PNAS

Supporting Information for

Temporal changes in plasma membrane lipid content induce endocytosis to regulate developmental epithelial-to-mesenchymal transition

Michael L. Piacentino*, Erica J. Hutchins, Cecelia J. Andrews, and Marianne E. Bronner

*Corresponding Author: Michael L. Piacentino
Email: michaelpiacentino@jhmi.edu

This PDF file includes:

Figures S1 to S5
Tables S1
SI References

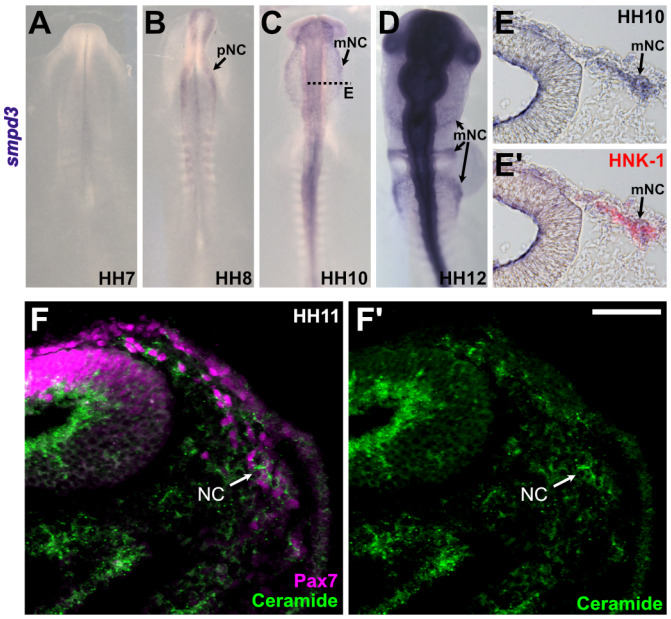

Fig. S1. *Smpd3* gene expression is detected in premigratory and migratory cranial neural crest cells. *In situ* hybridization shows that *Smpd3* expression is absent at early stages (**A**), initiates in specified, premigratory neural crest cells (**B**), and is maintained during migration (**C,D**). **E**, Transverse section of embryos at stage HH10 (**C**, dashed line) shows *Smpd3* expression in migrating cranial neural crest cells, immunolabeled by the surface antigen HNK-1. HH, Hamburger Hamilton stage; pNC, premigratory neural crest; mNC, migratory neural crest. See also Fig. 1. **F**, Immunolabeling reveals enrichment of the lipid ceramide in Pax7-labeled migrating neural crest cells at stage HH11, as ell as along the apical surface of the neural tube. Scale bar represents 50 μm.

