nature portfolio

Peer Review File

SAMD9 senses cytosolic double-stranded nucleic acids in epithelial and mesenchymal cells to induce antiviral immunity

Corresponding Author: Dr Siyuan Ding

This manuscript has been previously reviewed at another journal. This document only contains information relating to versions considered at Nature Communications.

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)
The authors have now submitted a thoroughly revised version of their manuscript, addressing most of the critical points. However, I have two remaining concerns that need to be resolved before the manuscript can be published with claims fully supported by the data:

Since the authors suggest that SAMD9 is a PRR that signals via TBK1, the authors should conduct co-IP studies in which they show the association of SAMD9 and TBK1 upon stimulation.

The authors indicate that tRNA cleavage is not required for SAMD9's IFN-inducing activity. This is currently supported by the K350A mutant (revision Figure 4). This is a very important piece of information that needs to be integrated into the manuscript (I would even suggest putting this into the primary figure set).

Reviewer #2

(Remarks to the Author)
Hou et al have substantially addressed the original reviewers comments by: (1) Being more specific on their claims as to SAMD9 sensing nucleic acid (revised title) (2) Providing further experiments delineating the relationship between SAMD9 and known NA sensors (3) Providing further insights on how SAMD9 signals to IFN (4) More specific data on NA binding (5) Interpretation of in vivo data improved (6) Suitably circumspect in the Discussion as to how exactly SAMD9 signals.

Reviewer #3

(Remarks to the Author)
I have reviewed their previous manuscript entitled 'SAMD9 is a sensor for cytosolic double-stranded DNA and double-stranded RNA' before. I found many improvements in the current manuscript compared to the previous one; however, there are still unclear points that the authors should address or further investigate. Specific comments are listed below.

Specific comments

1 The authors have changed the title, but the abstract content does match the title and either the title or the abstract should be changed. In addition, when the authors keep the title, figures especially Figure 2 including a lot of biochemical assays for dsDNA should be rearranged.

2 They clearly show in Figure S4 that SAMD9 overexpression induces IFNL3 and CCL5 in a STING-independent manner.

Furthermore, based on the result of Figure 1 E and F, SAMD9 activates the MAVS-TBK1 dependent signaling. In Figure 1I, the authors suggest that SAMD9 functions independently of RIG-I and MDA5. In this case, how can SAMD9 activate MAVS in the absence of CARD? Can SAMD9 overexpression induce IFNL3 and CCL5 in RIG-I/MDA5 DKO cells?

3 Positive-stranded ssRNA viruses such as ZIKA and CHIKV (examined in Figure 3F) are generally known to accumulate dsRNA during their replication (Barnard et al, J Gen Virol 2023). Can the authors stain cells infected with these viruses by dsRNA antibodies (J2 etc)? Further careful infectious dose titration will be required to determine the susceptibility to these positive-stranded ssRNA viruses that will generate dsRNA.

Response to the reviewers' comments on the *Nature Communications* manuscript (ID: NCOMMS-24-77815-T, Hou G, *et al.*, SAMD9 senses cytosolic double-stranded nucleic acids in epithelial and mesenchymal cells to induce antiviral immunity).

Below please find our point-by-point response.

Reviewer #1 (Remarks to the Author):

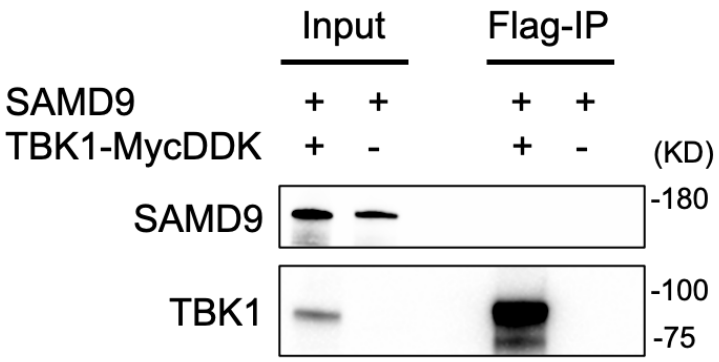
Q: The authors have now submitted a thoroughly revised version of their manuscript, addressing most of the critical points.

A: We are pleased to learn that reviewer 1 found that our manuscript "a thoroughly revised version" and "addressing most of the critical points". To strengthen our conclusions, we have now provided new experimental evidence, which is elaborated below. We hope these additional experiments addressed the concerns.

However, I have two remaining concerns that need to be resolved before the manuscript can be published with claims fully supported by the data:

Q1: Since the authors suggest that SAMD9 is a PRR that signals via TBK1, the authors should conduct co-IP studies in which they show the association of SAMD9 and TBK1 upon stimulation.

