THE EMBO JOURNAL

Manuscript EMBO-2011-79268

Regulation of mammalian transcription by Gdown1 through a novel steric crosstalk revealed by cryo-EM

Yi-Min Wu, Jen-Wei Chang, Chun-Hsiung Wang, Yen-Chen Lin, Pei-lun Wu, Shih-hsin Huang, Chia-Chi Chang, Xiaopeng Hu, Averell Gnatt, and Wei-Hau Chang

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Review timeline:

Submission date:	25 August 2011
Editorial Decision:	29 September 2011
Revision received:	10 April 2012
Editorial Decision:	04 May 2012
Revision received:	25 June 2012
Editorial Decision:	26 June 2012
Accepted:	03 July 2012

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.)

1st Editorial Decision 29 September 2011

Thank you for submitting your manuscript for consideration by The EMBO Journal. I have now received the final report from the three referees who evaluated your study and I enclose their comments below. As you will see the referees express interest in the localisation of Gdown1 on RNAP II, however they require significant additional experimental analysis to strengthen the main conclusions. It is important to focus on additional structural and biochemical evidence for the localisation of Gdown1 and also structural analysis of the complete TFIIF complex bound to the polymerase. Finally, additional evidence for the competition between Gdown1 and TFIIF should be provided. I realize that the referees request a significant number of experiments but on reflection they are central to the main conclusions of the study and in the end will significantly strengthen it and are therefore required. Should you be able to address these concerns we would be happy to consider a revised version of the manuscript for publication in The EMBO Journal.

I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript. When you submit a revised version to the EMBO Journal, please make sure you upload a letter of response to the referees' comments. Please note that when preparing your letter of response to the referees' comments that this will form part of the Review Process File, and will therefore be available online to the community.

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

Yours sincerely,
Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

Gdown1 is an interesting new component of some RNA polymerase II preparations fro mammalian cells. Relatively little is known about this protein, but it appears to repress transcription and confer a requirement for the Mediator complex. The manuscript by Wu et al contributes with important data and an interesting model that helps to explain the molecular role of Gdown1 in transcription initiation. The manuscript is well written and easy to follow. The data are new, interesting, and will for sure be of interest to a wider audience.

However, I do have one major concern that the authors must address. In their manuscript, they compare cryo-EM results form RNA polymerase II in different conformations. In this way, they identify the location of Gdown1 in the complex, which leads to a series of interesting conclusions. However, the way the localization of Gdown1 is predicted is a bit shaky, since this is a very small subunit (43 kDa). Given the size of the RNA polymerase II complexes studied, Gdown1 only corresponds to a small percentage of the total volume. What makes the localization even more hazardous is the low resolution (24 ≈). The authors must therefore use an alternative technique to support their claims regarding the localization of Gdown1. The obvious and best choice would be a simple cross-linking experiment, which could identify the RNA polymerase II subunits that interact with Gdown1. Alternatively, they could solve the structure in complex with an antibody against Gdown1, which would further support the localization of the protein.

Referee #2 (Remarks to the Author):

The work described by Wu et al., aims at a structural description of the bovine RNA polymerase II associated to the regulatory subunit/factor Gdown1 by cryo electron microscopy and image analysis. The authors analyze the structure of purified RNA PolII in negatively stained and cryo-negatively stained conditions; and reach a structure that is comparable to yeast RNA pol II but cannot clearly define the Gdown1 binding site because of low occupancy. To increase Gdown1 occupancy on RNA pol II, recombinant protein is added in excess which improved Gdownd1 visibility that appears as a protein density that closes over the DNA binding cleft and holding DNA in place thus gives a hint to the increased transcriptional efficiency. The Gdown1 binding is believed to cover the RAP74 binding site on the RNA pol II surface.
In general this reviewer finds that the experimental details are not described precisely and that the reader lacks important information to assess the exposed results.
1- The biochemical characterization of the analyzed complexes is not shon. The reader cannot Judge whether Gdown1 is stoickiometrically associated to the RNA polII, which is the reason suggested for its poor detection in the first experiment. In the original publication by Hu et al., 2006 the Gdown1 protein appeared to be present in stoickoimetric amounts and resulted in a strongly reduced transcriptional activity. Here the authors add large amount of Gdown1 in order to detect its binding. It is important to show that this interaction is specific and that it results in an even stronger reduced a transcriptional activity than RNA Pol II(G) otherwise one might conclude that the detected binging site is non-specific.

