

# Enteric viruses replicate in salivary glands and infect through saliva

<https://doi.org/10.1038/s41586-022-04895-8>

Received: 22 September 2020

Accepted: 23 May 2022

Published online: 29 June 2022

 Check for updates

S. Ghosh<sup>1</sup>, M. Kumar<sup>1</sup>, M. Santiana<sup>1</sup>, A. Mishra<sup>1</sup>, M. Zhang<sup>1</sup>, H. Labayo<sup>1</sup>, A. M. Chibly<sup>2</sup>, H. Nakamura<sup>3</sup>, T. Tanaka<sup>3</sup>, W. Henderson<sup>4</sup>, E. Lewis<sup>5,6</sup>, O. Voss<sup>7</sup>, Y. Su<sup>8,9</sup>, Y. Belkaid<sup>5,6</sup>, J. A. Chiorini<sup>3</sup>, M. P. Hoffman<sup>2</sup> & N. Altan-Bonnet<sup>1</sup>✉

Enteric viruses like norovirus, rotavirus and astrovirus have long been accepted as spreading in the population through fecal–oral transmission: viruses are shed into feces from one host and enter the oral cavity of another, bypassing salivary glands (SGs) and reaching the intestines to replicate, be shed in feces and repeat the transmission cycle<sup>1</sup>. Yet there are viruses (for example, rabies) that infect the SGs<sup>2,3</sup>, making the oral cavity one site of replication and saliva one conduit of transmission. Here we report that enteric viruses productively and persistently infect SGs, reaching titres comparable to those in the intestines. We demonstrate that enteric viruses get released into the saliva, identifying a second route of viral transmission. This is particularly significant for infected infants, whose saliva directly transmits enteric viruses to their mothers' mammary glands through backflow during suckling. This sidesteps the conventional gut–mammary axis route<sup>4</sup> and leads to a rapid surge in maternal milk secretory IgA antibodies<sup>5,6</sup>. Lastly, we show that SG-derived spheroids<sup>7</sup> and cell lines<sup>8</sup> can replicate and propagate enteric viruses, generating a scalable and manageable system of production. Collectively, our research uncovers a new transmission route for enteric viruses with implications for therapeutics, diagnostics and importantly sanitation measures to prevent spread through saliva.

Saliva forms the first line of defence against many pathogens entering through the oral route and can reflect an individual's clinical state<sup>9,10</sup>. Saliva testing is commonly used to diagnose several viruses that infect SGs, such as Epstein–Barr virus, rabies virus, herpes simplex virus and both severe acute respiratory syndrome coronavirus 1 and 2 (refs. <sup>2,3,9–11</sup>). Norovirus, rotavirus and astrovirus genomic RNA have been frequently detected in saliva in symptomatic and asymptomatic individuals<sup>12–14</sup>. These observations were interpreted as gut contaminants because these viruses are thought to mainly transmit through the fecal–oral route by ingestion of contaminated food and water and productively replicate in the intestines<sup>1,15–18</sup>.

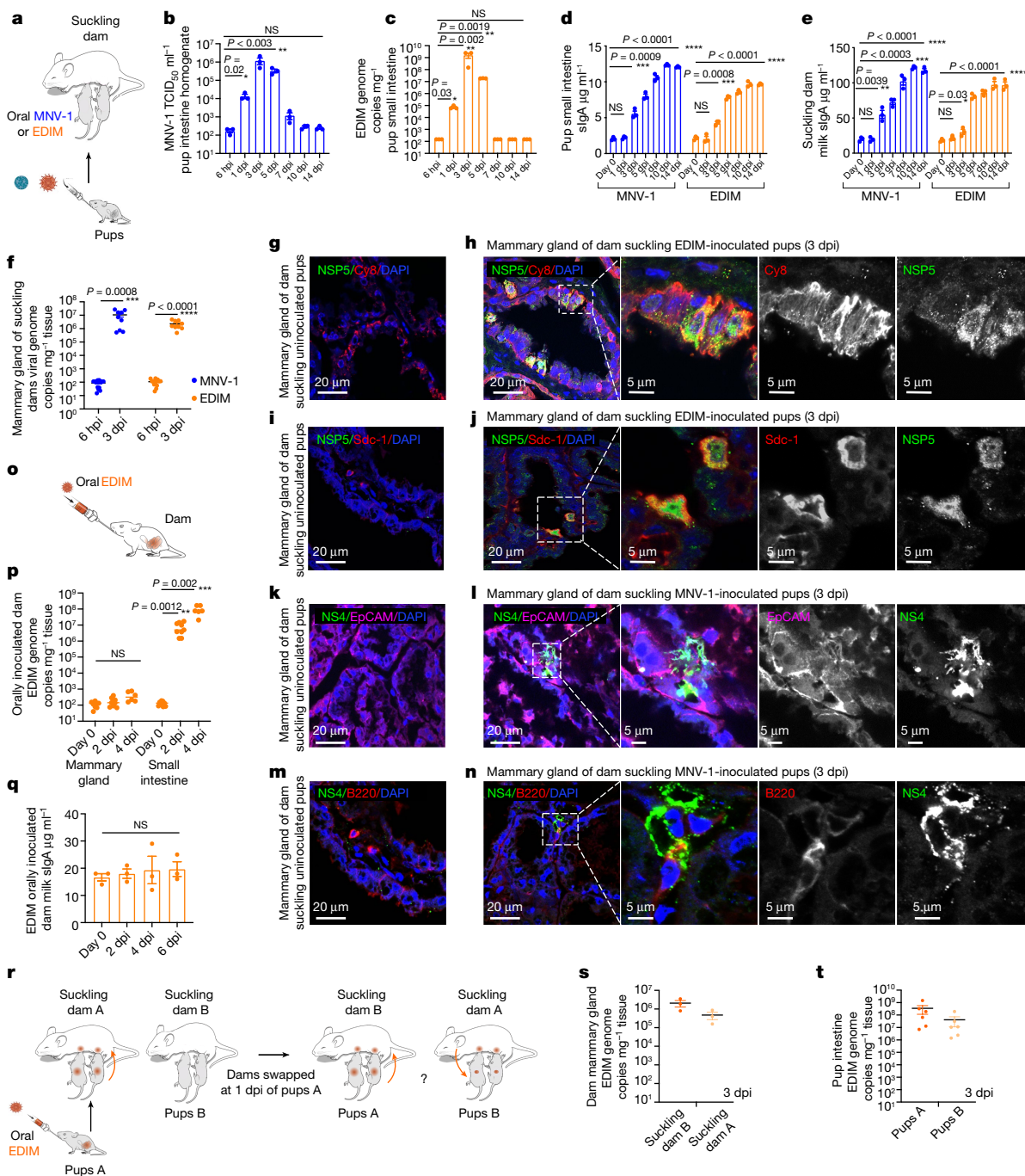
## Suckling transmits enteric viruses to mothers

Neonatal mouse pups (less than 10 days old) are excellent models for studying enteric infections because their immature digestive tracts and immune systems make them susceptible to viruses<sup>19,20</sup>. Indeed, intestinal enteric infections were readily detectable when pups were orally inoculated with murine norovirus 1 (MNV-1) or rotavirus (epizootic diarrhoea of infant mice (EDIM)) (Fig. 1a) and replication measured by either median tissue culture infectious dose (TCID<sub>50</sub>) (MNV-1)

or quantitative PCR (qPCR) (EDIM). Robust intestinal replication of MNV-1 (Fig. 1b) and EDIM (Fig. 1c) was observed in pup intestines with both viruses peaking between 3 and 5 days post-inoculation (dpi) and clearing by 7–10 dpi. Similar findings were also obtained in adult mice (Extended Data Fig. 1a,b).

Like all infant mammals, pups rely on suckling milk to obtain critical immune components such as secretory IgA (sIgA), which is produced by the mammary glands of lactating mothers (called dams in mice)<sup>4–6</sup>. Milk sIgA protects pups, who are not capable of producing their own sIgA until close to weaning, by helping them combat infections in the gut<sup>4–6</sup>. We observed a rapid spike in pup small intestine sIgA levels starting at 3 dpi of the pup with either MNV-1 or EDIM (Fig. 1d), which correlated with a rapid spike in the dam's milk sIgA levels (Fig. 1e). To start with, all dams used in these experiments were EDIM/MNV-1 seronegative (not shown). When we isolated dam mammary glands, we found an approximate 10<sup>5</sup>-fold increase in MNV-1 and EDIM genomic RNA abundances over input, indicating mammary enteric virus replication (Fig. 1f). This was confirmed by immunostaining dam mammary glands with antibodies against EDIM and MNV-1 non-structural replication proteins NSP5 and NS4 respectively, which identified epithelial cells lining the milk ducts (Fig. 1g,h,k,l) and B cells (Fig. 1i,j,m,n) as replication sites for EDIM and MNV-1.

<sup>1</sup>Present address: Laboratory of Host-Pathogen Dynamics, National Heart Lung and Blood Institute, National Institutes of Health, Bethesda, MD, USA. <sup>2</sup>Matrix and Morphogenesis Section, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD, USA. <sup>3</sup>AAV Biology Section, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD, USA. <sup>4</sup>Faculty of Nursing, University of Connecticut, Storrs, CT, USA. <sup>5</sup>Metaorganism Immunity Section, Laboratory of Host Immunity and Microbiome, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, USA. <sup>6</sup>Microbiome Program, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, USA. <sup>7</sup>Department of Microbiology and Immunology, University of Maryland School of Medicine, Baltimore, MD, USA. <sup>8</sup>Advanced Imaging and Microscopy Resource, National Institutes of Health, Bethesda, MD, USA. <sup>9</sup>Laboratory of High Resolution Optical Imaging, National Institute of Biomedical Imaging and Bioengineering, National Institutes of Health, Bethesda, MD, USA. ✉e-mail: [nihal.altan-bonnet@nih.gov](mailto:nihal.altan-bonnet@nih.gov)

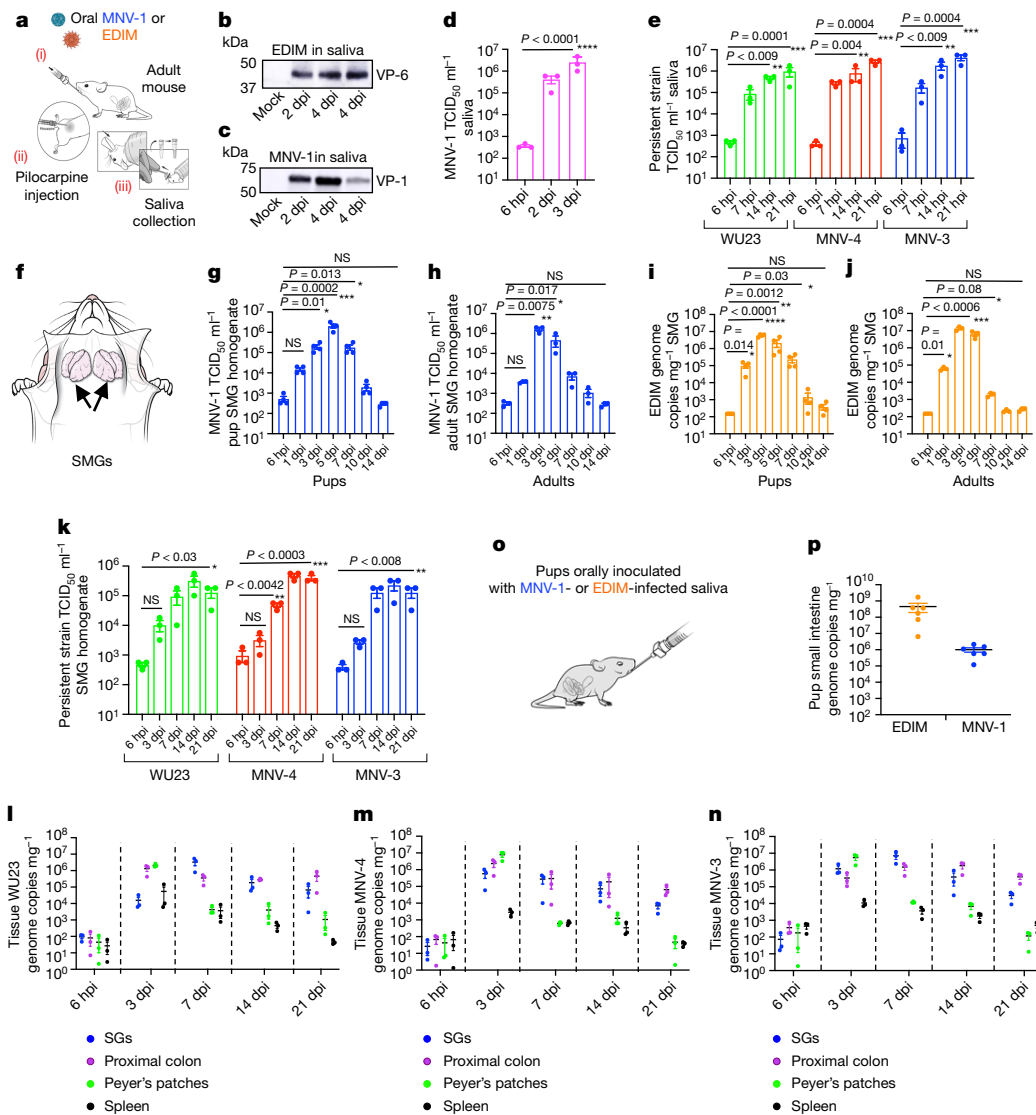


**Fig. 1 | Suckling infected pups directly transmit enteric viruses to mothers' mammary glands.** **a**, Mouse pups orally inoculated with MNV-1 or EDIM were left to nurse with mothers (dams). **b**, MNV-1 titres in pup intestines post-inoculation. **c**, EDIM replication in pup intestines post-inoculation. **d**, Intestinal sIgA of MNV-1- and EDIM-infected pups. **b–d**,  $n = 3$  number of biologically independent experiments, seven animals per experiment; each dot represents one animal. **e**, Milk sIgA of dams ( $n = 3$ , three dams per virus; each dot represents an experiment). **f**, Viral replication in dam mammary glands suckling MNV-1- or EDIM-inoculated pups ( $n = 3$ , 23 animals per virus; each dot represents one animal). **g–n**, Immunostaining of mammary glands of dams suckling uninoculated or MNV-1- or EDIM-inoculated pups. **o**, Ten-day post-partum dams were orally inoculated with EDIM. **p**, EDIM replication in mammary glands and intestines of **o** ( $n = 3$ , eight animals, three each for 6 hpi

and 2 dpi and two for 4 dpi per experiment; each dot represents one animal). **q**, Milk sIgA from dams orally inoculated with EDIM ( $n = 3$ , each dot represents an experiment). **r**, Pup/dam swap experiment scheme. **s, t**, EDIM levels in the mammary glands of dams (**s**) and small intestines of pups (**t**) ( $n = 3$ , each experiment included two cages and each cage consisted of one dam and two pups; each dot represents one animal). Data are the mean  $\pm$  s.e.m. **b–d, e, f, p, q**, Two-tailed unpaired *t*-test. Statistical information is found in Supplementary Table 4. The 6-hpi (**b, c, f**) and day-0 (**d, e, p, q**) values were taken as the starting input values. Calculations of the limit of detection (LOD) for the TCID<sub>50</sub> and qPCR are described in the Methods. In Figs. 1–3, the LOD for TCID<sub>50</sub> per millilitre was approximately 10<sup>2</sup>; the LOD for the qPCR data was 78  $\pm$  9 (s.e.m.) genome copies per milligram tissue. All micrograph experiment reproducibility information is found in the Methods.