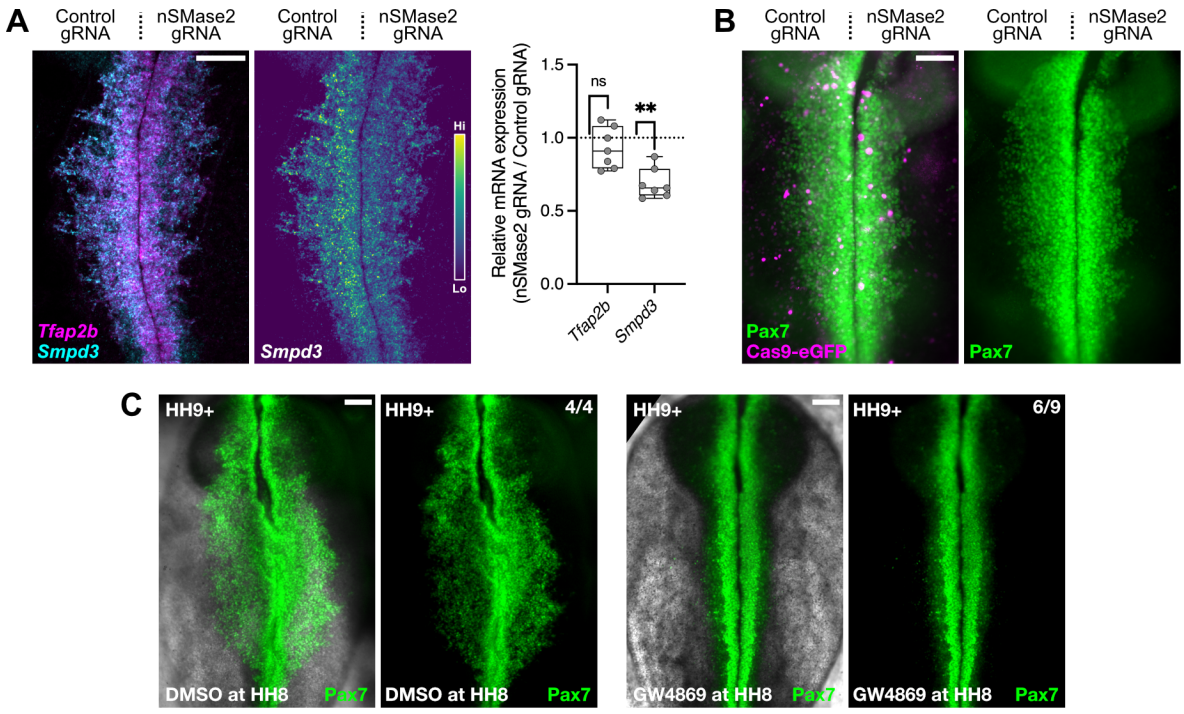

Fig. S2. CRISPR/Cas9-mediated nSMase2 knockout and neutral sphingomyelinase inhibition phenocopy nSMase2 MO. A, Embryos were electroporated with a Cas9-eGFP-expressing construct, together with U6.3-driven control- or nSMase2-targeting gRNAs. At HH9, embryos were fixed and processed for HCR to detect expression of *Smpd3* transcripts and the neural crest marker *Tfap2b*. Box plot displaying relative fluorescent intensity shows that, compared with control gRNA, nSMase2 gRNA does not affect *Tfap2b* expression but does knock down *Smpd3* transcript levels (n=7 embryos). **B**, Pax7 staining in nSMase2 CRISPR-electroporated embryos reveals a significant reduction in cranial neural crest migration area (See also Fig. 2B for quantitation). **C**, Wild type embryos were treated with DMSO (left) or the neutral sphingomyelinase inhibitor GW4869 (50 µm, right) at stage HH8 (prior to neural crest EMT) and incubated until stage HH9+ (migratory stage). Pax7 staining reveals that DMSO treatment did not prevent neural crest EMT (n=4/4), while global GW4869 treatment bilaterally inhibited EMT (n=6/9). Scale bars represent 100 µm. ns, non-significant, $^{**}p < 0.01$; two-tailed paired *t*-test. See also Fig. 2.

