Title:

Importin β1 targeting by Hepatitis C Virus NS3/4A Protein Restricts IRF3 and NF-κB Signaling of IFNB1 Antiviral Response

Authors: Bridget Gagné, Nicolas Tremblay, Alex Y Park, Martin Baril and Daniel Lamarre

Article Type: Original Research

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Decision and Reviews

Dear Dr. Lamarre

Thank you for submitting your manuscript "KPNB1-Associated Proteins Control IRF3 and NF-κB p65 Signalling of IFNB1 Antiviral Response" for consideration for publication in Traffic. I asked two colleagues who are experts in the field to review the paper and their verbatim comments are appended below. Both referees have made suggestions to correct and improve the presentation of your manuscript. In addition, referee 2 raises concerns about why you have used SENDV to screen for HCV-cell interacting partners, and why you have chosen to focus on IFNB1. You will need to address these concerns before I can reconsider this paper for publication.

Although I cannot accept your manuscript for publication at this point, I believe that you will be able to address the referees concerns and I look forward to receiving your revised manuscript. To expedite handling when you resubmit please be sure to include a response outlining how you have addressed each of the referees' concerns.

Sincerely,

Trina A. Schroer, Ph.D.
Co-Editor

Referee's Comments to the Authors

Referee: 1

Comments to the Author
The authors performed knockdown studies of previously reported HCV-host interactors that they had determined from LC/MS-MS analysis. They tracked the effects of antiviral innate immune response upon knockdown of 132 potential HCV-host interactor genes. They obtained a list of positive and negative regulators of antiviral response, and gene ontology analysis of these regulators shows that Karyopherin nuclear transport receptors, their regulators and nucleoporins are associated with the most highly enriched terms. Therefore, they focused on silencing 4 Karyoperins (KPNB1, TNPO1, XPO1 and CSE1L) and Ran, which were all on their list of positive regulators of antiviral response, and found that only KPNB1 knockdown increased viral replication. They also performed an RNAi screen of 60 proteins associated with the NPC and nuclear transport to probe effects on nuclear localization of IRF3 and NF-κB

p65 upon viral infection. The effects of the knockdowns seem rather small, with KPNB1 knockdowns being show the largest decreases in % of cells with nuclear IRF3 or p65 (30-40% for IRF3 and 15-30% for p65). The effects of silencing individual KPNA isoforms on IRF3 nuclear localization range from negligible (KNPA1 and KPNA6) to small 15-20% decreases (KPNA2, KPNA3 and KPNA4) while effects on p65 nuclear localization is more uniform and convincing at 20-30% for all 6 KPNA isoforms. The authors then showed results for knockdowns of other Karyopherins, mRNA exporters, Ran and its regulators and nucleoporins.

The work is interesting and important in highlighting the importance of the nuclear transport machinery in controlling viral infection induced nuclear localization of IRF3 and p65, hence antiviral response and potentially viral replication.

More detailed comments:

1) The organization of the Results section is a bit odd and perhaps could be optimized for maximum clarity. It is odd that they started with a screen, followed by specific studies of a few positive regulators and then back to a screen of many proteins associated with the nuclear transport machinery. Interspersed in there are specific studies of viral replication, apoptosis upon KPN1 knockdown along with binding of KPN1 to NS3. The order of results makes for difficult reading. Would it be better to proceed from knockdown screen of HCV-host interactors to the screen of 60 proteins associated with the NPC and nuclear transport and then because the effects of KPNB1 knockdown are the largest, to then focus on KPNB1-specific studies.
Minor concerns:

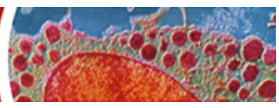
2) The 2nd sentence of Introduction is not quite accurate. The NPC does not contain 30 nucleoporins and nucleocytoplasmic transporter proteins. The nuclear transport machinery does. The NPC has multiple copies of 30 different nucleoporins. Similarly, the authors frequently refer to the NPC components and their transporters. They seem to refer to transporters for NPC components so the sentences need to be revised to uncouple the two as the transporters transport hundreds if not thousands of cellular proteins.
3) Lines 10-15 of page 2: “Macromolecules can only cross …… for this process (Cook et al, 2007).” Is inaccurate and completely outdated by >15 years. There is a family of >20 Karyopherin proteins that mediate nuclear import, export and bidirectional transport. Please read recent reviews on this topic.
4) The next sentence is also wrong – Kap-alpha binds specifically to only KPNB1. No other transporter uses Kap-alpha as an adaptor to bind classical-NLS containing cargoes.
5) Page 3, middle of 1st paragraph – it is unclear what the “membranous web” refers to.
6) The sentence “However, although IRF3 …… and Nups involved in the process are yet to be determined.”, in the 1st paragraph on the right column of page 3, is also inaccurate as it is very well established that the Kap-beta carrier for any Kap-alpha is KPNB1 and not any other Kap-beta.
7) Discussion is too long.