We tested whether replication in mammary glands and the rapid surge in milk sIgA was due to dams being infected by their pups via the conventional fecal–oral route because of possible coprophagia in the

shared living space. The entero–mammary pathway during pregnancy and lactation is well known to lead to transfer of immune cells, although not viruses, from the gut to the mammary gland on gut infection or



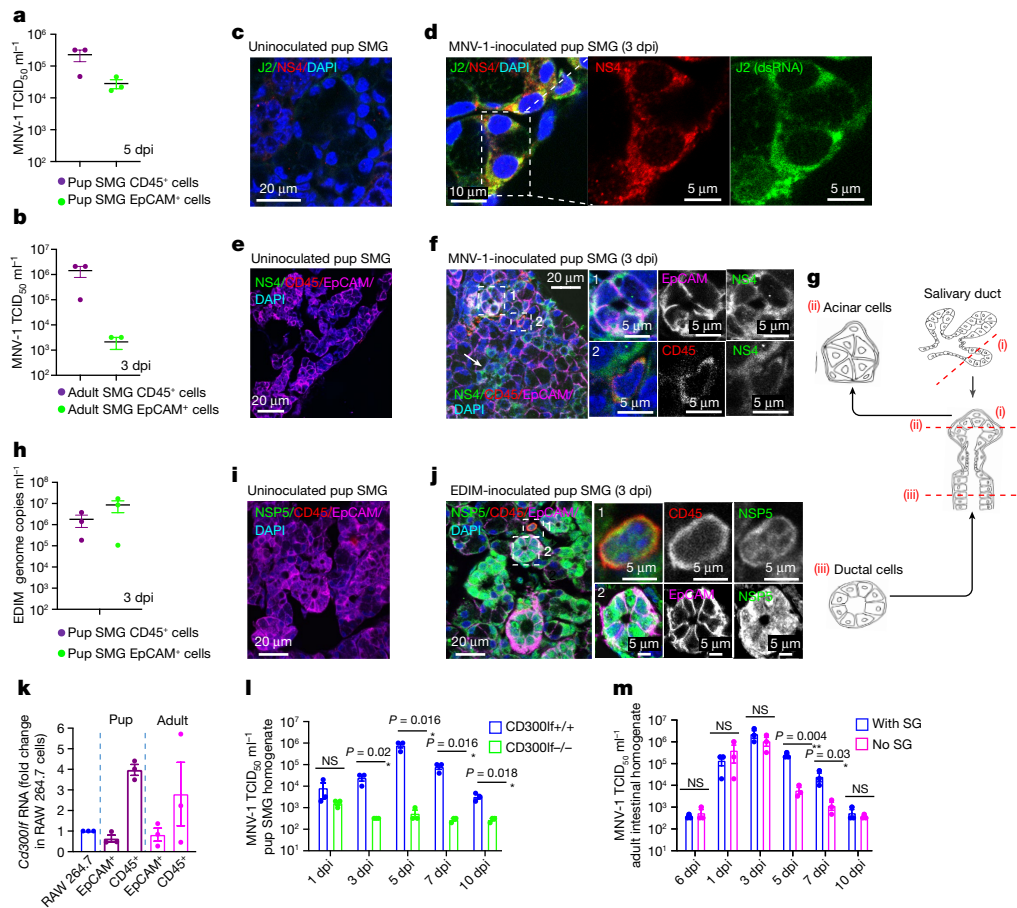
**Fig. 2 | Enteric viruses replicate acutely and persistently in SGs and are transmitted through saliva.** **a**, Schematic of saliva collection. **b,c**, Saliva collected from EDIM-infected (**b**) or MNV-1-infected (**c**) mice probed with anti-VP-6 and anti-VP-1, respectively ( $n = 3$ , five animals per experiment). **d**, MNV-1 titres in saliva ( $n = 3$ , three animals per experiment; each dot represents an experiment). **e**, WU23, MNV-4 and MNV-3 titres in saliva ( $n = 3$ , four animals per experiment; each dot represents an experiment). **f**, Schematic of mouse SMGs. **g,h**, MNV-1 titres in pup (**g**) and adult (**h**) SMGs ( $n = 3$ ). **i,j**, EDIM replication in pup (**i**) ( $n = 4$ ) and adult (**j**) SMGs ( $n = 3$ ). **g-j**, Each data point represents one animal, seven animals per experiment. **k**, WU23, MNV-4 and MNV-3 titres in SMGs

( $n = 3$ , five animals per experiment; each dot represents one animal). **l-n**, Replication in the SGs, proximal colon, Peyer's patches and spleen of adult mice inoculated with WU23 (**l**), MNV-4 (**m**) and MNV-3 (**n**) ( $n = 3$ , five animals per experiment; each dot represents data from one animal). **o**, Schematic of oral inoculation with infected saliva. **p**, Viral replication in the small intestines of pups orally inoculated with infected saliva ( $n = 3$ , each dot represents one animal, total six animals). Pups were also inoculated with uninfected saliva for baseline reference. Data are the mean  $\pm$  s.e.m. **d,e,g-j,k**, Two-tailed unpaired *t*-test. Statistical information is in Supplementary Table 4. For gel source data, see Supplementary Figure 1. The input for **d,e,g-k,l-n**, is 6 hpi.

vaccination<sup>4</sup>. We orally inoculated EDIM seronegative dams (pup-free) with EDIM at 10 days post-partum (Fig. 1o) and measured the levels of EDIM genomic RNA in mammary glands and small intestine (Fig. 1p) and milk sIgA (Fig. 1q). In contrast to dams suckling infected pups (Fig. 1d-f), there was no sIgA surge in the milk of orally inoculated dams (Fig. 1q); their mammary glands did not show any detectable viral RNA (Fig. 1p) whereas their small intestines had a 10<sup>6</sup>-fold increase in viral RNA by 4 dpi (Fig. 1p).

To further interrogate this mode of viral transfer, mouse pups were orally inoculated with EDIM (pups A) and placed back for suckling on their mother (dam A). At 1 dpi, their mother was swapped with a foster dam (dam B) from a cage of uninoculated pups (pups B). Dam A was placed with and suckled pups B whereas pups A were suckled by dam B (Fig. 1r). At 3 dpi, all animals from both cages were killed and

virus replication in the dam mammary glands (Fig. 1s) and pup small intestines (Fig. 1t) was measured. We found a 10<sup>4</sup>-fold increase in viral genome levels in the mammary glands of both dams A and B (Fig. 1s) and a 10<sup>6</sup>-fold increase in the genome levels in the small intestines of both pups A and B (Fig. 1t) over input (approximately 10<sup>2</sup> genome copies per milligram tissue). This indicated that both dams A and B had been infected by suckling the directly inoculated pups A, with dam A infected by 1 dpi with pups A and dam B in the days after. Pups B were probably infected by suckling from dam A's infected mammary glands or from her feces. Collectively, these findings indicated that enteric viruses backflow from pups to their mothers' mammary glands through suckling, leading to both an in situ mammary gland infection and rapid triggering of milk sIgA surge that may contribute to clearing the pups' infection<sup>6</sup>.



**Fig. 3 | Murine norovirus and rotavirus replicate in the epithelial and immune cells of SMGs.** **a, b**, MNV-1 titres in epithelial (EpCAM<sup>+</sup>) and immune (CD45<sup>+</sup>) cells sorted from the SMGs of inoculated pups (**a**) and adults (**b**) ( $n = 3$ , each dot represents data from two animals, six animals in total). **c–f**, Immunostaining of SMGs from uninoculated and MNV-1-inoculated pups with anti-NS4, anti-J2 (dsRNA), anti-EpCAM and anti-CD45. MNV-1 replication in acinar (arrows and box 1) and immune cells (box 2). **g**, Schematic of salivary duct structure. **h**, EDIM replication in EpCAM<sup>+</sup> and CD45<sup>+</sup> cells sorted from the SMGs of inoculated pups ( $n = 3$ , each dot represents two animals, six animals in total). **i, j**, Immunostaining of SMGs from uninoculated (**i**) and EDIM-inoculated (**j**) pups with anti-NSP5, anti-CD45 and anti-EpCAM. EDIM replication in

immune (box 1) and ductal cells (arrows and box 2). **k**, *Cd300lf* expression in SMGs ( $n = 3$ , three adults and three pups per experiment). RAW264.7 and HeLa cells were used as positive and negative controls, respectively. **l**, MNV-1 titres in the SMGs of inoculated *Cd300lf*<sup>-/-</sup> and *Cd300lf*<sup>+/+</sup> mice ( $n = 3$ , each dot represents one animal, five animals per experiment for each group). **m**, MNV-1 titres in the intestines of inoculated SG-removed or SG-intact adult mice ( $n = 3$ , each dot represents one animal, six animals per experiment for each group). **a, b, h, k**, Gating strategies are depicted in Extended Data Fig. 3. Data are the mean  $\pm$  s.e.m. **l, m**, Two-tailed unpaired *t*-test between two groups for each time point. Statistical information is given in Supplementary Table 4. **m**, Input is 6 hpi. All micrograph experiment reproducibility information is found in the Methods.

### Enteric viruses are transmitted through saliva

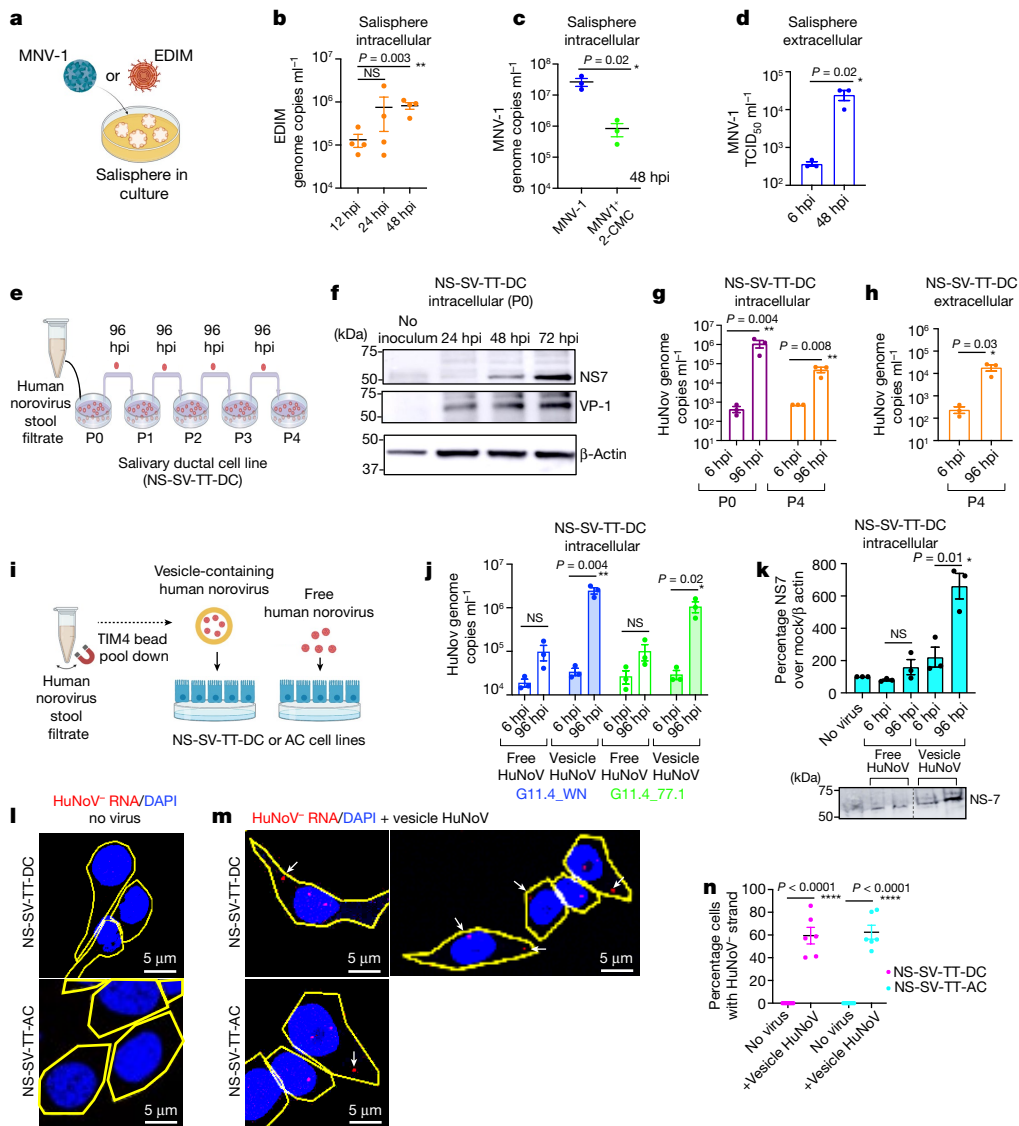
We tested whether saliva could be the conduit for enteric viral transmission to mammary glands during suckling. We extracted saliva from adult mice (as it was easier than doing so from pups)<sup>21</sup> orally inoculated with EDIM or MNV-1 (Fig. 2a). Immunoblotting with anti-EDIM rotavirus VP6 (Fig. 2b) and anti-MNV-1 VP1 (Fig. 2c) showed each virus to be shed in saliva starting at 2 dpi; MNV-1 TCID<sub>50</sub> measurements showed titres at 3 dpi to be approximately 10<sup>4</sup>-fold higher than input (6 h post-inoculation (hpi)) (Fig. 2d). Whereas MNV-1 and EDIM infections are acute and cleared in 7–10 days (ref.<sup>20</sup>) (Fig. 1b,c and Extended Data Fig. 1a,b), MNV-3, MNV-4 and the less-well-characterized WU23 murine norovirus strains persistently infect the proximal colon and shed into feces for weeks<sup>22–25</sup>. We inoculated mice with these strains and found that all were also persistently shed in saliva for at least 3 weeks after inoculation, with titres approximately 10<sup>3</sup>-fold higher than input (6 hpi) (Fig. 2e).

### Enteric virus replication in SGs

The major SG complex (Fig. 2f) consists of parotid, sublingual and submandibular glands (SMG)<sup>26,27</sup>. SMGs were chosen as a representative

SG because they are the largest and provide basal salivary secretion. After oral inoculation of pups and adults with the MNV-1, MNV-3, MNV-4 or WU23 strains, an approximate 10<sup>4</sup>-fold increase in titres over each virus respective input level (6 hpi) was measured in the SMGs (Fig. 2g,h,k). Replication was also sensitive to 2'-C-methylcytidine (2-CMC) norovirus polymerase inhibitor<sup>28</sup> (Extended Data Fig. 1c). Similarly, in the SMGs of EDIM- and murine astrovirus-inoculated<sup>29</sup> mice, approximately 10<sup>5</sup>- and 10<sup>3</sup>-fold increases in viral genome copies were measured, respectively (Fig. 2i,j and Extended Data Fig. 1d). Notably, for MNV-3, MNV-4 and WU23, the level and duration of SMG replication was comparable to that of the proximal colon (Fig. 2l–n). By contrast, viral RNA levels were significantly lower and ultimately cleared from Peyer's patches and spleen (Fig. 2l–n). We also tested the CR6 murine norovirus, which shares with MNV-3 and MNV-4 critical sequence motifs in its VP1 and NS1/2 proteins needed for systemic infection and persistence, respectively<sup>25,30</sup>. CR6 did not replicate in SMGs (Extended Data Fig. 2), pointing to differences among persistent murine norovirus strains.