2- The authors use the wording Cryo EM which is misleading since it refers to unstained cryo EM. The authors used a variation of cryo negative stain (the reference cited does not describe the technique actually used by the authors).

3- For the electron microscopy part the authors claim that 3-D reconstruction was done using either the ab-initio method or the reference-based method, but the reader cannot infer which method has been used for which reconstruction. In any case the reference-based approach in which the yeast X-

ray structure is injected should be avoided since it creates too much bias and sentences such as "The reliability of those structural models is verified by the outcome of virtually identical, p15" have little meaning since the same structure might have been used for angular assignments.

4- The class averages shown in figure 1B show very little significant information such as the position of the groove, the position of the RPB4-7 subunits that form an extended stalk. The average images appear constituted by dots which mimic the granularity of the carbon film more than reflecting the signal from RNA polymerase. The authors should show a comparison between these original class averages and reprojections of the final 3-D model which would be more informative.

5- The authors identify a small density difference that accounts for 5% of the expected mass of Gdown1 without showing any statistically validated difference map. The authors should show a difference map with a 3sigma threshold and explain the positive and negative differences, a labeling experiment should be provided to show that this density is due to Gdown1.

6- The authors do not exploit the interesting observation that Gdown1 displaces the RNA Pol II monomer/dimer equilibrium. The presence of both forms in the same experiment would certainly improve the significance of any density difference because the experimental conditions since staining and defocus values would be identical. Moreover the location of Gdown1 should explain the interference with the dimerization (covers the dimerisation interface or induced a conformational change). In addition the quality of the dimer reconstruction should be improved because of better alignment.

7- The gel-shift experiments with Rap74 are not convincing, the very high mobility shift is likely to represent aggregated RNA polII. The excess of Rap74 is not detected on the gel and the complex presumably formed between RNA pol II and RAP74 is not characterized biochemically (gel filtration and SDS PAGE to show stable and equimolar association of Pol II with RAP74). The conditions in which the RNA PolII-RPA74 complexes were observed are not indicated (negative stain at room temperature?). The results obtained are in sharp disagreement with the previously published results from the Asturias group since the major density difference is not found over the Rpb2 lobe and protrusion domain. The previous results were obtained in unstained cryoEM conditions and are therefore likely to be more informative (although controversed in the field).

8- The most spectacular observation is the cryo negatively stained reconstruction of bovine RNA Pol II supplemented with a large excess of Gdown1. The density termed "a" bridges Rpb5 with the Rpb1 clamp domain and density "b" protrudes from the Rpb2 protrusion domain. I have three major concerns with this additional densities attributed to Gdown1: (1) the "a" domain but not the "b" domain is clearly seen in reconstruction 1 (RNA Pol II(G)) which was proposed to be substoickiometric for Gdown1. (2) an additional density of similar size is clearly visible in reconstruction 3 at the opposite to the Rpb4/7 stalk. This domain should be mentioned and would probably appear on a density difference map. (3) The connectivity between the "a" and "b" domains is unfortunately missing and considering point (1) it cannot be excluded that Gdown1 has multiple binding sites on RNA Pol II. Again a biochemical characterization of the complexes is needed.

9- I found that the dimerization readout to show that RAP74 and Gdonwn1 have overlapping binding sites quite indirect. Why not show biochemically (gel filtration + SDS PAGE) that once RAP74 is bound, Gdown1 cannot and vice versa.

10- Figure 5C needs more explanations and cannot be understood as such. If Gdown1 bridges over the DNA binding cleft it should impair DNA binding and prevent or inhibit transcription at the early stage while possibly stimulating transcription in the elongation phase. Can the assay discriminate between these two effects.

The data presented needs significant revision and additional experimental data before being resubmitted. A better biochemical characterization the RNA Pol II complexes is needed and a more carefull interpretation of the density difference maps.

Referee #3 (Remarks to the Author):

In this manuscript, the authors report the cryo EM structures of bovine RNA pol II, pol II + Gdown1 and pol II + the TFIIF large subunit Rap74. Their results suggest that Gdown binds to a site on polymerase that overlaps where TFIIF binds and that the two factors would compete for polymerase. The authors supply some evidence for this competition and propose a model that makes sense with the known biochemical properties. This is an interesting topic and the authors have made a good start in examining this question. Unfortunately, at this stage, the data are not convincing and a number of important questions remain which the authors must address.