Referee: 2

Comments to the Author
KPNB1-Associated Proteins Control IRF3 and NF-κB p65
Signalling of IFNB1 Antiviral Response

This study is based on a previous publication by the same authors where they identified cellular interaction partners of HCV (NS3/4A) with cellular transport proteins (Germain et al., 2014). Here, they follow up their findings by concentrating on a subset of HCV interactors. They use SENDV-infected cells which are transfected with siRNA to knockdown the HCV interacting cellular factors as well as with a reporter construct under an interferon promoter. Finally, the authors show that knockdown of importin-ß1 (IFNB1) reduces IRF-3 and NFkB p65 nuclear translocation which further correlates with increased viral replication and reduced interferon induction.

Major comments:
The study suffers from two major set-backs:
#1: First, as it is written, the previous findings on HCV cellular interactors and the current findings on cellular genes that control interferon induction seem detached. It is not clear why the authors have used SENDV and not HCV. One possibility to get a logical connection could be a confirmation of their findings by using HCV and looking for the effects on viral replication in the siRNA setting used.
#2: It is also not clear why the authors have concentrated on importin-ß1 (IFNB1) among all the cellular HCV interactors they have identified. It is not surprising that key transcription factors are not transported into the cell



nucleus if the major receptor controlling the classical cellular import machinery (importin-α/ß) is shut off. This fact is not even discussed in the manuscript and requires careful consideration.

Specific comments:
#1:The authors should use the new nomenclature “importin-α or importin-β” and not karyopherin (KPNA or KPNB1)
#2: first paragraph of the result section: the rational of choosing select host genes is not clear. First the authors state that 426 proteins have been identified of which only 13 were described as modulators of HCV replication. Then, they move on saying that they have concentrated on 132 proteins. This does not make sense.
#3: page 3, section “RNAi screen targeting the NPC and its transport Proteins“: here, the authors state that it is not clear which importin-α isoform mediated NFkB interaction. In the Fagerlund et al papers, it is nicely shown that NFkB interaction is most efficient with importin-α3 compared to other isoforms. The authors should at least discuss this discrepancy.
#4: Figure 4a: why is p65 nuclear localization not affected upon importin-ß1 silencing?
#5: Figure 5: silencing of importin-α3 should lead to decreased p65 nuclear localization. How do the authors explain this?
#6: Figure 6: the authors need to show whether silencing of one importin-α isoform affects the expression of other importins as an essential control.

Author Rebuttal

Dear Editor,

Please find enclosed a revised copy of the manuscript TRA-16-0564 with the revised title “Importin β1 targeting by Hepatitis C Virus NS3/4A Protein Restricts IRF3 and NF-κB Signaling of IFNB1 Antiviral Response" that addresses the specific points made by reviewers. We thank the reviewers for their diligent review of our manuscript as well as for their useful suggestions to correct and to improve the presentation of the manuscript. We have carefully considered most of the reviewers’ comments and have addressed the majority of these points. We believe these modifications have substantially improved the scientific content and presentation of this manuscript, and that it is suitable for publication in Traffic.

What follows is a summary of significant revisions and a point-by-point response to the reviewers’ comments.

Summary of significant revisions:
• In reply to reviewer’s comments, appropriate changes have been made to the Introduction for an accurate description of the current state of knowledge, to the organization of Results section and Figures to facilitate the overall understanding of the manuscript, and we have substantially shortened the discussion section to focus on key findings.
• Figure 8 is new data that provide evidence of a role of NS3/4A in HCV infection that triggers the cleavage of IMPβ1 and inhibits nuclear transport to disrupt IFNB1 production.
• Appropriate changes have been made to the text of Results section to explain the use of SeV infection model instead of HCV infection, which facilitates experiments and analysis for RNAi gene silencing screening of proteins that modulate the RIG-I like receptor signaling pathway and prevent interference of HCV proteins with early MAVS signaling.
• The study of IMPβ1/KPNB1 is now more evident as the decrease of SeV-mediated IFNB1 production upon its knockdown is phenocopied by expression of NS3/4A that triggers the cleavage of IMPβ1 and inhibits nuclear transport of IRF3/NFkB, providing evidence of a direct link to HCV and the discovery of a novel strategy to evade innate immune response.

