

Spatial remodeling of sphingomyelin synthesis by Zika virus drives neuroinflammation and cortical developmental injury

Xinyan Hao^{1,2,6}, Tao Yao^{4,5,6}, Zijian Zhao^{1,2}, Jianfei Zheng^{1,3}, Pei Chang^{1,2}, Yifei Pei^{1,2}, Shulin Li^{1,3}, Yingying Guo^{1,3}, Bo Shan^{4,5*} and Min Zhang^{1,2*}

¹ State Key Laboratory of Membrane Biology, Tsinghua University, Beijing, 100084, China.

² School of Pharmaceutical Sciences, Tsinghua Medicine, Tsinghua University, Beijing, 100084, China.

³ Tsinghua-Peking Center for Life Sciences, School of Life Sciences, Tsinghua University, Beijing, 100084, China.

⁴ Zhejiang Key Laboratory of Pancreatic Disease, The First Affiliated Hospital, Zhejiang Key Laboratory of Frontier Medical Research on Cancer Metabolism, and Institute of Translational Medicine, Zhejiang University School of Medicine, Hangzhou, Zhejiang, 310029, China.

⁵ Institute of Fundamental and Transdisciplinary Research, Cancer Center, Zhejiang University, Hangzhou, Zhejiang, China.

⁶ These authors contributed equally to this work.

* Correspondence: boshan@zju.edu.cn, zhangmin143@mail.tsinghua.edu.cn

ABSTRACT

Neuroinflammation can disrupt brain development, yet the cellular mechanisms driving pathogenic inflammatory responses in the developing brain remain poorly understood. Here, we identify ectopic sphingomyelin synthesis at the endoplasmic reticulum–Golgi intermediate compartment (ERGIC) as a mechanism linking Zika virus (ZIKV) infection to neuroinflammation and cortical developmental injury. Using super-resolution imaging and lipidomics, we show that the viral protein NS4A

redirects sphingomyelin synthase 1 (SGMS1) and ceramide transfer protein (CERT1) to the ERGIC, establishing ectopic sphingomyelin synthesis hub. The resulting sphingomyelin accumulation at the ERGIC promotes homo-oligomerization and activation of TMED10, an ERGIC-resident protein translocator that mediates unconventional protein secretion, thereby driving interleukin-1 β (IL-1 β) release and neuroinflammation. In mice, inhibition of sphingomyelin synthesis attenuated IL-1 β release and neuroinflammation while preserving cortical development. Furthermore, functional mutational screening identified key TMED10 residues required for sphingomyelin sensing. Disruption of this lipid-sensing mechanism via point mutation suppressed ZIKV-induced inflammatory responses *in vitro* and ameliorated neuroinflammation and cortical developmental defects *in vivo*. These findings establish an ERGIC sphingomyelin–TMED10 axis linking spatial lipid remodeling to pathogenic inflammatory signaling and identify a potential therapeutic target for inflammation-driven neurodevelopmental injury.

INTRODUCTION

Inflammation is an evolutionarily conserved response that protects tissues from infection and injury by coordinating pathogen clearance and restoration of homeostasis^{1,2}. The developing brain is particularly vulnerable to inflammation because neural progenitor proliferation, differentiation and tissue organization must be precisely coordinated during development^{3,4}. Consequently, inflammatory signals that are transient or protective in mature tissues can disrupt developmental programs

when encountered during critical windows⁵⁻⁷. Understanding how protective inflammatory responses become pathogenic in the developing brain is essential for defining the mechanisms underlying inflammation-associated neurodevelopmental injury.

Zika virus (ZIKV), a mosquito-borne flavivirus, provides a striking example of this vulnerability. The 2015-2016 epidemic established a causal link between maternal ZIKV infection and microcephaly, as well as other neurological abnormalities collectively referred to as congenital Zika syndrome (CZS)^{8,9}. Although impaired proliferation and survival of neural progenitor cells (NPCs) are recognized contributors to cortical underdevelopment¹⁰⁻¹³, these cell-autonomous defects do not fully account for the inflammatory and developmental pathology associated with ZIKV infection.

ZIKV infection is also associated with prominent neuroinflammation in the developing brain, including microglial activation and inflammatory programs characterized by cytokine production and type I interferon signaling¹⁴⁻²⁰. Although these responses initially contribute to antiviral defense, sustained or excessive inflammatory signaling can compromise NPC function and disrupt the developmental environment of the fetal brain^{4,19,21}. However, how ZIKV infection engages the cellular machinery that drives pathogenic inflammatory signaling remains poorly understood.

Here, we identify spatial remodeling of sphingomyelin metabolism at the ER–Golgi intermediate compartment (ERGIC) as a previously unrecognized mechanism linking

ZIKV infection to unconventional secretion of IL-1 β . We further show that this pathway drives neuroinflammation and cortical developmental injury *in vivo*. These findings reveal how virus-induced remodeling of host membrane metabolism can be translated into pathogenic inflammatory signaling during brain development.

RESULTS

ZIKV-induced IL-1 β drives neuroinflammation and cortical developmental injury

Given the prominent neuroinflammatory response in the developing brain following ZIKV infection^{17,22,23}, we first asked whether pro-inflammatory factor release is induced during congenital infection and whether it contributes to neurodevelopmental injury. To address this, we employed an *in utero* infection model by intracerebroventricular injection of ZIKV into embryonic mouse brains at E13.5, a critical period of cortical neurogenesis^{10,24-26}. Analysis of the culture medium of brain explants at E16.5 revealed increased release of multiple inflammatory cytokines, including IFN- γ , IL-6 and TNF- α , with IL-1 β showing a particularly pronounced increase (Fig. 1a, b; Supplementary information, Fig. S1a).

Microglia are a major source of IL-1 β in the central nervous system^{20,27-29}. Consistent with the *in vivo* findings, primary microglia showed strong dose-dependent IL-1 β secretion upon ZIKV infection (Supplementary information, Fig. S1b). This occurred without substantial caspase-3 activation or LDH release, indicating that IL-1 β release was not accompanied by overt cell death (Supplementary information, Fig. S1b).

Moreover, ZIKV-induced IL-1 β secretion remained intact in microglia lacking gasdermin D (GSDMD), an executor of pyroptosis³⁰⁻³³, demonstrating that ZIKV-induced IL-1 β release does not rely on canonical pyroptotic mechanisms (Supplementary information, Fig. S1c).

We next tested whether IL-1 β contributes causally to ZIKV-associated cortical developmental injury. ZIKV infection resulted in reduced head size and weight, decreased cortical thickness and loss of SOX2-positive NPCs, accompanied by transcriptional activation of inflammatory pathways and suppression of neurodevelopmental programs (Fig. 1c-j; Supplementary information, Fig. S1d, e). Blocking IL-1 signaling with IL-1 receptor antagonist (IL-1RA)³⁴ largely reversed these abnormalities, restoring cortical architecture, NPC abundance and associated transcriptional programs (Fig. 1d-j; Supplementary information, Fig. S1d, f). Conversely, intracerebroventricular administration of recombinant IL-1 β was sufficient to induce inflammatory responses and restrict cortical development, recapitulating key morphological features of ZIKV-associated brain injury (Fig. 1k-q). Thus, IL-1 β is not merely associated with ZIKV-induced neuroinflammation but represents a critical mediator of cortical developmental injury. These findings prompted us to investigate how ZIKV infection induces IL-1 β secretion in the developing brain.

NS4A is a viral determinant of pathogenic IL-1 β secretion

To identify viral determinants responsible for aberrant IL-1 β secretion, we screened

ZIKV membrane proteins using an established HEK293T-based unconventional secretion system that stably expresses mature IL-1 β (mIL-1 β)³⁵. After confirming that mIL-1 β secretion in this system responds to ZIKV infection in a manner similar to endogenous IL-1 β in embryonic brain microglia (Supplementary information, Fig. S2a), we identified the non-structural protein NS4A as the most potent inducer of IL-1 β secretion (Fig. 2a; Supplementary information, Fig. S2b, c). NS4A similarly stimulated IL-1 β secretion in primary microglia, independently of GSDMD (Supplementary information, Fig. S2d). In contrast, the closely related NS4A protein from dengue virus serotype 2 (DENV2) had little effect on IL-1 β secretion (Supplementary information, Fig. S2e), suggesting that this activity is not a general property of flaviviral NS4A proteins.

Deletion analysis mapped the IL-1 β -inducing activity of NS4A to its N-terminal α 1 helix (Supplementary information, Fig. S2f-h). Mutational screening within this region identified NS4A-M7A as a loss-of-function mutant that failed to induce IL-1 β secretion (Supplementary information, Figs. S2i, S3a). Expression of NS4A-WT in the embryonic brain induced robust inflammatory responses and cortical developmental abnormalities, whereas NS4A-M7A had little effect (Supplementary information, Fig. S3b-k). Consistent with these findings, recombinant ZIKV strain carrying the M7A mutation (ZIKV-NS4A^{M7A}) failed to trigger IL-1 β secretion in microglia or embryonic brains (Fig. 2b, c) and did not induce neuroinflammation or cortical developmental defects *in vivo* (Fig. 2d-l). Together, NS4A is a viral determinant that couples ZIKV

infection to pathogenic IL-1 β secretion and cortical developmental injury.

NS4A couples sphingomyelin metabolism to unconventional IL-1 β secretion

NS4A localized predominantly to the ERGIC (Supplementary information, Fig. S4a, b), a membrane compartment implicated in TMED10-dependent unconventional protein secretion (THU)³⁵. NS4A co-localized with TMED10 at the ERGIC (Supplementary information, Fig. S4c), and genetic ablation of TMED10 abolished IL-1 β secretion induced by either NS4A or ZIKV (Supplementary information, Fig. S4d, e), indicating that NS4A engages the THU pathway. Notably, NS4A-M7A retained ERGIC localization and association with TMED10 at levels comparable to NS4A-WT (Supplementary information, Fig. S4a-c, f), indicating that loss of IL-1 β -inducing activity cannot be explained by defective ERGIC targeting or TMED10 association. These findings suggested that NS4A requires additional ERGIC factors to activate IL-1 β secretion.

To identify such factors, we combined proximity labeling with ERGIC-enriched membrane proteomics. TurboID labeling of NS4A-WT, which retained full IL-1 β -inducing activity (Supplementary information, Fig. S5a), identified proteins differentially associated with NS4A-WT and NS4A-M7A (Fig. 3a; Supplementary information, Table S1). In parallel, mass spectrometry of ERGIC-enriched membrane fractions from cells expressing NS4A-WT, NS4A-M7A or vector control identified proteins selectively enriched at the ERGIC by NS4A-WT (Fig. 3b; Supplementary information, Table S2). The intersection of these datasets yielded seven candidates,

among which sphingomyelin synthase 1 (SGMS1) and ceramide transfer protein (CERT1) were prominent hits with established roles in sphingomyelin metabolism (Fig. 3c)³⁶⁻³⁸. Both proteins associated with NS4A-WT but not NS4A-M7A, as confirmed by co-immunoprecipitation (Supplementary information, Fig. S5b, c). Depletion of either SGMS1 or CERT1 markedly reduced IL-1 β secretion induced by NS4A or ZIKV (Supplementary information, Fig. S5d-h).

We next tested whether sphingomyelin metabolism is functionally required for this response. Expression of sphingomyelin-degrading enzyme SMPD2, pharmacological inhibition of sphingomyelin synthesis with Malabaricone C³⁹ or inhibition of CERT1 with HPA-12⁴⁰, reduced NS4A- or ZIKV-induced IL-1 β secretion (Fig. 3d; Supplementary information, Fig. S5i-k). *In vivo*, perturbation of sphingomyelin synthesis also attenuated ZIKV-associated cortical developmental abnormalities (Fig. 3e-j). These findings establish sphingomyelin metabolism as a functional component of NS4A-driven IL-1 β secretion and link viral membrane remodeling to inflammatory injury in the developing brain.

To place these findings in the cellular context of ZIKV infection, we reanalyzed published single-cell transcriptomic datasets from ZIKV-infected mouse fetal brains⁴¹. ZIKV infection was associated with marked microglial expansion and increased transcriptional programs related to unconventional secretion, inflammation and sphingomyelin metabolism (Supplementary information, Fig. S6a, b; Fig. 3k). Reclustering of microglia identified a distinct MG2 population enriched for THU

activity, IL-1 signaling and sphingomyelin metabolic programs, together with strong antiviral signatures (Fig. 3l-p). Independent analysis of ZIKV-infected human fetal brain explants revealed similar enrichment of unconventional secretion, inflammatory and sphingolipid metabolic programs (Supplementary information, Fig. S6c, d). Together, these analyses place the sphingomyelin–THU program within ZIKV-responsive microglia and support its relevance to human fetal brain infection.

NS4A redirects sphingomyelin synthesis to the ERGIC

Sphingomyelin synthesis occurs predominantly at the trans-Golgi network (TGN)^{42,43}. Interestingly, NS4A expression induced relocalization of both SGMS1 and CERT1 to the ERGIC, where they accumulated to form an ectopic lipid metabolic hub, accompanied by a corresponding reduction of these proteins at the TGN (Fig. 4a-d). As a result, immunofluorescence analysis revealed increased sphingomyelin levels specifically at the ERGIC following NS4A expression or ZIKV infection (Fig. 4e, f). Biochemical fractionation of ERGIC membranes confirmed the enrichment of SGMS1, CERT1 and sphingomyelin at this compartment (Fig. 4g, h). Lipidomic analysis further revealed a broad increase in sphingomyelin species together with elevated ceramide, consistent with enhanced delivery of ceramide to the ERGIC by CERT1 (Fig. 4i-l; Supplementary information, Table S3). None of these changes was observed with the NS4A-M7A mutant (Fig. 4a-l), linking NS4A-dependent ERGIC remodeling to sphingomyelin accumulation.

CERT1- mediated ceramide transport normally depends on binding of its PH domain

to PI4P at the TGN^{37,44}. We found that the ERGIC was also enriched in PI4P (Supplementary information, Fig. S7a), and inhibition of PI4KIII β with T-00127-HEV1 (HEV1)⁴⁵ reduced NS4A-induced IL-1 β secretion (Supplementary information, Fig. S7b). NS4A also associated with the PH domain of CERT1 (Supplementary information, Fig. S7c), suggesting that NS4A-dependent CERT1 recruitment to the ERGIC contributes to local sphingomyelin production. Together, these findings show that NS4A spatially redistributes the sphingomyelin biosynthetic machinery to the ERGIC, creating a localized lipid environment associated with IL-1 β secretion.

ERGIC sphingomyelin activates TMED10-mediated IL-1 β secretion

To determine whether sphingomyelin accumulation at the ERGIC is sufficient to activate THU-dependent IL-1 β secretion, we employed a Retention Using Selective Hooks (RUSH) system to selectively reposition SGMS1 from the Golgi to the ERGIC. An ERGIC-localized streptavidin-TMED10 Δ GD hook retained SBP-tagged SGMS1 at the ERGIC, whereas addition of biotin released SGMS1 to the Golgi⁴⁶ (Fig. 5a). ERGIC retention of SGMS1 increased local sphingomyelin abundance and markedly enhanced IL-1 β secretion, whereas release of SGMS1 to the Golgi restored both sphingomyelin distribution and IL-1 β secretion toward basal levels (Fig. 5b, c). To directly monitor cargo entry into TMED10-containing vesicles, we utilized a GFP complementation assay (Fig. 5d). Consistent with the secretion phenotype, ERGIC-retained SGMS1 robustly drove IL-1 β translocation, an effect that was

completely abrogated upon SGMS1 release (Fig. 5e). Thus, localized sphingomyelin production at the ERGIC is sufficient to promote TMED10-dependent cargo translocation and IL-1 β secretion.

We next asked how ERGIC sphingomyelin activates TMED10. Retention of SGMS1 at the ERGIC markedly increased TMED10 homo-oligomerization while reducing its association with other TMED family members (Fig. 5f). Consistent with a role for sphingomyelin in this process, expression of the sphingomyelinase SMPD2 reduced NS4A-induced TMED10 self-association (Fig. 5g). These findings indicate that ERGIC sphingomyelin enrichment promotes a shift in TMED10 oligomeric state toward homo-oligomerization, enabling TMED10-dependent cargo translocation and IL-1 β secretion.