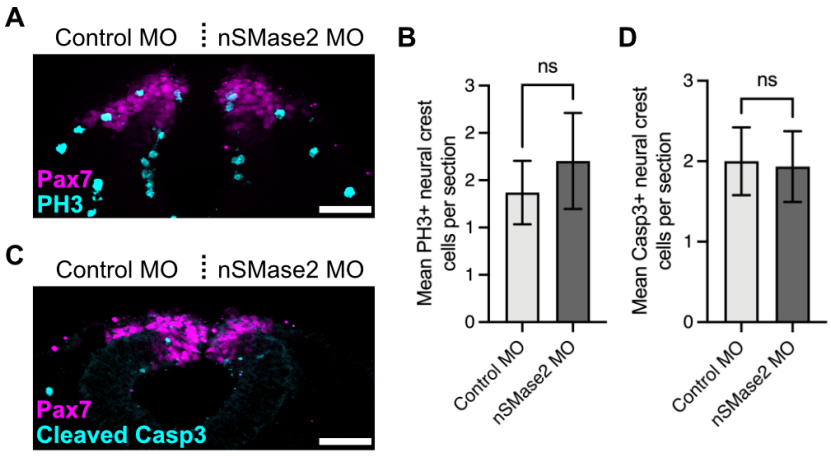

Fig. S3. **nSMase2 knockdown does not affect neural crest proliferation or survival.** Gastrulating chick embryos were electroporated with a non-binding control morpholino (Control MO, left) and nSMase2-targeting morpholino (nSMase2 MO, right), and where subsequently immunostained for the neural crest marker Pax7 and the mitotic marker phospho-histone H3 (PH3, **A**), or for the apoptosis indicator, activated caspase 3 (cleaved Casp3, **C**) in section at the HH9+. Quantitation of PH3+ (n=27 sections from 9 embryos, **B**) and Casp3+ (n=15 sections from 5 embryos, **D**) cells show no significant change between nSMase2 knockdown and the contralateral control. Displayed is the mean with error bars reflecting SEM. Scale bars represent 50 μm. ns, non-significant; two-tailed paired *t*-test.

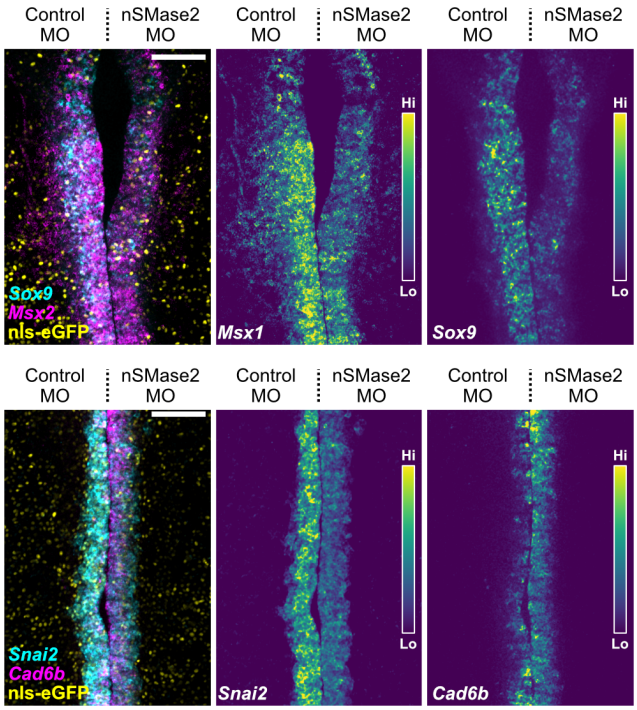

Fig. S4. nSMase2 knockdown disrupts activation of the EMT transcriptional program.
Representative images of nSMase2 knockdown embryos co-electroporated with nls-eGFP to label transfected cells, followed by HCR analysis for expression of neural crest markers *Msx1*, *Sox9*, *Snai2*, and *Cad6b* at stage HH9-. Scale bars represent 100 μm. See quantitation in Fig. 3A.