In addition to suckling, we tested whether saliva could transmit infection to others through the conventional oral route. We orally inoculated pups with saliva obtained from MNV-1- or EDIM-infected



**Fig. 4 | Enteric viruses replicate in salispheres and SG cell lines.**

**a**, Schematic of MNV-1 or EDIM inoculation in salispheres. **b**, EDIM genome copies in EDIM-inoculated salisphere lysates ( $n = 4$ ). **c**, MNV-1 replication in salispheres with 2-CMC ( $n = 3$ ). **d**, MNV-1 titres in MNV-1-inoculated salisphere supernatants ( $n = 3$ ). **e**, NS-SV-TT-DC inoculation scheme with HuNoV. **f**, Immunoblot of PO cell lysates probed with anti-NS7 and anti-VP1.  $\beta$ -Actin was used as the loading control ( $n = 3$ ). **g, h**, HuNoV genomic RNA levels in P0 and P4 cell lysates (**g**) and P4 supernatants (**h**) ( $n = 3$ ). **i**, Scheme of vesicle pull-down with TIM-4 beads from stool filtrate<sup>11</sup>. **j**, HuNoV genomic RNA levels in cell lysates of vesicle-cloaked or free virus-inoculated NS-SV-TT-DC cultures ( $n = 3$ ). **k**, Cellular lysates of vesicle-cloaked or free virus-inoculated NS-SV-TT-DC cultures were analysed by immunoblotting with anti-NS7 ( $n = 3$ ) and anti- $\beta$ -actin. **l, m**, FISH with probes against the (-) HuNoV strand was performed on uninoculated (**l**) and vesicle-cloaked HuNoV (GII.4-WN)-inoculated (**m**) NS-SV-TT-DC and NS-SV-TT-AC cell cultures. Cell outlines are shown in yellow; the arrows point to (-) HuNoV RNA. **n**, Percentage of NS-SV-TT-DC and NS-SV-TT-AC cells with (-) HuNoV strand FISH staining ( $n = 6$ ). Data are the mean  $\pm$  s.e.m. **b–d, g, h, j, k, n**, Each dot represents a biologically independent experiment; two-tailed unpaired  $t$ -test between two groups. Statistical information is shown in Supplementary Table 4. **d, g, h, j, k**, The input was 6 hpi. **b, c, g, h, j**, The LOD for qPCR was  $10^2$  genome copies  $\text{ml}^{-1}$ . **d**, The LOD for TCID<sub>50</sub> per millilitre was  $2 \times 10^2$ . All micrograph experiment reproducibility information is found in the Methods. For gel source data, see Supplementary Figure 1.

adults (Fig. 2o) and measured intestinal viral genome levels at 3 dpi. This showed significant EDIM and MNV-1 replication (Fig. 2p), as in pups orally inoculated with fecal EDIM or MNV-1 (Fig. 1b,c).

### Replication in immune and epithelial cells

TCID<sub>50</sub> measurements showed significant MNV-1 titres in the CD45<sup>+</sup> immune and EpCAM<sup>+</sup> (epithelial cell adhesion molecule) epithelial SMG cell populations (Fig. 3a,b and Extended Data Fig. 3). This was confirmed by coimmunostaining SMGs with antibodies against MNV-1 replication reporters NS4 and double-stranded RNA (dsRNA) (J2) and epithelial (EpCAM) and immune cell (CD45) markers (Fig. 3c–f). NS4 staining was localized to acinar epithelial cells (Fig. 3f,g), marked with NKCC1 (Extended Data Fig. 1e,f). EDIM also replicated in both CD45<sup>+</sup> and EpCAM<sup>+</sup> SMG cell populations (Fig. 3h), but replication in the latter was mainly in ductal epithelial cells (Fig. 3g,i,j).

*Cd300lf*, encoding the functional intestinal receptor for all known murine norovirus strains<sup>23,31</sup>, was also highly expressed in SMG EpCAM<sup>+</sup> and CD45<sup>+</sup> cell populations (Fig. 3k). *Cd300lf*<sup>-/-</sup> mouse pups inoculated with MNV-1 did not show any productive infection of their SGs (Fig. 3l) indicating that CD300lf receptors are essential for SMG infection by

murine noroviruses. Notably, partial extraction of the SGs from adult mice before oral MNV-1 inoculation led to faster clearing of the intestinal infection (Fig. 3m). These data, together with MNV-3/MNV-4/WU23 persistence (Fig. 2e,k–n) and slower clearance of acute viruses from SMGs (Fig. 2g–j) relative to intestines (Fig. 1b,c), indicates that SGs may act as enteric virus reservoirs.

### Ex vivo salivary replication models

In vitro models of virus replication are important tools for studying viral life cycles, therapeutics and vaccines. We investigated whether spheroids assembled from murine SG cells (salispheres)<sup>7</sup> could be used as ex vivo culture systems for murine enteric viruses. In EDIM-inoculated salispheres, we measured a tenfold increase in EDIM genomic RNA copies between 12 hpi and 48 hpi (Fig. 4a,b). As salispheres endogenously express CD300lf (Extended Data Fig. 4a), we inoculated them with MNV-1. This resulted in 2-CMC-sensitive replication (Fig. 4c) and MNV-1 egress into culture supernatants at 48 hpi (Fig. 4d). CR6, which had not replicated in SMGs in vivo (Extended Data Fig. 2), robustly replicated in salispheres (Extended Data Fig. 4b–d).

## Salivary cell lines replicate human norovirus

Human enteroid and B cell culture models currently used to replicate human noroviruses (HuNoVs) require supplements (bile acids and microbiota) and are costly<sup>32,33</sup>. Therefore, we tested whether HuNoV could replicate in human SG cell lines. We chose SV40-transformed adherent salivary cell lines, NS-SV-TT-DC (ductal) and NS-SV-TT-AC (acinar), that routinely grow as a monolayer<sup>8</sup> and adapted them to serum-free media. The ductal NS-SV-TT-DC cells were inoculated with a fixed volume of HuNoV stool filtrate (GII.4 Sydney) (Fig. 4e), which was washed off at 6 hpi. The PO lysate collected at intervals post-wash and immunoblotted with antibodies against capsid VP1 and polymerase NS7 showed time-dependent increases in both proteins over input (6 hpi), indicating replication (Fig. 4f). Next, HuNoV was sequentially passaged four times through NS-SV-TT-DC cells, each time inoculating the supernatant from the previous passage in a new culture, washing off inoculum at 6 hpi and collecting the cell lysates or supernatants at 96 hpi (Fig. 4e) to measure HuNoV genomic copies (Fig. 4g,h). In PO, we measured an approximate 1,000-fold increase in intracellular HuNoV (genome copies per millilitre) between 6 and 96 hpi. Although by P4 replication had attenuated, with intracellular and extracellular virus levels approximately 100-fold (Fig. 4g) and tenfold higher, respectively, than the inputs for each (6 hpi) (Fig. 4h), the replication measured approximated those reported in enteroid cultures<sup>33</sup>.

Rotaviruses and noroviruses egress from cells non-lytically as viral clusters inside vesicles<sup>18</sup>. Compared to free fecal viruses (for example, due to vesicle lysis), vesicle-cloaked viruses are protected from fecal proteases and nucleases, enabling high multiplicities of infection (MOIs) and leading to enhanced infectivity<sup>18,34,35</sup>. Indeed, only vesicle-cloaked, HuNoV-enriched inocula replicated efficiently in the ductal and acinar salivary cell lines as evidenced by qPCR, immunoblots for viral non-structural proteins (NS7, NS6) and single-molecule fluorescence in situ hybridization (FISH)<sup>34</sup> against the (–) replicative HuNoV RNA strands (Fig. 4i–n and Extended Data Fig. 4f,g). This finding may have implications for enhancing infectivity in other HuNoV culture models<sup>32,33</sup>.

Annually, norovirus, rotavirus and astrovirus combined infect approximately one billion people across developed and developing countries, leading to significant morbidity and mortality. The diverse sanitation practices do not support the fecal–oral route as the sole route of their transmission. We have shown that SGs are significant replication sites for these viruses on par with the intestines; saliva transmits infection to others including nursing mothers. Furthermore, SGs may act as reservoirs, thus continuing to spread enteric viruses by saliva in the absence of diarrhoea. Our results bring focus on the enteric viral infection of SGs and saliva as a potentially more significant transmission route through talking, coughing, sneezing and kissing compared to the accepted transmission mode, fecal contamination. Hence our findings indicate that sanitation measures in addition to ones preventing fecal spread may be needed to prevent transmission of enteric viruses in the population.

## Online content

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at <https://doi.org/10.1038/s41586-022-04895-8>.

1. Petri, W. A. Jr et al. Enteric infections, diarrhea, and their impact on function and development. *J. Clin. Invest.* **118**, 1277–1290 (2008).

- Posse, J. L., Dios, P. D. & Scully, C. in *Saliva Protection and Transmissible Diseases* (eds Posse, J. L., Dios, P. D. & Scully, C.) 53–92 (Academic Press, 2017).
- Huang, N. et al. SARS-CoV-2 infection of the oral cavity and saliva. *Nat. Med.* **27**, 892–903 (2021).
- Rodríguez, J. M., Fernández, L. & Verhasselt, V. The gut–breast Axis: programming health for life. *Nutrients* **13**, 606 (2021).
- Rogier, E. W. et al. Secretory antibodies in breast milk promote long-term intestinal homeostasis by regulating the gut microbiota and host gene expression. *Proc. Natl Acad. Sci. USA* **111**, 3074–3079 (2014).
- Blutt, S. E., Miller, A. D., Salmon, S. L., Metzger, D. W. & Conner, M. E. IgA is important for clearance and critical for protection from rotavirus infection. *Mucosal Immunol.* **5**, 712–719 (2012).
- Vining, K. H. et al. Neurturin-containing laminin matrices support innervated branching epithelium from adult epithelial spheroids. *Biomaterials* **216**, 119245 (2019).
- Azuma, M., Tamatani, T., Kasai, Y. & Sato, M. Immortalization of normal human salivary gland cells with duct-, myoepithelial-, acinar-, or squamous phenotype by transfection with SV40 ori- mutant deoxyribonucleic acid. *Lab. Invest.* **69**, 24–42 (1993).
- Lee, J. M., Garon, E. & Wong, D. T. Salivary diagnostics. *Orthod. Craniofac. Res.* **12**, 206–211 (2009).
- Lima, D. P., Diniz, D. G., Moimaz, S. A. S., Sumida, D. H. & Okamoto, A. C. Saliva: reflection of the body. *Int. J. Infect. Dis.* **14**, e184–e188 (2010).
- Corstjens, P. L., Abrams, W. R. & Malamud, D. Saliva and viral infections. *Periodontol.* **2000** **70**, 93–110 (2016).
- Pisanic, N. et al. Minimally invasive saliva testing to monitor norovirus infection in community settings. *J. Infect. Dis.* **219**, 1234–1242 (2019).
- Kirby, A., Dove, W., Ashton, L., Hopkins, M. & Cunliffe, N. A. Detection of norovirus in mouthwash samples from patients with acute gastroenteritis. *J. Clin. Virol.* **48**, 285–287 (2010).
- Anfruns-Estrada, et al. Detection of norovirus in saliva samples from acute gastroenteritis cases and asymptomatic subjects: association with age and higher shedding in stool. *Viruses* **12**, 1369 (2020).
- de Graaf, M., van Beek, J. & Koopmans, M. P. Human norovirus transmission and evolution in a changing world. *Nat. Rev. Microbiol.* **14**, 421–433 (2016).
- Crawford, S. E. et al. Rotavirus infection. *Nat. Rev. Dis. Primers* **3**, 17083 (2017).
- Karst, S. M., Wobus, C. E., Goodfellow, I. G., Green, K. Y. & Virgin, H. W. Advances in norovirus biology. *Cell Host Microbe* **15**, 668–680 (2014).
- Santiana, M. et al. Vesicle-cloaked virus clusters are optimal units for inter-organismal viral transmission. *Cell Host Microbe* **24**, 208–220.e8 (2018).
- Wolf, J. L., Cukor, G., Blacklow, N. R., Dambrauskas, R. & Trier, J. S. Susceptibility of mice to rotavirus infection: effect of age and administration of corticosteroids. *Infect. Immun.* **33**, 565–574 (1981).
- Roth, A. N. et al. Norovirus infection causes acute self-resolving diarrhea in wild-type neonatal mice. *Nat. Commun.* **11**, 2968 (2020).
- Bagavant, H. et al. A method for the measurement of salivary gland function in mice. *J. Vis. Exp.* **131**, 57203 (2018).
- Arias, A., Bailey, D., Chaudhry, Y. & Goodfellow, I. Development of a reverse-genetics system for murine norovirus 3: long-term persistence occurs in the caecum and colon. *J. Gen. Virol.* **93**, 1432–1441 (2012).
- Graziano, V. R. et al. CD300lf conditional knockout mouse reveals strain-specific cellular tropism of murine norovirus. *J. Virol.* **95**, e01652-20 (2021).
- Hsu, C. C., Riley, L. K., Wills, H. M. & Livingston, R. S. Persistent infection with and serologic cross-reactivity of three novel murine noroviruses. *Comp. Med.* **56**, 247–251 (2006).
- Walker, F. C. et al. Norovirus evolution in immunodeficient mice reveals potentiated pathogenicity via a single nucleotide change in the viral capsid. *PLoS Pathog.* **17**, e1009402 (2021).
- Knosp, W. M., Knox, S. M. & Hoffman, M. P. Salivary gland organogenesis. *Wiley Interdiscip. Rev. Dev. Biol.* **1**, 69–82 (2012).
- Patel, V. N. & Hoffman, M. P. Salivary gland development: a template for regeneration. *Semin. Cell Dev. Biol.* **25–26**, 52–60 (2014).
- Rocha-Pereira, J. et al. The viral polymerase inhibitor 2'-C-methylcytidine inhibits Norwalk virus replication and protects against norovirus-induced diarrhea and mortality in a mouse model. *J. Virol.* **87**, 11798–11805 (2013).
- Cortez, V. et al. Characterizing a murine model of astrovirus using viral isolates from persistently infected immunocompromised mice. *J. Virol.* **93**, e00223-19 (2019).
- Nice, T. J., Strong, D. W., McCune, B. T., Pohl, C. S. & Virgin, H. W. A single-amino-acid change in murine norovirus NS1/2 is sufficient for colonic tropism and persistence. *J. Virol.* **87**, 327–334 (2013).
- Orchard, R. C. et al. Discovery of a proteinaceous cellular receptor for a norovirus. *Science* **353**, 933–936 (2016).
- Jones, M. K. et al. Human norovirus culture in B cells. *Nat. Protoc.* **10**, 1939–1947 (2015).
- Ettayebi, K. et al. Replication of human noroviruses in stem cell-derived human enteroids. *Science* **353**, 1387–1393 (2016).
- Chen, Y.-H. et al. Phosphatidylserine vesicles enable efficient en bloc transmission of enteroviruses. *Cell* **160**, 619–630 (2015).
- Kerviel, A., Zhang, M. & Altan-Bonnet, N. A new infectious unit: extracellular vesicles carrying virus populations. *Annu. Rev. Cell Dev. Biol.* **37**, 171–197 (2021).