TMED10 response to sphingomyelin drives ZIKV-associated neuroinflammation and cortical injury

We next sought to define the molecular basis by which TMED10 responds to sphingomyelin. Using a rapid secretion assay based on complementary NanoLuc luciferase complementation⁴⁷, we performed systematic mutational analysis of the TMED10 transmembrane domain. Three transmembrane residues—G199, T202 and Q204—were identified as contributors to TMED10-mediated IL-1 β secretion (Fig. 6a, b; Supplementary information, Fig. S8a). Among these, Q204 was particularly important, as its mutation markedly impaired SGMS1-induced IL-1 β secretion (Fig. 6c). Structural modelling⁴⁸ placed several TMED10 transmembrane residues in

proximity to sphingomyelin, with Q204 positioned to form a hydrogen bond with the sphingomyelin carbonyl group, suggesting a potential role in lipid recognition (Fig. 6d). Consistent with this model, the Q204L mutation markedly impaired TMED10 homo-oligomerization, a key hallmark of TMED10 activation (Fig. 6e). TMED10-Q204L also failed to support IL-1 β release induced by either NS4A expression or ZIKV infection (Supplementary information, Fig. S8b, c). Similarly, primary microglia from TMED10-Q204L knock-in mice showed markedly reduced IL-1 β secretion following ZIKV infection or NS4A expression (Fig. 6f; Supplementary information, Fig. S8d).

We next examined whether this sphingolipid-TMED10 response contributes to ZIKV-induced brain injury *in vivo*. TMED10-Q204L mice were largely protected from ZIKV-induced disruption of cortical development, with preserved cortical architecture, reduced neuroinflammation and maintenance of neural progenitor populations (Fig. 6g-m). Although Q204L mice showed modest baseline changes in inflammatory and neurodevelopmental gene expression, the inflammatory response and suppression of neurodevelopmental programs induced by ZIKV were markedly attenuated compared with WT controls.

Together, these findings identify TMED10 as a lipid-responsive component of the unconventional secretion machinery that links ZIKV-induced ERGIC sphingomyelin remodeling to IL-1 β secretion, neuroinflammation and neurodevelopmental injury.

DISCUSSION

Our study identifies a mechanism by which ZIKV converts a host inflammatory response into developmental brain injury. We show that the viral protein NS4A redirects sphingomyelin synthesis to the ERGIC, where local lipid remodeling engages TMED10 and promotes unconventional secretion of IL-1 β . This pathway links viral membrane remodeling to microglial inflammatory activation and cortical developmental injury (Fig. 7). More broadly, our findings identify spatial organization of membrane metabolism as an upstream mechanism that can determine the inflammatory response of infected cells.

Mechanistically, NS4A coordinates the sphingomyelin biosynthetic machinery with the unconventional secretion pathway at the ERGIC. NS4A recruits SGMS1 and CERT1 to this compartment and promotes a local sphingomyelin environment that favors TMED10 homo-oligomerization and IL-1 β release. This spatial coupling provides a mechanism by which a viral membrane protein can convert changes in membrane organization into inflammatory signaling without broadly altering cellular lipid metabolism. Although NS4B and other ZIKV proteins have been implicated in more global lipid remodeling^{49,50}, our findings highlight a distinct mechanism in which the spatial organization of lipid metabolism determines inflammatory output.

The functional consequence of this pathway is particularly important in the developing brain. Although microglia can protect the developing brain by clearing infected cells^{41,51}, our data identify a distinct pathogenic state characterized by enhanced THU activity, sphingomyelin metabolism and IL-1 signaling. The emergence of this state

may reflect the ability of microglia to sense virus-induced changes in membrane organization and translate them into pathological inflammatory signaling. Importantly, IL-1 β was both necessary and sufficient to reproduce key features of ZIKV-associated cortical injury, whereas disrupting TMED10 sphingomyelin sensing reduced IL-1 β release and preserved cortical development *in vivo*. These findings place IL-1 β downstream of the viral membrane response but upstream of cortical developmental injury, providing a mechanistic link between the pathogenic microglial state and impaired neurodevelopment. More broadly, microglial activation is not intrinsically protective or pathogenic; rather, its consequences depend on the intensity, timing and cellular context of the inflammatory response. Such context dependence may contribute to the heterogeneous neurodevelopmental outcomes following fetal ZIKV exposure.

Our findings further suggest that inflammation-driven cortical developmental injury may be amenable to intervention by targeting the pathway that links cellular stress to pathogenic IL-1 β release. Inhibition of sphingomyelin synthesis, CERT1-dependent lipid transport or TMED10 sphingomyelin sensing reduced IL-1 β release, while disruption of the TMED10 lipid-response site protected cortical development *in vivo*. Rather than broadly suppressing immune responses, targeting this pathway could potentially attenuate the pathogenic branch of microglial activation while preserving upstream host-defense mechanisms. In particular, disrupting TMED10 lipid sensing may provide a strategy to uncouple excessive IL-1 β release from the initiating

infectious or inflammatory stimulus. This possibility may extend beyond congenital ZIKV infection to other conditions in which excessive IL-1 β signaling contributes to cortical developmental injury, although the therapeutic window, tissue specificity and safety of targeting this pathway during development remain to be determined.

ACKNOWLEDGEMENTS

M.Z. is supported by National Natural Science Foundation of China (32530024), National Key R&D Program of China (2024YFA1802601, 2025YFA1804303), National Natural Science Foundation of China (32370728, 32450388) and Tsinghua University Dushi Program. B.S. is supported by National Key R&D Program of China (2022YFA1104102), National Natural Science Foundation of China (32571287, 82270926).

We thank Dr. Feng Shao (National Institute of Biological Sciences, China), Dr. Xiao-Yu Hu (Westlake University, China), Dr. Yong-Qiang Deng (Southern Medical University, China), Dr. Gong Cheng, Dr. Qiang Ding and Dr. Li Yu (Tsinghua University, China) for providing materials and reagents. We thank Dr. Liang Ge (Tsinghua University) for helpful suggestions on the study. We thank Dr. Song-Hai Shi (Tsinghua University, China) for *in utero* intracerebroventricular injection assistance. We thank Li-Na Xu and Yu-Pei Jiao at Facility center of Metabolomics and lipidomics in Tsinghua University for their help with the LC-MS/MS experiments.

AUTHOR CONTRIBUTIONS

B.S. and M.Z. conceptualized the study. X.H., T.Y., J.Z., Y.G., B.S. and M.Z. conceived the experiments and wrote the manuscript. X.H., Z.Z., Y.P. and S.L. carried out the cell biology and biochemistry experiments. X.H. performed ZIKV infection, AAV delivery, lentiviral transduction, mouse and primary cell experiments. T.Y. and P.C. analyzed and visualized the RNA-seq data. B.S. and M.Z. supervised the project and provided funding.

COMPETING INTERESTS

Authors declare that they have no competing interests.

ADDITIONAL INFORMATION

Supplemental information

Table S1. Proteomics of NS4A-WT and NS4A-M7A interactomes by proximity labeling.

Table S2. Proteomics of ERGIC-enriched membrane fractionations from cells expressing control, NS4A-WT or NS4A-M7A.

Table S3. Lipidomics of lipid species and sphingomyelin subtypes in ERGIC-enriched fractions from HEK293T cells expressing empty vector, NS4A-WT or NS4A-M7A or infected with mock, ZIKV-NS4A^{WT} or ZIKV-NS4A^{M7A}.

REFERENCES

1. Mogensen, T.H. Pathogen Recognition and Inflammatory Signaling in Innate Immune Defenses. *Clin Microbiol Rev* **22**, 240–273 (2009).
2. Medzhitov, R. Origin and physiological roles of inflammation. *Nature* **454**, 428–435 (2008).
3. Ostrem, L.M., *et al.* Fetal brain response to maternal inflammation requires microglia. *Development* (2024).

4. Otero, A.M. & Antonson, A.M. At the crux of maternal immune activation: Viruses, microglia, microbes, and IL-17A. *Immunol Rev* **311**, 205–223 (2022).
5. Couch, A.C.M., Berger, T., Hanger, B., Matuleviciute, R. & Vernon, A.C. Maternal Immune Activation Primes Deficiencies in Adult Hippocampal Neurogenesis. *Brain Behav Immun*, 410–422 (2021).
6. Martín-Guerrero, S.M., *et al.* Maternal immune activation imprints translational dysregulation and differential MAP2 phosphorylation in descendant neural stem cells. *Mol Psychiatr* **30**, 2994–3007 (2025).
7. Assir, M.Z.K., *et al.* Modeling maternal immune activation in 3D ex vivo human fetal brain cerebroids reveals IL-17A-driven disruption of cortical development. *Nat Neurosci* **29**, 2456–2468 (2026).
8. Mlakar, J., *et al.* Zika Virus Associated with Microcephaly. *New Engl J Med* **374**, 951–958 (2016).
9. Rasmussen, S.A., Jamieson, D.J., Honein, M.A. & Petersen, L.R. Zika Virus and Birth Defects - Reviewing the Evidence for Causality. *New Engl J Med* **374**, 1981–1987 (2016).
10. Li, C., *et al.* Zika Virus Disrupts Neural Progenitor Development and Leads to Microcephaly in Mice *Cell Stem Cell* **19**, 672–672 (2016).
11. Tang, H.L., *et al.* Zika Virus Infects Human Cortical Neural Progenitors and Attenuates Their Growth. *Cell Stem Cell* **18**, 587–590 (2016).
12. Komarasamy, T.V., Adnan, N.A.A., James, W. & Balasubramaniam, V.R.M.T. Zika Virus Neuropathogenesis: The Different Brain Cells, Host Factors and Mechanisms Involved. *Front Immunol* **13**, 773191 (2022).
13. Garcez, P.P., *et al.* Zika virus impairs growth in human neurospheres and brain organoids. *Science* **352**, 816–818 (2016).
14. Wang, J., *et al.* Zika virus infected primary microglia impairs NPCs proliferation and differentiation. *Biochem Bioph Res Co* **497**, 619–625 (2018).
15. Hassaan, N.A. & Xing, L. The mechanisms of Zika virus-induced neuropathogenesis. *Braz J Microbiol* **55**, 3933–3943 (2024).
16. de Sousa, J.R., *et al.* Inflammasome activation results in severe damage to the central nervous system in fatal Zika virus microcephaly cases. *Cytokine* **111**, 255–264 (2018).
17. Lima, M.C., *et al.* The Transcriptional and Protein Profile From Human Infected Neuroprogenitor Cells Is Strongly Correlated to Zika Virus Microcephaly Cytokines Phenotype Evidencing a Persistent Inflammation in the CNS. *Front Immunol* **10**(2019).
18. Nascimento-Carvalho, G.C., *et al.* Zika-exposed microcephalic neonates exhibit higher degree of inflammatory imbalance in cerebrospinal fluid. *Sci Rep-Uk* **11**, 8474 (2021).
19. Dang, J., *et al.* Zika Virus Depletes Neural Progenitors in Human Cerebral Organoids through Activation of the Innate Immune Receptor TLR3. *Cell Stem Cell* **19**, 258–265 (2016).

20. Lum, F.M., *et al.* Zika Virus Infects Human Fetal Brain Microglia and Induces Inflammation. *Clin Infect Dis* **64**, 914–920 (2017).
21. Xu, Y.-P., *et al.* Zika virus infection induces RNAi-mediated antiviral immunity in human neural progenitors and brain organoids. *Cell Research* **29**, 265–273 (2019).
22. Jeong, G.U., *et al.* Zika Virus Infection Induces Interleukin-1 β -Mediated Inflammatory Responses by Macrophages in the Brain of an Adult Mouse Model. *J Virol* **97**, e0055623 (2023).
23. Wang, W.B., *et al.* Zika virus infection induces host inflammatory responses by facilitating NLRP3 inflammasome assembly and interleukin-1 β secretion. *Nature Communications* **9**, 106 (2018).
24. Miner, J.J., *et al.* Zika Virus Infection during Pregnancy in Mice Causes Placental Damage and Fetal Demise. *Cell* **165**, 1080–1090 (2016).
25. Yockey, L.J., *et al.* Vaginal Exposure to Zika Virus during Pregnancy Leads to Fetal Brain Infection. *Cell* **166**, 1247–1256.e1244 (2016).
26. Cugola, F.R., *et al.* The Brazilian Zika virus strain causes birth defects in experimental models. *Nature* **534**, 267–271 (2016).
27. Liu, X.Y. & Quan, N. Microglia and CNS Interleukin-1: Beyond Immunological Concepts. *Front Neurol* **9**(2018).
28. Muzio, L., Viotti, A. & Martino, G. Microglia in Neuroinflammation and Neurodegeneration: From Understanding to Therapy. *Front Neurosci-Switz* **15**(2021).
29. Monif, M., *et al.* Interleukin-1 β has trophic effects in microglia and its release is mediated by P2X7R pore. *J Neuroinflamm* **13**, 173 (2016).
30. He, W.T., *et al.* Gasdermin D is an executor of pyroptosis and required for interleukin-1 β secretion. *Cell Research* **25**, 1285–1298 (2015).
31. Kayagaki, N., *et al.* Caspase-11 cleaves gasdermin D for non-canonical inflammasome signalling. *Nature* **526**, 666–671 (2015).
32. Shi, J.J., *et al.* Cleavage of GSDMD by inflammatory caspases determines pyroptotic cell death. *Nature* **526**, 660–665 (2015).
33. Lieberman, J., Wu, H. & Kagan, J.C. Gasdermin D activity in inflammation and host defense. *Sci Immunol* **4**, eaav1447 (2019).
34. Lei, J., *et al.* IL-1 receptor antagonist therapy mitigates placental dysfunction and perinatal injury following Zika virus infection. *Jci Insight* **4**, e122678 (2019).
35. Zhang, M., *et al.* A translocation pathway for vesicle-mediated unconventional protein secretion. *Cell* **181**, 637–652 (2020).
36. Huitema, K., van den Dikkenberg, J., Brouwers, J.F.H.M. & Holthuis, J.C.M. Identification of a family of animal sphingomyelin synthases. *Embo J* **23**, 33–44 (2004).
37. Hanada, K., *et al.* Molecular machinery for non-vesicular trafficking of ceramide. *Nature* **426**, 803–809 (2003).

38. Hannun, Y.A. & Obeid, L.M. Sphingolipids and their metabolism in physiology and disease. *Nat Rev Mol Cell Bio* **19**, 175–191 (2018).
39. Othman, M.A., *et al.* Malabaricone C as Natural Sphingomyelin Synthase Inhibitor against Diet-Induced Obesity and Its Lipid Metabolism in Mice. *Acs Med Chem Lett* **10**, 1154–1158 (2019).
40. Yasuda, S., *et al.* A novel inhibitor of ceramide trafficking from endoplasmic reticulum to the site of sphingomyelin synthesis *J Biol Chem* **288**, 24162–24162 (2013).
41. Abdelbasset, M., *et al.* Differential contributions of fetal mononuclear phagocytes to Zika virus neuroinvasion versus neuroprotection during congenital infection. *Cell* **187**, 7511–7532 (2024).
42. Yeang, C., Ding, T.B., Chirico, W.J. & Jiang, X.C. Subcellular Targeting Domains of Sphingomyelin Synthase 1 and 2. *Nutr Metab* **8**, 89 (2011).
43. Subathra, M., Qureshi, A. & Luberto, C. Sphingomyelin Synthases Regulate Protein Trafficking and Secretion. *Plos One* **6**, e23644 (2011).
44. Kumagai, K. & Hanada, K. Structure, functions and regulation of CERT, a lipid-transfer protein for the delivery of ceramide at the ER-Golgi membrane contact sites. *Febs Lett* **593**, 2366–2377 (2019).
45. Arita, M., *et al.* Phosphatidylinositol 4-Kinase III Beta Is a Target of Enviroxime-Like Compounds for Antipoliavirus Activity. *J Virol* **85**, 2364–2372 (2011).
46. Zheng, J.F., *et al.* TMEDs mediate versatile cargo transport in vesicle-dependent unconventional secretion. *J Cell Biol* **225**, e202503075 (2025).
47. Schwinn, M.K., *et al.* CRISPR-Mediated Tagging of Endogenous Proteins with a Luminescent Peptide. *ACS chemical biology*, 467 (2017).
48. Passaro, S., *et al.* Boltz-2: Towards Accurate and Efficient Binding Affinity Prediction. *bioRxiv*, 2025.2006.2014.659707 (2025).
49. Leier, H.C., *et al.* A global lipid map defines a network essential for Zika virus replication. *Nature Communications* **11**, 3652 (2020).
50. Diop, F., *et al.* Zika virus infection modulates the metabolomic profile of microglial cells. *Plos One* **13**, e0206093 (2018).
51. Hatton, C.F. & Duncan, C.J.A. Microglia Are Essential to Protective Antiviral Immunity: Lessons From Mouse Models of Viral Encephalitis. *Front Immunol* **10**(2019).
52. Sun, Y.X., *et al.* A dual role of ERGIC-localized Rabs in TMED10-mediated unconventional protein secretion. *Nat Cell Biol* **26**, 1623–1623 (2024).
53. L.J. REED , H.M. A SIMPLE METHOD OF ESTIMATING FIFTY PER CENT ENDPOINTS. *American Journal of Epidemiology* **27**, 493–497 (1938).
54. Zeng, J.X., *et al.* The Zika Virus Capsid Disrupts Corticogenesis by Suppressing Dicer Activity and miRNA Biogenesis. *Cell Stem Cell* **27**, 618–632.e619 (2020).

