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hCAF1/CNOT7 regulates interferon signaling by targeting STAT1

Clément Chapat, Chloé Kolytcheff, Muriel Le Romancer, Didier Auboeuf, Pierre De La Grange, Kamel Chettab, Stéphanie Sentis, and Laura Corbo

Corresponding author: Laura Corbo, Université Lyon 1

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Editor: Anne Nielsen

1st Editorial Decision 17 September 2012

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by two referees whose comments are shown below.

As you will see from the reports, both referees express great interest in your findings, but they also raise important concerns that will have to be addressed in an adequate manner before a revised version of the manuscript can be submitted. Most importantly, both referees emphasize that additional experimental data is required to support the proposed model for the CAF1-STAT1 interaction as a regulator of interferon signaling.

Given the referees' positive recommendations, we offer you the opportunity to submit a revised version of the manuscript, addressing the comments of both reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance or rejection of your manuscript will therefore depend on the completeness of your responses to the full satisfaction of the referees in this revised version. Please do not hesitate to contact me if you have questions related to the review process and the requests made by the referees.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Peer-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://www.nature.com/emboj/about/process.html

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.

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

REFEREE REPORTS:

Referee #1:

This is an interesting study that implicates CAF1 in a novel role in regulating IFN responses through physical interaction with STAT1 protein. Overall I find the work to be generally well-designed and the manuscript clearly presented. I do have several points that should be addressed to clarify aspects of the study and provide somewhat clearer support for the conclusions drawn:

1. Fig. S1 - error bars and statistics are needed to support the significance of the small ~2X increases seen in most of the mRNAs assayed in MCF7 cells.
2. Fig. 2E: Could the 'resistance' to viral infection observed in these KD cells simply be due to the increased apoptosis and cell death that would indirectly result in decreased virus yields? I think that one needs to be careful in drawing conclusions when infecting KD cells that are exhibiting growth defects and increased rates of dying. If this concern is valid, then claiming that the CAF1 KD cells mimics IFN based on this assay is probably not appropriate.
3. Fig. 3: The mRNA decay that is being observed appears to only be linear between 0 and the first time point (2hr). Beyond that, little decay appears to be occurring. Actinomycin D transcriptional shut off can have dramatic effects on cell physiology after a few hours, so the earlier time points of such an analysis are always more accurate. Therefore I would encourage the authors to re-visit this experiment and increase the number of time points between 0-2 hrs to determine more accurate decay rates (rather than relying on 2 points as in the current graphs). Furthermore, if the 0-2 hr region is expanded, the effects observed on STAT1 mRNA decay may be similar to the other mRNAs (rather than different as currently concluded).
4. Fig. 3B: These data would be stronger if P body numbers were presented quantitatively with error bars.
5. Fig. 3: The authors conclude that the decrease in P bodies is consistent with impaired deadenylation - but the authors are seeing increased decay rates in the CAF1 KD which would infer precisely the opposite - increased deadenylation. To address this conundrum, why don't the authors measure deadenylation rates directly rather than making inferences about them?
6. Fig. 4A and B: These data would be improved if error bars and statistics were added.
7. Fig. 6: Is the interaction between CAF1 and STAT1 sensitive to RNAse? Given the RNA associations of CAF1, it is probably a good idea to formally rule out RNA bridging.
8. Fig. 6/7: Do antibodies specific for other components of the CAF/CCR4-NOT complex also co-IPPT STAT1? The result would be important to address in the model whether the full complex is interacting with STAT1 as depicted or whether CAF1 is acting independently of the full complex.

Referee #2:

The data in this paper show that hCAF1 binds to STAT1 and inhibits the expression of a subset of STAT1 target genes. It is a significant finding that hCAF1 acts as a negative regulator of STAT1 and its target gene expression, but the mechanism is not obvious. The authors pass over several of their results showing discrepancies from the model described in Fig 7. The data should be re-interpreted in order to fit an adequate model explaining the mechanism of the negative regulation of IFN signaling induced by hCAF1.

Specific comments
1. In order to understand the function of hCAF1 in IFN signaling, basic studies about hCAF1 regulation in response to IFN should be shown. Many negative regulators of IFN signaling, like

SOCS1, are up-regulated by IFN and their protein products bind to major molecules of the Jak-STAT pathway to inhibit it. These basic studies should answer the following questions; Is the expression of hCAF1 down-regulated in response to IFN? Or is the dissociation of hCAF1 from STAT1 accelerated by IFN? Does IFN induce any modification of hCAF1 protein? Or does the IFN-induced phosphorylation of STAT1 decrease its affinity to hCAF1 protein?

2. Figure 4A shows that the phosphorylation of STAT1 in response to IFN is decreased in hCAF1 knock-down cells, but the expression of STAT1 target genes is increased (Fig 4B). These data clearly show that the expression of these genes in Fig 4B is not regulated by P-STAT1. These data cannot support the model in Fig 7, and I cannot find any data in the paper that do support it.