**Publisher's note** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

© This is a U.S. Government work and not under copyright protection in the US; foreign copyright protection may apply 2022

## Methods

### Experimental models

**Cell culture.** For murine norovirus propagation and performing TCID<sub>50</sub>, RAW264.7 cells (ATCC TIB-71, research resource identifier: CVCL\_0493) were grown in 10% FCS (catalogue no. EF-0500-A; Atlas Biologicals) supplemented with DMEM (catalogue no. 11995-065; Thermo Fisher Scientific). HeLa cells were used for checking the baseline level of CD300lf expression. HeLa cells (ATCC CCL-2) were maintained in DMEM supplemented with 10% FCS and 2 mM L-glutamine. All cell lines were maintained in incubators at 37 °C and 5% CO<sub>2</sub>. NS-SV-DC and NS-SV-AC cells have been described previously<sup>8</sup>. *Mycoplasma*-free cells were established and grown in serum-free medium of KGM-2 Keratinocyte Growth Medium-2 BulletKit (Lonza) consisting of KBM-2 Basal Medium and KGM-2 SingleQuots Supplements (Lonza). Identity was established by short tandem repeat analysis and found to be distinct from other published profiles at the D16S539 locus, and the lines are referred to as NS-SV-TT-DC and NS-SV-TT-AC. SG organoid culture or salispheres were processed and propagated as per the established protocol<sup>26</sup>.

**Mouse model.** Specific-pathogen-free 6-week-old BALB/c adult female mice and litters of 10-day-old BALB/c pups including males and females were used for the experiments mentioned; 10-day-old C57BL/6J (stock no. 000664) and B6.129S(Cg)-*Stat1*<sup>tm1Dlv</sup>/J (stock no. 012606) mice were purchased from The Jackson Laboratory. C57BL/6J-CD300lfem1Cbwi/J breeding pairs were a gift from C. B. Wilen (Yale School of Medicine). The *Cd300lf*<sup>-/-</sup> allele was created by H. W. Virgin (Washington University at St Louis) using CRISPR-Cas9 endonuclease-mediated genome editing in C57BL/6J mouse zygotes. The B6 *Ifnar1*<sup>-/-</sup> mouse breeding pairs were a gift from D. Verthelyi (U.S. Food and Drug Administration (FDA)). The animals were maintained at the National Heart, Lung, and Blood Institute (NHLBI) animal care facility and 10-day-old mouse pups were used for the experiments. Genotyping for all breeding pairs and mouse pups was done by Transnetyx using tail cuts to confirm the knockout gene. All animals were tested to be seronegative for EDIM, MNV-1 and murine astroviruses. All BALB/c adults and pups were either purchased from The Jackson Laboratory (strain no. 000651) or housed and bred in-house (animals aged more than 6 weeks) in accordance with the procedure outlined in the guide for the Care and Use of Laboratory Animals under an animal study proposal approved by the NHLBI Animal Care and Use Committee.

In brief, the animal facility temperature was maintained at 22 °C with 40–50% humidity. Animals were housed in ventilated racks and the cages were supplied with hardwood bedding and nest packs. Animals were provided the NIH 31 feed and autoclaved water. A 6:00–18:00 light cycle was followed. Animals were infected randomly without any bias as to sex, and all animal experiments were validated double blinded. The age of the animals selected for each experiment is given in the figure legends. All animal experiments were performed in an American Association for Accreditation of Laboratory Animal Care (AAALAC)-accredited animal facility. Animals were killed as per the AAALAC guidelines: adult animals were killed by CO<sub>2</sub> exposure (USP Grade A) at 3 l min<sup>-1</sup> and mouse pups were killed by cervical dislocation. It took approximately 2–3 min to anaesthetize the animals with a 10–30% filled CO<sub>2</sub> chamber, at which time lack of respiration and faded eye colour were observed. CO<sub>2</sub> flow was maintained for a minimum of 1 min after respiration ceased.

### Virus stock

**Murine rotavirus.** The EDIM strain, a gift from H. Greenberg (Stanford University), was propagated in BALB/c mice. The DD<sub>50</sub> (the virus dose determined empirically to cause diarrhoea in 50% of mouse pups) of the stock in 10-day-old mouse pups was 10<sup>5</sup> plaque-forming units. Five-day-old mouse pups were orally inoculated with 100-fold-diluted stock virus in PBS; at 2 dpi, when the animals started to have diarrhoea, they were killed by cervical dislocation. Intestines were surgically extracted and tissues were homogenized in M199 medium and centrifuged

at 5,000 r.p.m. for 10 min at 4 °C to isolate the virus from tissue. The stock virus was aliquoted and stored at –80 °C until ready to use.

**Murine norovirus.** MNV-1 (ref. <sup>36</sup>) was a gift from K. Green (NIAID) and CR6 was a gift from M. Baldrige (Washington University at St Louis). Strains were propagated in RAW264.7 cells according to a previously published protocol<sup>36</sup>. In brief, 1 × 10<sup>7</sup> cells were seeded in 150-mm cell culture dishes and incubated for 48 h. Cells were infected with MNV-1 or CR6 at an MOI of 0.1 and incubated for 1 h at 37 °C and 5% CO<sub>2</sub>. Finally, cells were rinsed and further incubated with pre-warmed serum-free medium until the cytopathic effect (CPE) was visible. Cell cultures were freeze-thawed three times, medium was collected, and cell debris was removed by centrifugation (1,000g for 15 min). The TCID<sub>50</sub> per millilitre was calculated to be 8 × 10<sup>7</sup>. Cleared stock virus was aliquoted and stored at –80 °C until ready to use.

WU23 (8.75 × 10<sup>9</sup> TCID<sub>50</sub> ml<sup>-1</sup>) and MNV-3 (5.79 × 10<sup>10</sup> TCID<sub>50</sub> ml<sup>-1</sup>) were gifts from S. Karst, Department of Molecular Genetics and Microbiology, University of Florida College of Medicine; MNV-4 (1 × 10<sup>7</sup> TCID<sub>50</sub> ml<sup>-1</sup>) was a gift from C. Wobus, Department of Microbiology and Immunology, University of Michigan Medical School. Both were similarly propagated in RAW cells.

**Murine astrovirus.** The strain, a natural isolate denoted as murine astrovirus-Y, was a gift from S. Compton (Yale School of Medicine). The virus was propagated as per the published protocol<sup>37</sup>. In brief, 20 µl of murine Astrovirus-strain Y was orally inoculated into 6-week-old BALB/c mice. At 5 dpi, mice were killed and the colon was extracted and processed as a 10% homogenate in DMEM. Pooled colon homogenates were clarified by centrifugation and sterilized by passing through a 0.22-µm filter; stocks were stored at –80 °C.

**HuNoV.** HuNoV GII.4-77.1 (NIHIC 77.1) was a gift from K. Green (NIAID) and GII.4-WN (Sydney Strain) was a gift from W. A. Henderson (University of Connecticut). The GII.4-77.1 stool sample was first filtered through a 70-µm sieve to eliminate fats and other debris, followed by a finer filtration through a 20-µm sieve and lastly a 0.3-µm sieve to eliminate bacteria. The filtrate was then concentrated and diluted in 500 µl of PBS after ultracentrifugation at 100,000g for 1.5 h. Thereafter the stock was inoculated into NS-SV-TT-DC cells for 6 h, which were then washed with PBS twice and then incubated in fresh culture medium for 96 h. This generation of cells receiving the stock virus is denoted as P0. Virus was collected from P0 after 96 h and centrifuged at 3,000g for 15 min to eliminate cell debris. The supernatant obtained was used to infect a fresh cell culture (P1) that was incubated for 96 h; the virus was passaged for three more generations, termed P2–P4.

**Vesicle isolation from human stool samples.** All stools were diluted to 50% vol/vol with Dulbecco's PBS (DPBS). Then 1-ml aliquots of diluted stools were subjected to differential centrifugation to clear the stool of debris at 4 °C as follows: 500g for 10 min; 1,000g for 10 min; 2,000g for 10 min; 4,000g for 10 min; 7,000g for 10 min three times. Pellets of stool debris were discarded and supernatant was transferred to a new microcentrifuge tube. Vesicles were isolated from cleared stool solutions using the MagCapture Exosome Isolation Kit PS (catalogue no. 293-77601; Fujifilm Wako) according to the manufacturer specifications with the following modifications: cleared stool solutions were subjected to three sequential pulldowns using 120 µl of beads for each pulldown at 4 °C in a rotator at 15 r.p.m. The first pulldown was performed overnight and the rest for 2–3 h. Vesicles were eluted twice in 2 × 50 µl of elution buffer at 37 °C for 10 min. The isolated vesicles from all pulldowns were combined and stored at 4 °C until needed for the infectivity assays. The rest of the fraction was labelled as free virus and stored similarly.

### Virus infection

**Cell culture.** Viruses to infect the salispheres of MNV-1 and CR6 were inoculated at an MOI = 1; for EDIM, 20 µl of stock was added in a 24-well

## Article

format and incubated for 2 h before cells were washed with PBS three times (each wash involving centrifugation at 1,000 r.p.m. for 5 min and resuspension of pellets with PBS) and replaced with fresh medium. Thereafter, cells were incubated for various time points, collected for RNA isolation and processed for qPCR analysis. Vesicles and free virus fractions obtained from human stool samples were tested for infectivity in the NS-SV-TT-DC and NS-SV-TT-AC cell lines. Cells were inoculated with 10  $\mu$ l of each fraction and incubated for 6 h before washing and replacing with fresh medium. Thereafter, cells were incubated for 96 h and further processed for RNA and protein extraction for qPCR and immunoblotting; for staining and the FISH assay, cells were fixed with 4% paraformaldehyde (PFA) for 15 min at room temperature and washed three times with PBS.

**Mouse adult and pup infections.** For all adult infections with EDIM, MNV-1, CR6, WU23, MNV-3 and MNV-4, 6-week-old mice (for BALB/c, *Stat1*<sup>-/-</sup> and *Irfar1*<sup>-/-</sup>) were inoculated pre-orally with a tenfold dilution of the stock virus (EDIM) or  $6 \times 10^6$  TCID<sub>50</sub> units of stock murine noroviruses (MNV-1, CR6, WU23, MNV-3 and MNV-4) in a volume of 100  $\mu$ l. For all experiments involving pups, pups were infected at 10 days of age with EDIM (100-fold dilution of stock) or MNV-1 ( $10^5$  TCID<sub>50</sub> units) or murine astrovirus (tenfold dilution of stock) with units of each virus strain in a volume of 100  $\mu$ l delivered by oral gavage using a 22-gauge plastic feeding tube. Mock-infected mice or pups were inoculated with an equal volume of DMEM or PBS. After infection, one set of animals was always killed to collect tissue samples at 6 hpi to measure viral input to tissue; the rest were killed at the indicated time points as per the experiments. Tissues were stored at -20 °C for RNA isolation or kept in 4% PFA at 4 °C overnight for cryosectioning and immunostaining. Similarly, 10-day-old C57BL/6j *Cd300*<sup>f/f</sup> and wild-type C57BL/6j mouse pups were inoculated with MNV-1 (dosage as mentioned earlier) to measure viral replication in SMGs and small intestines.

**Milk extraction from dams.** Milking of dams was started a day before inoculation of the pups with MNV-1/EDIM. In brief, dams were separated from pups 2 h before milking. First, dams were injected with 2 IU kg<sup>-1</sup> of oxytocin (catalogue no. O3251; Sigma-Aldrich). After 5 min, with gentle squeezing of the nipples, milk was extracted and collected by suction using a 200- $\mu$ l pipette as described in ref. <sup>38</sup>. Dams were placed back in the cages with the pups after the procedure.

**Foster mother experiment.** Ten-day-old pups were orally inoculated with EDIM virus; 24 hpi the natural dam was replaced with a foster dam introduced from an age-matched uninfected cage. At the same time, the natural mother from the cage of the infected pups was introduced to feed the uninfected pups from the foster dam cage. After swapping dams between these cages, animals were monitored for 2 days; at 3 dpi, pups and dams from both cages were killed for tissue isolation (pups: small intestine; dams: mammary gland) to quantify viral replication by qPCR.

**2-CMC treatment.** For MNV-1 replication validation in SMGs, 2-CMC, a nucleoside polymerase inhibitor of MNV-1 replication, was used to monitor viral replication in 10-day-old mouse pups in vivo and in salisphere culture in vitro. The dosage in vivo and in vitro was adapted from an established protocol with a few modifications<sup>28</sup>. In brief, for in vivo treatment, administration was initiated by an intraperitoneal injection of 50  $\mu$ g kg<sup>-1</sup> day<sup>-1</sup> dissolved in PBS, divided into two daily treatments 1 day before oral inoculation of MNV-1 until 5 dpi (time determined for maximum replication in SMGs). To compare the effects of the dosage, a mock group was only administered 2-CMC; another group inoculated with MNV-1 received a similar volume of PBS intraperitoneally twice a day. At 5 dpi, pups were killed and tissues (SMGs and small intestine) were extracted for viral quantification. For the in vitro analysis, salisphere cultures received 12.5  $\mu$ g ml<sup>-1</sup> of 2-CMC

dissolved in dimethylsulfoxide (DMSO) 1 h before MNV-1 inoculation. After 2 h of inoculation, the inoculum was removed and the cultures were washed as mentioned earlier and plated with fresh medium along with 2-CMC and monitored for 48 h. Spheres were later collected for RNA extraction. A mock group (without MNV-1 inoculation) was treated with DMSO.

**Salivectomy or SG removal from BALB/c adult mice.** The surgical area was sanitized with povidone-iodine or chlorhexidine scrub and prepped by alternating three times with alcohol and povidone-iodine or chlorhexidine solution. Then the 6-week-old mice were anaesthetized with isoflurane (1–3%) delivered through a gas anaesthesia machine vaporizer with an appropriate anaesthetic gas scavenging system. Animals were intubated with a nose cone for gas delivery after induction in an inhalation anaesthetic chamber. Ketamine and xylazine (80–120 mg kg<sup>-1</sup> ketamine and 5–25 mg kg<sup>-1</sup> xylazine) were injected intraperitoneally to anaesthetize the animals. Once they were anaesthetized, hair was removed from the ventral cervical area and ophthalmic ointment was applied to the eyes. The animal was then placed on a heated water blanket on the operating table. The animal was placed in dorsal recumbency and the surgical site was disinfected using chlorhexidine scrub followed by 70% alcohol. This sequence was repeated three times, alternating between the scrub solution and alcohol. The surgical site was then aseptically draped. A ventral midline cervical incision was made in the skin, which was dissected free of the underlying tissue and retracted laterally. Using a blunt dissection instrument, submandibular and sublingual SGs were separated from the connective and surrounding tissue holding them in place. Once the glands were free, the ducts were cut or cauterized to complete the removal. Both pairs of glands were removed as a single unit. The left and right parotid glands were then individually removed in an analogous manner. The skin incision was closed with a simple interrupted or subcutaneous suture pattern. Bupivacaine was infiltrated along the incision line for pain relief. The animal was placed in a warmed cage for recovery and returned to the regular housing cage when mobile. Warmed fluids (2–3 ml) were given subcutaneously to ensure adequate hydration. Analgesics (bupivacaine, up to 2 mg kg<sup>-1</sup> given intradermally or topically on the incision site) were administered to alleviate pain during the post-operative period (3 days). MNV-1 was inoculated on the fifth day after the procedure and the animal monitored for viral replication for 10 days; animals were killed at the intervals mentioned earlier. Age-matched control groups without salivectomy were used to compare the effect of the surgical removal of SGs on MNV-1 infection.