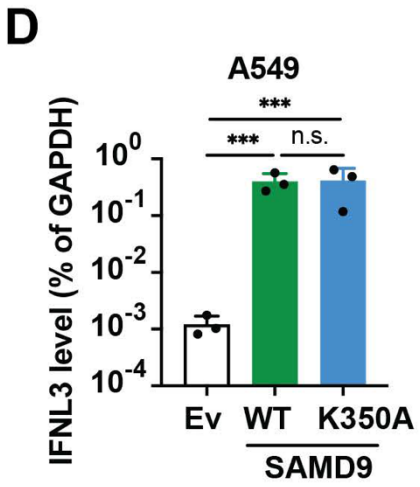
A1: We agree with the reviewer that it is important to test the interaction between SAMD9 and TBK1. We performed a co-IP assay in HEK293 cells, in which we co-transfected plasmids encoding SAMD9 with TBK1. While we failed to detect any interaction between SAMD9 and TBK1 (Revision Figure 1), the exact molecular mechanisms by which SAMD9 mediates IFN signaling via MAVS and TBK1 will be addressed in future projects.



Revision Figure 1. Immunoblotting analysis of anti-Flag antibody immunoprecipitates of cell lysates from HEK293 cells co-transfected with untagged SAMD9 and Flag-tagged TBK1.

Q2: The authors indicate that tRNA cleavage is not required for SAMD9's IFN-inducing activity. This is currently supported by the K350A mutant (revision Figure 4). This is a very important piece of information that needs to be integrated into the manuscript (I would even suggest putting this into the primary figure set).

A2: We thank the reviewer for your thoughtful suggestion on our K350A mutant overexpression data. We now moved it into Supplementary Figure 2D.



New Supplementary Figure 2D. A549 cells transfected with Ev, SAMD9-Myc-DDK (WT), or SAMD9-K350A-Myc-DDK (K350A) plasmid for 24 hr and the mRNA levels of IFNL3 measured by qRT-PCR.

Reviewer #2 (Remarks to the Author):

Q: Hou et al have substantially addressed the original reviewers comments by: (1) Being more specific on their claims as to SAMD9 sensing nucleic acid (revised title) (2) Providing further experiments delineating the relationship between SAMD9 and known NA sensors (3) Providing further insights on how SAMD9 signals to IFN (4) More specific data on NA binding (5) Interpretation of in vivo data improved (6) Suitably circumspect in the Discussion as to how exactly SAMD9 signals.

A: We appreciate the reviewer 2 found that we "have substantially addressed the original reviewer's comments".

Reviewer #3 (Remarks to the Author):

Q: I have reviewed their previous manuscript entitled 'SAMD9 is a sensor for cytosolic double-stranded DNA and double-stranded RNA' before. I found many improvements in

