nature portfolio

Peer Review File

GGCX promotes Eurasian avian-like H1N1 swine influenza virus adaption to interspecies receptor binding

Corresponding Author: Professor Hongbo Zhou

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In this study, Zou et al. conducted a genome-wide CRISPR/Cas9 knockout screen utilizing EA H1N1 SIV in porcine kidney cells and found that knockout GGCX inhibited the carboxylation modification of viral HA and the adhesion of progeny viruses, ultimately impeding the replication of EA H1N1 SIV. Further, they found that GGCX is the determinant of the HA D225E substitution of EA H1N1 SIV. Take together, the authors demonstrate that GGCX supports EA H1N1 SIV adaption for binding to α-2,6 SA receptor. They should, however, address the comments raised below, specifically, the data on the role of GGCX for influenza virus infection thus far are restricted to one cell line (PK-15 cell) of porcine kidney, which is not the primary site of influenza virus replication, and more strains need to be tested to validate the conclusion.

Major Comments
1. Several genome-wide CRISPR screens have been performed by different laboratories to identify the host factors that promote or suppress influenza virus replication. Was GGCX found as a hit in any of other studies?
2. It is not rigorous to draw the conclusions that “GGCX promotes Eurasian avian-like H1N1 swine influenza virus adaption to interspecies receptor binding” using one strain of EA H1N1 SIV (HuB/H1N1). As shown in figure 6b, there are many EA H1N1 swine influenza viruses that naturally bear 225G in the HA protein. Is GGCX-mediated HA carboxylation important for other H1N1 viruses? Does GGCX promote the change from D or G to E at position 225 in the HA protein of those EA H1N1 viruses that naturally bear 225D or 225G.
3. Why does the authors select PK-15 cells, a porcine kidney cell line, for CRISPR screens? The lung but not kidney is the primary target for influenza virus infection. Based on the data from the human protein atlas (https://www.proteinatlas.org/ENSG00000115486-GGCX/tissue), GGCX is expressed in almost all human tissues. Whether human GGCX has a similar function to swine GGCX? Does GGCX catalyze the substitution adaption of EA H1N1 SIV HA in a respiratory epithelia cell line, such as A549 cells? The authors should choose at least one respiratory epithelia cell line to confirm their findings.
4. Is GGCX present in avian cells? If so, whether avian GGCX has a similar function to swine GGCX?
5. In this manuscript, HuB/H1N1 is mainly used in vitro experiments, why is HN/H1N1 used in vivo studies (line 723, figure 2)? Could the authors elucidate the rationale behind the selection of HN/H1N1 for in vivo experiments and delineate the specific differences between HuB/H1N1 and HN/H1N1 strains?
6. The authors quantified the progeny Gla and Glu viruses by absolute quantitative real-time PCR. Does the abrogation of GGCX-mediated HA carboxylation reduce the quantity of HA incorporated into virions?
7. In figure 1c-d, the absence of GGCX significantly decreased the replication of different subtype influenza viruses. Consequently, the quantities of NP and HA present in the cell lysates of GGCX-KO cells are anticipated to be less than those in WT cells under the condition where the expression level of GAPDH is equivalent. However, in figure 3b and 3f, it seems that the quantities of NP and HA present in the cell lysates of GGCX-KO cells and WT cells are similar, why?

Minor Comments
1 In figure 1a, the labeled “HuB” should be “HuB/H1N1”.
2 In figure 6c, the numbers depicted on the Y-axis of the graph are not properly aligned.