55. Kim, J.Y., Grunke, S.D., Levites, Y., Golde, T.E. & Jankowsky, J.L. Intracerebroventricular Viral Injection of the Neonatal Mouse Brain for Persistent and Widespread Neuronal Transduction. *Jove-J Vis Exp*, 51863 (2014).
56. Jang, A. & Lehtinen, M.K. In utero intracerebroventricular delivery of adeno-associated viral vectors to target mouse choroid plexus and cerebrospinal fluid. *Star Protoc* **4**, 101975 (2023).
57. Rathore, A.P.S., Saron, W.A.A., Lim, T., Johan, N. & St John, A.L. Maternal immunity and antibodies to dengue virus promote infection and Zika virus-induced microcephaly in fetuses. *Sci Adv* **5**, eaav3208 (2019).
58. Yuan, L., *et al.* A single mutation in the prM protein of Zika virus contributes to fetal microcephaly. *Science* **358**, 933–936 (2017).
59. Chen, T., *et al.* ImageGP 2 for enhanced data visualization and reproducible analysis in biomedical research. *Imeta* **3**, e239 (2024).
60. Cho, K.F., *et al.* Proximity labeling in mammalian cells with TurboID and split-TurboID. *Nat Protoc* **15**, 3971–3999 (2020).
61. Ge, L., Melville, D., Zhang, M. & Schekman, R. The ER-Golgi intermediate compartment is a key membrane source for the LC3 lipidation step of autophagosome biogenesis. *Elife* **2**, e00947 (2013).
62. Ulmer, C.Z., Jones, C.M., Yost, R.A., Garrett, T.J. & Bowden, J.A. Optimization of Folch, Bligh-Dyer, and Matyash sample-to-extraction solvent ratios for human plasma-based lipidomics studies. *Anal Chim Acta* **1037**, 351–357 (2018).
63. Reichl, B., *et al.* Evaluation and optimization of common lipid extraction methods in cerebrospinal fluid samples. *J Chromatogr B* **1153**, 122271 (2020).
64. Maekawa, M. & Fairn, G.D. Molecular probes to visualize the location, organization and dynamics of lipids. *J Cell Sci* **127**, 4801–4812 (2014).
65. Deng, Y., Rivera-Molina, F.E., Toomre, D.K. & Burd, C.G. Sphingomyelin is sorted at the trans Golgi network into a distinct class of secretory vesicle. *Mol Biol Cell* **27**, 6677–6682 (2016).
66. Hao, Y.H., *et al.* Integrated analysis of multimodal single-cell data. *Cell* **184**, 3573–3587 (2021).
67. Stuart, T., *et al.* Comprehensive Integration of Single-Cell Data. *Cell* **177**, 1888–1902 (2019).
68. Stokes, C., *et al.* The human neural cell atlas of Zika virus infection in developing brain tissue. *Cell Rep Med* **6**, 102189 (2025).
69. Subramanian, A., *et al.* Gene set enrichment analysis: A knowledge-based approach for interpreting genome-wide expression profiles. *P Natl Acad Sci USA* **102**, 15545–15550 (2005).
70. Liberzon, A., *et al.* Molecular signatures database (MSigDB) 3.0. *Bioinformatics* **27**, 1739–1740 (2011).

71. Arthur Liberzon, C.B., Helga Thorvaldsdóttir, Mahmoud Ghandi, Jill P Mesirov, Pablo Tamayo. The Molecular Signatures Database (MSigDB) hallmark gene set collection. *Cell systems* **1**, 417–425 (2025).

MATERIALS AND METHODS

Mice

All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Tsinghua University (permit number: 24-ZM1). Mice were housed in ventilated cages under specific pathogen-free (SPF) or ABSL-2 conditions, with controlled temperature (20-26°C), humidity (40-70%), and a 12 h light-dark cycle, and were provided with food and water ad libitum. C57BL/6J mice were obtained from the Laboratory Animal Resources Center at Tsinghua University. *TMED10*^{Q204L/Q204L} knock-in mice (C57BL/6J) were created using CRISPR-Cas9-mediated targeting (GemPharmatech Co. Ltd). C57BL/6J mice were obtained from the Laboratory Animal Resources Center at Tsinghua University. GSDMD whole-body knockout mice were originated from Dr. Feng Shao (National Institute of Biological Sciences, Beijing) as previously described³². *TMED10* inducible whole-body knockout mice were generated via crossbreeding of *TMED10*^{fl/fl} with Cre-ERT (provided by Dr. Xiao-Yu Hu, Westlake University). Pregnant dams were intraperitoneally injected with tamoxifen (50 mg/kg body weight) or corn oil every other day from E10.5 to E16.5. The genotyping primers were listed in Table S4.

Cell culture and transfection

WT and *TMED10*-KO HEK293T, HeLa, COS-7 (provided by Dr. Yi-Guo Wang, Tsinghua University), Vero E6 cells (provided by Dr. Qiang-Feng Zhang, Tsinghua University) were maintained in DMEM supplemented with 10% FBS at 37°C in 5% CO₂ as described previously^{35,41,52}. Primary microglia prepared from mouse fetal brains were maintained in DMEM/F12 supplemented with 10% FBS, 1% Glutamax

(Gibco, 35050061) at 37°C in 5% CO₂. For immunofluorescence, cells were grown on coverslips (CITOGLAS). Transfection of plasmids into cells was performed using PEI (Polysciences, Inc.) for HEK293T and Sage LipoPlus (Sage Chemical Co. Ltd.) for HeLa according to the manufacture's protocols.

Plasmids and shRNA oligos

The TMED10, Str-TMED10ΔGD, LgBiT, mL-1β, ERGIC53, TGN46 expression and purification plasmids were described previously^{35,46}. ZIKV viral proteins (proM-E, E, NS2A, NS2B-NS3, NS4A and NS4B) were PCR-amplified from ZIKV strain FSS13025 cDNA template (kindly provided by Dr. Gong Cheng, Tsinghua University) and inserted into FUGW vector with an N-terminal FLAG for mammalian cell expression. V5-, HA-, TurboID-tagged NS4A-WT and NS4A-M7A constructs were generated by PCR amplification from FLAG-NS4A template and cloned into the FUGW vector. SGMS1, SMPD2 and CERT1 were PCR amplified from cDNA and inserted into FUGW vectors with a C-terminal HA tag. SGMS1-SBP was generated by PCR amplification from SGMS1 template and cloned into the FUGW vector. NS4A, TMED10, CERT1 truncations and mutations were generated by mutagenesis PCR. pET28a-Eqt-SM-FLAG was a gift from Dr. Yong-Qiang Deng, Southern Medical University. IL-1RA was PCR amplified from cDNA and inserted into pET28a for protein purification in *E. coli*.

For shRNAs, the plasmids (pLKO.1 lentivirus vector as a backbone) were purchased from the Center of Biomedical Analysis, Tsinghua University. The targeting sequences of these shRNAs were shown in the Table S5.

Reagents and antibodies

We obtained anti-FLAG agarose (A2220), anti-V5 agarose (A7345), anti-HA agarose (A2095)- and biotin (B4501) from Sigma; protease inhibitor cocktail (11836145001)

and phosphatase inhibitors (4906845001) from Roche; Phenylmethylsulfonyl fluoride (PMSF, ST505) from Beyotime; Opti-Prep (1893) from Serumwerk; Ni Sepharose (17-5268-01) from Cytiva; Streptavidin Beads 6FF (SA021025) from Smart-Lifesciences; IL-1 β ELISA Kit (KE10003) from Proteintech; antigen retrieval solution (ZLI-9064) from ZSGB-BIO; Malabaricone C (Mal C, HY-N8518) and T-00127_HEV1 (HEV1, HY-108313) from MCE; HPA-12 (H1553) from TCI; Furimazin (F882901) from Macklin.

We purchased mouse anti-V5 (V8012; IF, 1:500), anti-FLAG (F3165; WB, 1:5,000; IF, 1:500), anti-T7 (69522; WB, 1:2,000), rabbit anti-ERGIC53 (E1031; WB, 1:2,000) antibodies from Sigma; rabbit anti-V5 (13202; WB, 1:1,000; IF, 1:500), anti-HA (3724; WB, 1:5,000; IF, 1:500), anti-GSDMD (39754; WB, 1:2,000), anti-Caspase-3 (9662; WB, 1:2,000) antibodies from CST; mouse anti-tubulin (200608; WB, 1:10,000) and anti-actin (200068-8F10; WB, 1:5,000) antibodies from Zen Bioscience; rabbit anti-IL-1 β (ab9722; WB, 1:5,000) antibody from Abcam; goat anti-IL-1 β (BAF401; WB, 1:2,000) antibody from R&D; mouse anti-ERGIC53 (ENZ-ABS300-0100; IF, 1:200) antibody from Enzo; rabbit anti-ZIKV-NS4A (GTX133704; WB, 1:1,000; IF, 1:100) and anti-ZIKV-Envelope (GTX634155; WB, 1:1,000) antibodies from Gentex; rabbit anti-TMED10 (15199-1-AP; WB, 1:3,000), anti-CERT1 (15191-1-AP; WB, 1:2,000), anti-SGMS1 (19050-1-AP; WB, 1:500), anti-SOX2 (66411-1-Ig; IF, 1:500), mouse anti-TMED10 (67876-1-Ig; IF, 1:200), rabbit anti-ERGIC53 (13364-1-AP; IF, 1:200) antibodies from Proteintech; rat anti-SOX2 (HA601396; IF, 1:500) antibody from HUABIO; goat anti-rabbit IgG Alexa Fluor Plus 647 (A32733), anti-mouse IgG Alexa Fluor Plus 647 (A32728), anti-rabbit IgG Alexa Fluor 568 (A-11011), anti-mouse IgG Alexa Fluor 568 (A-11004), anti-rabbit IgG Alexa Fluor Plus 488 (A32731), anti-mouse IgG Alexa

Fluor Plus 488 (A32723) from Invitrogen.

Protein purification

IL-1RA, mL-1 β and Eqt-SM-FLAG were purified with Ni Sepharose (Cytiva, 17-5268-01) and pGEX4T1-GST-LgBiT with Glutathione beads as previously described^{34,35,46}. Briefly, protein expression was induced through the addition of IPTG (100 μ M) to BL21 *E. coli* cells for 18 h at 16°C. Next, the bacteria were collected and lysed in lysis buffer (2 $\times$ PBS, 10 mM imidazole, 0.5 mg/mL lysozyme, 0.3 mM DTT and 1 mM PMSF) on ice for 30 min. The lysates were sonicated and centrifuged at 20,000 rpm for 1 h. The supernatant was incubated with Ni Sepharose or Glutathione Agarose at 4°C for 2 h. The beads were collected and washed with 2 $\times$ PBS containing 0.1% Tween 20 and 25 mM imidazole for His proteins or 1 $\times$ PBS containing 0.1% Tween 20 for GST proteins. Proteins were eluted with elution buffer (2 $\times$ PBS and 250 mM imidazole for His proteins or 50 mM Tris pH 8.0, 250 mM KCl and 25 mM glutathione for GST proteins), concentrated using Amicon ultra centrifugal filters (Merck, UFC901096) and desalted by PD MiniTrap G-25 (Cytiva, 55965-84-9). The endotoxin was removed by incubating purified proteins with endotoxin removal resin (Thermo Scientific, 88274) overnight at 4°C. The purified proteins were snap-frozen in liquid nitrogen and stored at -80°C.

ZIKV preparation

ZIKV strain SPH2015 (GenBank: KX197192) was kindly provided by Dr. Qiang Ding from Tsinghua University. ZIKV stain FSS13025 (GenBank: KU955593) was recovered from an infectious cDNA clone from Dr. Gong Cheng. To produce viral stocks, Vero cells were infected with 10 mL of diluted virus (MOI = 0.05) for 2 h, after which another 5 mL of medium was added. Culture supernatants were harvested 4 d post-infection and filtered through 0.45 μ m syringe filters⁵⁹. Viral stocks were

aliquoted and stored at -80°C. Viral titers (expressed as TCID₅₀/mL) were determined by cytopathic effect (CPE) in Vero cells using serial dilutions and calculated by the Reed-Muench method⁵³.

The ZIKV-NS4A^{M7A} (M7A substitution in NS4A) mutant strain was constructed through mutagenic PCR using the ZIKV FSS13025 clone as a template and confirmed the mutation by DNA sequencing⁶¹. The resulting plasmid was linearized via restriction endonuclease digestion and purified by Phenol/Chloroform extraction. Viral RNA was synthesized by *in vitro* transcription using the HiScribe T7 ARCA mRNA Kit (NEB, E2065S) and then purified. The RNA was transfected into Vero cells using Lipofectamine 3000 (Biomed, TG111-01) and recombinant virus was harvested from culture supernatants 4 d post-transfection.

ZIKV infection, AAV delivery and lentiviral transduction

ZIKV-NS4A^{WT} and ZIKV-NS4A^{M7A} used in Fig.2b-l; Supplementary information, Fig.S4a; Fig. 4f, h, j, l were derived from the FSS13025 strain. All other experiments used the SPH2015 strain. Pregnant dams were infected with ZIKV at E13.5 by *in utero* intracerebroventricular injection⁵⁴⁻⁵⁶ of 200-350 PFU in 1 µL PBS (with or without 1% DMSO), delivered alone or together with IL-1RA (0.25 µg), HPA-12 (50 µM) or Mal C (25 µM). Mock-infected dams received PBS injections. Dams were euthanized at E16.5 for tissue collection as previously described. Primary microglia and HEK293T cells were infected with ZIKV at MOI = 2 or at the indicated MOIs at 37°C. The inoculum was then replaced with fresh medium containing 2% FBS and cells were harvested 36-48 h post-infection.

AAV serotype 9 vectors expressing GFP, V5-tagged NS4A-WT or NS4A-M7A under a CMV promoter were generated by OBiO Tech, Inc., China. For AAV delivery, 1 µL of the AAV-GFP, AAV-NS4A-WT or AAV-NS4A-M7A vector (2×10¹² vg/mL) was

injected into embryos by *in utero* intracerebroventricular injection at E13.5. Fetal brains were collected at E18.5 for downstream analyses.

Lentiviral transduction was employed to overexpress NS4A-WT or NS4A-M7A or SMPD2 in primary microglia; mScarlet-OSBP-PH and GFP-ERGIC53 in COS-7 cells; and to knock down SGMS1 or CERT1 in HEK293T cells and primary microglia. Lentiviral particles were generated as previously described⁴² by transfecting HEK293T cells with FUGW-based overexpression plasmids (co-transfected with VSV-G and psPAX2) or pLKO.1-based shRNA knockdown plasmids (co-transfected with pMD2.G and psPAX2). Viral supernatants were collected 72 h post-transfection and used to transduce the indicated cells.

Secretion determination

To assess cytokine secretion from ZIKV-infected fetal brain tissues, brain tissues collected at E16.5 were transferred into serum-free DMEM/F12 and incubated at 37°C in 5% CO₂ for 6 h. The culture medium was collected and concentrated using 10 kDa Amicon Ultra centrifugal filters. IL-1 β release was quantified via ELISA (Proteintech; KE10003), and broader cytokine profiling was performed using a Mouse Magnetic Luminex Assay (Universal Biotech Co., Ltd.) according to the manufacturer's instructions.

To determine IL-1 β secretion from microglia, primary microglia isolated from fetal brains using a CD11b-positive Microglia Isolation Kit (RWD Life Science; K1306-10) were cultured for 5 d in DMEM/F12 with 10% FBS and 1% GlutaMAX, and then incubated in fresh medium with indicated drugs for 8 h. The supernatant was subsequently collected, concentrated, and analyzed for IL-1 β secretion using the aforementioned ELISA kit.

To assess IL-1 β secretion from HEK293T cells, cells expressing mL-1 β -FLAG,

mIL-1 β -HA or mIL-1 β -HiBiT were transfected with the indicated plasmids. For analysis of mIL-1 β -FLAG and mIL-1 β -HA secretion, the culture medium was replaced with EBSS containing the indicated treatments and cells were incubated for 1 h. The culture medium was then collected and concentrated, while the corresponding cell pellets were lysed directly in SDS-PAGE loading buffer. Secreted and intracellular mIL-1 β were analyzed by immunoblotting. For analysis of mIL-1 β -HiBiT secretion, 30 μ L of culture medium was incubated with Furimazine (10 nM final concentration) and 5 ng of recombinant LgBiT protein in a final volume of 60 μ L at room temperature for 10 min. Luminescence was measured using a SPARK Multimode Plate Reader (TECAN). Corresponding cell pellets were lysed directly in SDS-PAGE loading buffer for analysis of intracellular mIL-1 β -HiBiT.