3. The authors explain in the text and Fig 7 that hCAF1 binds to STAT1 in the cytoplasm, but Fig 1B (hCAF1 expression), Fig 4D (STAT1 expression), and Fig 6C (co-localization of hCAF1 and STAT1) clearly show that hCAF1 is located in the nucleus and the interaction between hCAF1 and STAT1 may be found in the nucleus as well as the cytoplasm in the absence of IFN. The interaction seems to be lost when cells are stimulated with IFN (Fig 6Cb).

4. Cheon and Stark (2009) show that U-STAT1 induces a subset of IFN-induced genes even in the absence of IFN. This subset of genes is the same set of genes that is up-regulated in DNA damage resistant cancer cells studied by the Weichselbaum group. The set of IFN-induced genes found in this study (Fig 1C) also contains the same subset of genes. hCAF1 knock-down increases the expression of this subset of genes regardless of IFN stimulation, but not the expression of other IFN stimulated genes (for example, SOCS1, IRF1, TAP1 etc), suggesting that hCAF1 is a negative regulator of U-STAT1 rather than P-STAT1.
5. The introduction is far too long and too complex. The authors should make the points essential for understanding their data succinctly and save the complications for the discussion.
6. hCAF1 has several activities. Do the authors have any information about which activity is required for the functions they now describe? This aspect should certainly be discussed.
7. Important connections between STATs and chromatin structure have been made by Yan et al (FASEB J. 25, 232-241) and by Christova et al (J. Cell Sci. 120, 3262-3270. It would be good if the authors could put their current observations into the context of this literature.

1st Revision - authors' response 09 December 2012

Point-by-point response to the reviewers:

Referee #1

We would like to thank referee 1 for his/her favourable comments on our paper.

1. Fig. S1 - error bars and statistics are needed to support the significance of the small ~ small ~2X increases seen in most of the mRNAs assayed in MCF7 cells.

We apologize for the important lack. Statistics and error bars are now presented.

2. *Fig. 2E: Could the 'resistance' to viral infection observed in these KD cells simply be due to the increased apoptosis and cell death that would indirectly result in decreased virus yields? I think that one needs to be careful in drawing conclusions when infecting KD cells that are exhibiting growth defects and increased rates of dying. If this concern is valid, then claiming that the CAF1 KD cells mimics IFN based on this assay is probably not appropriate*.

We agree with the referee and we are sorry for not including this control in the first manuscript. We have clarified this important point, adding in Fig.2 E (right panel) data showing that the growth rate and the viability of $hCAF1^{kd}$ and control cells are not affected in the course of the viral infection. We think that we can conclude that CAF1kd cells are more resistant to viral infection than the control cells.

3. Fig. 3: The mRNA decay that is being observed appears to only be linear between 0 and the first time point (2hr). …… Therefore I would encourage the authors to re-visit this experiment and increase the number of time points between 0-2 hrs to determine more accurate decay rates (rather than relying on 2 points as in the current graphs). Furthermore, if the 0-2 hr region is expanded, the effects observed on STAT1 mRNA decay may be similar to the other mRNAs (rather than different as currently concluded).

As judiciously suggested, we have redone the kinetic experiments increasing the time points of actinomicin treatment between 0 and 2 hrs. The results, now illustrated in a new version of Figure 3A, confirm the results obtained before (now presented in the Supplementary data Fig. 2) and support our previous conclusions. In fact the decay rates of IFI27, IFITM1 and IFI6 mRNAs was significantly lower in hCAF1 knockdown cells than in control cells. By contrast, the stability of the STAT1 mRNA was not significantly different from that of control cells. On the basis of these results, we can reasonably conclude that hCAF1 regulates IFN-induced genes by different mechanisms besides the control of mRNA turnover.

4. . Fig. 3B: These data would be stronger if P body numbers were presented quantitatively with error bars.

The quantification of the number of signals per cell was performed on 100 cells using the Image J software and presented as mean± sem in the right panel of the figure 3B (the p-value was determined by Student's t-test).

5. Fig. 3: The authors conclude that the decrease in P bodies is consistent with impaired deadenylation - but the authors are seeing increased decay rates in the CAF1 KD which would infer precisely the opposite - increased deadenylation. To address this conundrum, why don't the authors measure deadenylation rates directly rather than making inferences about them?

We apologize for this misunderstanding, probably due to the lack of clarity in our manuscript. In fact CAF1^{kc} cells show decreased amount of P-bodies and decreased decay rate (or increased mRNA stability), consistent with impaired deadenylation. We hope that this conclusion is clearer in the revised version of the manuscript.