Reviewer #2

(Remarks to the Author)
The study by Zou et al. tries to identify host receptors required for replication of the Eurasian avian-like swine H1N1 lineage by utilizing a CRISPR-KO screening approach in porcine cells. Among previously identified proteins (validating their screening approach) they found GGCX to be an essential host factor for these viruses. The authors convincingly show, that GGCX is required for replication of these viruses in porcine cells as well as in infected mice. Furthermore, they show protein-protein interaction between GGCX and the viral HA protein, which leads to enhanced carboxylation of HA. By preparing viral stocks on both WT and GGCX-KO cells they can further show, that this carboxylation seems to be required for α-2,6-SA-, but not α-2,3-SA-binding. They were able to attribute this particular phenotype to carboxylation of the HA 225E mutation. Finally, they show that GGCX facilitates the acquisition of HA 225E in passaging experiments, thereby shedding light on the α-2,3-SA to α-2,6-SA receptor switch in the evolution of this porcine IAV lineage.
The presented study is interesting and the experiments are well-thought-out. The necessity of GGCX for IAV infection is convincingly shown. The authors mainly attribute this effect to the reduced carboxylation of HA proteins on the progeny viruses. If this was the case, however, it remains unclear why the GGCX-KO cells would show up in the CRISPR-screen since they should undergo full rounds of infections (unlike e.g. SLC35A1-KO cells). The authors briefly address the potential of host-protein carboxylation in the discussion (lines 332-336), but this should be experimentally addressed. We therefore raise the following concerns that should be addressed before publication:

Major concerns:
To show that the GGCX-KO cells are not inherently worse susceptible to viral infection, synchronized single-cycle infections of HuB/H1N1 (produced on WT- and GGCX-KO-cells as used in Figure 4e) should be performed on WT-and GGCX-KO cells. Intracellular viral RNA and viral protein levels should be determined before newly synthesized virions are released to exclude (or show) additional effects of the GGCX-KO on viral replication.

Figure 1F,G: it is unclear what timepoint is shown and what MOI was used for the infection. If this is a later timepoint (36+hpi) the virus titers are highly reduced to what is shown in the growth kinetics with WT cells. It would therefore be nice to see GGCX protein levels in wt cells and FLAG-GGCXreconstituted GGCX-KO cells in the same Western blot using GGCX antibody to compare protein levels.

Minor concerns:
Line 91: the CRISPR KO screen was performed in pig cells, not in pigs
Lines 145-146: the phenotype in GGCX-KD mice might suggest, that this is not only a porcine host factor

Reviewer #3

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have added new data and clarified aspects of the text. By doing so,the revised version of the article perfectly answers the questions I asked.

Reviewer #2

(Remarks to the Author)
We appreciate the effort of the authors and think that the quality of the manuscript has greatly improved with the additional experiments and changes in the text. However, the fact that GGCX shows up in the KO-screen is still puzzling if GGCX mainly affected the next round of infection (as mentioned in ll 208-209), particularly because WT virus was used for every round of infection in the initial screen.
The fact that supplementation of GGCX leads to a decrease in the interferon response might indicate that GGCX-KO cells should have higher levels of interferon response. Unfortunately this was not shown in Figure 1 of the rebuttal letter. If this was indeed the case, this would provide a more convincing reason for the GGCX-KO cells to show up in the screen. The figure should therefore be included in the manuscript. It does, however, need to be carefully discussed with the new data of Supp Figure 4, since the early stages of infection don't seem to be affected at all (at least when looking at NP).

Reviewer #3

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

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The images or other third party material in this Peer Review File are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder.
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Point-by-point response to the reviewers' comments and concerns

Comments from Reviewer 1

Comment 1: In this study, Zou et al. conducted a genome-wide CRISPR/Cas9 knockout screen utilizing EA H1N1 SIV in porcine kidney cells and found that knockout GGCX inhibited the carboxylation modification of viral HA and the adhesion of progeny viruses, ultimately impeding the replication of EA H1N1 SIV. Further, they found that GGCX is the determinant of the HA D225E substitution of EA H1N1 SIV. Take together, the authors demonstrate that GGCX supports EA H1N1 SIV adaption for binding to α-2,6 SA receptor. They should, however, address the comments raised below, specifically, the data on the role of GGCX for influenza virus infection thus far are restricted to one cell line (PK-15 cell) of porcine kidney, which is not the primary site of influenza virus replication, and more strains need to be tested to validate the conclusion.