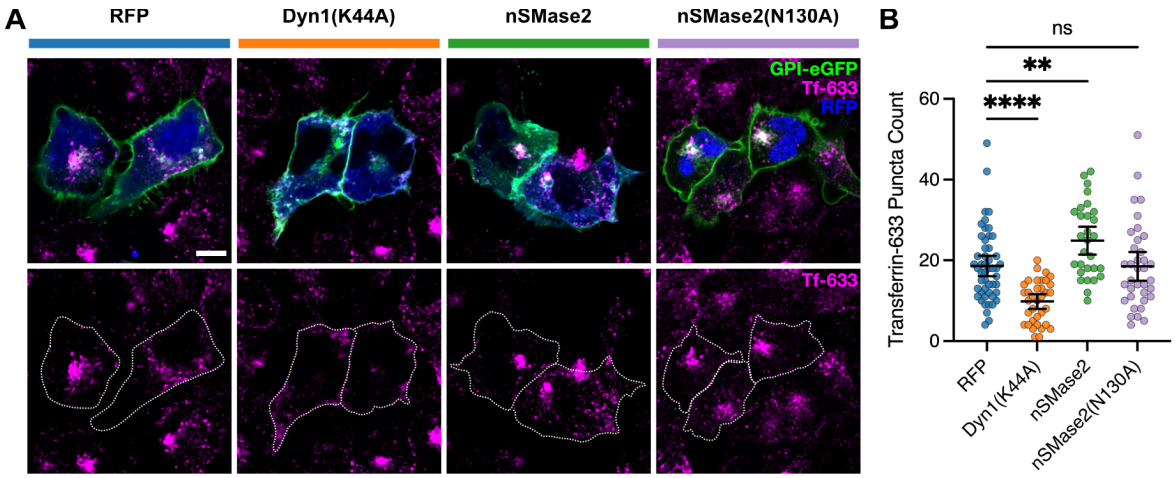

Fig. S5. nSMase2 catalytic activity is sufficient to induce endocytosis. A, Epithelial cells (U2OS) were transfected with GPI-eGFP to label the plasma membrane and the indicated overexpression constructs. After 24 hours, transfected cells were subjected the Transferrin-633-conjugate endocytosis assays, then fixed and visualized by confocal microscopy. **B**, Dot plot displaying the number of fluorescent Tf-633 puncta within individual transfected cells (n=50 RFP, 35 Dyn1(K44A), 29 nSMase2, and 36 nSMase2(N130A) cells). Overlayed bars display mean and 95% confidence intervals. ns not significant, ** $p<0.01$, **** $p<0.0001$; one-way ANOVA ($p<0.0001$) with Dunnett's multiple comparison analysis. White dotted lines indicate cell borders.

Table S1. Key Resources

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal IgG1 anti-Pax7	Developmental Studies Hybridoma Bank (DSHB)	Cat# PAX7; RRID:AB_528428
Mouse monoclonal IgM anti-HNK-1	Developmental Studies Hybridoma Bank (DSHB)	Cat# 3H5, RRID:AB_2314644
Mouse monoclonal IgG1 anti-Cad6B	Developmental Studies Hybridoma Bank (DSHB)	Cat# CCD6B-1, RRID:AB_531766
Rabbit monoclonal anti-Laminin (LAMA1)	Sigma-Aldrich	Cat# L9393, RRID:AB_477163
Rabbit polyclonal anti-Sox9	Millipore	Cat# AB5535, RRID:AB_2239761
Mouse monoclonal IgG1 anti-FLAG (Clone M2)	Sigma-Aldrich	Cat# F1804, RRID: AB_262044
Mouse monoclonal IgM anti-Ceramide (Clone MID 15B4)	Sigma-Aldrich	Cat# C8104, RRID:AB_259087
Rabbit polyclonal anti-phospho-Histone H3 (Ser10)	Millipore	Cat# 06-570, RRID:AB_310177
Rabbit polyclonal anti-Caspase-3, activated form	R & D Systems	Cat# AF835, RRID:AB_2243952
Rabbit monoclonal anti-Slug (Snai2, Clone C19G7)	Cell Signaling Technologies	Cat# 9585, RRID:AB_2239535
Goat polyclonal anti-GFP	Rockland	Cat# 600-101-215M, RRID:AB_2612804
Rabbit polyclonal anti-RFP/DsRed	MBL International	Cat# PM005, RRID:AB_591279
Chemicals, Peptides, and Recombinant Proteins		
Chemical: GW4869	Sigma-Aldrich	Cat# D1692
Peptide: Transferrin from Human Serum, Alexa Fluor 633 Conjugate	ThermoFisher Scientific	Cat# T23362
Dextran, Alexa Fluor 488; 3,000 MW, Anionic	ThermoFisher Scientific	Cat# D34682
C12 Ceramide (d18:1/12:0)	Avanti Polar Lipids	Cat# 860512
Deposited Data		
Bulk RNA-Seq: FACS-isolated chicken cells (neural crest at 5-6 somite stage, 8-10 somite stage, non-neural crest)	(1)	BioProject: PRJNA497574
Bulk RNA-Seq: Dissected chicken somites (epithelial somite stage, maturing somite stage)	(2)	BioProject: PRJNA602335
Experimental Models: Cell Lines		
Human: U2OS Cell Line	ATCC	Cat# HTB-96, RRID:CVCL_0042
Experimental Models: Organisms/Strains		
Fertilized chicken eggs	Sunstate Ranch, Sylmar, CA	