Point-by-point response to reviewer comments

Reviewer #1
The authors performed knockdown studies of previously reported HCV-host interactors that they had determined from LC/MS-MS analysis. They tracked the effects of antiviral innate immune response upon knockdown of 132 potential HCV-host interactor genes. They obtained a list of positive and negative regulators of antiviral response, and gene ontology analysis of these regulators shows that Karyopherin nuclear transport receptors, their regulators and nucleoporins are associated with the most highly enriched terms. Therefore, they focused on silencing 4 Karyoperins (KPNB1, TNPO1, XPO1 and CSE1L) and Ran, which were all on their list of positive regulators of antiviral response, and found that only KPNB1 knockdown increased viral replication. They also performed an RNAi screen of 60 proteins

associated with the NPC and nuclear transport to probe effects on nuclear localization of IRF3 and NF-κB p65 upon viral infection. The effects of the knockdowns seem rather small, with KPNB1 knockdowns being show the largest decreases in % of cells with nuclear IRF3 or p65 (30-40% for IRF3 and 15-30% for p65). The effects of silencing individual KPNA isoforms on IRF3 nuclear localization range from negligible (KNPA1 and KPNA6) to small 15-20% decreases (KPNA2, KPNA3 and KPNA4) while effects on p65 nuclear localization is more uniform and convincing at 20-30% for all 6 KPNA isoforms. The authors then showed results for knockdowns of other Karyopherins, mRNA exporters, Ran and its regulators and nucleoporins.

The work is interesting and important in highlighting the importance of the nuclear transport machinery in controlling viral infection induced nuclear localization of IRF3 and p65, hence antiviral response and potentially viral replication.

Major concerns:
1. The organization of the Results section is a bit odd and perhaps could be optimized for maximum clarity. It is odd that they started with a screen, followed by specific studies of a few positive regulators and then back to a screen of many proteins associated with the nuclear transport machinery. Interspersed in there are specific studies of viral replication, apoptosis upon KPN1 knockdown along with binding of KPN1 to NS3. The order of results makes for difficult reading. Would it be better to proceed from knockdown screen of HCV-host interactors to the screen of 60 proteins associated with the NPC and nuclear transport and then because the effects of KPNB1 knockdown are the largest, to then focus on KPNB1-specific studies.

As suggested by the reviewer, we made major changes to the organization of the Results section and conducted additional experiments. We now describe our data in the following order:
1. Effect of silencing HCV-interacting proteins on IFNB1 antiviral response
2. Gene Ontology analysis
3. Microscopy-based high content screening of nuclear transport of IRF3 and NF-κB p65 upon viral infection
4. Analysis of IMP-β NTRs and Nups Knockdown on virus-mediated IRF3/NF-κB p65 nuclear translocation and IFNB1 production
5. HCV NS3/4A-mediated cleavage of IMPβ1 inhibits nuclear transport of IRF3 and NF-κB p65 to evade IFNB1 production

Minor concerns:
2) The 2nd sentence of Introduction is not quite accurate. The NPC does not contain 30 nucleoporins and nucleocytoplasmic transporter proteins. The nuclear transport machinery does. The NPC has multiple copies of 30 different nucleoporins. Similarly, the authors frequently refer to the NPC components and their transporters. They seem to refer to transporters for NPC components so the sentences need to be revised to uncouple the two as the transporters transport hundreds if not thousands of cellular proteins.

We agreed with the reviewer and have removed the sentence. We substantially modified the text of the introduction in response to comments 2-4 to more accurately describe the current state of knowledge, and to harmonize protein name with the recommended nomenclature (specific comment 1 of reviewer 2).

3) Lines 10-15 of page 2: “Macromolecules can only cross …… for this process (Cook et al, 2007).” Is inaccurate and completely outdated by >15 years. There is a family of >20 Karyopherin proteins that mediate nuclear import, export and bidirectional transport. Please read recent reviews on this topic.

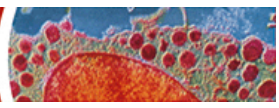
We agreed with the reviewer and have removed the sentence.

4) The next sentence is also wrong – Kap-alpha binds specifically to only KPNB1. No other transporter uses Kap-alpha as an adaptor to bind classical-NLS containing cargoes.

We agreed with the reviewer and have removed the sentence.

5) Page 3, middle of 1st paragraph – it is unclear what the “membranous web” refers to.

This is now clarified in the new version. We have added the following lines to the Introduction section: “Indeed, Nups were reported to accumulate in virus-induced endoplasmic reticulum (ER)-derived membranous structures where HCV replication occurs (so-called membranous webs), indicating that Nups can gate these compartments to promote viral replication and to prevent cytosolic RIG-I-like receptor (RLR) sensing of viral RNA”



6) The sentence “However, although IRF3 …… and Nups involved in the process are yet to be determined.”, in the 1st paragraph on the right column of page 3, is also inaccurate as it is very well established that the Kap-beta carrier for any Kap-alpha is KPNB1 and not any other Kap-beta.