Response: We appreciate your positive comments and constructive suggestions regarding our manuscript. We have carefully addressed your concerns and responded to each comment point by point, as shown below and in the revised manuscript.

In response to your specific concerns about the host cells primarily infected by influenza viruses, we have further investigated the role of GGCX in IAV replication within its natural reservoir. Our study included a pig trachea epithelial cell line (newborn pig trachea cells - NPTr) and human A549 cells. Notably, GGCX knockout significantly decreased the replication of multiple IAV strains in both NPTr and A549 cells (Revised figure S3). To further validate our conclusion that GGCX mediates the HA 225E substitution of EA H1N1 SIV, we applied additional NPTr cell lines and more strains, including natural HA 225D and 225G bearing viruses (WZ/H1N1 and SD/H1N1). Our results show that GGCX can also promote the HA 225E substitution for natural HA 225D and 225G bearing viruses in both PK-15 and NPTr cells (Revised figure 6f-g).

Major Comments

Comment 2: Several genome-wide CRISPR screens have been performed by different laboratories to identify the host factors that promote or suppress influenza virus replication. Was GGCX found as a hit in any of other studies?

Response: Thank you for your comment. Several genome-wide CRISPR screens have been conducted to identify host factors that promote or suppress influenza virus replication, primarily focusing on human or avian influenza viruses in corresponding host cells. Only one study had explored swine influenza virus in porcine cells. However, due to differences in the virus strains used, there were limited shared host factors identified. Upon comparison, we found that GGCX is a novel host factor identified exclusively in our study, with no previous reports of its involvement in influenza virus replication. We have added a discussion to the revised manuscript (lines 335-337) addressing this point.

Comment 3: It is not rigorous to draw the conclusions that "GGCX promotes Eurasian avian-like H1N1 swine influenza virus adaption to interspecies receptor binding" using one strain of EA H1N1 SIV (HuB/H1N1). As shown in figure 6b, there are many EA H1N1 swine influenza viruses that naturally bear 225G in the HA protein. Is GGCX-mediated HA carboxylation important for other H1N1 viruses? Does GGCX promote the change from D or G to E at position 225 in the HA protein of those EA H1N1 viruses that naturally bear 225D or 225G.

Response: Thank you for your constructive comments. To further confirm the role of GGCX in the evolutionary adaptation of EA H1N1 SIV, we utilized natural HA 225D and 225G bearing viruses (WZ/H1N1 and SD/H1N1) to infect wild-type (WT) and GGCX-knockout (KO) PK-15 or NPTr cells. The progeny viruses were then sequenced sequentially after serial passage. Our results revealed that the natural HA 225D and 225G bearing viruses proliferating from WT PK-15 and NPTr cells can partially revert to HA 225E by the fifth round, whereas those from GGCX-KO PK-15 and NPTr cells remained unchanged (Revised figure 6f-g and lines 301-309). This suggests that the role of GGCX in promoting Eurasian avian-like H1N1 swine influenza virus adaptation to interspecies receptor binding may be widely applicable.

Comment 4: Why does the authors select PK-15 cells, a porcine kidney cell line, for CRISPR screens? The lung but not kidney is the primary target for influenza virus infection. Based on the data from the human protein atlas (https://www.proteinatlas.org/ENSG00000115486-GGCX/tissue), GGCX is expressed

in almost all human tissues. Whether human GGCX has a similar function to swine GGCX? Does GGCX catalyze the substitution adaption of EA H1N1 SIV HA in a respiratory epithelia cell line, such as A549 cells? The authors should choose at least one respiratory epithelia cell line to confirm their findings.