1. The authors claim that the extra density on pol is due to two separate domains of Gdown. The authors should put a probe of some sort on one or both of these domains so that they can conclusively show that this is due to Gdown rather than some conformational shift in pol or some other unexpected explanation.

2. From the structure of the dimerization domain of TFIIF (Gaiser et al 2000) and the biochemical mapping of TFIIF on pol (Eichner et al 2010; Chen et al 2010) it is clear that the large TFIIF subunit (Rap74) will be almost entirely unstructured in the absence of the smaller subunit Rap30. The authors should repeat their EM analysis using complete TFIIF since their current structure is likely not meaningful.

3. The biochemical competition studies in Fig 5B are not at all convincing. The authors need another assay to show if this is the case. Also in Fig 5A, there is no information on what is varying in lanes 4-7.

4. I'm not an EM expert, but 13,000 particles seems like it is on the very low end of other cryo EM papers I have read for structures of this size and complexity. What is the accepted number of particles that are needed for analysis?

1st Revision - authors' response 10 April 2012

Response to Reviewers' remarks

Referee #1 (Remarks to the Author):

Gdown1 is an interesting new component of some RNA polymerase II preparations for mammalian cells. Relatively little is known about this protein, but it appears to repress transcription and confer a requirement for the Mediator complex. The manuscript by Wu et al contributes with important data and an interesting model that helps to explain the molecular role of Gdown1 in transcription initiation. The manuscript is well written and easy to follow. The data are new, interesting, and will for sure be of interest to a wider audience.

However, I do have one major concern that the authors must address. In their manuscript, they compare cryo-EM results form RNA polymerase II in different conformations. In this way, they identify the location of Gdown1 in the complex, which leads to a series of interesting conclusions. However, the way the localization of Gdown1 is predicted is a bit shaky, since this is a very small subunit (43 kDa). Given the size of the RNA polymerase II complexes studied, Gdown1 only corresponds to a small percentage of the total volume. What makes the localization even more hazardous is the low resolution (24 Å). The authors must therefore use an alternative technique to support their claims regarding the localization of Gdown1. The obvious and best choice would be a simple cross-linking experiment, which could identify the RNA polymerase II subunits that interact with Gdown1. Alternatively, they could solve the structure in complex with an antibody against Gdown1, which would further support the localization of the protein.

Authors' answers: Thanks for the wonderful suggestions. We did as suggested.

We have performed antibody labeling negative stain EM experiments. To do so, we used polyclonal antibody against Gdown1 to decorate RNAPII-Gdown1 complex and the antibody signal was found near the Rpb1 jaw or the nearby Rpb2. Similar results were obtained by using a monoclonal antibody against GST fused to the N-terminus of Gdown1 (**Fig. 4D** in the revision). Those results support the localization of Gdown1 on Rpb1 jaw-Rpb5 shelf by difference mapping based on the new cryo unstained structures ~ 19 Å (**Fig. 4B** in the revision).

It is important to note that consistent results were obtained timely by others. In a very recent paper from Roeder's lab: "Transcriptional regulation by Pol II(G) involving Mediator and competitive interactions of Gdown1 and TFIIF with Pol II" (Jishage *et al*., *Mol Cell* 45: 51-63 (2012)), crosslink techniques were employed to show that Gdown1 directly interacts with Rpb1 subunit and Rpb5 subunit, and the contact was assigned to be at the Rpb1 jaw-Rpb5 shelf.

Referee #2 (Remarks to the Author):

The work described by Wu et al., aims at a structural description of the bovine RNA polymerase II associated to the regulatory subunit/factor Gdown1 by cryo electron microscopy and image analysis. The authors analyze the structure of purified RNA Pol II in negatively stained and cryo-negatively stained conditions; and reach a structure that is comparable to yeast RNA pol II but cannot clearly define the Gdown1 binding site because of low occupancy. To increase Gdown1 occupancy on RNA pol II, recombinant protein is added in excess which improved Gdownd1 visibility that appears as a protein density that closes over the DNA binding cleft and holding DNA in place thus gives a hint to the increased transcriptional efficiency. The Gdown1 binding is believed to cover the RAP74 binding site on the RNA pol II surface.
In general this reviewer finds that the experimental details are not described precisely and that the reader lacks important information to assess the exposed results.

