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# Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Research policies, see our Editorial Policies and the Editorial Policy Checklist.

## Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

| n/a | Confirmed | |
|---|---|---|
| ☐ | ☒ | The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement |
| ☒ | ☐ | A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly |
| ☐ | ☒ | The statistical test(s) used AND whether they are one- or two-sided<br>*Only common tests should be described solely by name; describe more complex techniques in the Methods section.* |
| ☒ | ☐ | A description of all covariates tested |
| ☒ | ☐ | A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons |
| ☐ | ☒ | A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals) |
| ☐ | ☒ | For null hypothesis testing, the test statistic (e.g. *F*, *t*, *r*) with confidence intervals, effect sizes, degrees of freedom and *P* value noted<br>*Give P values as exact values whenever suitable.* |
| ☒ | ☐ | For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings |
| ☒ | ☐ | For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes |
| ☒ | ☐ | Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated |

*Our web collection on statistics for biologists contains articles on many of the points above.*

## Software and code

Policy information about availability of computer code

| | |
|---|---|
| Data collection | No software for data collection was used. |
| Data analysis | MATLAB scripts for designing and analyzing mutate-and-map, mutate-and-rescue, and SARS-CoV-2 SHAPE datasets is available on https://github.com/DasLab/NA_thermo<br>MATHLAB R2021a, Prism9, RNAstructure v5.2, Instructions for download and installation of FAST algorithm with RNA structure are available at https://med.stanford.edu/glennlab/download.html<br>The HiTRACE software package version 2.0 was used to analyze CE data (both MATLAB toolbox and web server available) and can be found at https://github.com/ribokit/HiTRACE |

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

## Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

Chemical mapping datasets for mutate-and-map and mutation/rescue experiments are available at http://rmdb.stanford.edu)80, accession codes:

PSL2IAV_1M7_0001, PSL2IAV_RSQ_0001.
All the data that support the findings of this study are available from the corresponding author upon reasonable request.

# Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

# Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| | |
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| Sample size | No sample size was statistically predetermined. Given the reproducibility of cell culture experiments, it was predetermined that a minimum of at least n=2 would provide sufficient information to reach meaningful data conclusions. Where possible, in vitro sample size was n=3 or more. For in vivo experiments the number of animals per group in IAV experiments with BALB/c mice was determined using G*power statistical software with the following parameters: Effect size- 1.8; Power- 90%, Type 1 error- and determined to be 7. For SARS-COV-2 animal experiment power was reduced to 80% and was acceptable considering the short supply of hamsters, group size was determined to be 5. |
| Data exclusions | No data was excluded in this manuscript. |
| Replication | Experiments herein underwent multiple replication studies, depending on the nature of the experiment (in vitro vs in vivo), and all replication attempts were successful.<br>Influenza animal studies were replicated at least twice, SARS-CoV-2 animal studies were performed once.<br>In instances where higher variability resulted, an increased number of replicates was employed to assure findings.<br>All SARS-CoV-2 studies performed in BSL3 (in vitro and in vivo) performed only once with multiple biological replicates due to limited access to these facilities. |
| Randomization | All mouse studies were randomized. We assigned equal number of mice randomly into each group. Weights were determined only after assignment to groups and prior to infection. |
| Blinding | Animal experiments were randomized blindly. The investigator was conducting the experiments, therefor data collection and analysis could not be blinded. |

# Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

## Materials & experimental systems

| n/a | Involved in the study |
|---|---|
| ☐ | ☒ Antibodies |
| ☐ | ☒ Eukaryotic cell lines |
| ☒ | ☐ Palaeontology and archaeology |
| ☐ | ☒ Animals and other organisms |
| ☒ | ☐ Human research participants |
| ☒ | ☐ Clinical data |
| ☒ | ☐ Dual use research of concern |

## Methods

| n/a | Involved in the study |
|---|---|
| ☒ | ☐ ChIP-seq |
| ☐ | ☒ Flow cytometry |
| ☒ | ☐ MRI-based neuroimaging |