Response: Thank you for your constructive comments. Based on our primary study and other reported studies suggesting that influenza virus can easily replicate in PK-15 cells, we hypothesized that there may exist key host genes involved in influenza virus infection. In fact, our group also conducted a CRISPR screening for influenza virus in NPTr cells, and we found that most of the top-scoring hit genes (SLC35A1, GGCX, RBM6, ADD1, et al) were the same between the two screens using different cells, which supported the evidence that PK-15 is a reliable cell model for influenza virus infection.

As suggested, we employed CRISPR-Cas9 technology to generate GGCX-knockout human A549 cells, a primary target for influenza virus, and examined the effects on influenza A virus (IAV) replication. Notably, knockout of GGCX significantly decreased the replication of multiple IAV strains in A549 cells (Revised figure S3g-h and lines 126-132). To further investigate whether human GGCX played a similar role in progeny IAV binding, we utilized progeny Gla and Glu viruses propagated from IAV-infected wild-type (WT) and GGCX-knockout (KO) A549 cells to infect WT and GGCX-KO A549 cells. Subsequent visualization of viral hemagglutinin (HA) binding activities revealed that the progeny Glu viruses exhibited lower binding activity compared to the Gla viruses in both WT and GGCX-KO A549 cells (Revised figure S5 and lines 220-226). Thus, our results demonstrated that human GGCX exhibits a similar function to swine GGCX, highlighting its conserved role in IAV replication and underscoring the significance of GGCX as a host factor in influenza virus infection.

To determine whether GGCX catalyzes the substitution adaptation of Eurasian avian-like H1N1 (EA H1N1) swine influenza virus (SIV) HA in a respiratory epithelial cell line, we sequenced progeny viruses infecting GGCX-KO and wild-type (WT) NPTr cells sequentially after serial passage. The results showed that both mutant HA 225D and 225G viruses of HuN/H1N1 and natural HA 225D and 225G bearing viruses proliferating from WT NPTr cells can also partially revert to HA

225E in the fifth round, whereas the viruses proliferating from GGCX-KO NPTr cells remained unchanged. Therefore, our results demonstrated that GGCX can catalyze the substitution adaptation of EA H1N1 SIV HA in a respiratory epithelial cell line, NPTr, further corroborating our findings (Revised figure 6f-g and lines 297-309).

Comment 5: Is GGCX present in avian cells? If so, whether avian GGCX has a similar function to swine GGCX?

Response: Thanks for your comment. We investigated the expression pattern of GGCX in various avian species. By querying database information, we found that GGCX is expressed in duck cells but not in chicken cells. Therefore, we sought to determine the role of GGCX in duck cells during influenza virus replication. To achieve this, we transfected three siRNAs targeting GGCX into duck embryo fibroblast (DEF) cells and infected them with the YS/H5N1 virus at 24 hours post-transfection. The supernatants were collected at 24 hours post-infection (hpi), and virus titers were determined by $TCID_{50}$. Our results revealed that knockdown of GGCX in duck cells significantly decreased the replication of avian influenza virus. These findings suggest that avian GGCX may have a similar function to swine GGCX, highlighting its conserved role in influenza virus replication across different species (Revised figure S3i and lines 132-134).

Comment 6: In this manuscript, HuB/H1N1 is mainly used in vitro experiments, why is HN/H1N1 used in vivo studies (line 723, figure 2)? Could the authors elucidate the rationale behind the selection of HN/H1N1 for in vivo experiments and delineate the specific differences between HuB/H1N1 and HN/H1N1 strains?

Response: Thanks for your comment. In our *in vivo* experiments, we initially utilized the HuB/H1N1 strain to challenge mice. However, we found that even with high pfu, the HuB/H1N1 challenge did not cause lethality in mice. Therefore, we opted for an alternative human-isolated EA H1N1 SIV, namely HuN/H1N1, to conduct our *in vivo* experiments. The rationale behind this selection was twofold. Firstly, the homology of the viral HA protein between HuB/H1N1 and HuN/H1N1 is remarkably high, at 99%. Secondly, HuN/H1N1 has been shown to cause lethality in mice, making it a more suitable choice for our *in vivo* challenge experiments. In addition to this, we have also

investigated the effects of GGCX knockout on HuN/H1N1 replication *in vitro* using PK-15, NPTr, and A549 cells. Our results revealed that knockout of GGCX significantly decreased the replication of HuN/H1N1 *in vitro* (Revised figure 1c, figure S3d, and figure S3h).