**Saliva collection and infection.** Six-week-old female BALB/c mice were inoculated with EDIM or MNV-1 (five mice per experiment, three independent experiments) and the persistent strains WU23, MNV-3 and MNV-4 (five mice per experiment, three independent experiments). Two hours before saliva collection animals were deprived of food and water to avoid contamination in saliva during further processing. Isoflurane (1–4%) or sevoflurane (2–6%) delivered through a gas anaesthesia machine vaporizer with an appropriate anaesthetic gas scavenging system was used to anaesthetize the animals before saliva collection; 2% pilocarpine (national drug code: 61314-206-15) was injected intraperitoneally to stimulate salivation, followed by a 2-min wait. A cotton swab was inserted into the oral cavity over a 10 min period and then the wet swab was placed in a small hole punctured at the bottom of a 0.6-ml uncapped microfuge tube, which was placed further into a 2-ml tube. Then saliva was recovered by centrifugation for 2 min at 7,500g at 4 °C. As soon as mice recovered, they were returned to cages; saliva was similarly collected at 3 and 4 dpi. For immunoblotting, 40  $\mu$ l saliva was aliquoted for all the time points from each individual mouse; the rest of the saliva was pulled into a single tube to infect the pups. To test the infectivity of saliva collected from EDIM- or MNV-1-infected adult mice, 10-day-old pups were infected with a volume of 100  $\mu$ l saliva followed



by collection of the small intestines at different time points. RNA was isolated from these tissues to quantify virus replication.

### RNA isolation

The SGs and small intestines from 6-week-old adult mice and 10-day-old pups and mammary glands from dams extracted for the various experiments used in the study were first homogenized in DNA/RNA shield buffer provided with the Quick-RNA Miniprep Plus Kit (catalogue no. R2001; Zymo Research) supplemented with proteinase. Homogenized tissues were further digested in the buffer for 5 h at 55 °C and centrifuged at 5,000 r.p.m. for 5 min; RNA lysis buffer was added to the pellet and the pellet processed according to the manufacturer's instructions. RNA was eluted in RNase/DNase-free water provided in the kit. For the salispheres, after the incubation time points mentioned earlier, cells were spun down at 1,000 r.p.m. for 5 min, resuspended in PBS for washing and spun down at 1,000 r.p.m. for 5 min. The pellet was resuspended in 200 µl of RNA lysis buffer provided in Quick-RNA Microprep Plus Kit (catalogue no. R1050; Zymo Research) and processed for RNA isolation according to the manufacturer's instructions. Similarly, after incubation, NS-SV-TT-DC and NS-SV-TT-AC cells were collected and resuspended in 200 µl of RNA lysis buffer from the Quick-RNA Microprep Plus Kit and processed to isolate RNA.

### qPCR

**qPCR by SYBR Green.** For all animal tissue samples (Figs. 1–3 and Extended Data Figs. 1a,b,d and 2) RNA was isolated as mentioned earlier and cDNA was prepared using the Maxima First Strand cDNA Synthesis Kit for qPCR (catalogue no. K1672; Thermo Fisher Scientific). The primers used for EDIM, MNV-1, murine astrovirus, WU23, MNV-3, MNV-4 and CR6 are listed in Supplementary Table 2. cDNA obtained from the samples was run along with the corresponding primers and with SYBR Green (catalogue no. 1725124; Bio-Rad Laboratories) in the Roche LightCycler 96 System (catalogue no. 05815916001). The thermal cycling conditions included a pre-incubation step 95 °C for 90 s followed by 45 cycles at 95 °C for 10 s, 54 °C for 10 s and 72 °C for 110 s. Each sample were run in duplicate for each experiment. For EDIM, MNV-1, WU23, MNV-3, MNV-4 and CR6, standards were run along with each experiment to measure virus genome copies per milligram of tissue. A detailed description of the standard curve preparation for each virus is given in the section 'Standard curve preparation for EDIM, MNV-1, CR6, WU23, MNV-3, MNV-4'.

For murine astrovirus (Extended Data Fig. 1d), samples were analysed with the *Gapdh* housekeeping gene (Supplementary Table 2) along with a mock-infected sample. The fold change obtained was measured over the mock-normalized by cycle threshold (Ct) values obtained for the housekeeping gene.

**Standard curve preparation for EDIM, MNV-1, CR6, WU23, MNV-3 and MNV-4.** Amplicons for each primer set were synthesized by Integrated DNA Technologies; on the basis of the amount and length of amplicons, the number of copies (molecules) was calculated for each primer set:

$$\text{Number of copies (molecules)} = \frac{X \text{ ng} \times 6.0221 \times 10^{23}}{\left(\frac{N}{2}\right) \times 660 \text{ g mol}^{-1} \times 10^9 \text{ ng g}^{-1}}$$

where  $X$  is the amount of amplicons in nanograms,  $N$  is the length of the dsDNA amplicon,  $660 \text{ g mol}^{-1}$  is the average mass of 1 bp dsDNA and  $6.0221 \times 10^{23}$  is Avogadro's number.

Amplicons were resuspended in 1 ml of DNase/RNase-free water to obtain the copy number per millilitre for each primer set. Thereafter, each amplicon copy number per millilitre was serially diluted from 10 to  $10^{10}$  in tenfold increments and subjected to qPCR to obtain the

standard curves for each individual set of primers. The initial calculation of genome copies per millilitre indicated the number of viral genomes per millilitre based on standard curves that quantified viral genomes per millilitre of 10 µl of reaction volume used to run the qPCR. This viral genomes per millilitre unit is a standard unit of qPCR calculation used to quantify the total number of identified RNA copy numbers per millilitre of reaction volume in PCR. For tissues, we additionally included the weight of the tissues used to extract the RNA to make it genome copies per milligram of tissue, which signifies the estimate of viral genome copies in 1 mg of tissue. The Ct values obtained were plotted against the log of copy numbers per millilitre of each to derive the standard curve, which corresponds to the linear equation and  $R^2$  value. On the basis of the standard curve Ct values obtained from unknown samples, the copy number per millilitre was obtained for EDIM, MNV-1, CR6, WU23, MNV-3 and MNV-4:

$$\text{EDIM copy number per ml} = (-0.3278 \times \text{Ct}) + 13.288, R^2 = 0.99$$

$$\text{MNV1 copy number per ml} = (-0.3583 \times \text{Ct}) + 13.975, R^2 = 0.97$$

$$\text{CR6 copy number per ml} = (-0.4956 \times \text{Ct}) + 17.225, R^2 = 0.93$$

$$\text{WU23 copy number per ml} = (-0.46586 \times \text{Ct}) + 15.211, R^2 = 0.95$$

$$\text{MNV3 or MNV4 copy number per ml} = (-0.5919 \times \text{Ct}) + 16.114, R^2 = 0.97$$

Please refer to Supplementary Table 3 for the amplicons and standards.

**LOD for qPCR.** qPCR was performed on tissues (salivary, mammary, intestine) of both mock-inoculated (with PBS) and virus-inoculated (EDIM, MNV-1, MNV-3, MNV-4, WU23) animals, the latter at 6 hpi (input time point). The Ct values obtained were converted to genome copies per milligram of tissue using the standard curves for the corresponding primers. The copies per milligram of tissue for mock-infected animals ranged from 124 (upper limit) to 22 (lower limit) (mean = 78, s.e.m. = 9). This probably corresponds to false positive signals arising from non-specific binding of primers. The copies per milligram of tissue obtained from virus-inoculated animals (6 hpi) also had a similar range. Thus, the mean  $78 \pm 9$  (s.e.m) was taken as the LOD, above which qPCR-based viral RNA detection by a particular primer was deemed to be positive for the corresponding virus.

For HuNoV, qPCR was performed following two methods: (1) with SYBR Green and several GII primers probing for various regions of the whole genome (Fig. 4g,h) and (2) by using GII.4 primers with TaqMan probe (Supplementary Table 2). (1) RNA from AQ15 HuNoV-infected NS-SV-TT-DC cells was isolated as mentioned in the 'RNA isolation' section and cDNA was prepared using the Maxima First Strand cDNA Synthesis Kit for qPCR. cDNA derived from the samples was run along with the GII primers and SYBR Green in the PCR system. The GII whole genome (Supplementary Table 3) was run along with the samples as a standard because the primers recognize several regions of the whole genome. A standard curve was prepared in a similar way with the GII whole genome as outlined for the murine rotavirus and norovirus. GII copy number per millilitre was  $(-3.62 \times \text{Ct}) + 46.206, R^2 = 0.89$ . The Ct values obtained from running the samples were used to measure HuNoV genome copies per millileter in cell lysate or supernatant (Fig. 4g,h) using the standard curve obtained. (2) For the TaqMan probe, RNA isolation from cells inoculated with GII.4-77.1 (Fig. 4j) was performed similarly using the Quick-RNA Microprep Plus Kit. RNA was subjected to one-step qPCR by kit (TaqMan Fast Virus 1-Step Master Mix, catalogue no. 4444432; Thermo Fisher Scientific) using a TaqMan probe. The sequences of primers and probes are given in the Supplementary Tables 2 and 3. qPCR was performed by reverse transcription at 52 °C for 10 min, RT inactivation at 95 °C for 20 s and initiation of denaturation

## Article

followed by 45 cycles of amplification at 95 °C for 15 s, 60 °C for 60 s. (3) The standard for the GII Quant Primer amplicon was run alongside each experiment as stated in the previous section and the HuNoV copy number per millilitre was calculated based on the plotted standard curve. Information about primers, probes and amplicons is found in Supplementary Tables 2 and 3. The GII.4 copy number per millilitre was  $(-0.2673 \times Ct) + 13.453$ ,  $R^2 = 0.97$ .

For the *Cd300lf* analysis by qPCR (Fig. 3k and Extended Data Fig. 4a), cDNA was prepared similarly to the RNA isolated by the Quick-RNA Microprep Plus Kit from CD45<sup>+</sup>, EpCAM<sup>+</sup> RAW264.7 and HeLa cells (for the *Cd300lf* experiment) and processed for qPCR using the primers listed in Supplementary Table 2. The Ct value was obtained for each cell type and calculated over RAW264.7 as positive control by the dCt method. HeLa cells were used as the negative control because *Cd300lf* was undetectable in the analysis with the *Cd300lf* primer.

Murine norovirus and rotavirus replication in salispheres were also studied by qPCR (Fig. 4b and Extended Data Fig. 4c). RNA was isolated as mentioned in the 'RNA isolation' section from salispheres and cDNA was prepared in a similar manner to that used for the tissue samples. The cDNA obtained was run using the corresponding primers of EDIM, MNV-1 and CR6 (Supplementary Table 2) along with SYBR Green. The Ct values obtained were analysed along the standard curves obtained for each virus as mentioned in the 'Standard curve preparation for EDIM, MNV-1, CR6, WU23, MNV-3 and MNV-4' section and the viral genome copy number per millilitre was obtained for each sample.

### Immunofluorescence labelling

SMGs or mammary glands were collected from euthanized adult mice or pups. Samples were then fixed overnight at 4 °C in 4% PFA (catalogue no. 15710; Electron Microscopy Sciences). The fixed samples were further processed at the NHLBI Pathology Core: samples were incubated for 24 h in 30% sucrose solution at 4 °C and embedded in optimal cutting temperature compound before freeze sectioning on a microtome (Leica Cryo-start, catalogue no. CM3050S; Leica Biosystems). For all immunofluorescence staining, antigen retrieval was performed by incubating slides in Universal HIER antigen retrieval reagent (catalogue no. ab208572; Abcam) in a boiling water bath for 20 min. Samples were then cooled and tissue sections were permeabilized for 2 h at room temperature with 0.1% Triton X100 (catalogue no. T9284; Sigma-Aldrich) in DPBS (catalogue no. 14190-144; Thermo Fisher Scientific) supplemented with 10% FCS. Then samples were incubated overnight at 4 °C in primary antibodies diluted in DPBS supplemented with 10% FBS. Next, samples were rinsed three times with DPBS and incubated for 2 h at room temperature in fluorescently labelled secondary antibodies diluted in DPBS supplemented with 10% FCS. Finally, samples were rinsed three times with DPBS and mounted using Fluoromount-G containing 4',6-diamidino-2-phenylindole (DAPI) stain (catalogue no. 17984-24; Electron Microscopy Sciences). Imaging was performed on a Zeiss LSM 780 confocal laser scanning microscope and analysed with Zen Blue software (Zeiss Zen 3.1 Blue edition). After the incubation period, NS-SV-TT-DC cells were fixed with 4% PFA at room temperature for 15 min. Thereafter, PFA was removed and washed with PBS three times. Cells were then blocked with BSA supplemented with the permeabilization agent saponin for 1 h. Cells were then incubated with anti-VP1 for 1 h followed by washes and adding appropriate secondary antibody for 1 h. All incubations were done at room temperature. After the final washes, cells were mounted by mounting agent with DAPI and imaged as mentioned earlier. Details of the antibodies are listed in Supplementary Table 1.

### Immunoblots

All uncropped/unprocessed blots are supplied in Supplementary Fig. 1.

**Saliva.** For immunoblotting, 40 µl of saliva from uninfected and 2, 3 and 4 dpi samples were directly cooked in 4× Laemmli sample buffer

(catalogue no. 161-0747; Bio-Rad Laboratories); cultured cells were lysed and then centrifuged, and the collected supernatants were mixed with 4× Laemmli sample buffer. SDS-PAGE was carried out in all samples using 10% SDS-polyacrylamide gel electrophoresis (catalogue no. 4561034; Bio-Rad Laboratories) and the sample transferred to a nitrocellulose membrane (catalogue no. 1704159; Bio-Rad Laboratories). Then the membranes were blocked in 5% bovine serum albumin (BSA) (catalogue no. BP1600-100; Thermo Fisher Scientific) in Tris-buffered saline buffer (catalogue no. 1706435; Bio-Rad Laboratories) containing Tween-20 (TBST) (catalogue no. 9005-64-5; Affymetrix) for 1 h at room temperature. Thereafter, membranes were incubated overnight with primary antibodies for EDIM VP6 (gift from J. Patton, Indiana University) and MNV-1 VP1 (gift from K. Green, NIAID). Membranes were washed five times with TBST and probed with specific horseradish peroxidase-tagged secondary antibodies for 1 h. This was followed by washing steps and development with Lumigen ECL Ultra (TMA-6) (catalogue no. TMA-100; Lumigen). Blots were imaged using the Amersham Imager 600 (catalogue no. 29083461; GE Healthcare). Details of the antibodies are listed in Supplementary Table 1.

**Salisphere culture.** After incubating for 48 h with MNV-1 or CR6, cells were spun down at 1,000 r.p.m. for 5 min; the collected pellet was resuspended in 100 µl of lysis buffer (catalogue no. 559759; BD Biosciences) supplemented with protease inhibitor (catalogue no. A32955; Invitrogen) and subjected to trichloroacetic acid (TCA) (catalogue no. T6399-100G; Sigma-Aldrich) precipitation (1:4 vol/vol, 15 min at 4 °C). The TCA precipitate was washed twice with acetone and air-dried. Samples were dissolved in 4× Laemmli sample buffer and run in a 4–20% gradient gel (catalogue no. 4561094; Bio-Rad Laboratories). Transfer and blocking were carried out as mentioned earlier. Blots were probed against anti-VP1 and GAPDH was used as the loading control.

**NS-SV-TT-DC and NS-SV-TT-AC cell lines.** After the incubation period was over, cells were collected and subjected to TCA precipitation as stated in the 'Salisphere culture' section. Samples were run in 4–20% gradient gels and transferred to a nitrocellulose membrane for probing against anti-HuNoV NS7 and VP1 and to a polyvinylidene fluoride membrane (catalogue no. 1704157; Bio-Rad Laboratories) for anti-HuNoV NS6 (all antibodies were a gift from K. Green, NIAID). β-Actin was used as the loading control. The rest of the procedure was same as mentioned in the 'Salisphere culture' section. Densitometry to analyse the expression levels of all proteins was done using ImageJ software (bundled with 64-bit Java, 1.8.0\_172; National Institutes of Health). The measure intensities for the virus-infected samples were normalized over mock and over the corresponding loading control and represented on a graph.