Fertilized chicken eggs	AA Laboratory Eggs, Westminster, CA	
Oligonucleotides		
Morpholino: Control MO CCTCTTACCTCAGTTACAATTTATA	Gene Tools	N/A
Morpholino: nSMase2 MO GGTGTCACTGTGTCAAGCATCCATA	Gene Tools	N/A
CRISPR/Cas9 gRNA Target: Control GCACTGCTACGATCTACACC	(3)	N/A
CRISPR/Cas9 gRNA Target: *SMPD3* GCAATCTGCGCAGCCCGAGA	This paper	N/A
Primer: nSMase2 FWD -76: CCTCAGTGTTGCTATGGATGC	This paper	N/A
Primer: nSMase2 REV 894 T7: TAATACGACTCACTATAGGGAACTGCCAGAATCTCCATCC	This paper	N/A
Primer: nSMase2 ATG XhoI: ATATCTCGAGGCCACCATGGTTTTATATACTTCCCCAT	This paper	N/A
Primer: nSMase2 stop ClaI: ATATATCGATTTAAGGATCATCTTCTCCTGTAG	This paper	N/A
Primer: nSMase2 N130A REV: GCAGCAGGCAAACGGCGGCACTGCCAAAGC	This paper	N/A
Primer: nSMase2 N130A FWD: GCTTTGGCAGTGCCGCCGTTTGCCTGCTGC	This paper	N/A
Primer: nSMase2 2462 REV: CATGCTACTGATGCAAGCACG	This paper	N/A
Primer: nSMase2 ATG SphI: TAGAGCATGCTATTATGCCACCATGGTTTTATATAC	This paper	N/A
Primer: nSMase2 nostop ClaI: CTCCATCGATTCCTCCTCCAGGATCATCTTCTCCTGTAGAC	This paper	N/A
Primer: nSMase2 FLAG ClaI: GCTTATCGATTCACTTGTCGTCATCGTCTTTGTAGTCGCCGGAGCCAGGATCATCTTCTCCTGTAG	This paper	N/A
Primer: LRP6 Signal Peptide FWD: TTTGGCAAAGAATTGCTCGAGCCACCATGGGGGCCGTCCTGAGGAG	This paper	N/A
Primer: LRP6 Signal Peptide FLAG REV: GCAACAAAGGCTTGTCGTCATCGTCTTTGTAGTCCAACAAAGGGGCCGCTCTCA	This paper	N/A
Primer: LRP6 FLAG FWD: GCAACAAAGGCTTGTCGTCATCGTCTTTGTAGTCCAACAAAGGGGCCGCTCTCA	This paper	N/A
Primer: LRP6 REV: ATTCGATATCAAGCTTATCGTCAGGAGGAGTCTGTACAGG	This paper	N/A
Recombinant DNA		
Plasmid: pCI::nSMase2	This paper	N/A
Plasmid: pCI::nSMase2-FLAG	This paper	N/A
Plasmid: pCI::nSMase2(N130A)	This paper	N/A
Plasmid: pCAG::nSMase2-RFP	This paper	N/A
Plasmid: pCAG::2a-RFP	(4)	N/A
Plasmid: pCI::H2B-RFP	(5)	N/A