Comment 7: The authors quantified the progeny Gla and Glu viruses by absolute quantitative real-time PCR. Does the abrogation of GGCX-mediated HA carboxylation reduce the quantity of HA incorporated into virions?

Response: We appreciate your constructive comment. To address your concern, we collected the progeny viruses from the supernatants at 9 hours post-infection (hpi) and isolated their RNAs. Subsequently, we employed absolute quantitative real-time PCR to determine the quantities of progeny virions. As shown in the new figure S4e, we determined the CT values for each progeny virus. Our results indicated that the CT values between each progeny Gla and Glu viruses were nearly identical, suggesting that there were no significant differences in the quantity of progeny Gla and Glu virions produced. Therefore, our findings imply that the abrogation of GGCX-mediated HA carboxylation had no impact on the quantity of HA incorporated into virions (Revised figure S4e and lines 209-215).

Comment 8: In figure 1c-d, the absence of GGCX significantly decreased the replication of different subtype influenza viruses. Consequently, the quantities of NP and HA present in the cell lysates of GGCX-KO cells are anticipated to be less than those in WT cells under the condition where the expression level of GAPDH is equivalent. However, in figure 3b and 3f, it seems that the quantities of NP and HA present in the cell lysates of GGCX-KO cells and WT cells are similar, why?

Response: Thank you for your comment. As expected, accompanied by the decreased virus titers, we would also anticipate reduced expressions of viral NP and HA proteins. However, in revised figure 3b and 3f, we used viruses to infect wild-type (WT) and GGCX-knockout (KO) PK-15 cells, and subsequently collected cell lysates for co-immunoprecipitation (co-IP) assays at 9 hours post-infection (hpi). Notably, at 9 hpi, we did not observe any obvious differences in viral protein expression between virus-infected WT and GGCX-KO PK-15 cells (Revised figure S4b). This suggests that the

quantities of NP and HA present in the cell lysates of GGCX-KO cells and WT cells are similar. We have added more details to the revised figure legends (Revised in lines 846, 850, 854, 857, and 861).

Minor Comments

Comment 9: In figure 1a, the labeled "HuB" should be "HuB/H1N1".

Response: Thank you for your suggestion. We have incorporated the additional information into the revised figure 1a and figure S1, as requested.

Comment 10: In figure 6c, the numbers depicted on the Y-axis of the graph are not properly aligned.

Response: We apologize for the error. We have corrected the Y-axis labeling in the revised figure 6c to ensure accuracy and clarity.

Comments from Reviewer 2

Reviewer #2 (Remarks to the Author):

Comment 11: The study by Zou et al. tries to identify host receptors required for replication of the Eurasian avian-like swine H1N1 lineage by utilizing a CRISPR-KO screening approach in porcine cells. Among previously identified proteins (validating their screening approach) they found GGCX to be an essential host factor for these viruses. The authors convincingly show, that GGCX is required for replication of these viruses in porcine cells as well as in infected mice. Furthermore, they show protein-protein interaction between GGCX and the viral HA protein, which leads to enhanced carboxylation of HA. By preparing viral stocks on both WT and GGCX-KO cells they can further show, that this carboxylation seems to be required for α-2,6-SA-, but not α-2,3-SA-binding. They were able to attribute this particular phenotype to carboxylation of the HA 225E mutation. Finally, they show that GGCX facilitates the acquisition of HA 225E in passaging experiments, thereby shedding light on the α-2,3-SA to α-2,6-SA receptor switch in the evolution of this porcine IAV lineage.