### Secretory IgA measurement

Small intestine content was extracted from mouse pups and the amount was measured and made into a 10% solution in PBS; 100 µl solution was directly transferred to an antibody-coated 96-well format of ELISA Kit for Secretory Immunoglobulin A (sIgA) (catalogue no. SEA641Mu; Cloud-Clone Corporation) and measurements were carried out according to the manufacturer's instructions. Similarly, milk collected in 1 ml tubes was diluted to a 10% solution in PBS and processed for sIgA measurement with the kit. The concentrations for the samples were measured from the standard curve plotted using the standards provided in the kit in micrograms per millilitre.

### Cell sorting from SMGs

SMGs were extracted from animals after euthanization and homogenized in ice-cold PBS (supplemented with 10% FCS). The homogenate was centrifuged at 1,000 r.p.m. for 5 min at 4 °C to pellet down cells; the homogenate was further incubated for 20 min at 37 °C in 3 ml of Gentle Collagenase/Hyaluronidase solution (catalogue no. 07919; Stemcell

Technologies) with shaking. Thereafter, the solution was centrifuged again at 1,000 r.p.m. for 5 min at 4 °C to collect the pellet and discard the supernatant. The pellet was further trypsin-treated for 5 min at 37 °C and passed through a 70-µm filter to eliminate undissociated tissue. The filtrate was then treated with a 4:1 NH<sub>4</sub>Cl:PBS solution to eliminate blood cells and subjected to centrifugation at 1,000 r.p.m. for 5 min at 4 °C. After leaving the red layer of cells at bottom, the supernatant consisted of single cells isolated from tissue. Cells were counted and incubated with anti-EpCAM conjugated to allophycocyanin (catalogue No. 17-5791-82; Thermo Fisher Scientific) and anti-CD45 conjugated to phycoerythrin (catalogue no. 12-0451-82; Thermo Fisher Scientific) for 1 h at 4 °C. Cells were subsequently washed and stained with LIVE/DEAD Fixable Aqua Dead cell stain (catalogue no. L34957; Thermo Fisher Scientific). Resuspended cells were sorted on a FACS ARIA III (BD Biosciences) cell sorter equipped with 355 nM, 407 nM, 488 nM, 532 nM and 640 nM laser lines using the FACSDiva v.8.1 software at 70 psi pressure using a 70 µm nozzle. Debris was removed based on scattering properties using forward scatter and side scatter parameters. Live-gated cells were purified for leukocytes identified as CD45<sup>+</sup> EpCAM<sup>-</sup> live cells whereas CD45<sup>-</sup> EpCAM<sup>+</sup> cells were identified as epithelial cells (Extended Data Fig. 3). Purified populations were collected in 10% PBS supplemented with FCS for downstream analysis either by TCID<sub>50</sub> or qPCR.

### TCID<sub>50</sub> calculation

Saliva, SMGs from pups, proximal colon from adults, cells obtained from single-cell sorting from SMGs and supernatant from cultures inoculated with various murine norovirus strains were subjected to TCID<sub>50</sub> analysis for viral titre calculation. The collection and processing methods for each sample were mentioned earlier. In brief, samples were added to RAW264.7 cells microplated on 96-well plates (3 × 10<sup>4</sup> cells per well) and serially diluted in DMEM by tenfold from 10<sup>0</sup> to 10<sup>-10</sup>. Plates were incubated until CPE was observed (day 7). Supernatant was removed and stained with crystal violet (catalogue no. HT901; Sigma-Aldrich) for 10 min. After rinsing with water twice, CPE wells were counted. TCID<sub>50</sub> per millilitre was calculated as per refs.<sup>39,40</sup>. The LOD was determined as the lowest concentration of virus where at least 50% CPE was observed. The detailed calculations are supplied in the source files for the corresponding figures.

### FISH

The assay was performed using RNAscope (Advanced Cell Diagnostics) Multiplex Fluorescent Detection Kit v2 (catalogue no. 323110) and RNAscope probes custom-synthesized to detect negative sense human norovirus RNA gene region 5349–6428 of norovirus GII strain Hu/USA/2015/GII.P16-GII.4 Sydney/Pasadena. Cells adhered to coverslips were fixed 96 hpi in 4% PFA for 30 min. Pretreatment of the samples was performed by serially dehydrating the samples with ethanol followed by permeabilization with RNAscope protease III (catalogue no. 322340) at 1:5 dilution in PBS for 30 min at room temperature before hybridizing for 2 h at 40 °C with the FISH probe. The amplification and dye labelling steps were performed according to the RNAscope v2 assay protocol. Opal 570 dye (catalogue no. FP1488001KT; Akoya Biosciences) at 1:1,500 dilution in RNAscope TSA buffer (catalogue no. 322810) was used to visualize the FISH staining. Coverslips were mounted with Fluoromount-G mounting medium with DAPI (catalogue no. 00-4959-52; Thermo Fisher Scientific) and imaging was done using a Zeiss LSM 780 confocal microscope.

### Statistics and reproducibility

Prism v.8 (GraphPad Software) was used for the statistical analysis. Error bars denote the s.e.m. in all figures. All statistical tests were done by two-tailed unpaired *t*-test. The number of experiments performed including the number of animals involved in each experiment are stated

in the corresponding to figure legends. Statistical information can be found in Supplementary Table 4.

**Reproducibility of micrographs.** In Fig. 1g–n, representative images were obtained from five independent experiments for each virus infections (EDIM or MNV-1). Two mammary glands per animals were extracted from a total of five animals per virus and subjected to tissue sectioning and immunofluorescence analysis.

In Fig. 3c–f,i,j and Extended Data Fig. 1e,f, representative images were obtained from four independent experiments for each virus infections (EDIM or MNV-1). SMGs from a total of four pups per virus were subjected to tissue sectioning and immunofluorescence analysis.

In Fig. 4l,m, representative images were obtained from six biologically independent experiments from NS-SV-TT-DC and NS-SV-TT-AC infection with vesicle-cloaked HuNoV. After the incubation period, fixed cells were subjected to FISH analysis.

The scale bars for each micrograph panel are stated in the individual figure panels.

### Reporting summary

Further information on research design is available in the Nature Research Reporting Summary linked to this paper.

### Data availability

The data generated in this study are provided as source data files corresponding to each figure. All statistics performed and the exact *P* values are presented in Supplementary Table 4. Primary data associated with figures are available in the source files. Any additional datasets generated and/or analysed are available from the corresponding author on reasonable request.

- Sosnovtsev, S. V. et al. Cleavage map and proteolytic processing of the murine norovirus nonstructural polyprotein in infected cells. *J. Virol.* **80**, 7816–7831 (2006).
- Compton, S. R., Booth, C. J. & Macy, J. D. Murine astrovirus infection and transmission in neonatal CD1 mice. *J. Am. Assoc. Lab. Anim. Sci.* **56**, 402–411 (2017).
- Willingham, K. et al. Milk collection methods for mice and Reeves' muntjac deer. *J. Vis. Exp.* **89**, 51007 (2014).
- Ghosh, S. et al. β-Coronaviruses use lysosomes for egress instead of the biosynthetic secretory pathway. *Cell* **183**, 1520–1535 (2020).
- Reed, L. J. & Muench, H. A simple method of estimating fifty per cent endpoints. *Am. J. Hyg.* **27**, 493–497 (1938).

**Acknowledgements** We thank M. Baldridge (Washington University in St Louis), S. Karst (University of Florida), C. Wobus (University of Michigan) and K. Green (NIAID, National Institutes of Health (NIH)) for providing the murine norovirus strains; S. Compton (Yale University) for providing the murine astrovirus; H. Greenberg (Stanford University) for providing the EDIM; C. B. Wilen (Yale University) for providing the Cd300lf<sup>-/-</sup> mice; P. Matzinger (NIAID), C. B. Wilen, S. Compton, C. Wobus, K. Green, B. Kelsall (NIAID), G. Parra (FDA) and G. Altan-Bonnet (National Cancer Institute) for discussion and critical reading of the manuscript; H. Vishwasrao and H. Shroff (Advanced Imaging and Microscopy Resource, National Institute of Biomedical Imaging and Bioengineering (NIBIB), NIH) for support with imaging the SGs; the NHLBI flow cytometry, pathology and animal cores for technical support; and A. Hoofring (NIH Medical Arts) for the illustrations. N.A.-B., S.G., M.K., M.Z., H.L. and M.S. were supported by the NHLBI and NIH. E.L. and Y.B. were supported by NIAID. Y.S. was supported by the NIBIB. M.P.H., J.A.C., H.N. and T.T. were supported by National Institute of Dental and Craniofacial Research (NIDCR)/NIH intramural funds. J.A.C. was also supported by an NIDCR/NIH intramural grant no. 1ZIAD000695.

**Author contributions** S.G. and N.A.-B. designed the experiments and wrote the manuscript. S.G., M.K., M.S., A.M., M.Z., H.L., Y.S., E.L., H.N., T.T. and A.M.C. performed the experiments; W.H., O.V., Y.B., J.A.C. and M.P.H. provided critical reagents and contributed to the design of experiments.

**Competing interests** The authors declare no competing interests.

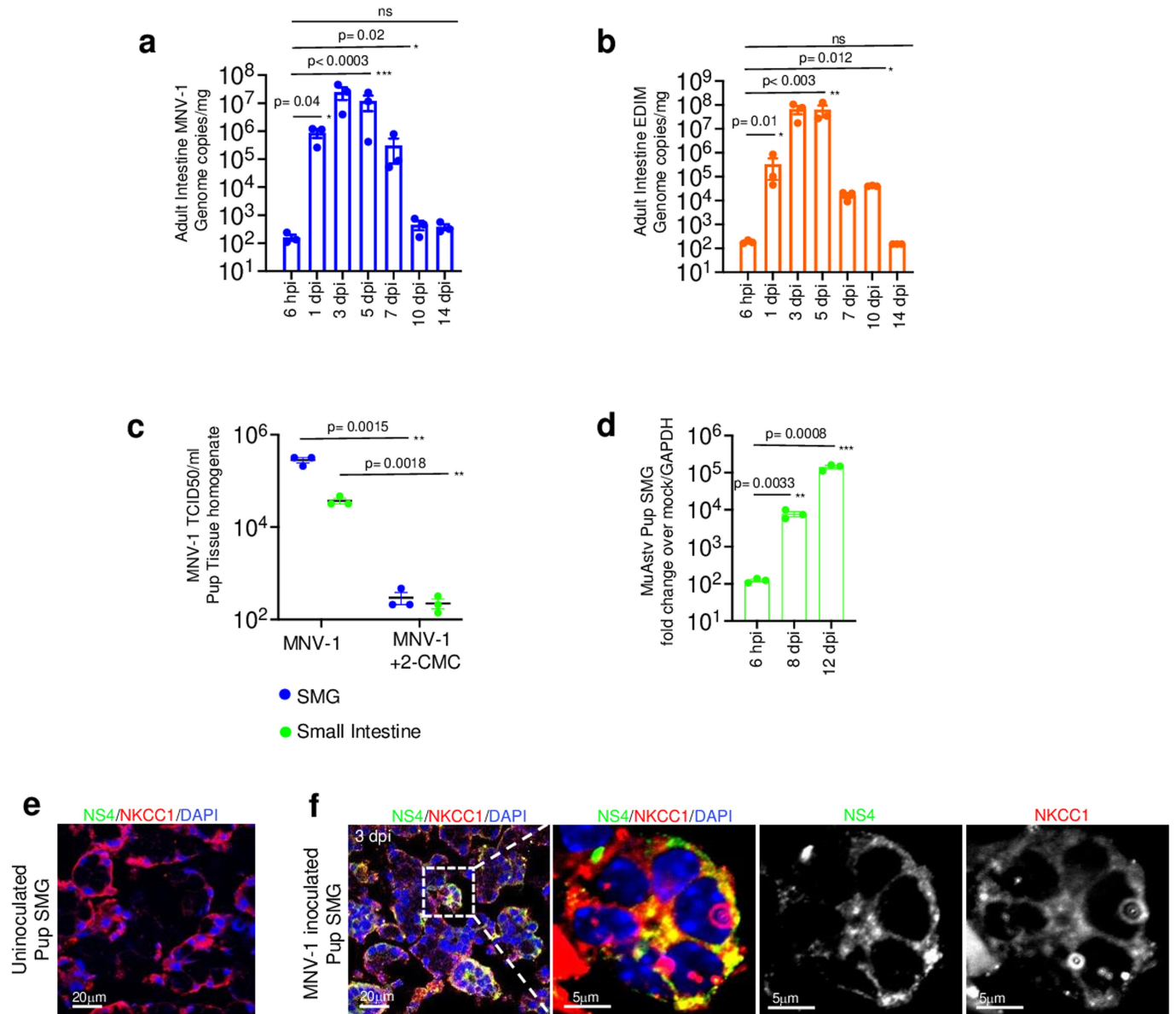
### Additional information

**Supplementary information** The online version contains supplementary material available at <https://doi.org/10.1038/s41586-022-04895-8>.

**Correspondence and requests for materials** should be addressed to N. Altan-Bonnet.

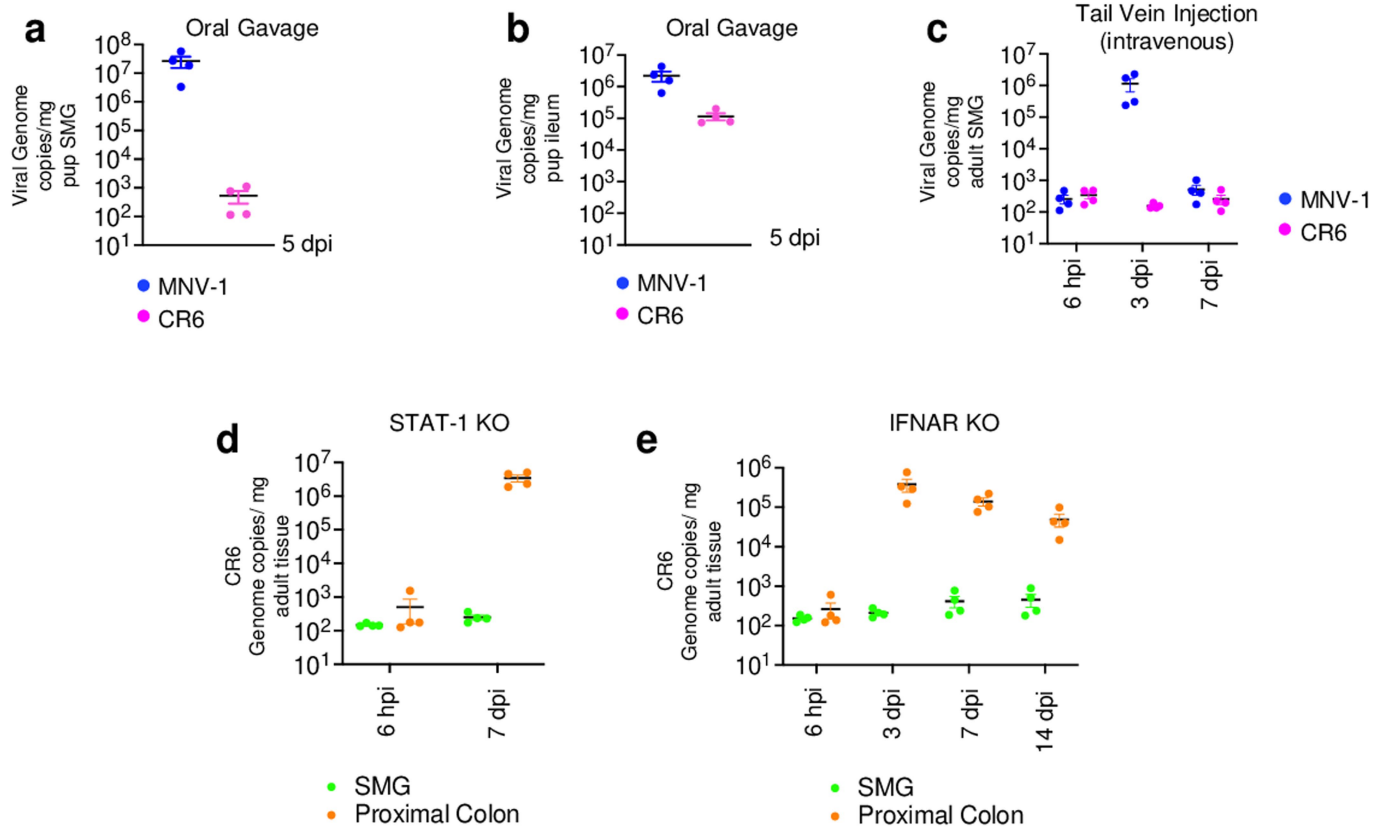
**Peer review information** Nature thanks Ian Goodfellow and the other, anonymous, reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