Answer: Thanks for so many wonderful remarks. We fixed all points mentioned as follows.

1- *The biochemical characterization of the analyzed complexes is not shown. The reader cannot judge whether Gdown1 is stoickiometrically associated to the RNA polII, which is the reason suggested for its poor detection in the first experiment. In the original publication by Hu et al., 2006 the Gdown1 protein appeared to be present in stoickoimetric amounts and resulted in a strongly reduced transcriptional activity. Here the authors add large amount of Gdown1 in order to detect its binding. It is important to show that this interaction is specific and that it results in an even stronger reduced a transcriptional activity than RNA Pol II(G) otherwise one might conclude that the detected binging site is non-specific.*

Answer: As suggested, we include an Coomassie-blue stained SDS-PAGE gel of native bovine RNAPII and RNAPII(G) in **Fig. 1A** (in the revision). The quantitation of the band intensity shows the stochiometry of the Rpb1, Rpb2 and Gdown1, roughly to be 08:1:0.7. The poor detection of Gdown1 on RNAPII(G) may comes from reasons other than sub- stoichiometry, for example, disorder. In addition, we did limited proteolysis of Gdown1 to enrich our biochemical characterization of Gdown1 (**Fig. 1C** in the revision). The results support that C-terminal half is largely unfolded, consistent with the notion by FoldIndex analysis (**Fig. 1D** in the revision). We thus re-address this issue in the revision. To address the unwanted non-specific issue, in the new unstained cryo-EM RNAPII-rGdown1, we have reduced the ratio of Gdown1 to RNAPII (4:1) to reconstitute of RNAPII(G). Such ratio is sufficient to turn the RNAPII dimers into monomers. This ratio was also used by others for completely inhibiting transcription (Jishage et al., (2012) *Mol Cell* 45, 51-63). By using 20ng of rGdown1, which is 4 times of RNAPII (50ng), Jishage et al., completely abolished the promoter-dependent transcription activity (see **Fig. 1B**, lane 4 in Jinshage et al).

2- *The authors use the wording Cryo EM which is misleading since it refers to unstained cryo EM. The authors used a variation of cryo negative stain (the reference cited does not describe the technique actually used by the authors).*

Answer: As suggested. We are now entitled to using "cryo-EM" because we have re-done our work with unstained specimens. As to cryo-negative staining using uranyl salts instead of molybdate, we now cite the correct one from Stark' s lab by Golas et al and also cite the one from Walz's lab by Ohi et al., who modified the techniques from Stark's lab.

3- *For the electron microscopy part the authors claim that 3-D reconstruction was done using either the ab-initio method or the reference-based method, but the reader cannot infer which method has been used for which reconstruction. In any case the reference-based approach in which the yeast X-ray structure is injected should be avoided since it creates too much bias and sentences such as "The reliability of those structural models is verified by the outcome of virtually identical, p15" have little meaning since the same structure might have been used for angular assignments.*

Answer: As suggested. We injected cryo-negative stained model for performing the angular reconstruction of the unstained cryo EM images. The cryo-stained model was obtained by injecting negative-stain RNAPII(G), which was obtained from common-line and showed no difference from RNAPII at low resolution.

4- *The class averages shown in figure 1B show very little significant information such as the position of the groove, the position of the RPB4-7 subunits that form an extended stalk. The average images appear constituted by dots which mimic the granularity of the carbon film more than reflecting the signal from RNA polymerase. The authors should show a comparison between these original class averages and reprojections of the final 3-D model which would be more informative.*

Answer: As suggested, 2D class averages were re-performed by CL2D on XMIPP and the characteristic RNAPII views showing groove or RPB4-7 stalk are now shown (**Fig. 2B** in the revision) in contrast to the corresponding re-projections of the final 3-D model of negative-stained RNAPII(G) with the corresponding orientation (**Fig. 2C** in the revision)

5- *The authors identify a small density difference that accounts for 5% of the expected mass of Gdown1 without showing any statistically validated difference map. The authors should show a difference map with a 3sigma threshold and explain the positive and negative differences, a labeling experiment should be provided to show that this density is due to Gdown1.*

Answer: As suggested. We subtracted the unstained RNAPII-elongation map from the RNAPII-Gdown1 map and showed the positive and negative difference above 5s (**Fig. 4B and 4C** in the revision). The positive difference on Rpb1 jaw to Rpb5 shelf is now assigned to Gdown1, consistent with our antibody labeling data (**Fig. 4D** in the revision) and supported by the crosslink data (Jishage et al., *Mol Cell* 45, 51-63 (2012)). The negative difference inside the DNA cleft may comes from nucleic acids themselves or from nucleic-acid induced protein motion, and the negative difference besides Rpb2 may come from RNAPII conformational change.