The presented study is interesting and the experiments are well-thought-out. The necessity of GGCX for IAV infection is convincingly shown. The authors mainly attribute this effect to the reduced carboxylation of HA proteins on the progeny

viruses. If this was the case, however, it remains unclear why the GGCX-KO cells would show up in the CRISPR-screen since they should undergo full rounds of infections (unlike e.g. SLC35A1-KO cells). The authors briefly address the potential of host-protein carboxylation in the discussion (lines 332-336), but this should be experimentally addressed. We therefore raise the following concerns that should be addressed before publication:

Response: We are grateful for your positive comments and constructive suggestions regarding our manuscript. We have carefully addressed each of your concerns and provided detailed responses below and in the revised manuscript.

Regarding your concern about the show up of GGCX-KO cells in the CRISPR screen, we acknowledge that our initial description may not have been sufficiently detailed or accurate. To clarify, we conducted CRISPR screening by infecting genome-scale PK-15 mutant cell libraries with HuB/H1N1. Surviving cells were collected and expanded for subsequent rounds of infection. Notably, in the subsequent rounds of infections, we repeatedly infected the surviving cells from the last round with wild-type (WT) HuB/H1N1, rather than the progeny virus from the previous round. After four rounds of screening, the SIV-resistance cells were isolated and analyzed, which ultimately led to the identification of the SIV-resistance GGCX-KO cells. We have added more information in the revised Materials and Methods section to provide further clarity (lines 95 and 454-459).

Regarding the potential of host-protein carboxylation, it is well established that GGCX catalyzes the post-translational carboxylation of several vitamin K-dependent (VKD) proteins, including coagulation factors, osteocalcin, and matrix Gla proteins. These VKD proteins have multiple functions in the host, highlighting the significance of GGCX-mediated carboxylation. In our study, we used BGLAP, a type of VKD protein, as a positive control (Figure 3b and 3f) and experimentally confirmed its carboxylation modification by GGCX. Furthermore, as shown in the figure below (Figure 1, provided for review only), we investigated the effect of GGCX overexpression on host innate immune responses and found that it can inhibit these responses, which provided an additional explanation for the promotion of IAV replication. However, the main role of GGCX in EA H1N1 SIV infection is catalyzing carboxylation modification of HA and promoting viral adaption to interspecies receptor binding.

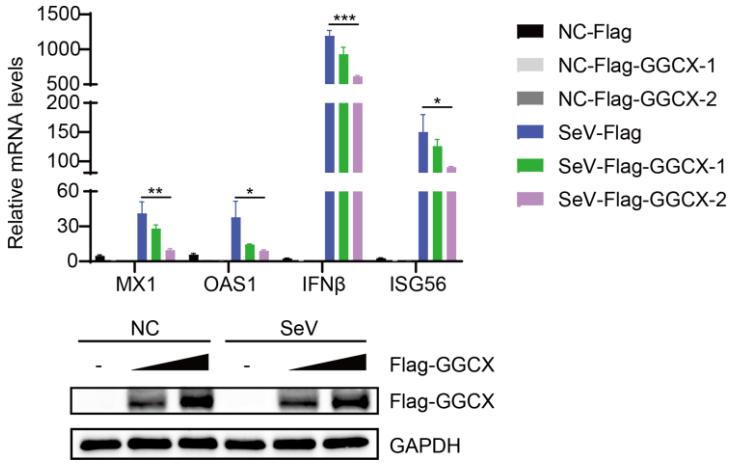


Figure 1. Effect of GGCX overexpression on host innate immunity response. PK-15 cells were transfected with increasing dose of exogenous Flag-GGCX for 24 h, followed by stimulated with SeV or not for 6 h. The mRNA levels of IFN-β and ISGs were detected using qRT-PCR. (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Major concerns:

Comment 12: To show that the GGCX-KO cells are not inherently worse susceptible to viral infection, synchronized single-cycle infections of HuB/H1N1 (produced on WT- and GGCX-KO-cells as used in Figure 4e) should be performed on WT-and GGCX-KO cells.