6. Fig. 4A and B: These data would be improved if error bars and statistics were added.
We added the error bars and statistics as requested.

7. . Fig. 6: Is the interaction between CAF1 and STAT1 sensitive to RNAse?

The interaction between hCAF1 and STAT1 is not mediated by RNA because RNase treatment of MCF7 cell lysates did not affect their co-precipitation. Fig. 6 C illustrates this result.

8. Fig. 6/7: Do antibodies specific for other components of the CAF/CCR4-NOT complex also co-IPPT STAT1? The result would be important to address in the model whether the full complex is interacting with STAT1 as depicted or whether CAF1 is acting independently of the full complex

This is indeed a key point. We have tested whether STAT1 interacted with other components of the CCR4-NOT complex using home-made polyclonal antibodies against several members of the CCR4-NOT complex, namely CCR4 and NOT1. The results obtained by immunoprecipitation assay were not convincing because hard to reproduce. These limits are probably due to the lack of strong antibodies to detect co-immunoprecipitation of endogenous proteins and/or to reveal a weak or indirect interaction. We therefore used the Proximity Ligation Assay, which is much more sensitive than IP, and we detected the interaction of STAT1 with NOT1 in the cytoplasm of MCF7 cells. These results are shown in the Supplemental Fig 9. We did not detect any interaction between STAT1 and CCR4 by PLA, consistent with GST-pull down results in Fig. 6 A. Although our overall data suggest that STAT1, *via* hCAF1 and NOT1, interacts with the full CCR4-NOT complex, we

would like to confirm the interaction between STAT1 and NOT1 using complementary approaches. For this reason, the CCR4-NOT complex is depicted in Figure 7 in light grey.

Referee #2

The data in this paper show that hCAF1 binds to STAT1 and inhibits the expression of a subset of STAT1 target genes. It is a significant finding that hCAF1 acts as a negative regulator of STAT1 and its target gene expression, but the mechanism is not obvious. The authors pass over several of their results showing discrepancies from the model described in Fig 7. The data should be re-interpreted in order to fit an adequate model explaining the mechanism of the negative regulation of IFN signalling induced by hCAF1.

The referee 2 is concerned by the model proposed in Fig. 7. In this model we illustrated how hCAF1 could control interferon signalling in normal cells under physiological conditions, not how hCAF1 depletion affects this pathway in hCAF1kd cells. We apologize for the confusion generated by the misinterpretation of this model. We hope that we clarified this in the revised version of manuscript (see answer to the referee comment 1 c)

Specific comments

1. *In order to understand the function of hCAF1 in IFN signalling, basic studies about hCAF1 regulation in response to IFN should be shown. ……These basic studies should answer the following questions*;

a) Is the expression of hCAF1 down-regulated in response to IFN?

hCAF1 expression in response to INF alpha and gamma, measured by q-RTPCR and by western blot using anti-CAF1 antibodies, was not affected. We apologize for the lack of description of these negative results, now illustrated in the Supplementary Figure 3 B.

b) *is the dissociation of hCAF1 from STAT1 accelerated by IFN? Does IFN induce any modification of hCAF1 protein? Or does the IFN-induced phosphorylation of STAT1 decrease its affinity to hCAF1 protein?*

Our data show the interaction between endogenous STAT1 and hCAF1 on resting MCF7, HeLa and U937 cell lines (Fig. 6 Da, Supplementary Fig. 7 and 8 a and c). INF treatment strongly reduced the interaction (Fig. 6 Db, Supplementary Fig. 8 b and c). Since the level of both proteins did not decrease after IFN treatment, hCAF1/STAT1 dissociation could be induced by the phosphorylation of STAT1 leading to its nuclear migration after IFN treatment, as shown in Figure 4 Db and c.
Currently no post-translational modifications of hCAF1 have been described, but we cannot rule out that INF can induce hCAF1 modifications. We have not analysed this possibility.

c) *Figure 4A shows that the phosphorylation of STAT1 in response to IFN is decreased in hCAF1 knock-down cells, but the expression of STAT1 target genes is increased (Fig 4B). These data clearly show that the expression of these genes in Fig 4B is not regulated by P-STAT1. These data cannot support the model in Fig 7, and I cannot find any data in the paper that do support it.*

We agree with the referee that in hCAF1 knockdown cells the expression of STAT1 target genes is not regulated by P-STAT1. This is also our conclusion. As discussed above, the model in Figure 7 seems to be confusing. So in the revised manuscript we have provided a novel version of this figure. In the panel A we now illustrate how hCAF1 depletion could functionally affect IFN pathway in resting hCAF1-knockdown cells. The panel B illustrates how hCAF1, through its interaction with STAT1, could regulate IFN signaling both in absence and after IFN induction. These two models are described and discussed in the novel version of the legend to figure 7 and in the revised discussion section.