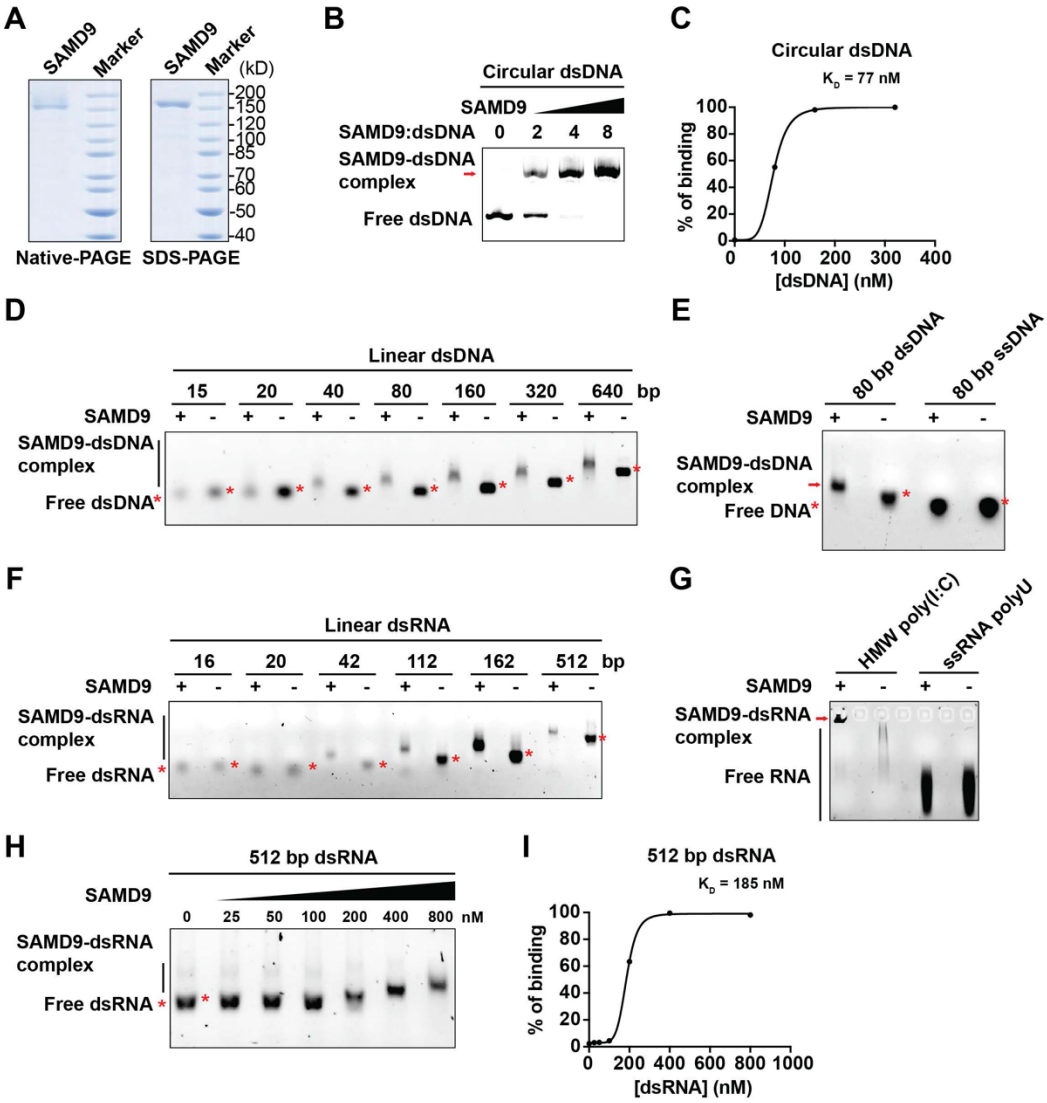
the current manuscript compared to the previous one; however, there are still unclear points that the authors should address or further investigate. Specific comments are listed below.

A: We are pleased to learn that the reviewer 3 acknowledged our revised manuscript “many improvements in the current manuscript compared to the previous one”. To address the potential concerns and weaknesses, we have now provided additional experimental data, which is elaborated below.

Specific comments

Q1: The authors have changed the title, but the abstract content does match the title and either the title or the abstract should be changed. In addition, when the authors keep the title, figures especially Figure 2 including a lot of biochemical assays for dsDNA should be rearranged.