Intracellular viral RNA and viral protein levels should be determined before newly synthesized virions are released to exclude (or show) additional effects of the GGCX-KO on viral replication.

Response: Thank you for your constructive suggestions. We have conducted additional experiments to address your concerns. Specifically, we infected wild-type (WT) and GGCX-knockout (KO) PK-15 cells with equivalent progeny Gla and Glu viruses and visualized viral HA binding activities. Our results showed that the Glu viruses of HuB/H1N1 and PR8/H1N1 exhibited lower binding activity compared to the Gla viruses in both WT and GGCX-KO PK-15 cells. In contrast, the progeny Gla and Glu viruses of SH13/H9N2 displayed identical binding activities in both WT and GGCX-KO PK-15 cells. These findings indicate that GGCX-KO cells are not inherently more susceptible to viral infection (Revised Figure 4b-d and lines 209-220).

To further exclude any additional effects of GGCX-KO on viral replication, we treated cells with Oseltamivir (OSTV) to prevent the release of newly synthesized virions. We then determined intracellular viral RNA and protein levels of viral NP in both virus-infected GGCX-KO and WT PK-15 cells during a single lifecycle of EA H1N1 SIV infection. Our results revealed no obvious differences in viral NP expression between these cell types when treated with OSTV (Revised Figure S4b-d and lines 204-209). These findings suggest that GGCX plays a specific role in catalyzing viral HA carboxylation, which affects the next round of EA H1N1 SIV infection.

Comment 13: Figure 1F,G: it is unclear what timepoint is shown and what MOI was used for the infection. If this is a later timepoint (36+hpi) the virus titers are highly reduced to what is shown in the growth kinetics with WT cells. It would therefore be nice to see GGCX protein levels in wt cells and FLAG-GGCX reconstituted GGCX-KO cells in the same Western blot using GGCX antibody to compare protein levels.

Response: Thank you for your comment and suggestion. In our experiments, we infected transfected cells with HuB/H1N1 at an MOI of 0.1, consistent with the conditions used in the growth kinetics assay with wild-type (WT) cells, and determined virus titers at 24 hours post-infection (hpi). We have added more details to the figure legend to clarify this point (Revised lines 817-818 and 822-823).

We noted that the virus titers were highly reduced compared to those observed in the growth kinetics assay with WT cells. This may be attributed to the fact that transfecting exogenous Flag-GGCX into WT and GGCX-knockout (KO) PK-15 cells affected the susceptibility of transfected cells to viral infection, resulting in lower virus titers.

Following your suggestions, we also determined GGCX protein expression in WT and exogenous Flag-GGCX-transfected cells, as well as Flag-GGCX-reconstituted GGCX-KO cells, using the same western blot. Our results showed that increased GGCX protein expression correlated with increased viral titers (Revised Figure 1f).

Minor concerns:

Comment 14: Line 91: the CRISPR KO screen was performed in pig cells, not in pigs

Response: Thank you for your comment. We have made the necessary correction and updated the text accordingly, as shown in line 91.

Comment 15: Lines 145-146: the phenotype in GGCX-KD mice might suggest, that this is not only a porcine host factor

Response: Thank you for your comment. We have revised the statement to accurately reflect our findings, and it now reads: "GGCX was identified as a host-dependent factor for EA H1N1 SIV infection" (Revised in lines 155-156).

Reviewer #3 (Remarks to the Author):

Comment 16: I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Response: We would like to express our sincere gratitude for your time and effort in reviewing our manuscript, as well as for your valuable and constructive comments.

Reviewer #1 (Remarks to the Author):

The authors have added new data and clarified aspects of the text. By doing so, the revised version of the article perfectly answers the questions I asked.

Response: We would like to express our sincere gratitude for your time and effort in reviewing our manuscript, as well as for your valuable and constructive comments.