6- *The authors do not exploit the interesting observation that Gdown1 displaces the RNA Pol II monomer/dimer equilibrium. The presence of both forms in the same experiment would certainly improve the significance of any density difference because the experimental conditions since staining and defocus values would be identical. Moreover the location of Gdown1 should explain the interference with the dimerization (covers the dimerisation interface or induced a conformational change). In addition the quality of the dimer reconstruction should be improved because of better alignment.*

Answer: As suggested. We obtained the 2D class average of the negative-stained dimer images by using CL2D (**Fig. 2E** in the revision) and compared it with 3D dimer model built from the 3D reconstruction RNAPII monomer (**Fig. 2F** in the revision) to find the interface belongs to Rpb3 or Rpb4. Such dimer is consistent with the one in an earlier study of yeast RNAPII with 2D electron crystallography by Darst and Kornberg. Our antibody labeling results show that an antibody against the Rpb3 subunit could induce a large fraction of RNAPII (~70%) to become monomer (**Supplementary Fig. 1C**). As to the findings from the difference mapping, there is weak Gdown1 density on the wall near Rpb3 ("region c" in **Fig. 4B** in the revision). Crosslink maps this region to the winged-helix of Tfg2/RAP30 and we speculate its unseen extension may cause the RNAPII dimer to turn into monomer.

7- *The gel-shift experiments with Rap74 are not convincing, the very high mobility shift is likely to represent aggregated RNA polII. The excess of Rap74 is not detected on the gel and the complex presumably formed between RNA pol II and RAP74 is not characterized biochemically (gel filtration and SDS PAGE to show stable and equimolar association of Pol II with RAP74). The conditions in which the RNA PolII-RPA74 complexes were observed are not indicated (negative stain at room temperature?). The results obtained are in sharp disagreement with the previously published results from the Asturias group since the major density difference is not found over the Rpb2 lobe and protrusion domain. The previous results were obtained in unstained cryo-EM conditions and are therefore likely to be more informative (although controversial in the field).*

Answer: As suggested. We replaced our mobility-shift experiments in native gel with the gel filtration assays followed by SDS PAGE (**Fig. 6** in revision) to demonstrate Gdown1 excludes TFIIF. It is noted that the conclusion Gdown1 excluding TFIIF have been reached by Roeder's lab using biochemical approaches (Jishage *et al*., *Mol Cell* 45, 51-63 (2012)), and by Price's lab (Cheng *et al*., *Mol Cell* 45, 38-50 (2012)). Here, we updated the unstained structure of RNAPII-TFIIF in the revision: the difference mapping showed the TFIIF densities tethering on RNAPII (**Fig. 5B** in revision), in keeping with crosslink results (Chen *et al (2010), EMBO* 29,717-726) and the antibody labeling showed the N-terminus of RAP74 near Rpb2. So what happened to Asturias's cryo-EM structure of yeast RNAPII (Chung *et al., Mol Cell* 12:1003-1013 (2003))? In Asturias's structure, Tfg1/RAP74 was assigned to be on the Rpb4/7 stalk, the opposite side of Rpb2. As a co-author of this work, I admit that the big density in the difference map of "RNAPII-TFIIF"-"RNAPII" on the Rpb4/7 was unfortunately derived using a 12-subunit yeast RNAPII cryo-EM structure that had a smaller Rpb4/7 volume than what is supposed to be (see Craighead, Chang and Asturias, *Structure* 10: 1117-1125 (2002)); moreover, at that time, it was a hidden assumption that significant fraction of Tfg1/RAP74 had a globular structure, leading to the interpretation of the difference density on Rpb4/7 belonged to Tfg1/RAP74. But now we understand Tfg1/RAP74 is largely disordered.