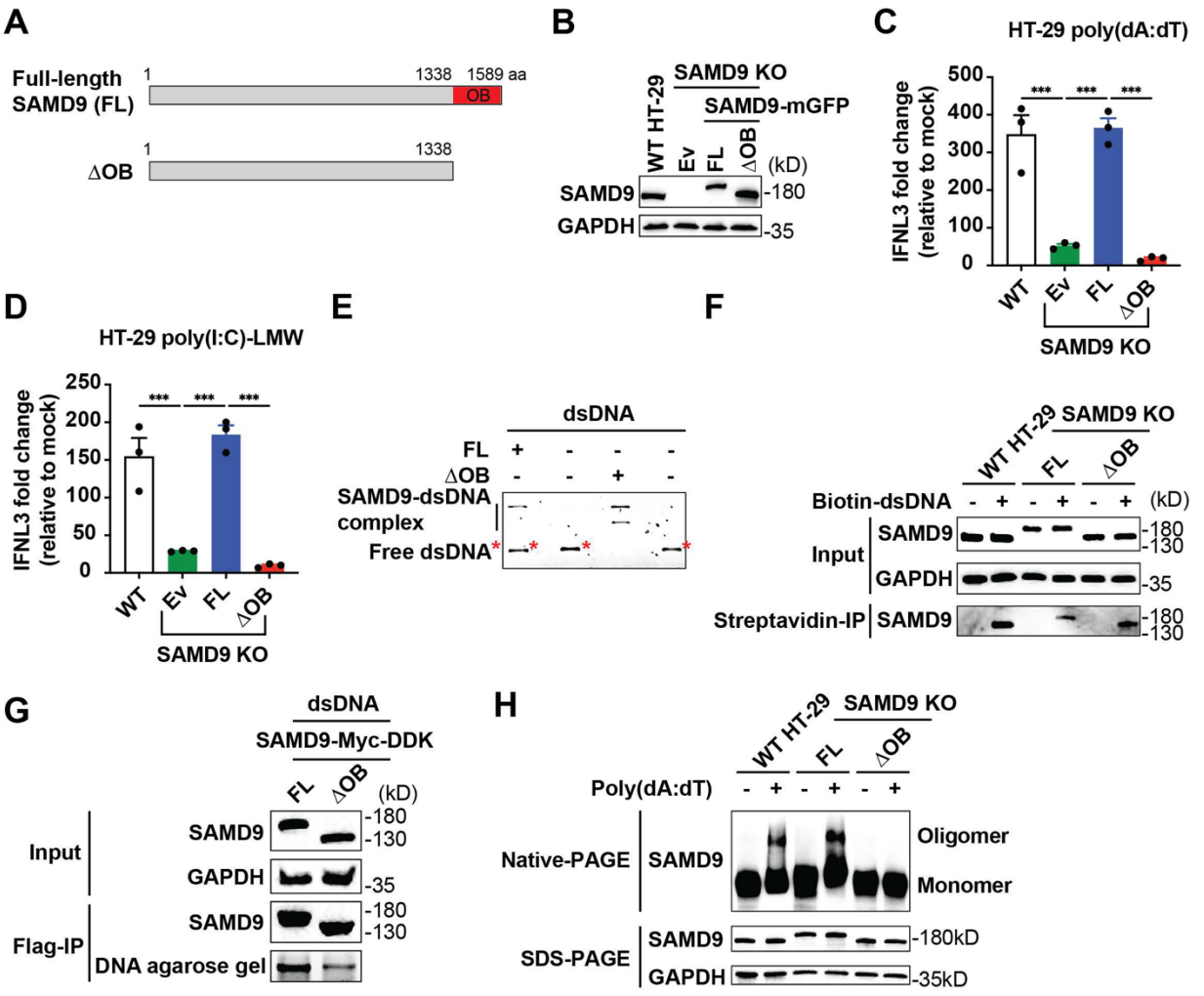
A1: We thank the reviewer for the constructive comments. We have modified both the title and the abstract. We also split the original Figure 2 into new Figure 3 and Figure 4 to separate the biochemistry from the functional data.



New Figure 3. SAMD9 binds to dsDNA and dsRNA

(A) Coomassie stain of His-SAMD9 protein analyzed by native-PAGE (left) or SDS-PAGE (right) electrophoresis.
(B) Electrophoretic mobility shift assay (EMSA) of circular plasmid dsDNA with an increasing amount of SAMD9.
(C) Measurement of the dissociation constant (K_D) of circular dsDNA with His-SAMD9, calculated based on a half-maximum binding on a curve fit.
(D) EMSA of SAMD9 with linear dsDNA fragments of indicated lengths.
(E) EMSA of His-SAMD9 with 80-bp dsDNA or 80-bp ssDNA.
(F) EMSA of SAMD9 with linear dsRNA fragments of indicated lengths.

(G) EMSA of His-SAMD9 with HMW poly(I:C) or single-stranded RNA polyuridine (ssRNA polyU).
(H) EMSA of His-SAMD9, provided in 2-fold serial dilutions, with 512-bp dsRNA.
(I) K_D measurements of 512 bp dsRNA with His-SAMD9, calculated based on a half-maximum binding on a curve fit.



New Figure 4. SAMD9 OB-fold domain is essential for IFN induction

(A) Schematic representation of SAMD9. FL: full-length; OB: oligonucleotide binding-fold domain.
(B) Validation of WT HT-29 and *SAMD9* KO HT-29 cells complemented with Ev, SAMD9-mGFP (FL), SAMD9-ΔOB-mGFP (ΔOB) by western blot with indicated antibodies.
(C) WT HT-29 and *SAMD9* KO HT-29 cells complemented with Ev, FL, or ΔOB transfected with poly(dA:dT) (1 μg/ml) for 24 hr and the IFNL3 mRNA level measured by qRT-PCR.