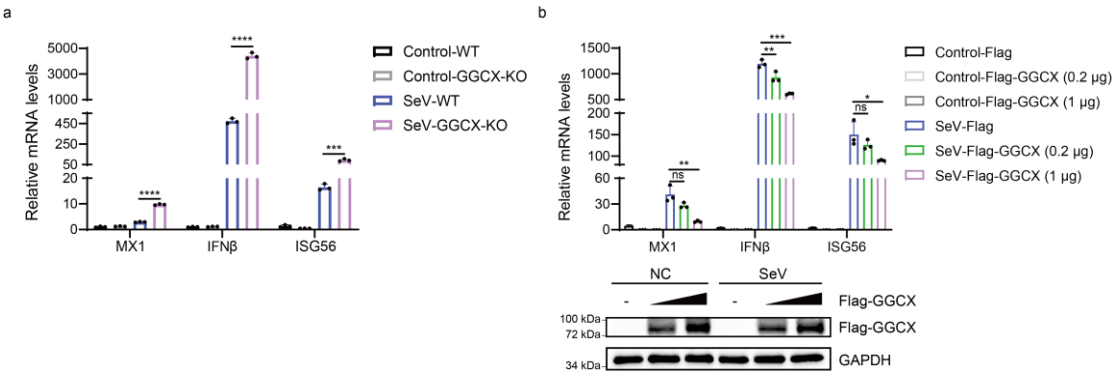
Reviewer #2 (Remarks to the Author):

We appreciate the effort of the authors and think that the quality of the manuscript has greatly improved with the additional experiments and changes in the text. However, the fact that GGCX shows up in the KO-screen is still puzzling if GGCX mainly affected the next round of infection (as mentioned in ll 208-209), particularly because WT virus was used for every round of infection in the initial screen.

The fact that supplementation of GGCX leads to a decrease in the interferon response might indicate that GGCX-KO cells should have higher levels of interferon response. Unfortunately this was not shown in Figure 1 of the rebuttal letter. If this was indeed the case, this would provide a more convincing reason for the GGCX-KO cells to show up in the screen. The figure should therefore be included in the manuscript. It does, however, need to be carefully discussed with the new data of Supp Figure 4, since the early stages of infection don't seem to be affected at all (at least when looking at NP).

Response: Thank you for your constructive comments. According to your suggestions, the effect of GGCX knockout on interferon responses was also determined. As expected, GGCX-KO cells exhibited higher levels of interferon response, providing an additional explanation for the shown up of GGCX in the screen and the inhibition of IAV replication. The results have been included in Supplementary Figure 7. However, knockout of GGCX exhibited limited effects on immune responses (Supplementary Fig. 7), as well as illustrated by the results that there was no discernible effect on the initial round of viral infection (Supplementary Fig. 4). Our

data suggest that GGCX plays a dominant role as an underlying driver of the HA D225E substitution in EA H1N1 SIV, catalyzing the carboxylation modification of viral HA 225E and ultimately determining the receptor binding preference of the virus. We have discussed in lines 376-382.



Supplementary Fig. 7 The role of GGCX in host innate immunity response. (a) GGCX-KO and WT PK-15 cells were stimulated with SeV or not for 6 h. The mRNA levels of IFN-β and ISGs were detected using qRT-PCR. (b) PK-15 cells were transfected with increasing dose of exogenous Flag-GGCX for 24 h, followed by stimulated with SeV or not for 6 h. The mRNA levels of IFN-β and ISGs were detected using qRT-PCR. Error bar in panels a and b indicates the standard deviation. The data shown in a and b are means ± SD. Statistical analysis was performed using unpaired, two-tailed Student's *t*-test (n = 3 biologically independent experiments). (ns, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$). Exact *P* values are available in Source Data. Source data are provided as a Source Data file.

Reviewer #3 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who

co-review manuscripts.

Response: We would like to express our sincere gratitude for your time and effort in reviewing our manuscript, as well as for your valuable and constructive comments.