8- *The most spectacular observation is the cryo negatively stained reconstruction of bovine RNA Pol II supplemented with a large excess of Gdown1. The density termed "a" bridges Rpb5 with the Rpb1 clamp domain and density "b" protrudes from the Rpb2 protrusion domain. I have three major concerns with this additional densities attributed to Gdown1: (1) the "a" domain but not the "b" domain is clearly seen in reconstruction 1 (RNA Pol II(G)) which was proposed to be substoickiometric for Gdown1. (2) an additional density of similar size is clearly visible in reconstruction 3 at the opposite to the Rpb4/7 stalk. This domain should be mentioned and would probably appear on a density difference map. (3) The connectivity between the "a" and "b" domains is unfortunately missing and considering point (1) it cannot be excluded that Gdown1 has multiple binding sites on RNA Pol II. Again a biochemical characterization of the complexes is needed.*

Answer: As suggested. We replace the cryo-negative stained reconstructions by unstained cryo-EM structure and the new results from difference mapping show Gdown1 tethers on RNAPII while a major density is located at Rpb1 jaw-Rpb5 shelf (see **Fig. 4** in the revision). Biochemical characterization of stoichiometry was done as suggested (see **Fig. 1** and its legend in the revision).

9- *I found that the dimerization readout to show that RAP74 and Gdonwn1 have overlapping binding sites quite indirect. Why not show biochemically (gel filtration + SDS PAGE) that once RAP74 is bound, Gdown1 cannot and vice versa.*

Answer: As suggested. We replaced our native-gel based competition assay with the size exclusion gel filtration/SDS PAGE assay to show when Gdown1 is associated with RNAPII, TFIIF is not (**Fig. 6C** in the revision).

10- *Figure 5C needs more explanations and cannot be understood as such. If Gdown1 bridges over the DNA binding cleft it should impair DNA binding and prevent or inhibit transcription at the early stage while possibly stimulating transcription in the elongation phase. Can the assay discriminate between these two effects?*

Answer: In the revised unstained RNAPII-Gdown1 cryo-EM structure, the bridge disappears so there is no longer issue as to impairing DNA binding. The transcription assay in old **Figure 5C** used tail template and this assay is not specific to promoter recognition so it cannot discriminate these two effects. It is beyond our scope to carry promoter-specific assay in our lab. As to this issue,

please read the recent paper as to Gdown1's function in transcription initiation from Bob Roeder's lab (Jishage *et al., Mol Cell* 45, 51-63 (2012), and in transcription elongation from David Price's lab (Cheng *et al*., *Mol Cell* 45, 38-50 (2012). Both show Gdown1 excludes TFIIF, in keeping with our study.

The data presented needs significant revision and additional experimental data before being resubmitted. A better biochemical characterization the RNA Pol II complexes is needed and a more careful interpretation of the density difference maps.

Referee #3 (Remarks to the Author):

In this manuscript, the authors report the cryo EM structures of bovine RNA pol II, pol II + Gdown1 and pol II + the TFIIF large subunit Rap74. Their results suggest that Gdown1 binds to a site on polymerase that overlaps where TFIIF binds and that the two factors would compete for polymerase. The authors supply some evidence for this competition and propose a model that makes sense with the known biochemical properties. This is an interesting topic and the authors have made a good start in examining this question. Unfortunately, at this stage, the data are not convincing and a number of important questions remain which the authors must address.

Authors' answers: Thanks for the wonderful suggestion. We did as suggested. Please read the following points.

1. *The authors claim that the extra density on pol is due to two separate domains of Gdown1. The authors should put a probe of some sort on one or both of these domains so that they can conclusively show that this is due to Gdown1 rather than some conformational shift in pol or some other unexpected explanation.*

Answer: As suggested. We performed antibody labeling experiments to identify the location of Gdown1. To do so, we fused GST tag to the N-terminus of recombinant Gdown1, and by negative-stained EM, we found the N-terminus of Gdown1 is localized near Rpb1 jaw or Rpb2 (**Fig. 4D** in the revised manuscript), supporting the difference map analysis results (**Fig. 4B** in the revised manuscript). Importantly, it is noted that in a new paper titled "Transcriptional regulation by Pol II(G) involving Mediator and competitive interactions of Gdown1 and TFIIF with Pol II" was published from Bob Roeder's lab (Jishage *et al., Mol Cell* 45, 51-63 (2012)), in which crosslink techniques were employed to show that Gdown1 directly interacts with Rpb1 subunit and Rpb5 subunit, and the contact was assigned to be at the Rpb1 jaw-Rpb5 shelf, supporting our cryo-EM study.