We agreed with the reviewer and have removed the sentence.

7) Discussion is too long.

We have refocused the discussion to the key findings of the study, which has significantly reduced the length of the section.

Reviewer #2
This study is based on a previous publication by the same authors where they identified cellular interaction partners of HCV (NS3/4A) with cellular transport proteins (Germain et al., 2014). Here, they follow up their findings by concentrating on a subset of HCV interactors. They use SENDV-infected cells which are transfected with siRNA to knockdown the HCV interacting cellular factors as well as with a reporter construct under an interferon promoter. Finally, the authors show that knockdown of importin-ß1 (IFNB1) reduces IRF-3 and NFkB p65 nuclear translocation which further correlates with increased viral replication and reduced interferon induction.

Major comments:
The study suffers from two major set-backs:
1: First, as it is written, the previous findings on HCV cellular interactors and the current findings on cellular genes that control interferon induction seem detached. It is not clear why the authors have used SENDV and not HCV. One possibility to get a logical connection could be a confirmation of their findings by using HCV and looking for the effects on viral replication in the siRNA setting used.

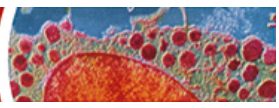
We clarify the use of SeV infection model instead of HCV infection, which facilitate experiments and analysis for RNAi gene silencing screening of proteins that modulate the RIG-I like receptor signaling pathway and prevent interference of HCV proteins with early MAVS signaling. The following sentences have been added to the first results section:

“However, HCV replication is often monitored in the RIG-I deficient Huh7.5 cell line such that one cannot assess if the viral-host protein interactions benefit the virus through subversion of the innate immune response resulting in increased replication. To test this hypothesis, we silenced 132 selective HCV-host interactors (Figure S1) using ~5 independent shRNA-expressing lentiviruses, and measured the induction of IFNB1 promoter-driven firefly luciferase upon SeV infection of A549 and HEK293T cells. We previously showed that SeV infection predominantly activates the RLR pathway in these cells, leading to the nuclear translocation of NF-κB and IRF3 transcription factors, induction of IFNB1 mRNA and secretion of IFN-β cytokine (Baril et al 2013).”

We also conducted additional experiments to confirm a role of the interaction of NS3/4A with IMPβ1 as a novel HCV strategy to evade IFNB1 production. We now provide evidence that expression of NS3/4A triggers the cleavage of IMPβ1 to phenocopy its silencing by inhibiting nuclear transport of IRF3 and NF-κB p65 to disrupt IFNB1 production. We further produced a mutated IMPβ1 variant that is resistant to the cleavage, which restores signalling and IFNB1 induction similarly to the treatment with BILN 2061 protease inhibitor, correlating with the disappearance of IMPβ1cleaved product.

2: It is also not clear why the authors have concentrated on importin-ß1 (IMPβ1) among all the cellular HCV interactors they have identified. It is not surprising that key transcription factors are not transported into the cell nucleus if the major receptor controlling the classical cellular import machinery (importin-α/ß) is shut off. This fact is not even discussed in the manuscript and requires careful consideration.

We now believe that the new data further emphasize why we focus on IMPβ1/KPNB1. Not only among NS3/4A-interacting proteins is IMPβ1 knockdown the one that showed the most significant decrease of innate response (IFNB1 and IFIT1), but more importantly its interaction with NS3/4A triggers the cleavage of IMPβ1 and inhibits nuclear transport of IRF3 and NF-κB providing evidence of a direct link to HCV infection. Furthermore, IMPβ1 is a major NTR that is targeting by many different viral proteins. Such strategy of restricting the nuclear translocation of IRF3 and NF-κB to evade IFNB1 production is now fully discussed for several viruses, resulting in a rapid blockage of early innate response conferring growth advantage to a large spectrum of RNA viruses. Finally, we believe to our



knowledge that there are no data describing a kinetics analysis of the transport of IRF3/NFKBp65 upon viral infection.

Specific comments:
1:The authors should use the new nomenclature “importin-α or importin-β” and not karyopherin (KPNA or KPNB1).

We agreed with the comments and have used the recommended nomenclature.

2: first paragraph of the result section: the rational of choosing select host genes is not clear. First the authors state that 426 proteins have been identified of which only 13 were described as modulators of HCV replication. Then, they move on saying that they have concentrated on 132 proteins. This does not make sense.