Cellular cytotoxicity was assessed by measuring lactate dehydrogenase (LDH) activity in the culture medium using an LDH Cytotoxicity Detection Kit (Thermo, 88953) according to the manufacturer's instructions.

Tissue preparation and histology

Cortical thickness was measured by Nissl staining as previously described^{57,58}. In brief, fetal brains were fixed in 4% paraformaldehyde (PFA) overnight at 4°C and cryopreserved in 30% sucrose at 4°C for 24-48 h. Brains were embedded in Tissue-Tek OCT compound (Sakura, 4583), snap-frozen on dry ice and stored at -80°C. Cryosections (10 μ m thickness) were cut on a Leica CM1950 cryostat. Sections were rinsed twice in distilled water (5 min each) and stained with Nissl Staining Solution (Beyotime, C0117) for 10 min at 50°C according to the manufacturer's instructions. Sections were then washed twice in distilled water, briefly immersed in 95% ethanol (~5 s) and washed twice in 70% ethanol. Dehydration was performed in 95% ethanol (two changes, 2 min each) followed by

clearing in xylene (two changes, 5 min each). Sections were mounted and imaged on a digital slide scanner (KF-PRO-120-HI, KFBIO). Cortical thickness was measured using K-Viewer and ImageJ.

RNA sequencing and data analysis

Total RNA was extracted from fetal brain tissues and mRNA was enriched for library construction using the VAHTS Universal V8 RNA-seq Library Prep Kit (Vazyme Biotech Co., Ltd, China). Libraries were sequenced on an Illumina NovaSeq X Plus 25B platform. High-quality reads were aligned to the reference genome using HISAT2 (v2.2.1) and gene expression levels were quantified as fragments per kilobase per million mapped reads (FPKM) using HTSeq (v0.6.1).

Differential expression analysis was performed using DESeq2 (v1.34.0) in R. Raw counts were filtered to remove lowly expressed genes (row sums ≤ 10 across all samples), normalized by the median-of-ratios method and fitted with a negative binomial generalized linear model. Wald tests were used to estimate $\log_2$ fold-changes between conditions, with shrinkage applied to dispersions and effect sizes. For pairwise comparisons, genes were ranked by DESeq2 $\log_2$ fold-change for downstream pathway analyses. For differential expression and pathway enrichment analyses between two groups, unpaired two-tailed Student's t-tests with Benjamini-Hochberg FDR correction were applied.

Gene set enrichment analysis (GSEA) was performed using GSEA software (v4.4.0). Normalized enrichment scores (NES) were computed for predefined gene sets from the Molecular Signatures Database (MSigDB), including Gene Ontology (GO) biological processes associated with cerebral cortex development and cellular response to interleukin-1 as well as MSigDB hallmark gene sets for inflammatory response. Terms with nominal $P < 0.05$ were considered significant. GO enrichment

analysis of upregulated and downregulated genes ($FDR < 0.05$, $P < 0.05$) was performed using DAVID Bioinformatics Resources (<https://davidbioinformatics.nih.gov/>). GO terms associated with inflammation and brain development with $P < 0.05$ were selected and enrichment plots were generated using ImageGP⁵⁹. Heatmaps and violin plots were generated using an online platform (<https://www.bioinformatics.com.cn>) and R. All analyses were performed in parallel for infected and control samples to minimize technical variation.

Immunofluorescence

Immunofluorescence was performed as previously described⁵². For cell imaging, cells grown on glass coverslips were fixed with pre-chilled methanol for 5 min on ice (for endogenous ERGIC53 staining) or with 4% PFA for 15 min at room temperature. Cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min, washed three times with PBS and blocked with 10% FBS in PBS for 1 h at room temperature. Cells were incubated with primary antibodies for 1 h at room temperature, washed three times with PBS and incubated with secondary antibodies for 1 h at room temperature. For sphingomyelin visualization, cells were additionally incubated with recombinant GFP-tagged non-toxic lysenin (NT-Lys; provided by Dr. Li Yu, Tsinghua University) in PBS for 15 min at room temperature. Fluorescence images were acquired using an Olympus FV3000 confocal microscope and quantified using ImageJ.

For cortical imaging, cryosections (8 μ m) were subjected to antigen retrieval at 95°C for 15 min and blocked with 3% BSA in PBS for 1 h at room temperature. Sections were incubated with anti-SOX2 primary antibody overnight at 4°C and subsequently stained with secondary antibody and DAPI for 1 h at room temperature. Whole-slide images were acquired on a Vectra Polaris automated imaging system (Akoya Biosciences) and quantified using Phenochart (Akoya Biosciences, v1.0.9) and

ImageJ.

For the fluorescence complementation assay, HEK293T cells expressing GFP11-TMED10 and mIL-1 β -GFP (1-10) were transfected with indicated plasmids. Following transfection, the cells were treated with or without 40 μ M biotin for 16 hours prior to harvesting. The GFP signal was detected using CytoFlex LX (Beckman) and analyzed using the FlowJo_V10 software (20,000-30,000 cells were analyzed in each group of each experiment).

3D-SIM and multi-SIM analysis

Cells were cultured on glass coverslips and processed for immunofluorescence as described above. To quantify co-localization of SGMS1 with ERGIC53, 3D-SIM image stacks were acquired along the *z*-axis using a Nikon A1/SIM/STORM system and volumetric distributions of SGMS1 and ERGIC53 signals were reconstructed to calculate the percentage of SGMS1 co-localizing with ERGIC53. Co-localization of NS4A with TMED10 or ERGIC53, SGMS1 with TGN46, and CERT1 with ERGIC53 or TGN46 was similarly analyzed using a multi-SIM system (NanoInsights). Co-localization analysis and quantification were performed using NIS-Elements AR (Nikon), NanoInsights reconstruction software, ImageJ and Imaris. All imaging experiments were performed at least three times and representative images are shown.

TurboID-based proximity labeling and co-immunoprecipitation

TurboID-based proximity labeling and co-immunoprecipitation (Co-IP) were performed as previously described⁶⁰. Briefly, cells transfected with FLAG-, V5- or TurboID-tagged NS4A-WT, NS4A-M7A or other indicated constructs were harvested 24 h post-transfection. For biotin labeling, cells were pre-treated with 50 μ M biotin for 10 min at 37°C prior to harvest. Cells were washed twice with ice-cold PBS, lysed on ice for 30 min in Co-IP buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM

EDTA, 0.5% NP-40, 10% glycerol) supplemented with protease inhibitor cocktail. Lysates were clarified by centrifugation, and the resulting supernatants were incubated with the appropriate agarose resins at 4°C for 3 h under continuous rotation. Bound complexes were washed five times with Co-IP buffer and either analyzed by immunoblotting or washed once with 50 mM ammonium bicarbonate buffer prior to proteomic analysis.

Membrane flotation assay

To enrich ERGIC membrane fraction, we performed membrane flotation as previously described⁶¹. Briefly, cells transfected with FLAG-tagged NS4A-WT or NS4A-M7A, or infected with ZIKV-NS4A^{WT} or ZIKV-NS4A^{M7A} were collected and resuspended in 3× the cell pellet volume of HB1 lysis buffer (20 mM HEPES-KOH, pH 7.2, 400 mM sucrose, 1 mM EDTA, protease and phosphatase inhibitors, 0.3 mM DTT) and then lysed by passing through a 22G needle until around 85% lysis analyzed by Trypan Blue staining. The homogenates were centrifuged at 3,000×g for 10 min, and then the supernatant was ultracentrifuged at 25,000×g for 20 min. The membrane pellet (P fraction) was suspended in 0.75 ml 1.25 M sucrose buffer and overlaid with 0.5 ml 1.1 M and 0.5 ml 0.25 M sucrose buffer (Sigma, Golgi isolation kit, GL0010). Centrifugation was performed at 120,000×g for 2 h (TLS 55 rotor, Beckman), after which the interface between 0.25 M and 1.1 M sucrose (L fraction) was collected. The L fraction was suspended in 1 ml 19% OptiPrep for a step gradient containing 0.5 ml 22.5%, 1 ml 19% (sample), 0.9 ml 16%, 0.9 ml 12%, 1 ml 8%, 0.5 ml 5% and 0.2 ml 0% OptiPrep each. Each density of OptiPrep was prepared by diluting 50% OptiPrep (20 mM Tricine-KOH, pH 7.4, 42 mM sucrose and 1mM EDTA) with a buffer containing 20 mM Tricine-KOH, pH 7.4, 250 mM sucrose and 1mM EDTA. The OptiPrep gradient was centrifuged at 150,000×g for 3 h (SW55 Ti

rotor, Beckman) and subsequently yields ten fractions from top to bottom. The combination of fractions 3 and 4, which correspond to ERGIC membranes, were collected and subjected to immunoblotting, dot blot, proteomics and lipidomic analyses.

Proteomic analysis

For interactome profiling of NS4A-WT and NS4A-M7A, NS4A expression of purified complexes were determined using WB. Protein including equal amounts of NS4A-WT and NS4A-M7A were submitted for LC-MS/MS analysis at the Protein Research and Technology Center, Tsinghua University. For proteomics of ERGIC-enriched membrane fractions, purified samples were resolved by SDS-PAGE and gel bands were excised and subjected to in-gel tryptic digestion prior to LC-MS/MS analysis. MS/MS spectra were searched against the Swiss-Prot human database using Proteome Discoverer with a false discovery rate (FDR) threshold of <1%. Proteins identified by at least one unique peptide were retained for further analysis.

Lipidomic analysis

ERGIC-enriched membrane fractions were collected from 2×10^8 HEK293T cells as described above. Lipids were extracted using a modified methyl-tert-butyl ether (MTBE)/methanol method^{70,71}. Briefly, membranes were resuspended in 300 μ L methanol and vortexed for 1 min, after which 1 ml MTBE and 250 μ L water were added sequentially with 1 min vortexing after each addition. The mixture was vortexed and allowed to stand for 5 min; this cycle was repeated three times. Samples were centrifuged at 8,000 rpm for 15 min and the upper organic layer was collected and dried under nitrogen. Lipid pellets were stored at -80°C and resuspended in 150 μ L dichloromethane/methanol (2:1, v/v) before LC-MS/MS analysis.

Lipids were separated by reverse-phase chromatography on a Cortecs C18 column (2.1×100 mm, $2.7 \mu\text{m}$; Waters). Mobile phase A consisted of water/acetonitrile (4:6, v/v) containing 10 mM ammonium acetate and mobile phase B consisted of acetonitrile/isopropanol (1:9, v/v). Gradient elution was performed at 0.28 mL/min with the column maintained at 40°C as follows: 0–3.0 min, 30% B; 4.5 min, 33% B; 7.0 min, 45% B; 8.0 min, 52% B; 11.0 min, 58% B; 14.0 min, 66% B; 17.0 min, 70% B; 21.0 min, 75% B; 23.0–30.0 min, 98% B; 30.5–35.0 min, 30% B. Data were acquired on an Orbitrap Exploris 240 mass spectrometer (Thermo Fisher Scientific) coupled to a Vanquish UHPLC system (Thermo Fisher Scientific).

Lipid identification and quantification were performed using LipidSearch 4.2 (Thermo Fisher Scientific) with mass tolerances of 8 ppm for precursors and 10 ppm for fragments and an m-score threshold of 5. Identifications were filtered to grades A–D; adducts monitored were $[\text{M}+\text{H}]^+$ and $[\text{M}+\text{NH}_4]^+$ in positive mode and $[\text{M}+\text{CH}_3\text{COO}]^-$ in negative mode. Sphingomyelin species were additionally identified and quantified using LipidSearch 5.0 with $[\text{M}+\text{HCO}_3]^-$ in negative mode. Only lipids with a chromatographic peak area exceeding 5×10^6 and a retention time deviation within ± 0.25 min were included for quantification. Differential profiles of phospholipids and sphingolipids were visualized as heatmaps to illustrate lipid signatures associated with ZIKV infection or with expression of Ctrl, NS4A-WT or NS4A-M7A.

Lipid extraction and dot blot of sphingomyelin

Total lipids were extracted from ERGIC-enriched membrane fractions as previously described^{46,62,63}. Membrane fractions collected from 6×10^7 cells were resuspended in PBS (1:1, v/v) and mixed with chloroform/methanol (chloroform: methanol: suspension = 8:4:3, v/v/v) by vortexing for 30 s. Mixtures were shaken at 37°C for 30

min and the chloroform phase was collected, evaporated under a stream of nitrogen and further dried at room temperature for 30 min. Dried lipids were dissolved in ethanol and phosphatidylcholine content was measured using a standard kit (Wako) for normalization. Equal amounts of lipids were spotted onto nitrocellulose membranes and dried at room temperature for 30 min. Membranes were blocked with 4% (w/v) fatty-acid-free BSA in TBS (20 mM Tris-HCl, 150 mM NaCl, pH 7.6) for 2 h and incubated sequentially with Eqt-SM-FLAG (2.5 μ g/mL), anti-FLAG primary antibody and HRP-conjugated secondary antibody, each in 4% BSA in TBST (TBS with 0.1% Tween-20) for 2 h^{64,65}. Membranes were washed three times with TBST (10 min each) and signals were detected by enhanced chemiluminescence.

RUSH system

HEK293T or HeLa cells were co-transfected with the ERGIC-hook strp-TMED10 Δ GD) and reporter SGMS1-SBP. To release SGMS1-SBP from the ERGIC, 40 μ M of biotin was added to the cultured cells for 16 h⁴⁶. Images were acquired using a multi-SIM system (NanoInsights). mIL-1 β secretion, fluorescence complementation and Co-IP were conducted in HEK293T cells following the procedures described above.

Public scRNA-seq datasets and analysis

Publicly available single-cell RNA sequencing (scRNA-seq) datasets were reanalyzed in R using Seurat^{66,67}, dplyr, tidyr, ggplot2, patchwork and scales. Mouse fetal-brain scRNA-seq data were obtained from Abdelbasset et al. (GEO: GSE267677)⁴¹, which profiled E16.5 fetal brains exposed to saline or ZIKV H/PF/2013 at E13.5. Human primary fetal neural cell scRNA-seq data were obtained from Stokes et al. (GEO: GSE238140)⁶⁸, which profiled primary human fetal neural cultures treated with mock medium, ZIKV/Brazil, or ZIKV/Cambodia-FSS13025.

Mouse fetal brain scRNA-seq reanalysis

The processed mouse fetal-brain Seurat object was used to define the major cellular landscape and condition-associated changes in cell composition. Experimental condition was encoded as mock or H/PF/2013. Broad cell-type labels from the reanalysis pipeline were harmonized into populations of NPC, neuron, microglia, monocyte, macrophage, astrocyte, vascular cell, ependymal cell, neutrophil, B cell, T cell and mixed. UMAP embeddings generated during the reanalysis pipeline were used for visualization with DimPlot. Cell type proportions were calculated within each condition by dividing the number of cells assigned to each annotated population to total annotated cells. For microglia-focused analysis, microglial cells were subsetted from the fetal-brain object and subjected to re-clustering, resulting in four microglial subclusters, MG1 to MG4.

Human fetal neural cell scRNA-seq reanalysis

For the human fetal neural cell analysis, raw scRNA-seq data of GSE238140 generated using 10x Genomics were filtered into feature-barcode matrices and imported into R with Read10X. Sample-level Seurat objects were generated for G001_mock, G002_BRZ and G003_FSS using Create SeuratObject. Human fetal neural cells were harmonized into neural stem cells, excitatory neurons, inhibitory neurons, astrocytes, oligodendrocytes and microglia. UMAP visualization was applied to display major cell populations. Module scores for the THU pathway, inflammatory response and sphingolipid metabolism were calculated using the AddModuleScore function in Seurat based on gene modules. For heat-map visualization, mean module scores were calculated for each cell type-condition group and converted to Z-scores across the displayed groups.

Gene module scoring, marker visualization and enrichment analysis

For gene module scoring, gene sets were obtained from the official Gene Set Enrichment Analysis (GSEA)/Molecular Signatures Database (MSigDB) collections⁶⁹⁻⁷¹. Single-cell gene-set scores were calculated in Seurat using AddModuleScore. For heat-map visualization, scores were averaged within each annotated microglia subcluster and converted to row Z-scores across MG1-MG4. For cell marker visualization, dot plots of cell marker genes were generated from scaled RNA expression using Seurat DotPlot. Genes of Sphingolipid metabolism include *Smpd1*, *Cers2*, *Cers5*, *Degs1*, *Sptlc1*, *Smpd3* and *Sgms1*; IL-1 β release genes include *Tmed10*, *Rab1a*, *Rab1b* and *Rab2a*; Inflammatory response genes include *Cxcl10*, *Il1 β* , *Nlrp3*, *Pycard*, *Casp1*, *Tnf* and *Ccl2*. Dot size represents the percentage of cells expressing indicated gene, and dot color represents scaled average expression. Gene-set enrichment analyses were performed using GSEA/MSigDB collections. Enriched terms were visualized as bubble plots in which dot size indicates the number of overlapping genes and dot color indicates enrichment *P* value.