Plasmid: pCI::nls-GFP	(6)	N/A
Plasmid: CMV::Dyn1(K44A)-mRFP	(7)	Addgene plasmid #55795
Plasmid: pTK-*FoxD3*-NC1.1m3::GFP	(8)	N/A
Plasmid: pTK-*FoxD3*-NC1.1m3::Dyn1(K44A)-mRFP	This paper	N/A
Plasmid: pCAG::GPI-eGFP	(9)	Addgene plasmid #32601
Plasmid: PM-eGFP	(10)	Addgene plasmid #21213
Plasmid: pCAG::PM-eGFP	This paper	N/A
Plasmid: TCF/Lef::GFP	(11)	Addgene plasmid #32610
Plasmid: M38-TOP::d2EGFP	Laboratory of Randall Moon	Addgene plasmid #17114
Plasmid: TCF/Lef::H2B-d2EGFP	This paper	N/A
Plasmid: BRE::GFP	(12)	N/A
Plasmid: BRE::H2B-d2EGFP	(4)	N/A
Plasmid: CMV::VSVG-LRP6	(13)	N/A
Plasmid: pCI::FLAG-LRP6	This paper	N/A
Plasmid: pCAG::Cas9	(3)	Addgene plasmid #99138
Plasmid: U6.3::empty	(3)	Addgene plasmid #99139
Plasmid: U6.3::Control gRNA	(3)	Addgene plasmid #99140
Plasmid: U6.3::*SMPD3* gRNA	This paper	N/A
Software and Algorithms		
Fiji v1.53c	(14)	https://imagej.net/Fiji
Bowtie2 v2.3.5.1	(15)	http://bowtie-bio.sourceforge.net/bowtie2/index.shtml
Cutadapt v2.10	(16)	https://cutadapt.readthedocs.io/en/stable/
FeatureCounts v2.0.1	(17)	http://subread.sourceforge.net/
R v3.6.1	(18)	https://www.r-project.org/
RStudio v1.2.1335	(19)	https://www.rstudio.com/
DESeq2	(20)	https://bioconductor.org/packages/release/bioc/html/DESeq2.html
Python v3.7.6	(21)	https://www.python.org/downloads/release/python-376/
Pandas v0.24.2	(22)	https://pandas.pydata.org/
Bokeh v1.4.0	(23)	https://docs.bokeh.org/en/latest/index.html
iqplot v0.1.6	(24)	https://iqplot.github.io/index.html
Scipy v1.5.2	(25)	https://scipy.org/

Scikit_posthoc v0.6.5	(26)	https://scikit-posthocs.readthedocs.io/en/latest/
Numpy v1.19.2	(27)	https://numpy.org/devdocs/index.html
Seaborn v0.11.0	(28)	https://seaborn.pydata.org/
Holoviews v1.13.3	(29)	https://holoviews.org/
GraphPad Prism 9 v9.2.0(283)		https://www.graphpad.com/

SI References

1. R. M. Williams, *et al.*, Reconstruction of the Global Neural Crest Gene Regulatory Network In Vivo. *Dev. Cell* **51**, 255-276.e7 (2019).

2. G. F. Mok, *et al.*, Characterising open chromatin in chick embryos identifies cis-regulatory elements important for paraxial mesoderm formation and axis extension. *Nat. Commun.* **12**, 1157 (2021).

3. S. Gandhi, M. L. Piacentino, F. M. Vieceli, M. E. Bronner, Optimization of CRISPR/Cas9 genome editing for loss-of-function in the early chick embryo. *Dev. Biol.* **432**, 86–97 (2017).

4. M. L. Piacentino, E. J. Hutchins, M. E. Bronner, Essential function and targets of BMP signaling during midbrain neural crest delamination. *Dev. Biol.* **477**, 251–261 (2021).

5. P. Betancur, M. Bronner-Fraser, T. Sauka-Spengler, Genomic code for Sox10 activation reveals a key regulatory enhancer for cranial neural crest. *Proc. Natl. Acad. Sci. U. S. A.* **107**, 3570–3575 (2010).

6. S. G. Megason, A. P. McMahon, A mitogen gradient of dorsal midline Wnts organizes growth in the CNS. *Development* (2002).