This section has being modified to clarify the goal of the study in testing a set of enriched HCV interactors in modulating innate immunity.

3: page 3, section “RNAi screen targeting the NPC and its transport Proteins“: here, the authors state that it is not clear which importin-α isoform mediated NFkB interaction. In the Fagerlund et al papers, it is nicely shown that NFkB interaction is most efficient with importin-α3 compared to other isoforms. The authors should at least discuss this discrepancy.

We agreed with the comments and have included in the discussion the following sentences:

“In our study, the depletion of IMP-α family members had varying effects on IRF3/NF-κB p65 nuclear localization but IFNB1 production was significantly decreased when IMPα1, IMPα4 and IMPα6 are individually silenced (Figures 3 and S3), which could be attributed to these 3 genes decreasing both IRF3 and NF-κB p65 nuclear translocation at 3 hours post-infection. NF-κB p65 was reported to be transported by IMPα3 and IMPα4 while a more recent study identified IMPα1 as the most critical adaptor for its nuclear translocation upon tumor necrosis factor-α (TNF-α) treatment (Fagerlund et al, 2005; Fagerlund et al, 2008; Liang et al, 2013). Our results largely support these studies with a predominant role of IMPα1 and IMPα4, except for the depletion of IMPα3 that may be over-compensating by other adaptors causing the increase of NF-κB p65 during viral infection.”

4: Figure 4a: why is p65 nuclear localization not affected upon importin-ß1 silencing?
In the section - Effects of NPC and transporter proteins knockdown on nuclear translocation of IRF3 and p65 - Three independent shRNAs targeting the main KAPβ import carrier KPNB1 significantly hindered the nuclear translocation of both IRF3 and NF-κB p65 when compared to the shRNA NT (Figure 4A).

We clarified this point by adding the following sentences in the Results section:
“Using three independent shRNAs specific to IMPβ1, we confirmed that it depletion significantly hindered IFNB1 induction of SeV-infected cells correlating with the reduced nuclear translocation of IRF3 and NF-κB p65 when compared to the shRNA NT (Figure 7A). Indeed, we showed that IMPβ1 knockdown led to a strong decrease in IRF3 nuclear translocation at 3 and 5 hours post-infection, before returning to normal levels at 8 and 10 hours post-infection. A similar pattern was observed for NF-κB p65 nuclear translocation, although with a less drastic decrease at 3 and 5 hours post-infection, and culminated with increased nuclear NF-κB p65 staining at 8 and 10 hours post-infection compared to that in presence of shRNA NT.”

5: Figure 5: silencing of importin-α3 should lead to decreased p65 nuclear localization. How do the authors explain this?

Our data is more in line with the data of Liang et al., 2013 describing that import of p65 mainly relies on IMPα1 (KPNA2) for TNF-α activation of A549 cells (the same cells used in our study).
While IMPα3 was previously reported by Fagerlund et al., 2005 to be the major IMPα family member responsible for the nuclear import of p65 upon its knockdown, it is clear that p65 needs more than one IMPβ and IMPα for its import with several reasons for a multi-pathway import of p65. Nevertheless, the ability of multiple importin receptors to recognize p65 potentially provides a multiply redundant transport system such that when one pathway is blocked, others can substitute for it.

In that context, we added the following sentences in the discussion: “Our results largely support these studies with a predominant role of IMPα1 and IMPα4, except for the depletion of IMPα3 that may be over-compensating by other adaptors causing the increase of NF-κB p65 during viral infection. Additionally, the need for fast activation upon viral

infection most not requires a process that is dependent on IMPα3 as its knockdown has no impact on IFNB1 production. ”

6: Figure 6: the authors need to show whether silencing of one importin-α isoform affects the expression of other importins as an essential control.

While we agreed that this is an important question to assess if silencing of one IMPα family member can affect the expression and function of others in a multi-pathway import of p65, we believe that this is out of the scope of this study as none of IMPα adaptors are identified as interactors of HCV proteins.

Sincerely yours,
Daniel Lamarre, PhD

Decision and Reviews

Dear Dr. Lamarre,

Thank you for submitting your revised manuscript “Importin β1 targeting by Hepatitis C Virus NS3/4A Protein Restricts IRF3 and NF-κB Signaling of IFNB1 Antiviral Response” to Traffic. I asked the referees to read the revised paper. The referees did not have comments for the author, but both share the view that you have addressed the concerns raised previously. I agree, and I am pleased to accept this paper for publication.

Sincerely,

Trina A. Schroer, Ph.D.
Co-Editor

Referee's Comments to the Authors

Referee: 1

Comments to the Author
(There are no comments.)

Referee: 2

Comments to the Author
(There are no comments.)