Biosafety statement

All experiments involving ZIKV or AAV were reviewed and approved by the Biosafety Committee of Tsinghua University. The generation of the ZIKV-NS4A^{M7A} mutant virus and all subsequent infection-related experiments were conducted in certified BSL-2 and ABSL-2 facilities, in strict compliance with institutional biosafety guidelines and standard operating procedures. According to the official classification of human pathogenic microorganisms published by the National Health Commission of the People's Republic of China (<http://www.nhc.gov.cn/wjw/c100375/202308/45b037124e0a437bb36fbc84332a9a74.shtml>), ZIKV is categorized as a BSL-2 pathogen. Accordingly, all relevant experiments were performed under appropriate containment conditions with the use of

suitable personal protective equipment. As a precautionary measure, we refrained from extensive testing of viral variants with the potential to expand tropism in human and murine cells.

Statistics

All data are presented as mean $\pm$ SEM. Statistical analyses were performed using two-tailed unpaired Student's t-test for comparisons between two groups and ANOVA for multiple-group comparisons, as detailed in the corresponding figure legends. All statistical analyses were carried out using GraphPad Prism 10.0 software. Experiments were independently repeated at least three times with comparable results; immunoblot data are representative of at least three independent biological replicates. Sample sizes were determined based on prior experience to ensure sufficient statistical power, and no statistical methods were used to predetermine sample sizes. Animals and samples were randomly assigned to experimental groups, and data collection and analysis were performed blinded to experimental conditions where applicable. Differences were considered statistically significant at $P < 0.05$, and exact P values are provided in the figures or figure legends.

DATA AVAILABILITY

All data supporting the findings of this study are available within the paper and its supplementary data. Further information and requests for reagents should be directed to Lead Contact, Min Zhang (zhangmin143@mail.tsinghua.edu.cn). Plasmids and cell lines generated in this study will be made available upon request. We may require a payment and/or a completed Materials Transfer Agreement in case there is potential for commercial application. Source data are provided with this paper.

CODE AVAILABILITY

All code reported in this manuscript are available on request.

Table S4. List of genotyping primers used in this study.

Gene	Sequences
<i>Gsdmd flox</i> (F)	TTCCCTCCAGCCCTACTTGC
<i>Gsdmd flox</i> (R)	TGAGCATCCTTCCCAACCTG
<i>Tmed10-Q204L</i> (F)	AGACCTATGGACAGAATGAGGCTAAC
<i>Tmed10-Q204L</i> (R)	AAGGACTTCAAGCAGTTGAGCACA

Table S5. Information about shRNA constructs used in this study.

shRNA	Sequences
Control shRNA	CGTGATCTTCACCGACAAGAT
<i>SGMS1</i> shRNA-1	GCGTTGGACAACAATAAAGAA
<i>SGMS1</i> shRNA-2	GCGAAGAATAATGAAGCTCAT
<i>SGMS1</i> shRNA-3	CCTCAGTAGGCAAGTTAAATA
<i>CERT1</i> shRNA-1	GCAAAGCGAGAGTATCCTAAA
<i>CERT1</i> shRNA-2	GCAACACTTTCTCATTGTATT

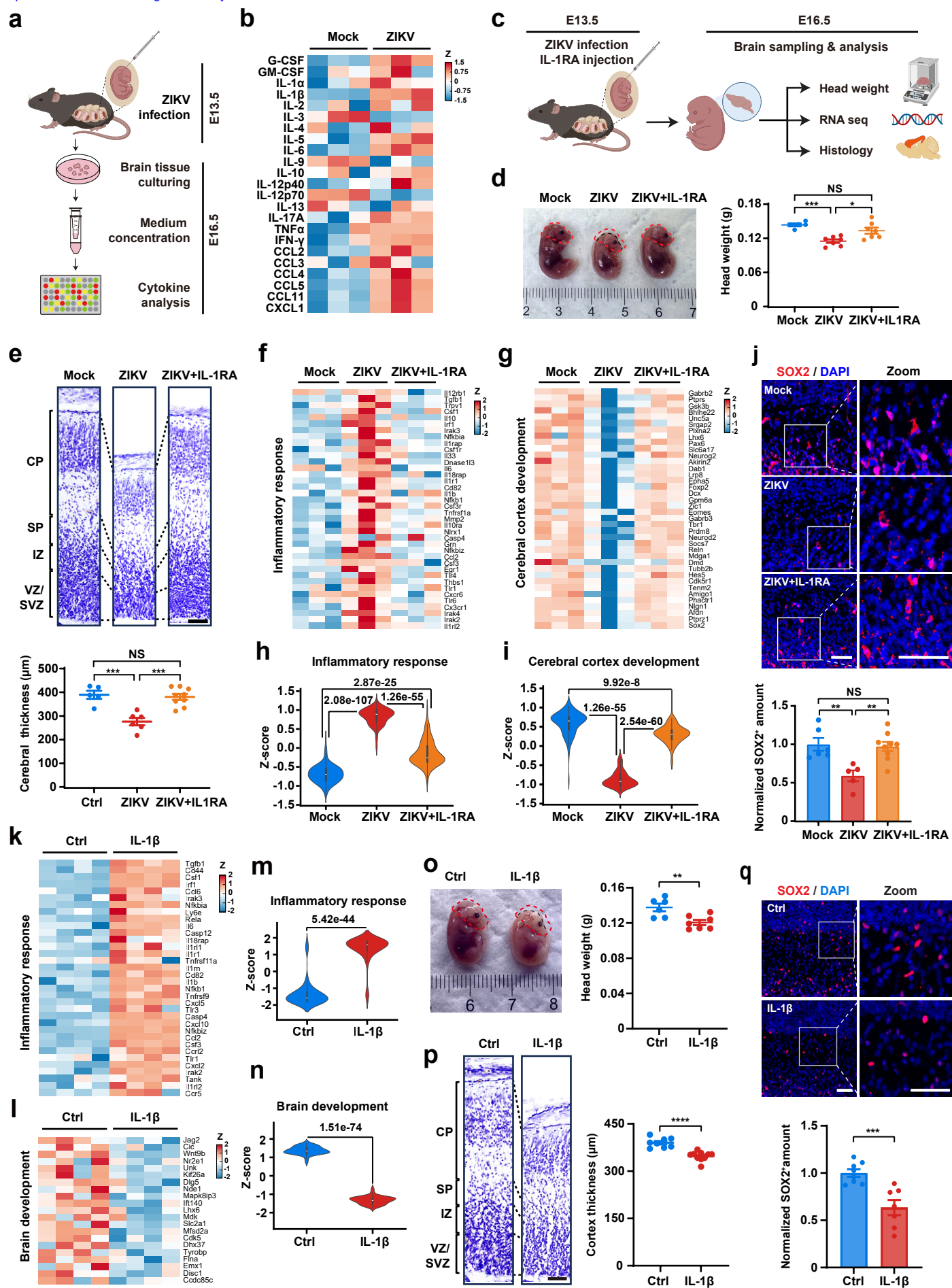


Figure 1

Fig. 1 | IL-1 β is responsible for ZIKV-induced neuroinflammation and cortical injury. a, Schematic of experimental design. Time-mated dams were injected intracerebroventricularly with PBS (mock) or ZIKV at E13.5. Fetal brains were collected at E16.5 and cultured for 6 h, and supernatants were collected for cytokine analysis. **b,** Heatmap of cytokine levels in fetal brain supernatants from (a) ($n = 3$). Concentrations were normalized and scaled from -1 to 1. **c,** Schematic of experimental design. Time-mated dams were injected intracerebroventricularly with PBS, ZIKV alone or ZIKV together with IL-1 receptor antagonist (IL-1RA; 0.25 μ g) at E13.5. Fetuses were collected at E16.5 for assessment of head size and weight, cortical histology, and bulk RNA-seq. **d,** Representative images of fetal heads (boundaries demarcated) and quantification of head weights ($n = 5-7$). **e,** Nissl staining of different cortical layers and quantification of cortical thickness. Cortical plate (CP), subplate (SP), intermediate zone (IZ), ventricular zone (VZ) and subventricular zone (SVZ) are indicated ($n = 5-9$). **f, g,** Heatmaps of representative genes associated with inflammatory response (f) and cortical development (g). **h, i,** Violin plots of indicated pathways from bulk RNA-seq. **j,** Representative images of cortices immunostained for SOX2 and DAPI, with quantification of SOX2⁺ NPCs normalized to mock ($n = 5-9$). **k-n,** RNA-seq analyses of fetal brains collected 6 h after dams received Ctrl (PBS) or mIL-1 β (10 ng) injection at E14.5. **k, l,** Heatmaps showing representative genes associated with inflammatory responses (k) and brain development (l). **m, n,** Violin plots showing the indicated pathways identified by bulk RNA-seq analysis. **o-q,** Analyses of E16.5 fetal brains from WT mice with Ctrl or mIL-1 β injection at E14.5. **o,** Representative images of fetal heads (boundaries demarcated) and quantification of head weights ($n = 6-7$). **p,** Nissl staining of cortical layers and quantification of cortical thickness ($n = 8-10$). **q,** Representative images of cortices immunostained for SOX2 and DAPI, with quantification of SOX2⁺ NPCs normalized to mock ($n = 7-8$). **e, j, p, q,** Scale bars, 50 μ m. Data are mean $\pm$ SEM (d, e, j, o, p, q). Statistical significance was assessed using one-way ANOVA with Tukey's multiple-comparison test (d, e, j) and unpaired two-tailed Student's *t*-test (h, i, m-q). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. NS, non-significant. Data are representative of at least three independent dams.

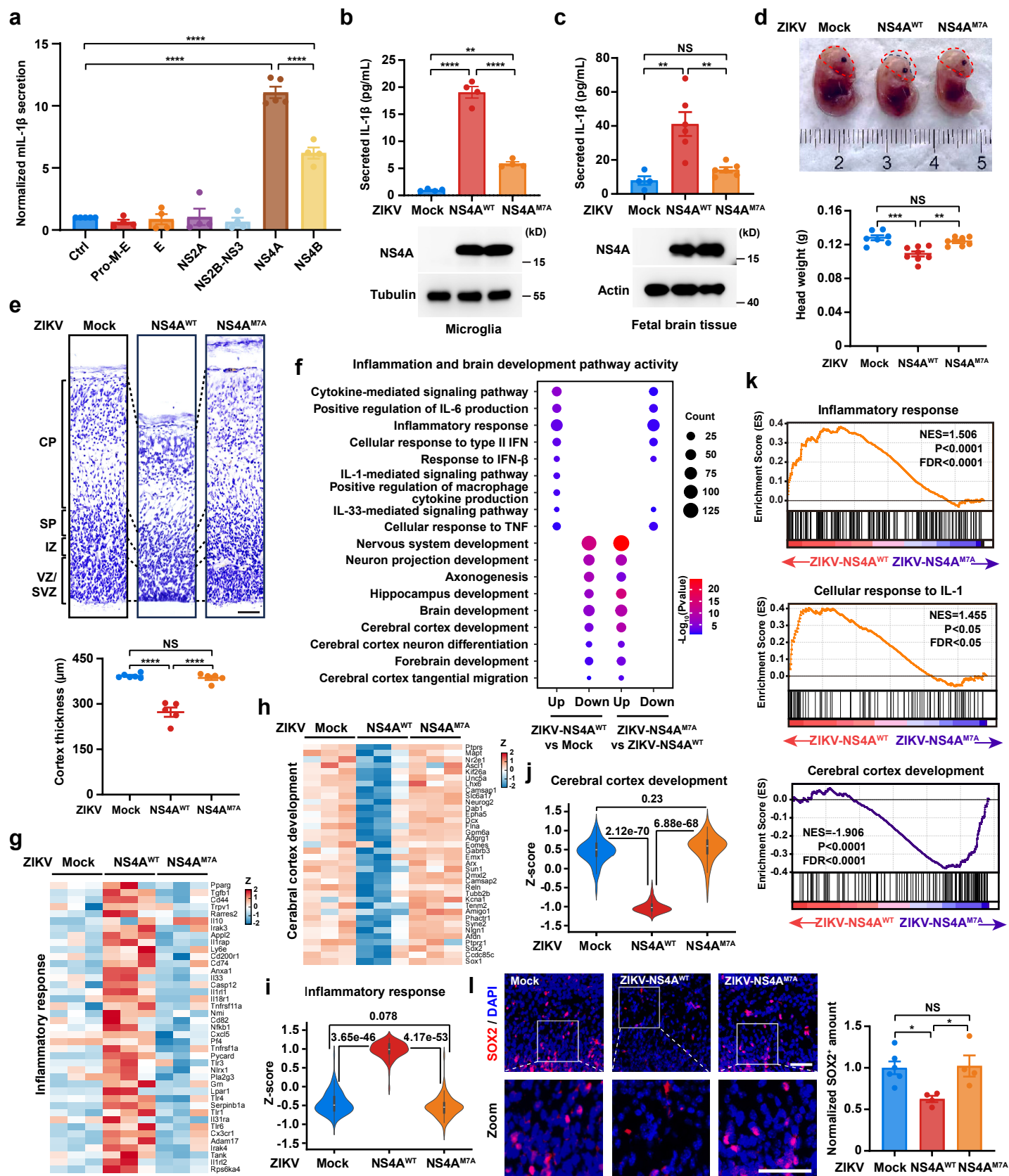


Figure 2

Fig. 2 | NS4A is responsible for ZIKV-induced IL-1 β secretion and neuroinflammation. **a**, Secretion of mIL-1 β in mIL-1 β -HA-expressing HEK293T cells transfected with empty vector or FLAG-tagged ZIKV proteins as indicated. mIL-1 β secretion was calculated and normalized. **b**, IL-1 β secretion measured by ELISA in primary microglia from E16.5 fetal brains of WT mice. Cells were infected with mock, ZIKV-NS4A^{WT} or ZIKV-NS4A^{M7A}. NS4A expression was verified. **c-l**, Analyses of E16.5 fetal brains from WT mice infected with mock, ZIKV-NS4A^{WT} or ZIKV-NS4A^{M7A} at E13.5. **c**, IL-1 β secretion measured by ELISA in fetal brain tissues ($n = 4-6$). **d**, Representative images of fetal heads (boundaries demarcated) and quantification of head weights ($n = 7-8$). **e**, Nissl staining of cortical layers and quantification of cortical thickness ($n = 5-6$). **f**, GO analysis of bulk RNA-seq data showing differentially enriched pathways associated with inflammation and cortical development ($P < 0.05$). Up, upregulated pathways; Down, downregulated pathways. **g,h**, Heatmaps of representative genes associated with inflammatory response (**g**) and cortical development (**h**). **i, j**, Violin plots of indicated pathways from bulk RNA-seq. **k**, GSEA of indicated pathways from bulk RNA-seq. **l**, Representative images of cortices immunostained for SOX2 and DAPI, with quantification of SOX2⁺ NPCs normalized to mock ($n = 4-6$). **e, l**, Scale bars, 50 μ m. Data are mean $\pm$ SEM (**a-e, l**). Statistical significance was assessed using one-way ANOVA with Tukey's multiple-comparison test (**b-e, l**) and unpaired two-tailed Student's *t*-test (**a, i-j**). * $p < 0.5$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. NS, non-significant. **a, b**, Data are representative of at least four independent experiments. **c-l**, Data are representative of at least three independent dams.