2. The authors explain in the text and Fig 7 that hCAF1 binds to STAT1 in the cytoplasm, but Fig 1B (hCAF1 expression), Fig 4D (STAT1 expression), and Fig 6C (co-localization of hCAF1 and STAT1) clearly show that hCAF1 is located in the nucleus and the interaction between hCAF1 and STAT1 may be found in the nucleus as well as the cytoplasm in the absence of IFN. The interaction seems to be lost when cells are stimulated with IFN (Fig 6 Cb).

We agree on the fact that figure 1B shows that hCAF1 localizes in both the cytoplasm and the nucleus of MCF7 cells. In contrast, Figure 4Da shows that in resting MCF7 cells, STAT1 localizes to the cytoplasm and that IFN treatment induced a nuclear translocation (figure 4 Db). The interaction between hCAF1 and STAT1 shown in figure 6 Ca (in the revised version fig; 6 Da) was performed in absence of IFN. The spot-like signals, indicating the interaction between endogenous hCAF1 and STAT1, appeared localized mainly in the cytoplasm of MCF7 cells in accordance with the cytoplasmic staining of STAT1 in resting MCF7 cells.
Since the image in figure 6 Da is recorded in the fixed plane, the position of certain spots inside the cell could be unclear. To overcome this drawback we performed z-stack projections using confocal microscopy. Multiple two-dimensional images (about 20) were obtained sequentially at different planes from the basal to the apical cell side (called a *z-stack* because they are collected along the z- or depth axis), to generate a reconstitution of a three-dimensional (3D) image (Supplementary Fig.7 A). In the Supplementary Fig. 7 B we can see a single median section cutting the nucleus, showing without ambiguity that the hCAF1/STAT1 interaction spots localize into the cytoplasm of resting MCF7 cells.

3. .Cheon and Stark (2009) show that U-STAT1 induces a subset of IFN-induced genes even in the absence of IFN. This subset of genes is the same set of genes that is up-regulated in DNA damage resistant cancer cells studied by the Weichselbaum group. The set of IFN-induced genes found in this study (Fig 1C) also contains the same subset of genes. hCAF1 knock-down increases the expression of this subset of genes regardless of IFN stimulation, but not the expression of other IFN stimulated genes (for example, SOCS1, IRF1, TAP1 etc), suggesting that hCAF1 is a negative regulator of U-STAT1 rather than P-STAT1.

TAP1 is present in the hierarchical clustering of gene expression profile in figure 1, and we confirmed by q-RT-PCR that this gene is up-regulated in hCAF1 knockdown cells. These results are shown in Fig.1 D. Conversely, the expression IRF1 and SOCS1 was not affected in these cells (Supplementary Fig. 1 B). Our interpretation of the results described in this manuscript is that hCAF1 acts as regulator of latent STAT1 function in resting cells, probably controlling STAT1 nuclear migration. Studies are in progress to determine whether hCAF1 is involved in the regulation of STAT1 phosphorylation and thereby its activation and migration.

4. *The introduction is far too long and too complex. The authors should make the points essential for understanding their data succinctly and save the complications for the discussion.*

We reduced and simplified the introduction, as demanded.

5. *hCAF1 has several activities. Do the authors have any information about which activity is required for the functions they now describe? This aspect should certainly be discussed.*

We think that hCAF1 has a dual function in the regulation of IFN signaling. In resting cells by its interaction with the latent form of STAT1 in the cytoplasm, hCAF1 controls STAT1 trafficking and protects the cells from undesirable stimulation. The hCAF1 activity involved in this function is not known. One possibility is that hCAF1 within the complex CCR4-NOT acts as a chaperone regulatory platform. Studies are in progress to determine whether hCAF1 is involved in the regulation of STAT1 phosphorylation and thereby its activation and migration.
In addition we cannot exclude that hCAF1 can also regulate chromatin architecture of some STAT1-target promoters in resting cells. This constitutes an attractive explanation for the changes in chromatin structure and histone modifications associated with hCAF1 knockdown. Indeed hCAF1 itself (Robin-Lespinasse et al., 2007) and several members of the CCR4-NOT complex have been shown to control histone modifications (Miller *et al.*, 2012).

The second role of hCAF1 concerns its contribution to the extinction of the IFN signal, through its deadenylase activity, by speeding up the degradation of some STAT1-regulated mRNAs

6. *Important connections between STATs and chromatin structure have been made by Yan et al (FASEB J. 25, 232-241) and by Christova et al (J. Cell Sci. 120, 3262-3270. It would be good if the authors could put their current observations into the context of this literature*.

The "Discussion" section has been amplified and partially rewritten in order to link our data with the literature data concerning STATs and chromatin structure.

Editorial Decision 04 January 2013

The paper has been re-reviewed by two original referees with no further comments.