# Antibodies

| | |
|---|---|
| Antibodies used | mouse anti-HA, Sinbiological, HB09AU0402-B (1:800); rabbit anti-PB2, Invitrogen PA5-3221 (1:1000); H-2Kd Influenza HA Tetramer-IYSTVASSL-PE, MBL Internationa, TB M520-1 (5 micro liter per 1x 10e6 cells ); APC/Cy7 anti-mouse CD8a, Biolegend, 100714 (2 micro-liter per 1x 10e6 cells ); PE/Cy7 anti-mouse CD3, Biolegend, 100220 (2 micro-liter per 1x 10e6 cells ); BD Horizon™ PE-CF594 Rat Anti-Mouse CD45, BD biosciences, 562420 (2 micro-liter per 1x 10e6 cells );Zombie Aqua™ Fixable Viability Kit, Biolegend, 423102 (1:500); Anti-Rabbit HRP (ThermoFisher, 65-6120); Goat-anti-Rabbit Alexa-Flour 488 (ThermoFisher, A-11008 ); mouse Anti-Actin (Sigma A2228) |
| Validation | Antibodies were purchased from Sinbiological, Invitrogen, MBL international, Biolegend, BD biosciences, ThermoFIsher and Sigma. Validation information is available from the manufacturers who provide references on their websites for the catalogue number listed listed above. See https://www.biolegend.com/ for Biolegend, https://www.thermofisher.com for Invitrogen and ThermoFisher, https://www.sigmaaldrich.com/ for Sigma, https://www.bdbiosciences.com/ for BD Biosciences, and https:// |

www.sinobiological.com/ for sinbiological.

## Eukaryotic cell lines

Policy information about cell lines

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| Cell line source(s) | MDCK- NBL2 (ATCC, CCL-34), HEK 293T (ATCC CRL-1573), VeroE6 (ATCC CRL-1586) Huh7.5_TMPRSS-ACE2 and A549-ACE2 (A gift from Dr. Timothy Sheahan); A549(ATCC, CCL-185); ATCC, A549-Dual (InvivoGen, a549d-nfis); THP1-Dual (InvivoGen, thpd-nfis) |
| Authentication | Cell lines were purchased from vendors and were not authenticated further.<br>Huh7.5_TEMPRSS-ACE2 and A549-ACE2 were generated by Dr. Sheahan and were not authenticated further. |
| Mycoplasma contamination | All cell lines were tested negatively for mycoplasma contamination. |
| Commonly misidentified lines<br>(See ICLAC register) | no commonly misidentified cell lines were used in this study |

## Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

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| Laboratory animals | BALB/c female mice, 6-8 weeks old (Jackson Laboratories)<br>Golden Syrian Hamsters strain LVG,10 week old females (Charles River)<br>Specific-pathogen-free chicken embryos (Charles River) |
| Wild animals | No wild animals were used in the study. |
| Field-collected samples | No field collected samples were used in the study. |
| Ethics oversight | Stanford University Administrative Panel on Laboratory Animal Care, Utah State University IACUC |

Note that full information on the approval of the study protocol must also be provided in the manuscript.

## Flow Cytometry

### Plots

Confirm that:

☒ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).

☒ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).

☐ All plots are contour plots with outliers or pseudocolor plots.

☒ A numerical value for number of cells or percentage (with statistics) is provided.

### Methodology

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| Sample preparation | Whole blood was collected from mice into heparinized tubes. The whole blood was overlaid on top Ficoll-Paque media and centrifuged at 400xg for 40 minutes at room temperature. The top layer containing plasma and platelets was removed and the peripheral blood mono-nuclear cells (PBMCs) at the interphase of the Ficoll layer were collected. The mononuclear cells were diluted in PBS and pelleted by centrifugation at 500 x g for 15 minutes following an additional wash with PBS. The pellet was suspended in PBS. Solenocytes were isolated by manual grinding of the spleen over a 40 m cell strainer. The cells were transferred several times through the strainer and processed further as described above. |
| Instrument | BD bioscience LSR II , see Online Methods |
| Software | Data was collected using the BD FACSDiva software (BD biosciences) and analyzed using FlowJo v10 |
| Cell population abundance | Percent of cell population is presented in Supplemental figure 18 |
| Gating strategy | FSC-H/FSC-A gate used to collect cells and a gate for live cells was then generated. The live cells were gated for CD3+/CD45+ cells and the positive cells were gated to determine the CD8+/HA-Tetramer+ cells. The gating strategy is exemplified in source data for Supplemental Figure 18 |

☒ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.