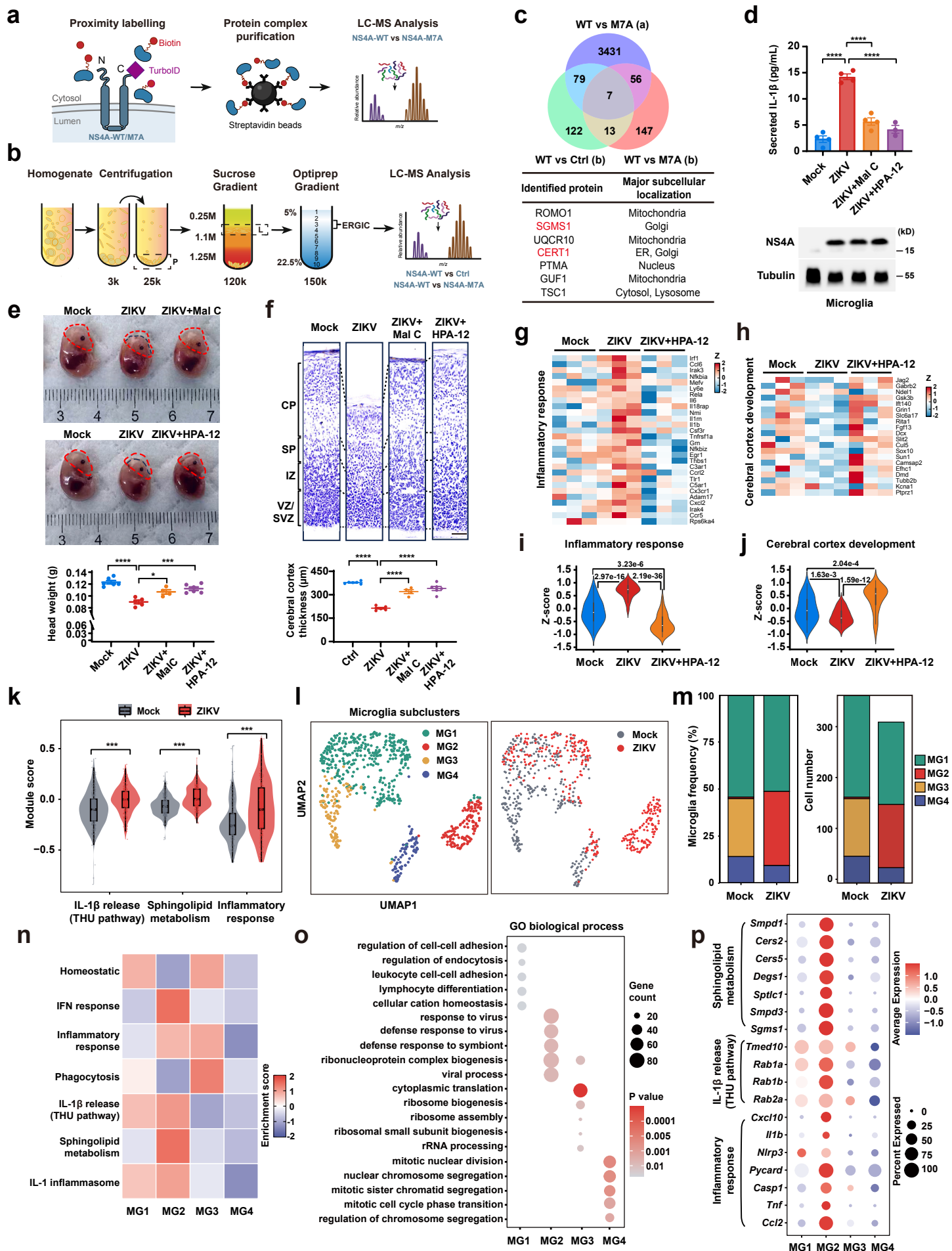


Figure 3

Fig. 3 | Sphingolipid metabolism is involved in ZIKV-induced neuroinflammation and cortical injury. **a**, Schematic of NS4A-WT and NS4A-M7A interactome identification using TurboID-based proximity labeling coupled with affinity purification and mass spectrometry. **b**, Schematic of ERGIC proteome identification using a membrane flotation assay coupled with mass spectrometry in cells expressing empty vector (Ctrl), NS4A-WT or NS4A-M7A. **c**, Venn diagram showing the overlap among differentially interacting proteins between NS4A-WT and NS4A-M7A from **(a)** (blue), differentially localized ERGIC proteins between Ctrl and NS4A-WT from **(b)** (green) and differentially localized ERGIC proteins between NS4A-WT and NS4A-M7A from **(b)** (red). Names and major subcellular localizations of the seven overlapping proteins are listed in the **Supplementary Table 1** and **Supplementary Table 2**. **d**, IL-1 β secretion measured by ELISA in primary microglia derived from E16.5 fetal brains of WT mice. Cells were infected with mock or ZIKV in the absence or presence of Malabaricone C (Mal C, 10 μ M) or HPA-12 (50 μ M). NS4A expression was verified by immunoblotting. Data are representative of at least three independent experiments. **e-j**, Analyses of E16.5 fetal brains from WT mice infected with mock or ZIKV in the absence or presence of Mal C (25 μ M) or HPA-12 (50 μ M) at E13.5. **e**, Representative images of fetal heads (boundaries demarcated) and quantification of head weights ($n = 4-6$). **f**, Nissl staining of cortical layers and quantification of cortical thickness ($n = 4-6$). Scale bar, 50 μ m. **g, h**, Heatmaps of representative genes associated with inflammatory response (**g**) and cerebral cortex development (**h**). **i, j**, Violin plots of indicated pathways from bulk RNA-seq. **k**, Violin plots showing module scores for IL-1 β release (THU pathway), sphingolipid metabolism and inflammatory response in microglia from mock- or ZIKV-infected fetal brains. Boxes indicate the median and interquartile range. **l, m**, UMAP visualization (**l**), frequency and absolute number (**m**) of microglial subclusters MG1-MG4. **n**, Heat map of functional program scores across microglial subclusters, shown as row-scaled Z-scores. **o**, GO analysis of biological-process enrichment analysis for each microglial subcluster. **p**, Dot plot showing the expression of signature genes related to sphingolipid metabolism, IL-1 β release (THU pathway) and inflammatory response across microglial subclusters. Dot size represents the proportion of cells expressing indicated genes; color indicates the row-scaled average expression level. Data are mean $\pm$ SEM (**d-f**). Statistical significance was assessed using one-way ANOVA with Tukey's multiple-comparison test (**d-f**), unpaired two-tailed Student's t -test (**i, j**) and two-sided Wilcoxon rank-sum test (**k**). * $p < 0.5$, *** $p < 0.001$, **** $p < 0.0001$. NS, non-significant. **e-j**, Data are representative of at least three independent dams.

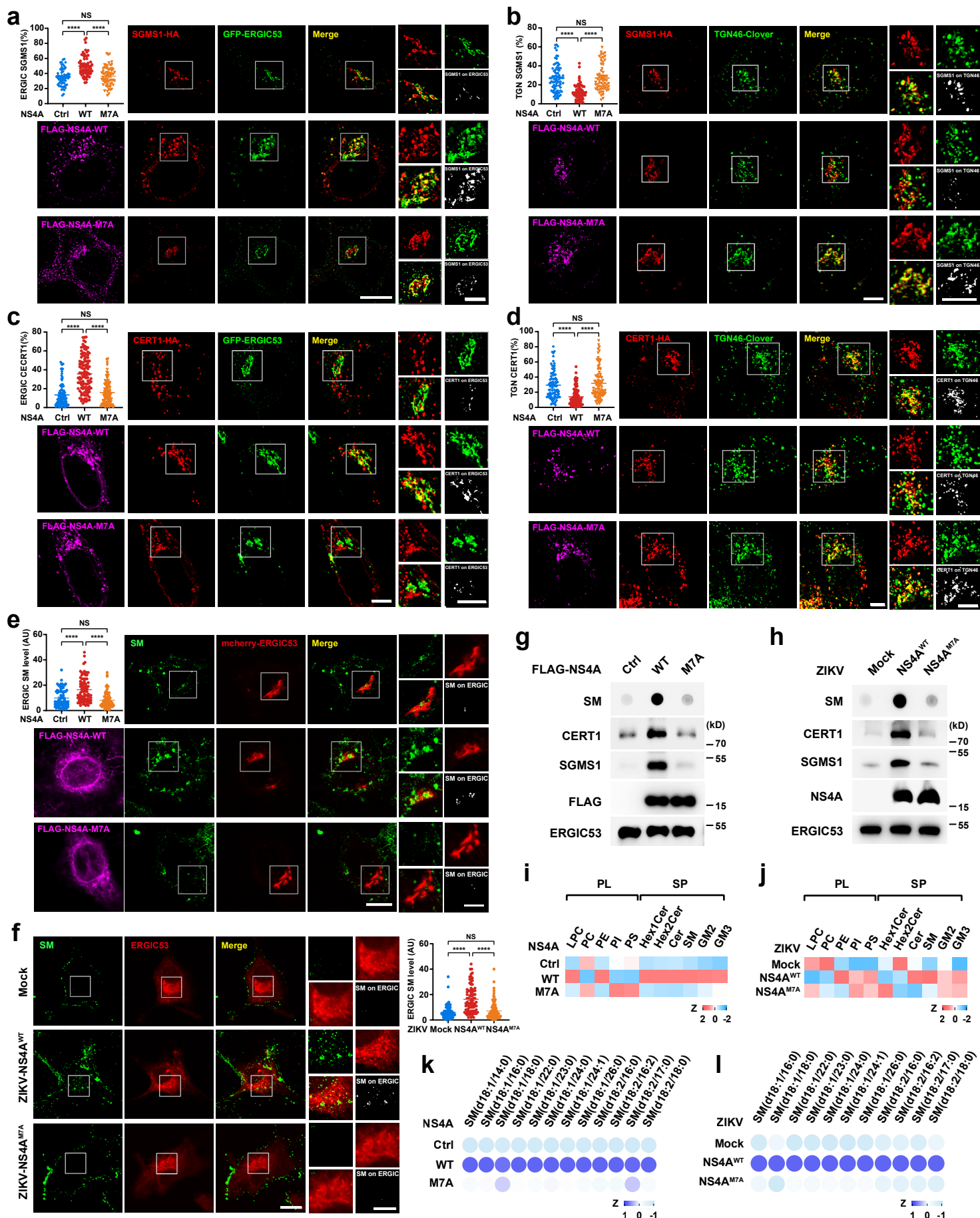


Figure 4

Fig. 4 | NS4A relocates SGMS1 and CERT1 to promote ectopic sphingomyelin synthesis at the ERGIC. **a**, SIM imaging of SGMS1 and ERGIC53 in HeLa cells expressing SGMS1-HA and GFP-ERGIC53 with or without FLAG-tagged NS4A-WT or NS4A-M7A. Percentage of SGMS1 on the ERGIC was quantified ($n = 61$ (Ctrl), 69 (NS4A-WT) and 64 (NS4A-M7A) cells). **b**, SIM imaging of SGMS1 and TGN46 in HeLa cells expressing SGMS1-HA and TGN46-Clover with or without FLAG-tagged NS4A-WT or NS4A-M7A. Percentage of SGMS1 on the TGN was quantified ($n = 80$ (Ctrl), 68 (NS4A-WT) and 62 (NS4A-M7A) cells). **c**, SIM imaging of CERT1 and ERGIC53 in HeLa cells expressing CERT1-HA and GFP-ERGIC53 with or without FLAG-tagged NS4A-WT or NS4A-M7A. Percentage of CERT1 on the ERGIC was quantified ($n = 109$ (Ctrl), 114 (NS4A-WT) and 101 (NS4A-M7A) cells). **d**, SIM imaging of CERT1 and TGN46 in HeLa cells expressing CERT1-HA and TGN46-Clover with or without FLAG-tagged NS4A-WT or NS4A-M7A. Percentage of CERT1 on the TGN was quantified ($n = 89$ (Ctrl), 106 (NS4A-WT) and 83 (NS4A-M7A) cells). **e, f**, Immunofluorescence of HeLa cells expressing mcherry-ERGIC53 and empty vector, FLAG-tagged NS4A-WT or NS4A-M7A (**e**) or primary microglia from E16.5 fetal brains infected with mock, ZIKV-NS4A^{WT} or ZIKV-NS4A^{M7A} (**f**). Sphingomyelin (SM) was detected by GFP-tagged NT-Lys. ERGIC SM levels from (**e**) ($n = 71$ (Ctrl), 90 (NS4A-WT) and 96 (NS4A-M7A) cells) and (**f**) ($n = 92$ (Mock), 85 (NS4A^{WT}) and 102 (NS4A^{M7A}) cells) were quantified. **g, h**, Dot blots of SM (detected with FLAG-tagged Equinatoxin II) and immunoblots of SGMS1 and CERT1 in ERGIC-enriched fractions from HEK293T cells transfected with empty vector, NS4A-WT or NS4A-M7A (**g**), or infected with mock, ZIKV-NS4A^{WT} or ZIKV-NS4A^{M7A} (**h**). **i, j**, Heatmaps of phospholipid (PL) and sphingolipids (SP) species in ERGIC-enriched fractions from HEK293T cells expressing empty vector, NS4A-WT or NS4A-M7A (**i**), or infected with mock, ZIKV-NS4A^{WT} or ZIKV-NS4A^{M7A} (**j**). Full lipid names are provided in **Supplementary Table 3**. **k, l**, Lipidomic profiles of SM subtypes in ERGIC-enriched fractions from HEK293T cells expressing empty vector, NS4A-WT or NS4A-M7A (**k**), or infected with mock, ZIKV-NS4A^{WT} or ZIKV-NS4A^{M7A} (**l**). **a-d**, Scale bars, 5 μ m. **e, f**, Scale bars, 10 μ m (overview) and 5 μ m (magnified views). Data are mean $\pm$ SEM (**a-f**). Statistical significance was assessed using one-way ANOVA with Tukey's multiple-comparison test (**a-f**). **** $p < 0.0001$. NS, non-significant. **a-h**, Data are representative of three independent experiments.

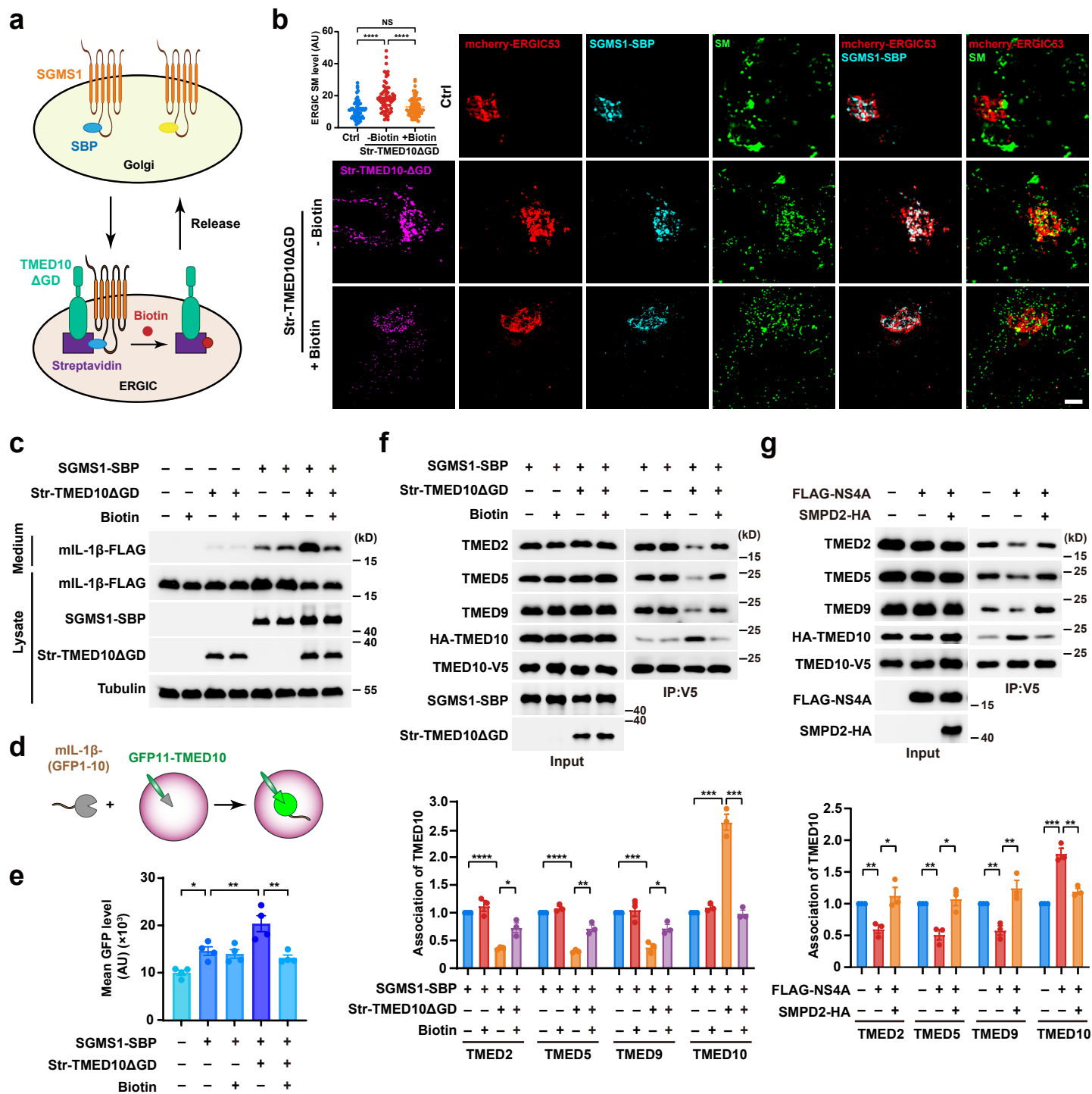


Figure 5

Fig. 5 | Sphingomyelin promotes TMED10-mediated IL-1 β secretion. **a**, Schematic diagram of the RUSH system to retain Golgi-localized SGMS1 to the ERGIC. SGMS1 fused with SBP was retained via binding to streptavidin connected to TMED10 Δ GD, an ERGIC-localized membrane protein deficient in unconventional protein secretion. Upon biotin addition, SGMS1 was released and trafficked back to the Golgi. **b**, SIM imaging of HeLa cells co-expressing mcherry-ERGIC53 and SBP-SGMS1-HA, without or with Str-TMED10 Δ GD-V5 expression, and treated without or with 40 μ M biotin for 16 h. SM was detected by GFP-tagged NT-Lys. Scale bar, 5 μ m. ERGIC SM levels ($n = 59$ (Ctrl), 61 (without biotin) and 65 (with biotin) cells) were quantified. **c**, Secretion of mIL-1 β in mIL-1 β -FLAG-expressing HEK293T cells combined with RUSH system. Cells were co-transfected without or with Str-TMED10 Δ GD-V5, and SBP-SGMS1-HA and treated without or with 40 μ M biotin for 16 h. **d**, **e**, Schematic diagram of the GFP complementation assay (**d**). Cells expressing GFP11-TMED10 and mIL-1 β -(GFP1-10) were transfected with or without SBP-SGMS1, Str-TMED10 Δ GD, and treated without or with 40 μ M biotin as indicated. Complemented GFP signals were analyzed by FACS analysis and quantified in (**e**). **f**, **g**, Co-IP from HEK293T cells co-expressing TMED10-V5 and HA-TMED10 without or with the indicated constructs. The association efficiency of TMED10-V5 with other subfamily members (TMED2, 5, and 9) or HA-TMED10 was quantified. Data are mean $\pm$ SEM (**e-g**). Statistical significance was assessed using one-way ANOVA with Tukey's multiple-comparison test (**e**) and unpaired two-tailed Student's t -test (**f**, **g**). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. NS, non-significant. Data are representative of three independent experiments.

