg^{2+} is able to partially activate switch 2 and to induce conformational strain on its interactions to domains II and III (Fig. S4), ii) domain III contacts switch 2 primarily through ionic interactions (Fig. 3B) which are destabilized at low temperatures (Elcock, 1998; Hendsch & Tidor, 1994), and iii) domain III is released from the G domain in molecule B of Sc-eIF5B · GDP for which crystals were obtained at 10 °C (Table I; Fig. S1E and S7A). Since switch 1 and the β13-β14 loop remain flexible in this structure, the release of domain III upon GDP binding results in a positive ΔASA corresponding to a positive contribution to ΔC_p as observed in the ITC experiments. The non-linear behaviour of ΔC_p above 10 °C would thus indicate

that domain III always has the propensity to be released in eIF5B·GDP due to the partial activation of switch 2 in the presence of Mg^{2+}, however, with a reduced tendency to do so with increasing temperatures at which the ionic interactions to the G domain become increasingly stable (Elcock, 1998; Hendsch & Tidor, 1994). In line with this interpretation, we found that the temperature dependency of ΔH does not break down at low temperatures when the ITC experiments are repeated with GDP in the absence of Mg^{2+} (Fig. 5A). Instead, ΔH plotted against the temperature results in a straight line with a slope (ΔC_p) of -102 $cal \cdot mol^{-1} \cdot K^{-1}$, which is comparable to that for the eIF5B·GDP complex in the presence of Mg^{2+} at higher temperatures (Table III).

Taken together, these data support the idea that the conserved Asp476 in the G3 motif plays a critical role in the reorganization of switch 2 in response to nucleotide binding and indicate a direct connection between the Mg^{2+} ion and the temperature dependency of ΔC_p in the eIF5B·GDP complex. The fact that GTP binding in contrast to GDP binding shows no temperature dependency of ΔC_p indicates that the interactions of domain III to domains I and II in apo eIF5B are broken upon GTP binding regardless of the temperature at which the reaction takes place. However, here the positive contribution to ΔC_p is compensated by the large negative contribution due to the burial of ASA in the GTP-bound G domain."

The authors go on to propose a what they call a dissociative switch mechanism that is based on the assumption that domain III is released from its interaction with domain I and II. However there is no structure that would back up this proposal and the evidence from ITC et al is at best questionable. For a full understanding of the role of eIF5B in joining the ribosomal subunits, the location of domain IV would also be very helpful but it is also not seen in the GTP-bound structure.

Reply:

As pointed out on pages 7 and 8, the stable interactions of domain III to domain I (G domain) are limited to very few specific contacts to residues of switch 2. In order to form these contacts, switch 2 has to adopt a specific, defined conformation that has so far not been observed in other translational GTPases (except the archeal ortholog). This conformation and consequently all those interactions that mediate the contact of domain III to domain I are completely lost upon the GTP induced conformational change in switch 2; the residues of switch 2 that interact with domain III in the apo state are simply not available any more for the same interactions (Figure 3B and S4). The new (GTP-) conformation of switch 2 is nearly identical to that of EF-Tu and other GTPases and thus no crystallographic artifact. This is direct structural evidence that domain III has to be released from its interactions to domain I upon GTP binding. The fact that domain III can easily loose all its contacts to domain I is furthermore directly visible from one of the eIF5B·GDP structures.

After its release, domain III either reassociates forming *new* stable interactions with the reorganized G domain, or it remains released and flexible. The ITC experiments point towards the latter case, where domain III in *free* eIF5B·GTP is unable to form contacts to the reorganized G domain that are stable enough to fix domains III and consequently also domain IV in one defined conformation.

The recently published cryo-EM model of eIF5B on the 80S ribosome (Fernandez et al, Science (2013)) clearly supports this assumption of a concerted reorganization of domains III and IV that requires the release of domain III from domains I and II: In the cryo-EM structure with eIF5B(domains I-IV), domain III is reorganized relative to the G domain in a way that all the contacts to domains I and II that existed in the apo structures of the protein have been lost (shown in the newly incorporated Figure 3D). In the same study, the cryo-EM structure of eIF5B in which only the G domain and domain II are visible furthermore shows that they are unable in their GDPCP-bound form to stabilize domains III and IV in a defined conformation even on the ribosome as long as the contact to the tRNA is not formed – in line with our assumption that domain III is released from its contacts to the G domain when the latter is bound to GTP (in this case GDPCP). A discussion of this structure was incorporated in the revised version of this manuscript (page 18; please also see response to referee #2)).

Some specific comments:

It would be easier if Figures 2 A,B,C would show the switches in the same colour, in a background of different colours for each structure

Reply:

We also considered this option. However, we decided to use different colours of the switch regions in the respective nucleotide states because it allows the superposition of the different structures without frequently changing their colours. Hence, the colouring for the different forms can stay the same as in Figure 2 in the other figures of the manuscript, which in our opinion makes it easier to identify them.

some Figures are way too busy, i.e. Fig. 2D, 3A,

Reply:

We tried to reduce the information content in Figure 2D. In Figure 3A we removed the structure of Ct-eIF5B·GDP from the superposition to make it less busy; moreover we deleted the inset and instead moved its information content to the separate Figure 3B.

Fig. 5 is fairly useless, we know how an ITC curve looks like and I trust that they are correctly interpreted, as shown in Table 2

Reply:

In showing some examples of the ITC curves we tried to conform to the conventions from other ITC studies (e.g. Hauryliuk et al, PNAS (2008); Kononenko et al, Nucleic Acids Res., (2010); Paleskava et al, J. Biol. Chem. (2012)). However, in the revised version of the manuscript we moved the ITC curves to the supplementary information (New Figure S6).

The paper needs some language correction, i.e. evolutionary conservatism (p3) is probably meant to mean conservation etc.

Reply:

Done

With these modifications and our responses to the referees' comments, we hope that the revised version of this manuscript is now acceptable for publication in The EMBO Journal. We would like to take this opportunity to thank the three anonymous referees for their comments and valuable suggestions on our manuscript.

2nd Editorial Decision 20 February 2014

Thank your for submitting the revised version of your manuscript to The EMBO Journal. The study has now been seen by one of the original referees (comments included below) and as you will see this person finds that all criticisms raised have been sufficiently addressed. I am therefore happy to inform you that your manuscript has been accepted for publication pending some technical amendments.

REFEREE REPORT:

Referee #1:

The authors answered all my concerns. There are still too many quotes, a few misprints and unclear sentences, but I believe this can be copy-edited at a later stage. The paper will stimulate the discussion among those working in translation initiation and on translation GTPases in general.