2. *From the structure of the dimerization domain of TFIIF (Gaiser et al 2000) and the biochemical mapping of TFIIF on pol (Eichner et al 2010; Chen et al 2010) it is clear that the large TFIIF subunit (Rap74) will be almost entirely unstructured in the absence of the smaller subunit Rap30. The authors should repeat their EM analysis using complete TFIIF since their current structure is likely not meaningful.*

Answer: As suggested. We renewed the cryo-EM reconstruction with entire TFIIF, namely RNAPII-TFIIF. The difference map analysis by subtracting a bovine RNAPII from the RNAPII-TFIIF indicates the locations of TFIIF densities, consistent with the biochemical mapping from Steve Hahn's lab (Eichner *et al* 2010*, EMBO J.* 29, 706-716*)* and crosslink from Patrick Cramer and Juri Rappsilber (Chen *et al 2010, EMBO J.* 29, 717-726) (**Fig. 5** in the revised manuscript).

3. *The biochemical competition studies in Fig 5B are not at all convincing. The authors need another assay to show if this is the case. Also in Fig 5A, there is no information on what is varying in lanes 4-7.*

Answer: As suggested. Fig. 5B native gel mobility competition assay is now replaced by a size exclusion chromatography (gel filtration) followed by SDS-PAGE and silver staining, which demonstrates Gdown1 competes out TFIIF on RNAPII (**Fig. 6** in the revised

manuscript). **Fig 5A** is now moved to **Supplementary Fig. 3**, Lanes 4-7 represent successive elution fractions from a GSH column of the rGdown1-Rpb5 complex captured by a nickel-NTA column in the previous step.

4. *I'm not an EM expert, but 13,000 particles seems like it is on the very low end of other cryo EM papers I have read for structures of this size and complexity. What is the accepted number of particles that are needed for analysis?*

Answer: In our revision, we increase particle numbers to ~25,000 for the cryo-EM RNAPII complexes to replace the ~13,000 particle cryo-negative stained structures. The resolution improves to 19 Å. To cite examples of particles with similar size and complexity, ~20,000 particle images were used for cryo-EM reconstruction of RNA polymerase I to a resolution of 17 Å (Kuhn *et al, Cell* 131, 1260-1272 (2007)), and also for cryo-EM reconstruction of RNA polymerase III to a result of 17 Å (Fernandez-Tornero *et al*, *Mol Cell* 25, 813-823 (2007)).

Resolution can get better as particle number is increased (see the analysis by Chang *et al, Structur*e 18, 17-27 (2010)). But how much more really depends on the specimen. In the case of a particle containing high symmetry, for instance, icosahedral virus, 13,000 would be enough to reach near-atomic resolution (please visit the websites of Wah Chiu at Baylor, Hong Zhou at UCLA, and Niko Grigorieff at Brandies). On the other hand, Eva Nogales and Robert Tijian have studied three distinct activator-TFIID complexes, all asymmetric particles, to 33 Å by using ~14,000 cryo-EM particle images (Liu *et al, Genes and Development* 23, 1510-1521(2009)).

2nd Editorial Decision 04 May 2012

Thank you again for submitting your revised manuscript, which has now been once more evaluated by the original referees 2 and 3. I am pleased to inform you that both of them consider the study singificantly improved and thus now in principle suitable for publication. Nevertheless, referee 3 retains some specific issues that I would like to ask you to address in a final round of minor revision (see below).

A few additional editorial points should also be incorporated in this final version:
- please ensure proper EMDB database submission of relevant structural data, as specified in our Guide to Authors
- please revise the gel/blot panels shown in Figure 1, which appear to be composites assembled from separate lanes without proper indication. Please also submit as accompanying 'source data' the original scans for these panels, indicating the origins of the individual lanes and confirming that they come from the same experiment/gel/exposure

Once we will have received your final version satisfactorily addressing these remaining issues, we should hopefully be able to swiftly proceed with acceptance and production of your manuscript. Should you need any further clarifications in this regard, please do not hesitate to contact me.

Yours sincerely,

Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #2 (Remarks to the Author):

The authors have considerably modified and improved the manuscript, included significant amount of new data that supports their model and improved the statistical manlysis of their data. To my

opinion the work is now suitable for publication in EMBO J.