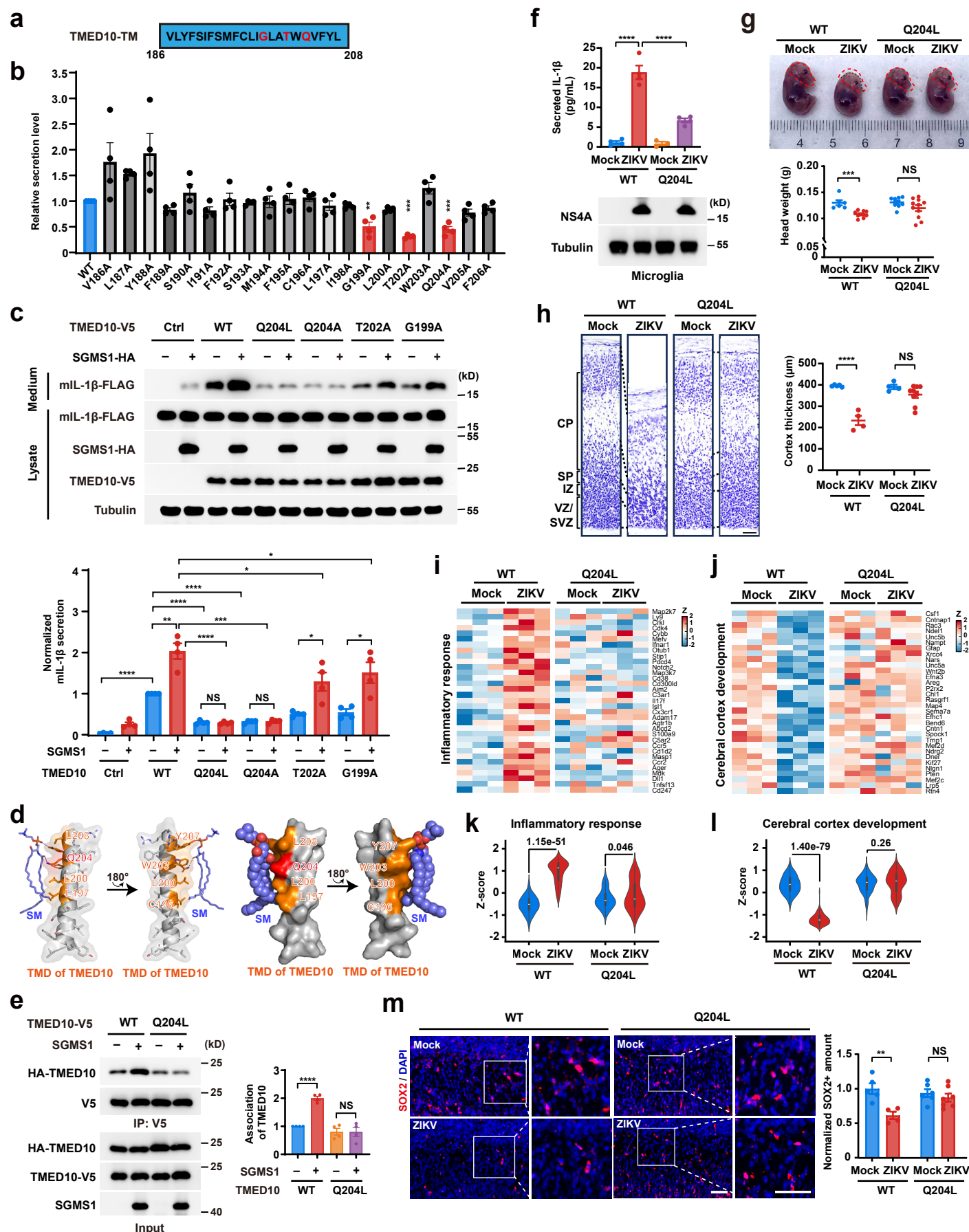


Figure. 6

Fig. 6 TMED10 response to sphingomyelin drives ZIKV-associated neuroinflammation and cortical injury. **a** Amino acid sequence of the TMED10 transmembrane domain (TMD). **b** Quantification of relative mIL-1 β secretion levels in TMED10-KO HEK293T cells expressing WT or indicated TMED10 mutants, as determined by a complementary NanoLuc luciferase secretion assay. **c** Secretion of mIL-1 β in TMED10-KO HEK293T cells co-expressing control, TMED10-WT or mutants without or with SGMS1-HA. mIL-1 β secretion was calculated and normalized. **d** Surface representation of the predicted interaction between C18:0 SM and TMD of TMED10 (aa186-210) generated by Boltz-2, involving multiple hydrophobic interactions and hydrogen bonds. SM is shown in blue, TMED10 in gray, the SM-TMED10 interface in orange, and residue Q204 is highlighted in red. pTM = 0.73. **e** Co-IP from HEK293T cells co-expressing V5- and HA-tagged TMED10 (WT or Q204L mutant) and without or with SGMS1-HA. The association efficiency of TMED10-V5 with HA-TMED10 was quantified. **f** IL-1 β secretion measured by ELISA in primary microglia from E16.5 fetal brains of WT or TMED10^{Q204L/Q204L} (Q204L) knock-in mice. Cells were infected with mock or ZIKV. Data are representative of at least three independent experiments. **g-m** Analyses of E16.5 fetal brains from WT or Q204L mice infected with mock or ZIKV at E13.5. Representative images of fetal heads (boundaries demarcated) and quantification of head weights ($n = 6-11$). **h** Nissl staining of cortical layers and quantification of cortical thickness ($n = 4-9$). **i, j** Heatmaps of representative genes associated with inflammatory response (**i**) and cerebral cortex development (**j**). **k, l** Violin plots of indicated pathways from bulk RNA-seq. **m**, Representative images of cortices immunostained for SOX2 and DAPI, with quantification of SOX2⁺ NPCs normalized to mock ($n = 4-7$). Scale bars, 50 μ m (**h, m**). Data are mean $\pm$ SEM (**b-c, e-h, m**). Statistical significance was assessed using unpaired two-tailed Student's t -test (**b, c, e-h, k-m**). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. NS, non-significant. Data are representative of three independent experiments in **b, c, e, f** or dams in **g-m**.

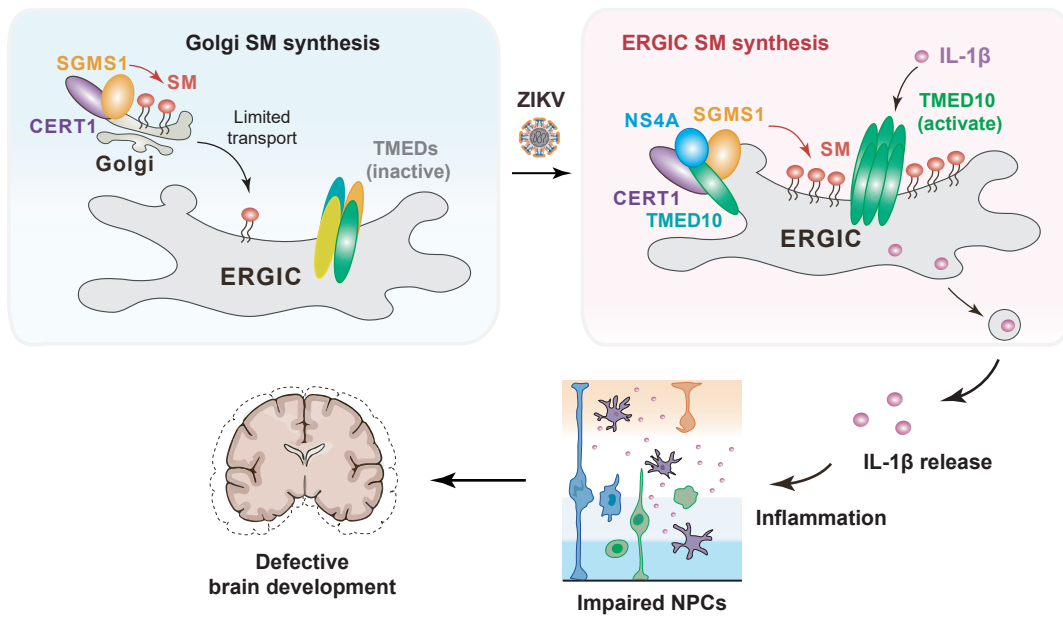


Figure 7

Fig. 7 | Model of ZIKV-induced ERGIC sphingomyelin remodeling, neuroinflammation and cortical developmental injury. Under normal conditions, sphingomyelin synthesis occurs primarily at the Golgi, with limited transport to ERGIC where TMED10 forms hetero-oligomeric complexes with other TMED family members, maintaining THU pathway in an inactive state. ZIKV infection relocates SGMS1 and CERT1 to ERGIC through NS4A protein interactions, establishing ectopic sphingomyelin synthesis at this compartment. Sphingomyelin accumulation triggers TMED10 homo-oligomerization and THU pathway activation, promoting IL-1 β secretion. IL-1 β induces neuroinflammation that impairs NPCs and disrupts cortical development.

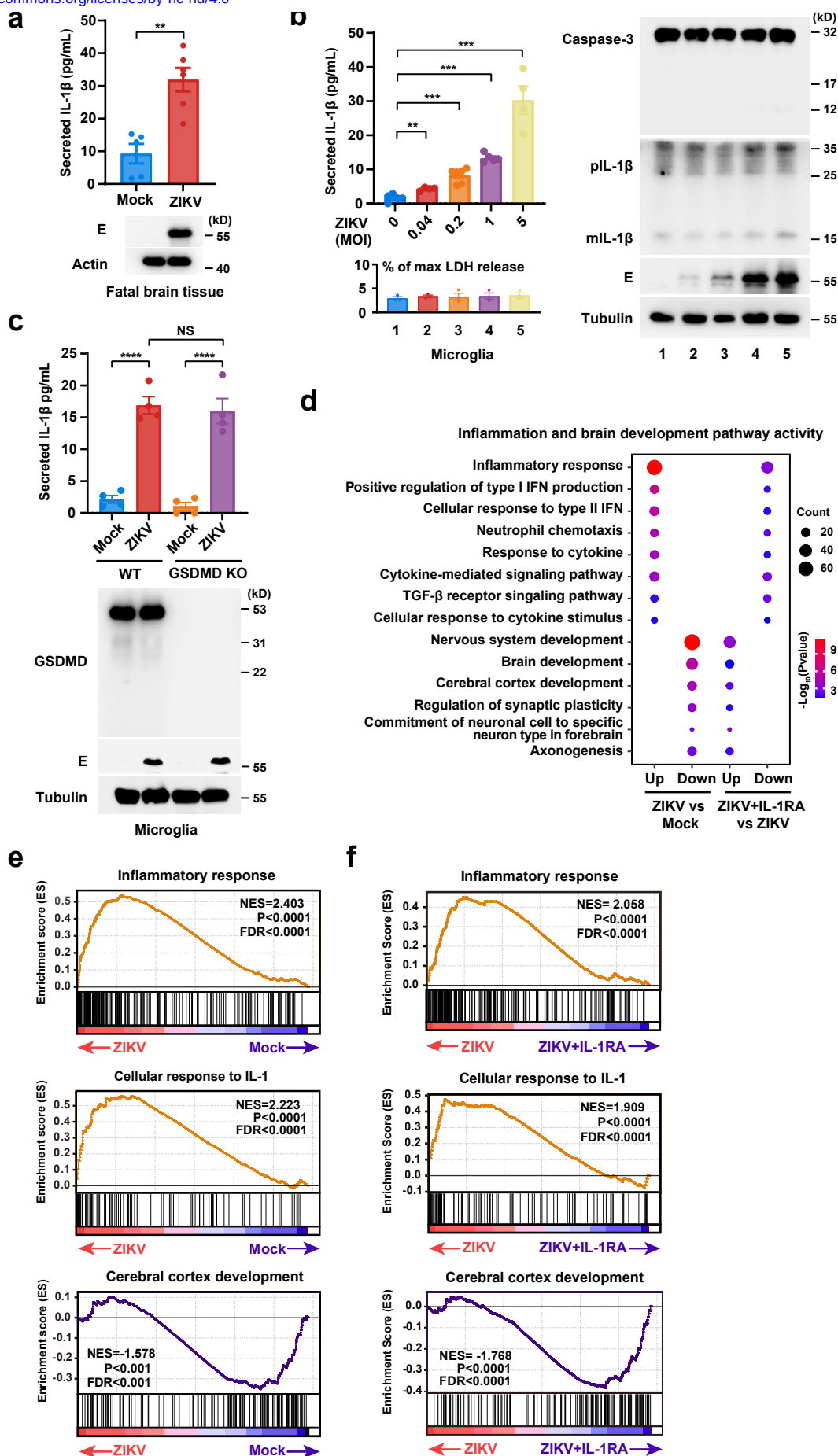


Figure S1

Fig. S1 | ZIKV infection induces IL-1 β secretion and neuroinflammation. **a**, IL-1 β secretion measured by ELISA in fetal brain tissues from WT mice infected with mock or ZIKV at E13.5 and collected at E16.5. ZIKV Envelope (E) protein expression was determined by immunoblotting. **b**, IL-1 β secretion measured by ELISA in primary microglia from E16.5 fetal brains of WT mice. Cells were infected with mock or increasing MOIs of ZIKV. Expression of IL-1 β , caspase-3 and ZIKV E protein were determined by immunoblotting. LDH release was quantified. **c**, IL-1 β secretion measured by ELISA in primary microglia from E16.5 fetal brains of wild-type (WT) or GSDMD-knockout (KO) mice infected with mock or ZIKV. GSDMD and ZIKV E protein expression were verified by immunoblotting. **d-f**, Analyses of E16.5 fetal brains from WT mice with mock-infection or ZIKV-infection in the presence or absence of IL-1RA. **d**, GO analysis of bulk RNA-seq data showing differentially enriched pathways associated with inflammation and cortical development ($P < 0.05$). Up, upregulated pathways; Down, downregulated pathways. **e**, **f**, GSEA of indicated pathways from bulk RNA-seq. Data are mean $\pm$ SEM (**a-c**). Statistical significance was assessed using unpaired two-tailed Student's *t*-test (**a**, **b**) and one-way ANOVA with Tukey's multiple-comparison test (**c**). ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. NS, non-significant. Data are representative of at least three independent experiments (**a-c**) or three independent dams (**d-f**).