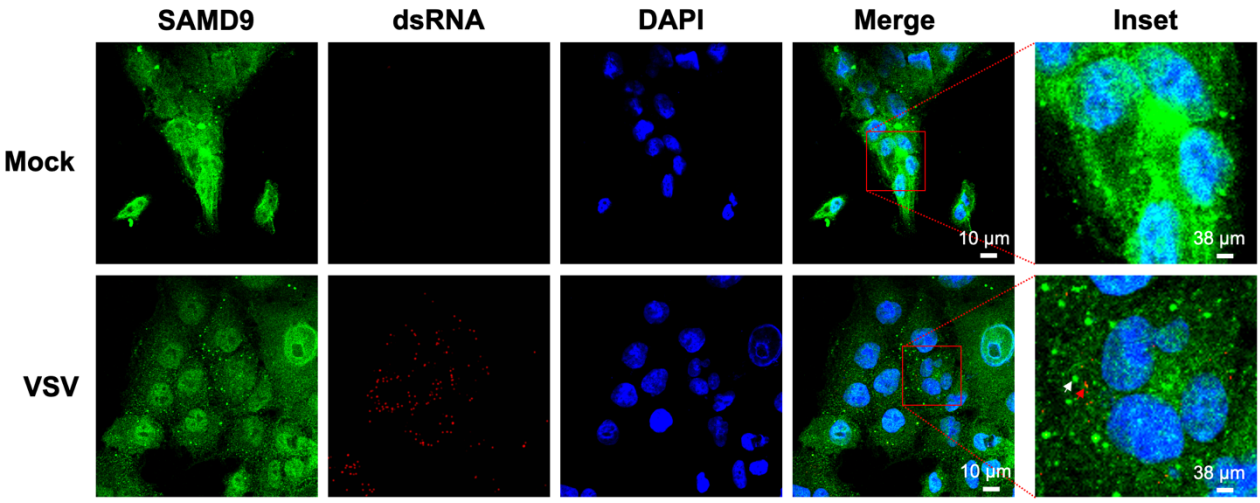
(D) WT HT-29 and *SAMD9* KO HT-29 cells complemented with Ev, FL, or ΔOB transfected with LMW poly(I:C) (1 μg/ml) for 24 hr and the IFNL3 mRNA level measured by qRT-PCR.
(E) EMSA of His-MBP-tagged SAMD9 FL or ΔOB proteins with circular dsDNA.
(F) Immunoblotting analysis of streptavidin immunoprecipitates of cell lysates from WT HT-29 and *SAMD9* KO HT-29 cells complemented with FL or ΔOB incubated with biotinylated 48,502-bp dsDNA.
(G) Immunoblotting analysis of anti-Flag antibody immunoprecipitates of cell lysates from HEK293 cells transfected with Myc-DDK-tagged SAMD9 FL or ΔOB.
(H) WT HT-29 and *SAMD9* KO HT-29 cells complemented with FL or ΔOB transfected with poly(dA:dT) (10 μg/ml) for 8 hr, and cell lysates harvested for native-PAGE or SDS-PAGE electrophoresis with indicated antibodies.

Q2: They clearly show in Figure S4 that SAMD9 overexpression induces IFNL3 and CCL5 in a STING-independent manner. Furthermore, based on the result of Figure 1 E and F, SAMD9 activates the MAVS-TBK1 dependent signaling. In Figure 1I, the authors suggest that SAMD9 functions independently of RIG-I and MDA5. In this case, how can SAMD9 activate MAVS in the absence of CARD? Can SAMD9 overexpression induce IFNL3 and CCL5 in RIG-I/MDA5 DKO cells?

A2: We appreciate the reviewer for the valuable comment. We made several attempts to examine whether SAMD9 interacts with MAVS or TBK1. As of now, we have no evidence of direct directions. As part of our future plan, we will dissect the downstream signaling pathway that connects SAMD9 to IFN activation.

Q3: Positive-stranded ssRNA viruses such as ZIKA and CHIKV (examined in Figure 3F) are generally known to accumulate dsRNA during their replication (Barnard et al, J Gen Virol 2023). Can the authors stain cells infected with these viruses by dsRNA antibodies (J2 etc)? Further careful infectious dose titration will be required to determine the susceptibility to these positive-stranded ssRNA viruses that will generate dsRNA.

A3: We appreciate the reviewer for suggesting this important experiment. We tested the dsRNA production by vesicular stomatitis virus (VSV), a negative-stranded ssRNA virus. By J2 monoclonal antibody staining, we detected dsRNA produced by VSV infection in A549 cells, but these signals did not appear to co-localize with SAMD9 (Revision Figure 2). It is possible that these viral replication machineries, which mostly take place in the perinuclear ER and ER-related membranes, are not immediately accessible to SAMD9. We acknowledge the reviewer's point, and we will further explore variations in viral dose and time points to determine whether additional phonotypes can be documented in the future studies.



Revision Figure 2. A549 cells infected with mock or VSV (MOI=5) for 5 hr, then fixed and stained for SAMD9 (green), dsRNA (red), nuclei (blue) by confocal microscopy. White arrow points to SAMD9 staining, red arrow points to dsRNA staining.