7. T. R. Hynes, S. M. Mervine, E. A. Yost, J. L. Sabo, C. H. Berlot, Live cell imaging of Gs and the beta2-adrenergic receptor demonstrates that both alphas and beta1gamma7 internalize upon stimulation and exhibit similar trafficking patterns that differ from that of the beta2-adrenergic receptor. *J. Biol. Chem.* **279**, 44101–44112 (2004).

8. M. S. Simões-Costa, S. J. McKeown, J. Tan-Cabugao, T. Sauka-Spengler, M. E. Bronner, Dynamic and differential regulation of stem cell factor FoxD3 in the neural crest is Encrypted in the genome. *PLoS Genet.* **8**, e1003142 (2012).

9. J. M. Rhee, *et al.*, In vivo imaging and differential localization of lipid-modified GFP-variant fusions in embryonic stem cells and mice. *Genesis* **44**, 202–218 (2006).

10. M. N. Teruel, T. A. Blanpied, K. Shen, G. J. Augustine, T. Meyer, A versatile microporation technique for the transfection of cultured CNS neurons. *J. Neurosci. Methods* **93**, 37–48 (1999).

11. A. Ferrer-Vaquer, *et al.*, A sensitive and bright single-cell resolution live imaging reporter of Wnt/ß-catenin signaling in the mouse. *BMC Dev. Biol.* **10**, 121 (2010).

12. G. Le Dréau, *et al.*, Canonical BMP7 activity is required for the generation of discrete neuronal populations in the dorsal spinal cord. *Development* **139**, 259–268 (2012).

13. B. T. MacDonald, M. V. Semenov, H. Huang, X. He, Dissecting molecular differences between Wnt coreceptors LRP5 and LRP6. *PLoS One* **6**, e23537 (2011).

14. J. Schindelin, *et al.*, Fiji: an open-source platform for biological-image analysis. *Nat. Methods* **9**, 676–682 (2012).

15. B. Langmead, S. L. Salzberg, Fast gapped-read alignment with Bowtie 2. *Nat. Methods* **9**, 357–359 (2012).

16. M. Martin, Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.journal* **17**, 10–12 (2011).

17. Y. Liao, G. K. Smyth, W. Shi, featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics* **30**, 923–930 (2014).

18. R. Ihaka, R. Gentleman, R: A Language for Data Analysis and Graphics. *J. Comput. Graph. Stat.* **5**, 299–314 (1996).

19. J. S. Racine, RSTUDIO: A platform-independent IDE for R and sweave. *Journal of Applied Econometrics* **27** (2012).

20. M. I. Love, W. Huber, S. Anders, Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* **15**, 550 (2014).

21. G. van Rossum, F. L. Drake, *Python 3 Reference Manual* (2009).

22. W. McKinney, P. D. Team, Pandas-Powerful python data analysis toolkit. *Pandas—Powerful Python Data Analysis Toolkit* **1625** (2015).

23. Bokeh Development Team, *Bokeh: Python library for interactive visualization* (2018).

24. J. S. Bois, justinbois/iqplot: 0.1.6 (2020) https:/doi.org/10.22002/D1.1614.

25. P. Virtanen, *et al.*, SciPy 1.0: fundamental algorithms for scientific computing in Python. *Nat. Methods* **17**, 261–272 (2020).

26. M. Terpilowski, scikit-posthocs: Pairwise multiple comparison tests in Python. *J. Open Source Softw.* **4**, 1169 (2019).

27. S. van der Walt, S. C. Colbert, G. Varoquaux, The NumPy Array: A Structure for Efficient Numerical Computation. *Computing in Science Engineering* **13**, 22–30 (2011).

28. M. Waskom, seaborn: statistical data visualization. *J. Open Source Softw.* **6**, 3021 (2021).

29. J.-L. Stevens, P. Rudiger, J. Bednar, HoloViews: Building complex visualizations easily for reproducible science in *Proceedings of the 14th Python in Science Conference*, (SciPy, 2015) https:/doi.org/10.25080/majora-7b98e3ed-00a.