**Reprints and permissions information** is available at <http://www.nature.com/reprints>.



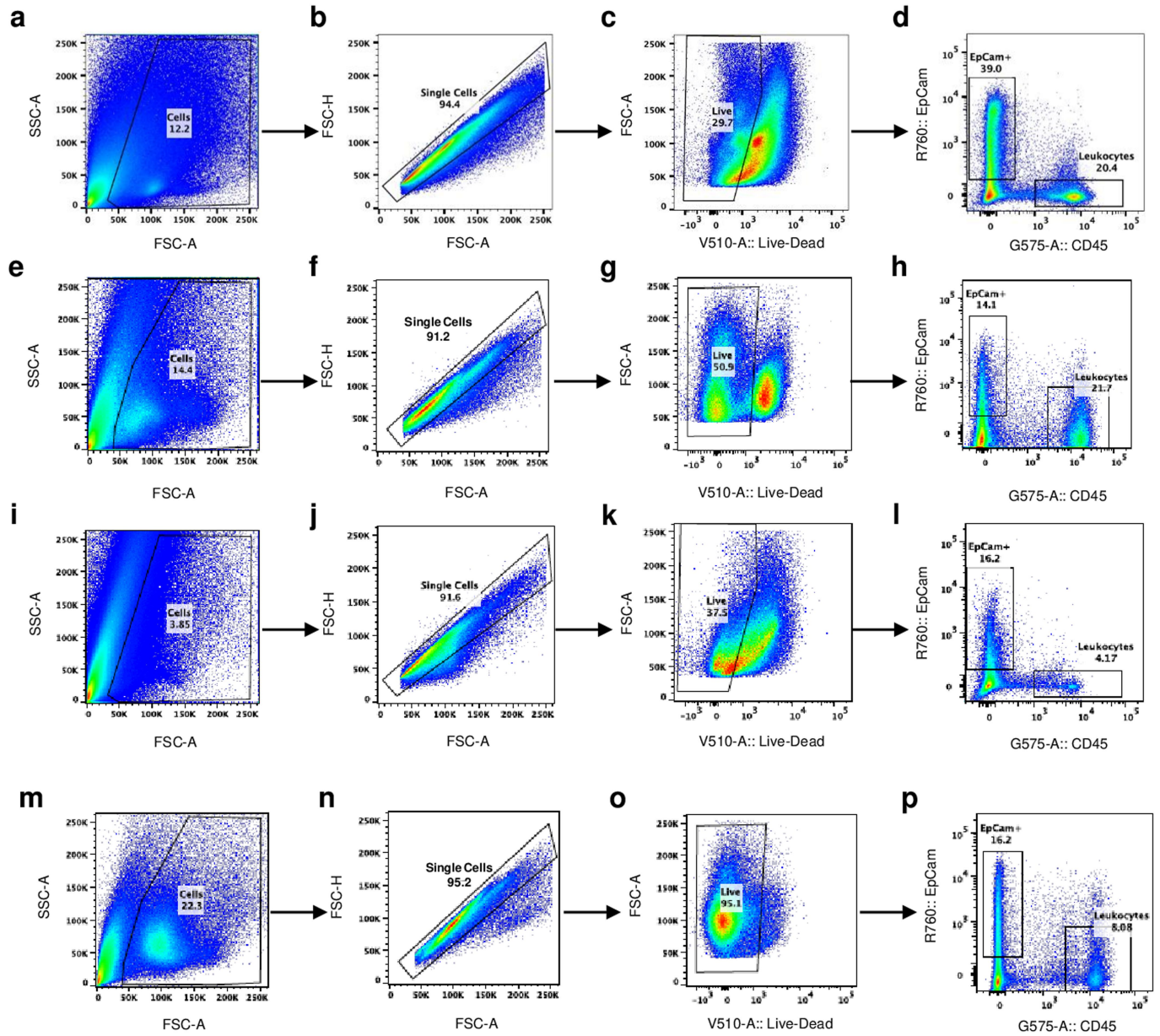
**Extended Data Fig. 1 | MNV-1, EDIM and Astrovirus replication in SMGs and Intestine.** **a**, MNV-1, and **b**, EDIM replication in adult intestines (from 3 independent experiments, each dot represents 1 animal, total 7 animals/experiment). Bar graphs are mean ± Standard Error of Mean (SEM). **c**, MNV-1 titers in SMGs and small intestine isolated from inoculated pups, +/- 2-CMC treatment (from 3 independent experiments, each dot represents an experiment, 3 animals/experiment). 3 animals/experiment were only treated with 2-CMC as a negative control. Dot plots are mean ± SEM. **d**, Murine Astrovirus genome copies in SMGs of inoculated pups (from 3 independent

experiments, total 3 animals/experiment). GAPDH was used as control for normalization. Bar graphs are mean ± SEM. **e**, **f** Immunostaining of SMGs obtained from uninoculated, and MNV-1 inoculated pups with anti-NS4 and anti-NKCC1. Depicted are representative images obtained from 4 independent experiments (total 4 pups). All statistical tests were performed by two-tailed unpaired t-test. Statistical information is in Supplementary Table 4. All micrograph experiment reproducibility information is in *Statistics and Reproducibility in Methods*. Input for **a**, **b**, **d** is 6 hpi. **c**, The LOD for TCID50 ml<sup>-1</sup> is 10<sup>2</sup>; **a**, **b** LOD for qPCR is 78 ± 9 (s.e.m.) genome copies mg<sup>-1</sup> tissue.



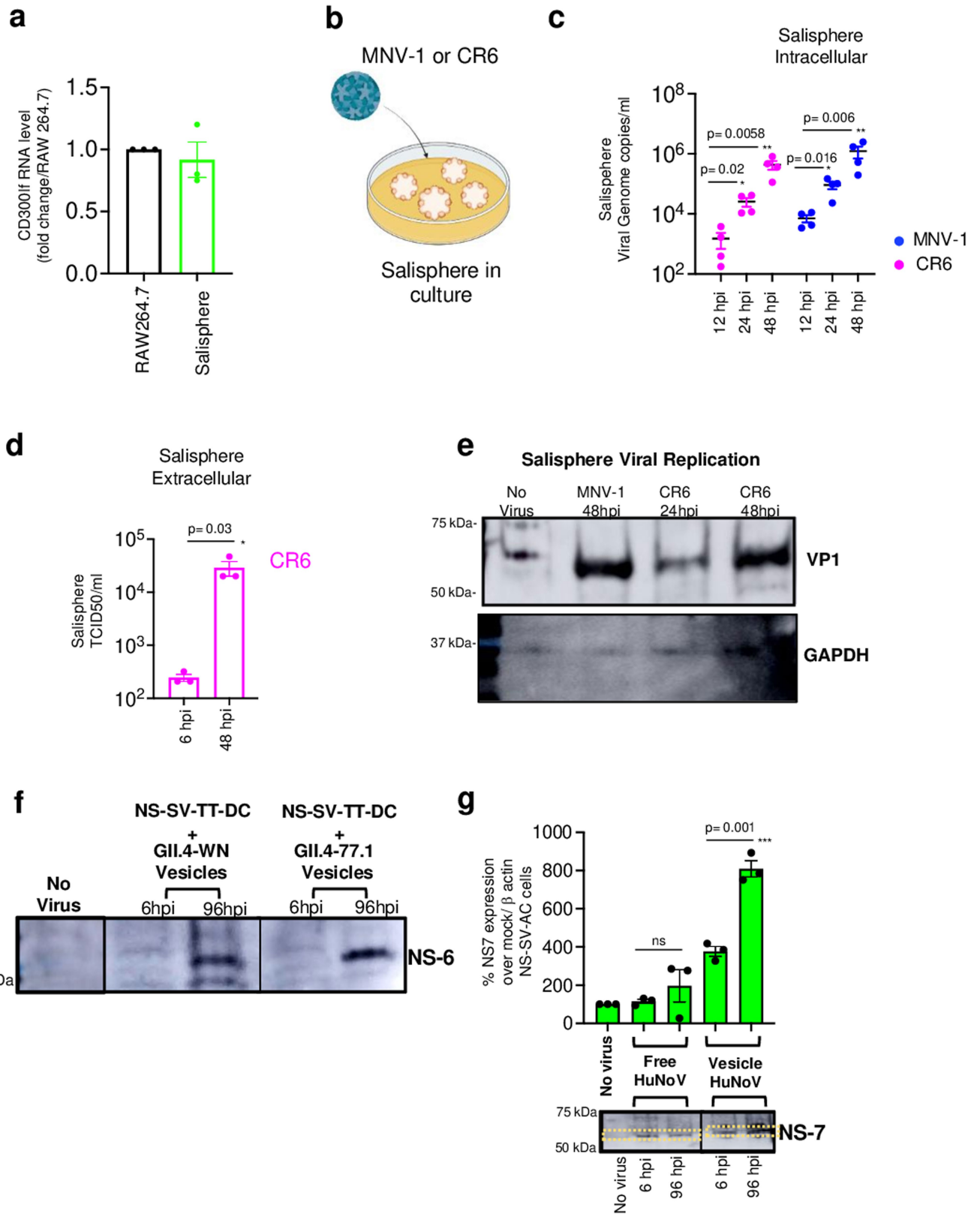
**Extended Data Fig. 2 | Investigating SMG infection of CR6.** MNV-1 and CR6 viral genome copies were measured in **a**, SMG and **b**, ileum of orally inoculated mice (n = 2, with 2 animals/experiment, each data point represents data from 1 animal. Data are mean ± SEM). Similarly in **c**, viral genome copies for MNV-1 and CR6 were measured in SMGs after tail vein injection of each virus (n = 4, with 3 animals/experiment each dot represents data from 1 animal. Data are

mean ± SEM). CR6 genome copies were measured in **d**, STAT-1, and **e**, IFNAR knockout mice. In both experiments animals were orally gavaged and SMG and proximal colon tissues monitored for replication. For **d**, n = 2 with 2 animals/experiment and **e**, n = 4 with 4 animals/experiment, each dot represents data from 1 animal. Data are mean ± SEM. Input for **c**, **d**, **e** is 6 hpi. **a-e**, The LOD for qPCR data is 78 ± 9 (s.e.m) genome copies mg<sup>-1</sup> tissue.



**Extended Data Fig. 3 | Gating strategies for cell sorting.** Panels **a–d**, **e–h**, **i–l** and **m–p** represents gating strategies for Fig. 3a, b, h and k. Briefly, **a** panel represents the gating for cells, eliminating the cell debris followed by single cell selection in **b**, eliminating doublets. Panel **c** represents gating strategy for live/

dead cells. Live cells selected from **c**, was subjected to cell sorting based on EpCam+ and CD45+ cells in **d**. Sorted cells obtained from **d** was analyzed in Fig. 3a. The same chronology is maintained for panels: **e–h**, **i–l** and **m–p**.



Extended Data Fig. 4 | See next page for caption.

# Article

**Extended Data Fig. 4 | Ex-vivo replication of MNV-1, CR6 and human norovirus.** **a**, CD300lf expression in uninoculated salispheres (from 3 independent). RAW246.7 cells were used as positive control for comparison. Bar graph is mean  $\pm$  SEM. **b**, Schematic of MNV-1/CR6 inoculation into salisphere culture. **c**, CR6 and MNV-1 replication in salispheres (from 4 independent experiments represented by a dot). Genome copies/ml was calculated based on standard curve plotted on log scale. Dot plots are mean  $\pm$  SEM. **d**, Supernatants collected from CR6 inoculated salispheres (3 independent experiments, each dot represents an experiment) and analyzed by TCID50 and plotted on log scale. Bar graphs are mean  $\pm$  SEM. **e**, Immunoblots of lysates from MNV-1 (48hpi) and CR6 (24 hpi & 48 hpi) inoculated salisphere cultures probed against VP-1 and GAPDH (blot representative of 2 independent experiments). **f**, Lysates from NS-SV-TT-DC cultures inoculated with vesicle-cloaked human noroviruses from

two separate isolates (GII.4-WN and GII.4-77.1) for 6 hpi and 96 hpi (but washed at 6 hpi) were probed with anti-NS6 antibody (from 3 independent experiments). **g**, Lysates from NS-SV-TT-AC cultures inoculated with vesicle-cloaked human noroviruses from isolate (GII.4-77.1) for 6 hpi and 96 hpi (but washed at 6 hpi) were probed with anti-NS7 and anti- $\beta$  actin (from 3 independent experiments represented by each dot) and analyzed by densitometry (dashed line box) and plotted as a bar graph; mean  $\pm$  SEM. Samples were derived from the same experiment and blots were processed in parallel. Statistical test was performed using two-tailed unpaired t-test between 6 hpi and 96 hpi for each inoculum groups. Statistical information is in Supplementary Table 4. For gel source data, see Supplementary Figure 1. Input for **d**, **g** is 6 hpi and **c** is 12 hpi. **c**, The LOD for qPCR data from salisphere was  $10^2$  genome copies  $\text{ml}^{-1}$ . **d**, The LOD for TCID50  $\text{ml}^{-1}$  was approximately  $2 \times 10^2$ .



## Reporting Summary

Nature Portfolio 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 Portfolio 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  
*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  
*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 was used for open source data collection

Data analysis Zen Blue (Zeiss Zenn 3.1 Blue edition) from Carl Zeiss was used for image analysis for images obtained with the Zeiss LSM780 confocal microscope. GraphpadPrism 8 software was used for plotting graphs and statistical analysis. Densitometry was carried out with GE Amersham Imager 600 software. Flow cytometry data was analyzed with FACSDIVA 8.1 software.

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 Portfolio [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 description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our [policy](#)

The authors declare that all data reported in this study are available within the paper and its supplementary information files.

## 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](https://www.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.

Sample size	All experiments were performed minimum 3 times for statistical significance. Total number of animals included in each experiment is mentioned in corresponding figure legends.
Data exclusions	No data was excluded
Replication	All experiments were replicated at least 3 independent times.
Randomization	Animals were chosen from a certain age-group that has been established as models for infection in lab. The infection model is unbiased for sex of animals used.
Blinding	Experiments and data analysis were independently performed by authors and cross- checked for conclusive interpretation.