Referee #3 (Remarks to the Author):

In response the reviewer's comments, the authors have made significant improvements in their revised manuscript. These include higher resolution cryo EM structures without negative stain, antibody localization of Gdown and TFIIF using antibodies, using complete TFIIF instead of just the large subunit and a much improved biochemical competition assay. Even though two papers have since been published on the competition of Gdown and TFIIF from Price and Roeder, this is a valuable contribution because it gives a structural basis for understanding the binding and role of Gdown. Two major points should be addressed by the authors before publication:

1) It is concerning that comparisons of the Gdown-Pol complex with Pol lacking Gdown are not comparing the same thing. The Gdown complex contains no nucleic acid while the pol II and pol II-IIF complexes contain nucleic acid elongation scaffolds. The authors need to provide some evidence that this difference is not affecting their difference density maps.

2) The text, especially the discussion, is much too long and will benefit from improving the writing to be more concise and improving english usage. Overall, the manuscript could easily be reduced by {greater than or equal to}30%.

2nd Revision - authors' response 25 June 2012

Response to Reviewers' remarks

Referee #3 (Remarks to the Author):

In response the reviewer's comments, the authors have made significant improvements in their revised manuscript. These include higher resolution cryo EM structures without negative stain, antibody localization of Gdown1 and TFIIF using antibodies, using complete TFIIF instead of just the large subunit and a much improved biochemical competition assay. Even though two papers have since been published on the competition of Gdown1 and TFIIF from Price and Roeder, this is a valuable contribution because it gives a structural basis for understanding the binding and role of Gdown1. Two major points should be addressed by the authors before publication:

1) It is concerning that comparisons of the Gdown-Pol complex with Pol lacking Gdown are not comparing the same thing. The Gdown complex contains no nucleic acid while the pol II and pol II-IIF complexes contain nucleic acid elongation scaffolds. The authors need to provide some evidence that this difference is not affecting their difference density maps.

Answer: As suggested. We have added a new result of the cryo-EM structure of RNAPII-Gdown1 with dsDNA/RNA (RNAPII-Gdown1 elongation complex) and subtracted the RNAPII-elongation map from the RNAPII-Gdown1 elongation map. Interestingly, the new positive map is virtually identical to the old one, generated by subtracting RNAPII elongation from RNAPII-Gdown1. As such, the concern as to the nucleic acids has been rigorously removed and it is legitimate to attribute the positive difference exclusively to Gdown1. Another interesting thing is that the new negative difference above 10s due to nucleic acid disappears. (**Figure 4** in the revision).

2) The text, especially the discussion, is much too long and will benefit from improving the writing to be more concise and improving english usage. Overall, the manuscript could easily be reduced by {greater than or equal to}30%.

Answer: As suggested. We have rewritten a more concise version.

3rd Editorial Decision 26 June 2012

Thanks for submitting your re-revised version, incorporating additional data in response to referee 3. With these modification, there are no more objections from the scientific side. However, I am afraid that the two editorial points I raised in my previous decision letter of May 4th remain still unaddressed:

- the manuscript text still lacks a reference to availability of structural data at EMBD, which is however a prerequisite for acceptance. To quote from our 'Guide to Authors':
"Structures of biological macromolecules solved by electron microscopy must be submitted to the EMDB database at http://emdatabank.org. For a brief description of the database, see Lawson et al. (2011) Nucleic Acids Res. 39: D456-D464."

- on close inspection, I am afraid that the problem with image assembly from apparently non-adjacent lanes in Figure 1 persists, and is not addressed by your submission of the gel used in (non-problematic) Figure 1A. In Figure 1B, however, there still appear to be visible separation lines between lanes 2 and 3, and between lanes 3 and 4 at least. We will thus need to see the original data from which this Figure panel was assembled, including explanations if necessary. Furthermore, if lanes have indeed been assembled together, this has to be made clearly visible in the figure and explained/justified in the figure legends.

I would appreciate if you could get back to me on these points at your earliest convenience.

Sincerely,

Editor
The EMBO Journal

Additional correspondence 28 July 2012

As suggested, we submitted our four cryo-EM structures to EMDB database The EMDataBank ACCESSION CODE are EMD-5440 ~ EMD-5443.

In figure 1B, we see the editors persistence at verifying the authenticity of our images in a positive light, and consider it helpful to prevent falsification of data. Image 1B is the original scan. The streaks that appear between lanes or in lane 5 itself, are the result of developer scratches of that particular exposure.

Please find the attached files as requested of the entire image.

Thank you for your help.