## 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	Methods
n/a	n/a
<input checked="" type="checkbox"/> Involved in the study	<input checked="" type="checkbox"/> Involved in the study
<input checked="" type="checkbox"/> Antibodies	<input type="checkbox"/> ChIP-seq
<input checked="" type="checkbox"/> Eukaryotic cell lines	<input checked="" type="checkbox"/> Flow cytometry
N/A <input type="checkbox"/> Palaeontology and archaeology	N/A <input type="checkbox"/> MRI-based neuroimaging
<input checked="" type="checkbox"/> Animals and other organisms	
N/A <input type="checkbox"/> Human research participants	
N/A <input type="checkbox"/> Clinical data	
N/A <input type="checkbox"/> Dual use research of concern	

## Antibodies

Antibodies used	Antibody Catalogue Number Company Syndecan-1 ab34164 Abcam (MA, USA) EDIM NSP5 EDIM VP6 Gift from John Patton's lab (Indiana University Bloomington, USA) MNV-1 VP1 MNV-1 NS4, Propol Human norovirus NS-7 Human norovirus VLP-1 Gift from Kim Green's lab (NIAID, NIH, Bethesda, USA) NKCC1 Gift from Matt Hoffman's lab (NIDCR, NIH, Bethesda, USA) Epcam-APC 17-5791-82 ThermoFisher CD45 12-0451-82 ThermoFisher Scientific B220 557390 Pharmingen
Validation	1. Syndecan-1: Mouse monoclonal [B-A38] to Syndecan-1. Validated Applications: Flow Cyt, IHC-P. PMID: 31226359 2. EDIM-NSP5: Guinea Pig. PMID: 17182692 3. EDIM-VP6: Guinea Pig. PMID: 30092198 4. MNV-1 VP-1: Guinea Pig. PMID: 30092198

5. MNV-1 Propol: Rabbit. PMID: 16873239
6. Human Norovirus NS-7, NS-6, VLP-1: Rabbit. PMID: 32488028
7. NKCC1: Goat. PMID: 30159893
8. Human Norovirus VP-1: Guinea Pig. PMID: 31551337
9. Epcam-APC: Rat. The G8.8 monoclonal antibody reacts with the 40 kDa mouse EPCAM (epithelial cellular adhesion molecule). Validated Applications: Flow, IHC-P,F, ChIP, Functional Assay. PMID: 31672973
10. CD-45: Rat. The 30-F11 monoclonal antibody reacts with all isoforms of mouse CD45, also known as Leukocyte Common Antigen (LCA). CD45 is expressed by all hematopoietic cells excluding mature erythrocytes and platelets. The cytoplasmic portion of CD45 has tyrosine phosphatase enzymatic activity and plays an important role in activation of lymphocytes. PMID: 30365542
11. B220: Rat. The RA3-6B2 monoclonal antibody specifically binds to an epitope on the extracellular domain of the transmembrane CD45 glycoprotein which is dependent upon the expression of exon A and specific carbohydrate residues. It is expressed on B lymphocytes at all stages from pro-B through mature and activated B cell, but it is decreased on plasma cells and a subset of memory B cells. The levels of CD45R expression on the B-cell lineage appear to be developmentally regulated.

## Eukaryotic cell lines

Policy information about [cell lines](#)

Cell line source(s)

RAW264.7 : ATCC TIB-71 RRID# CVCL\_0493  
 HeLa: ATCC, CCL-2  
 NS-SV-TT-DC nd NS-SV-AC: Gift from J.A. Chiorini Lab, AAV Biology Section, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD, USA. PMID: 7687310

Authentication

NS-SV-TT-DC authentication was STR fingerprinting.  
 NS-SV-AC authentication not carried out by us.

Mycoplasma contamination

Cell lines were negative for mycoplasma contamination.

Commonly misidentified lines  
 (See [ICLAC](#) register)

No commonly misidentified cell lines were used in this study.

## Palaeontology and Archaeology

Specimen provenance

*Provide provenance information for specimens and describe permits that were obtained for the work (including the name of the issuing authority, the date of issue, and any identifying information). Permits should encompass collection and, where applicable, export.*

Specimen deposition

*Indicate where the specimens have been deposited to permit free access by other researchers.*

Dating methods

*If new dates are provided, describe how they were obtained (e.g. collection, storage, sample pretreatment and measurement), where they were obtained (i.e. lab name), the calibration program and the protocol for quality assurance OR state that no new dates are provided.*

Tick this box to confirm that the raw and calibrated dates are available in the paper or in Supplementary Information.

Ethics oversight

*Identify the organization(s) that approved or provided guidance on the study protocol, OR state that no ethical approval or guidance was required and explain why not.*

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

## Animals and other organisms

Policy information about [studies involving animals](#); [ARRIVE guidelines](#) recommended for reporting animal research

Laboratory animals

All animals were maintained at NHLBI Animal Care Facility, Bethesda, USA.  
 Mouse, BALB/c (Stock: 000651), C57BL/6J (stock 000664), B6.129S(Cg)-Stat1tm1Dlv/J (Stock No: 012606) originally procured from Jacksons Laboratories.  
 Cd300lfem1Cbwi/J breeding pairs were a kind gift from Dr. Craig B. Wilen (Yale School of Medicine, New Haven, CT, USA). The Cd300lf-/- allele was created by Dr. Herbert W. Virgin (Washington University at Saint Louis) using CRISPR/cas9 endonuclease-mediated genome editing in C57BL/6J mouse zygotes.  
 B6.IFNAR-/- mice breeding pair were a kind gift from Dr. Daniela Verthelyi, Food and Drug Administration, MD, USA.

Wild animals

N/A

Field-collected samples

N/A

Ethics oversight

All animal experiments were performed in an American Association for the Accreditation of Laboratory Animal Care (AAALAC) accredited animal facility. Housing and breeding (animals that aged more than 6 weeks) in accordance with the procedure outlined in the guide for the Care and Use of Laboratory Animals under an animal study proposal approved by the NHLBI Animal Care and Use Committee.

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

## Human research participants

Policy information about [studies involving human research participants](#)

### Population characteristics

*Describe the covariate-relevant population characteristics of the human research participants (e.g. age, gender, genotypic information, past and current diagnosis and treatment categories). If you filled out the behavioural & social sciences study design questions and have nothing to add here, write "See above."*

### Recruitment

*Describe how participants were recruited. Outline any potential self-selection bias or other biases that may be present and how these are likely to impact results.*

### Ethics oversight

*Identify the organization(s) that approved the study protocol.*

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

## Clinical data

Policy information about [clinical studies](#)

All manuscripts should comply with the ICMJE [guidelines for publication of clinical research](#) and a completed [CONSORT checklist](#) must be included with all submissions.

### Clinical trial registration

*Provide the trial registration number from ClinicalTrials.gov or an equivalent agency.*

### Study protocol

*Note where the full trial protocol can be accessed OR if not available, explain why.*

### Data collection

*Describe the settings and locales of data collection, noting the time periods of recruitment and data collection.*

### Outcomes

*Describe how you pre-defined primary and secondary outcome measures and how you assessed these measures.*

## Dual use research of concern

Policy information about [dual use research of concern](#)

### Hazards

Could the accidental, deliberate or reckless misuse of agents or technologies generated in the work, or the application of information presented in the manuscript, pose a threat to:

- | No                       | Yes                      |                            |
|--------------------------|--------------------------|----------------------------|
| <input type="checkbox"/> | <input type="checkbox"/> | Public health              |
| <input type="checkbox"/> | <input type="checkbox"/> | National security          |
| <input type="checkbox"/> | <input type="checkbox"/> | Crops and/or livestock     |
| <input type="checkbox"/> | <input type="checkbox"/> | Ecosystems                 |
| <input type="checkbox"/> | <input type="checkbox"/> | Any other significant area |

### Experiments of concern

Does the work involve any of these experiments of concern:

- | No                       | Yes                      |   |
|--------------------------|--------------------------|---|
| <input type="checkbox"/> | <input type="checkbox"/> | Demonstrate how to render a vaccine ineffective                             |
| <input type="checkbox"/> | <input type="checkbox"/> | Confer resistance to therapeutically useful antibiotics or antiviral agents |
| <input type="checkbox"/> | <input type="checkbox"/> | Enhance the virulence of a pathogen or render a nonpathogen virulent        |
| <input type="checkbox"/> | <input type="checkbox"/> | Increase transmissibility of a pathogen                                     |
| <input type="checkbox"/> | <input type="checkbox"/> | Alter the host range of a pathogen  |
| <input type="checkbox"/> | <input type="checkbox"/> | Enable evasion of diagnostic/detection modalities                           |
| <input type="checkbox"/> | <input type="checkbox"/> | Enable the weaponization of a biological agent or toxin                     |
| <input type="checkbox"/> | <input type="checkbox"/> | Any other potentially harmful combination of experiments and agents         |

## ChIP-seq

### Data deposition

- Confirm that both raw and final processed data have been deposited in a public database such as [GEO](#).
- Confirm that you have deposited or provided access to graph files (e.g. BED files) for the called peaks.

#### Data access links

May remain private before publication.

For "Initial submission" or "Revised version" documents, provide reviewer access links. For your "Final submission" document, provide a link to the deposited data.

#### Files in database submission

Provide a list of all files available in the database submission.

#### Genome browser session

(e.g. [UCSC](#))

Provide a link to an anonymized genome browser session for "Initial submission" and "Revised version" documents only, to enable peer review. Write "no longer applicable" for "Final submission" documents.

### Methodology

#### Replicates

Describe the experimental replicates, specifying number, type and replicate agreement.

#### Sequencing depth

Describe the sequencing depth for each experiment, providing the total number of reads, uniquely mapped reads, length of reads and whether they were paired- or single-end.

#### Antibodies

Describe the antibodies used for the ChIP-seq experiments; as applicable, provide supplier name, catalog number, clone name, and lot number.

#### Peak calling parameters

Specify the command line program and parameters used for read mapping and peak calling, including the ChIP, control and index files used.

#### Data quality

Describe the methods used to ensure data quality in full detail, including how many peaks are at FDR 5% and above 5-fold enrichment.

#### Software

Describe the software used to collect and analyze the ChIP-seq data. For custom code that has been deposited into a community repository, provide accession details.

## 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

#### Sample preparation

SMGs were extracted from animals after euthanization and homogenized in ice-cold 1XPBS (supplemented with 10% FBS). Homogenate was centrifuged at X1000 rpm for 5 minutes at 4°C to pellet down cells which was further incubated for 20 minutes at 37°C in 3ml of Gentle Collagenase/Hyaluronidase solution (StemCell Technologies, Cambridge, MA, USA, Catalogue No. 07919) with shaking. Thereafter centrifuged the solution again at X1000 rpm for 5 minutes at 4°C to collect the pellet and discard the sup. The pellet was further trypsin treated for 5 minutes at 37°C and passed through 70mm filter to eliminate un-dissociated tissue. The filtrate was then treated with a 4:1 NH4Cl: PBS solution to eliminate blood cells and subjected to centrifugation at X1000 rpm for 5 minutes at 4°C. Leaving the red layer of cells at bottom the entire supernatant consists of single cell isolation from tissue. Cell number was counted and incubated with anti-EpCAM conjugated to APC (ThermoFisher Scientific, Catalogue No. 17-5791-82) and anti-CD45 conjugated to PE (ThermoFisher Scientific, Catalogue No. 12-0451-82) for 1 hour at 4°C. Cells were subsequently washed and stained with live/dead Aqua stain (ThermoFisher Scientific, Catalogue No. L34957). Resuspended cells were sorted on ARIAllu (BD) cell sorter equipped with 355nM, 407nM, 488nM, 532nM and 640nM LASER lines using FACSDIVA 8.1 software at 70 psi pressure using 70-micron nozzle. Debris were removed based on scattering properties using FSC and SSC parameters. Live gated cells were purified for Leukocytes identified as CD45+ EpCAM- live cells where as CD45-EpCAM+ cells were identified as epithelial cells.

#### Instrument

Resuspended cells were sorted on ARIAllu (BD) cell sorter equipped with 355nM, 407nM, 488nM, 532nM and 640nM LASER lines using FACSDIVA 8.1 software at 70 psi pressure using 70-micron nozzle.

#### Software

FACSDIVA 8.1 software

#### Cell population abundance

Cell population abundance: 105 cells were obtained in each group CD45 and Epcam positive from both adult and mouse pup submandibular glands.

Gating strategy

Debris were removed on the basis of scattering properties using FSC and SSC parameters. Live gated cells were purified for Leukocytes identified as CD45+ EpCAM- live cells where as CD45-EpCAM+ cells were identified as epithelial cells.

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

## Magnetic resonance imaging

### Experimental design

Design type

Indicate task or resting state; event-related or block design.

Design specifications

Specify the number of blocks, trials or experimental units per session and/or subject, and specify the length of each trial or block (if trials are blocked) and interval between trials.

Behavioral performance measures

State number and/or type of variables recorded (e.g. correct button press, response time) and what statistics were used to establish that the subjects were performing the task as expected (e.g. mean, range, and/or standard deviation across subjects).

### Acquisition

Imaging type(s)

Specify: functional, structural, diffusion, perfusion.

Field strength

Specify in Tesla

Sequence &amp; imaging parameters

Specify the pulse sequence type (gradient echo, spin echo, etc.), imaging type (EPI, spiral, etc.), field of view, matrix size, slice thickness, orientation and TE/TR/flip angle.

Area of acquisition

State whether a whole brain scan was used OR define the area of acquisition, describing how the region was determined.

Diffusion MRI

 Used

 Not used

### Preprocessing

Preprocessing software

Provide detail on software version and revision number and on specific parameters (model/functions, brain extraction, segmentation, smoothing kernel size, etc.).

Normalization

If data were normalized/standardized, describe the approach(es): specify linear or non-linear and define image types used for transformation OR indicate that data were not normalized and explain rationale for lack of normalization.

Normalization template

Describe the template used for normalization/transformation, specifying subject space or group standardized space (e.g. original Talairach, MNI305, ICBM152) OR indicate that the data were not normalized.

Noise and artifact removal

Describe your procedure(s) for artifact and structured noise removal, specifying motion parameters, tissue signals and physiological signals (heart rate, respiration).

Volume censoring

Define your software and/or method and criteria for volume censoring, and state the extent of such censoring.

### Statistical modeling & inference

Model type and settings

Specify type (mass univariate, multivariate, RSA, predictive, etc.) and describe essential details of the model at the first and second levels (e.g. fixed, random or mixed effects; drift or auto-correlation).

Effect(s) tested

Define precise effect in terms of the task or stimulus conditions instead of psychological concepts and indicate whether ANOVA or factorial designs were used.

Specify type of analysis:  Whole brain  ROI-based  Both

Statistic type for inference  
(See [Eklund et al. 2016](#))

Specify voxel-wise or cluster-wise and report all relevant parameters for cluster-wise methods.

Correction

Describe the type of correction and how it is obtained for multiple comparisons (e.g. FWE, FDR, permutation or Monte Carlo).

### Models & analysis

n/a | Involved in the study

  Functional and/or effective connectivity

  Graph analysis

  Multivariate modeling or predictive analysis

Functional and/or effective connectivity

*Report the measures of dependence used and the model details (e.g. Pearson correlation, partial correlation, mutual information).*

Graph analysis

*Report the dependent variable and connectivity measure, specifying weighted graph or binarized graph, subject- or group-level, and the global and/or node summaries used (e.g. clustering coefficient, efficiency, etc.).*

Multivariate modeling and predictive analysis

*Specify independent variables, features extraction and dimension reduction, model, training and evaluation metrics.*