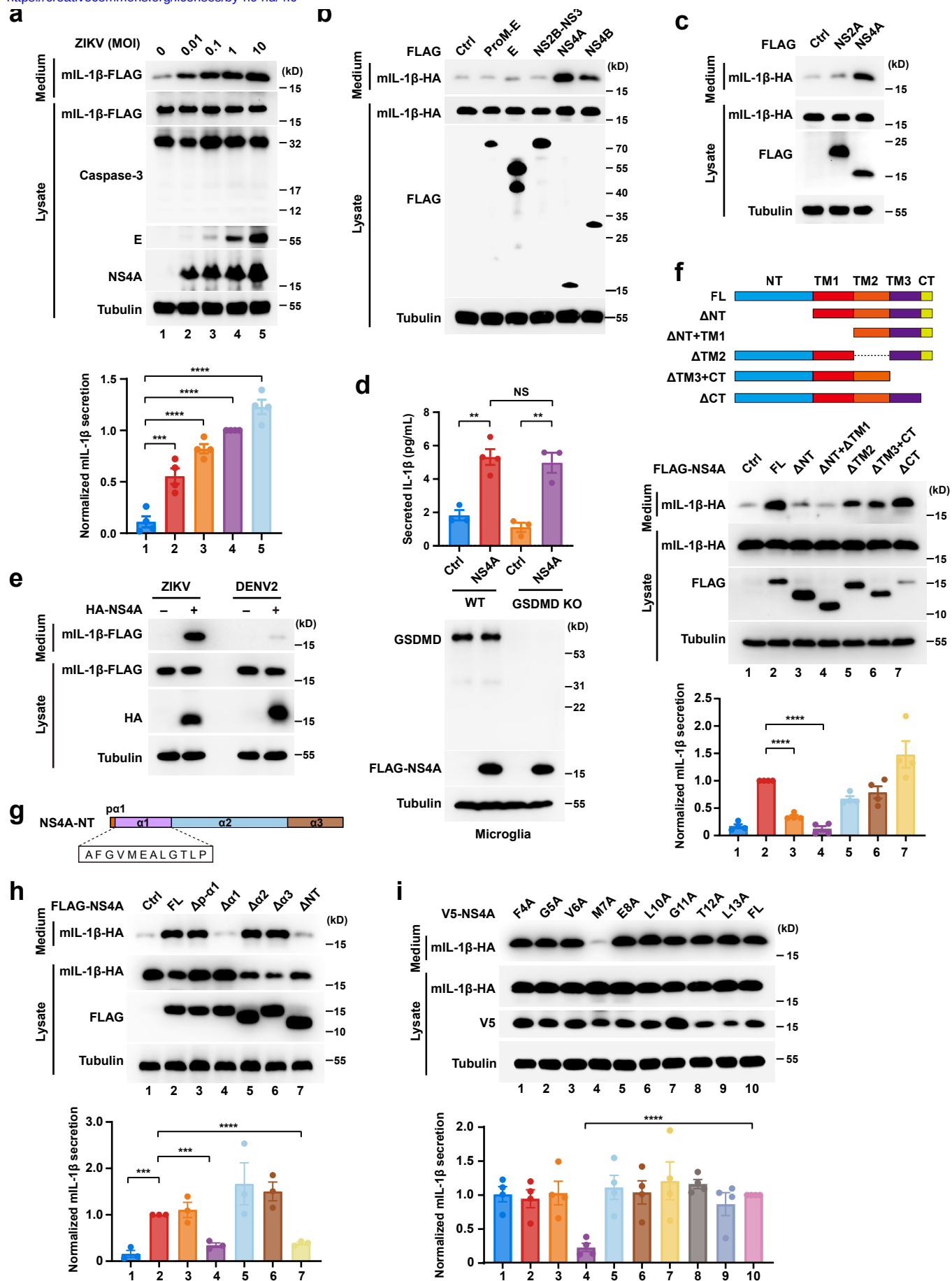


Figure S2

Fig. S2 | NS4A is a key regulator for ZIKV-induced IL-1 β secretion. **a**, Secretion of mIL-1 β in mIL-1 β -FLAG-expressing HEK293T cells infected with mock or increasing MOIs of ZIKV. **b, c**, Secretion of mIL-1 β in mIL-1 β -HA-expressing HEK293T cells transfected with FLAG-tagged ZIKV proteins as indicated. **d**, IL-1 β secretion measured by ELISA in primary microglia from E16.5 fetal brains of WT or GSDMD-KO mice ($n = 3-4$). Cells were transduced with lentivirus expressing Ctrl or FLAG-tagged NS4A. **e**, Secretion of mIL-1 β in mIL-1 β -FLAG-expressing HEK293T cells transfected with or without HA-tagged NS4A from ZIKV or DENV2. **f**, Schematic of full-length (FL) NS4A and truncation constructs (N-terminal (NT), transmembrane domain 1-3 (TM1-3) and C-terminal (CT)). Secretion of mIL-1 β in mIL-1 β -HA-expressing HEK293T cells transfected with FLAG-tagged FL NS4A or the indicated NS4A truncations. **g**, Schematic of NS4A-NT truncation constructs ($\alpha 1$, $\alpha 1$, $\alpha 2$ and $\alpha 3$) predicted by [PSIPRED Workbench \(ucl.ac.uk\)](https://psipred.ucl.ac.uk). The amino acid sequence of $\alpha 1$ is shown. **h, i**, Secretion of mIL-1 β in mIL-1 β -HA-expressing HEK293T cells transfected with FLAG- or V5-tagged FL NS4A or the indicated NS4A truncations (**h**) or point mutants (**i**). **a, f, h, i**, mIL-1 β secretion was calculated and normalized. Data are mean $\pm$ SEM (**a, d, f, h, i**). Statistical significance was assessed using unpaired two-tailed Student's t -test (**a, f, h, i**) or one-way ANOVA with Tukey's multiple-comparison test (**d**). ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. NS, non-significant. Data are representative of at least three independent experiments.

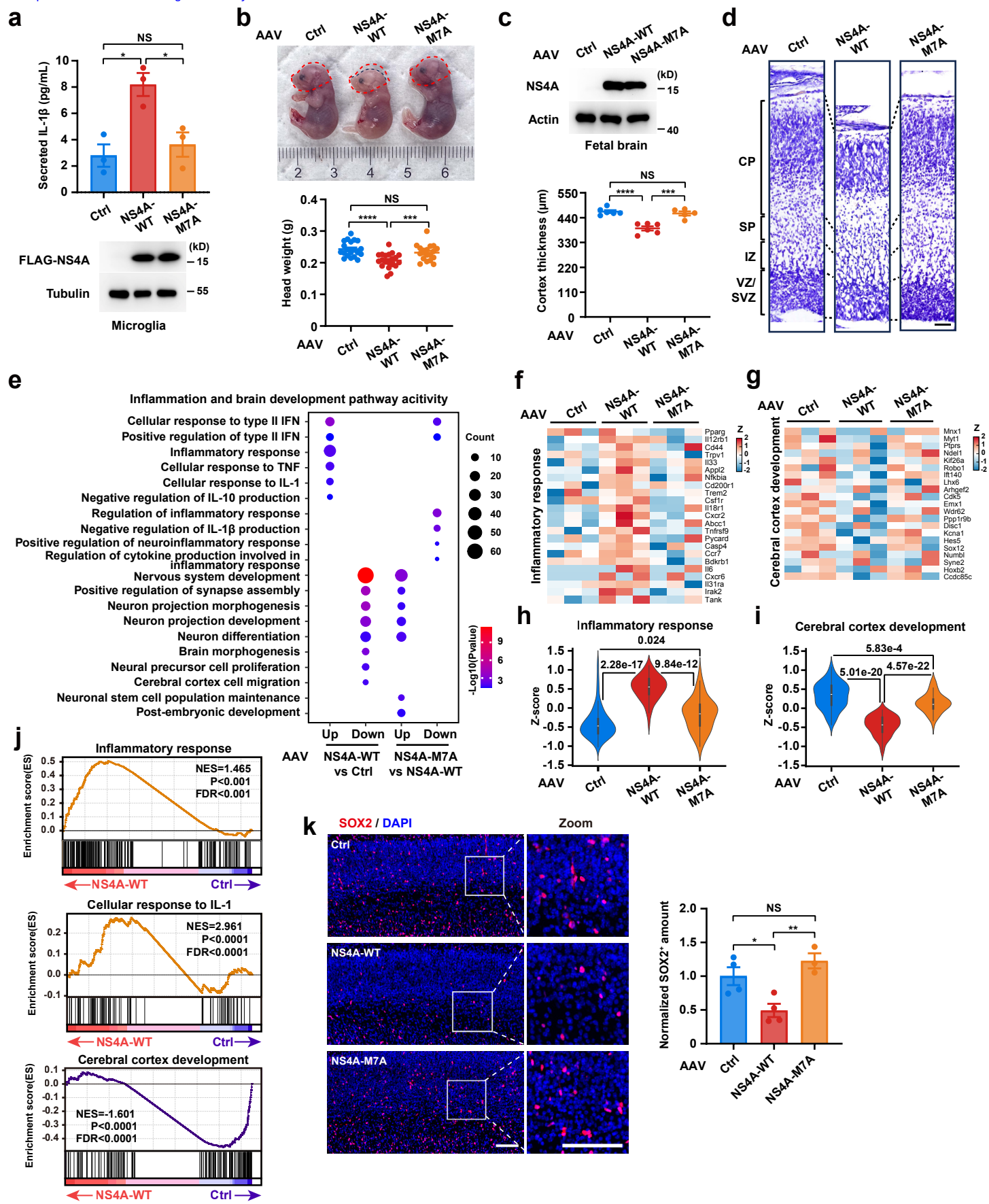


Figure S3

Fig. S3 | Expression of NS4A induces IL-1 β secretion and recapitulates ZIKV-induced neuroinflammation and cortical injury. **a**, IL-1 β secretion measured by ELISA in primary microglia from E16.5 fetal brains of WT mice. Cells were transduced with lentivirus expressing Ctrl, FLAG-tagged NS4A-WT or NS4A-M7A. NS4A expression was verified by immunoblotting. Data are representative of three independent experiments. **b-k**, Analyses of E18.5 fetal brains from WT mice injected intracerebroventricularly with AAV expressing GFP (Ctrl), NS4A-WT or NS4A-M7A at E13.5. **b**, Representative images of fetal heads (boundaries demarcated) and quantification of head weights ($n = 18-20$). **c**, NS4A-WT and NS4A-M7A expression from AAV were verified by immunoblotting. **d**, Nissl staining of cortical layers and quantification of cortical thickness ($n = 5-6$). Scale bar, 50 μm . **e**, GO analysis of bulk RNA-seq data showing differentially enriched pathways associated with inflammation and cortical development ($P < 0.05$). Up, upregulated pathways; Down, downregulated pathways. **f**, **g**, Heatmaps of representative genes associated with inflammatory response (**f**) and cerebral cortex development (**g**). **h**, **i**, Violin plots of indicated pathways from bulk RNA-seq. **j**, GSEA of indicated pathways from bulk RNA-seq. **k**, Representative images of cortices immunostained for SOX2 and DAPI, with quantification of SOX2⁺ NPCs normalized to Ctrl ($n = 3-4$). Scale bars, 100 μm . Data are mean $\pm$ SEM (**a**, **b**, **d**, **k**). Statistical significance was assessed using one-way ANOVA with Tukey's multiple-comparison test (**a**, **b**, **d**, **k**) and unpaired two-tailed Student's t -test (**h-i**). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. NS, non-significant. **b-k**, Data are representative of three independent dams.

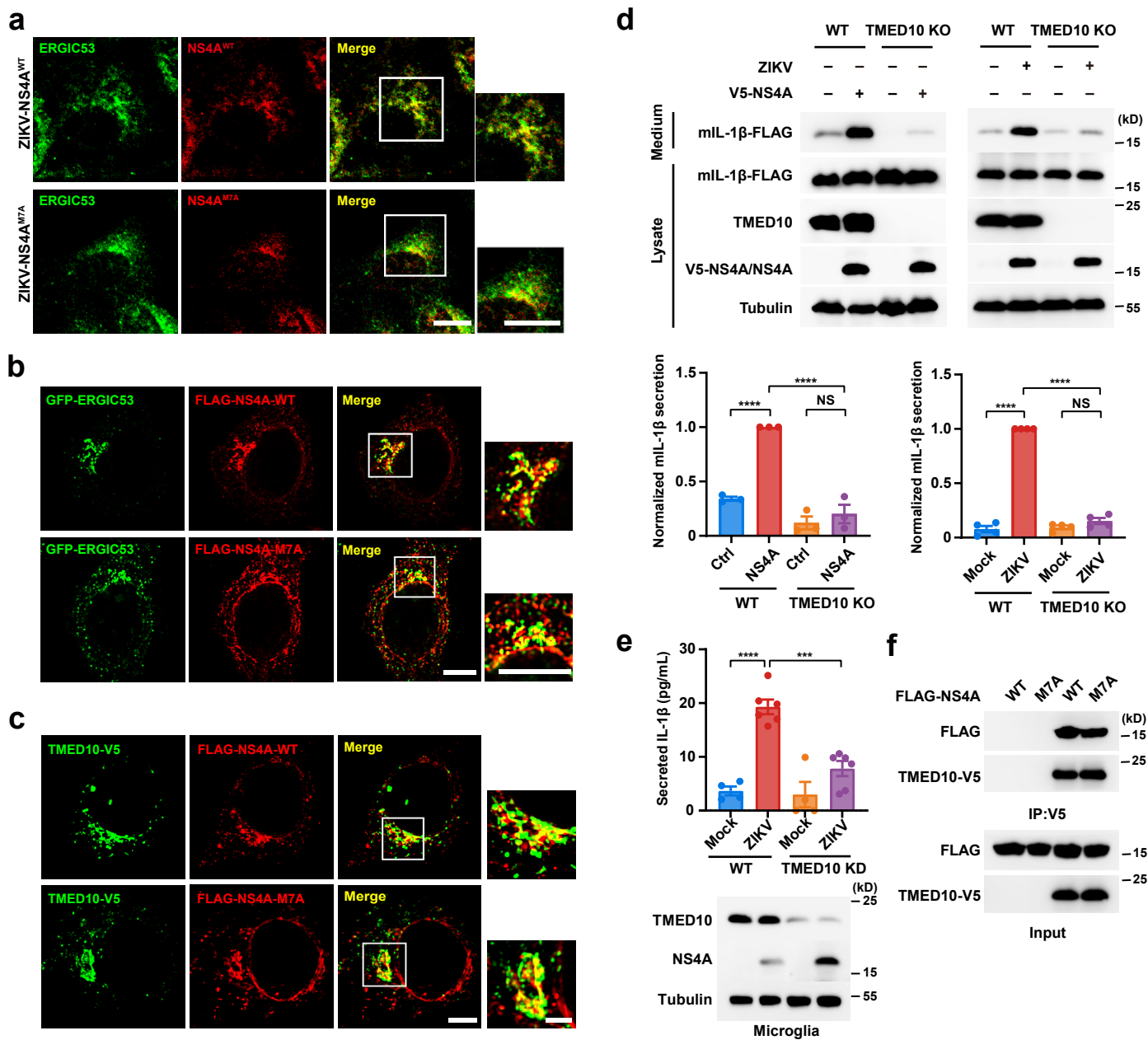


Figure S4

Fig. S4 | NS4A-regulated IL-1 β secretion depends on TMED10. **a**, Fluorescence imaging of NS4A and ERGIC53 in Vero E6 cells infected with mock, ZIKV-NS4A^{WT} or ZIKV-NS4A^{M7A}. Scale bars, 10 μ m. **b**, SIM imaging of NS4A and ERGIC53 in GFP-ERGIC53-expressing HeLa cells transfected with FLAG-tagged NS4A-WT or NS4A-M7A. Scale bars, 5 μ m. **c**, SIM imaging of NS4A and TMED10 in HeLa cells co-transfected with TMED10-V5 and either FLAG-tagged NS4A-WT or NS4A-M7A. Scale bars, 5 μ m (overview) and 2 μ m (magnified view). **d**, Secretion of mIL-1 β in mIL-1 β -FLAG-expressing WT or TMED10-KO HEK293T cells transfected with V5-NS4A or infected with ZIKV. mIL-1 β secretion was calculated and normalized. **e**, IL-1 β secretion measured by ELISA in primary microglia from E16.5 fetal brains of WT or TMED10-KD mice ($n = 4-6$). Cells were infected with mock or ZIKV. TMED10 and NS4A expression were verified by immunoblotting. **f**, Co-IP from HEK293T cells transfected with TMED10-V5 alone or together with FLAG-tagged NS4A-WT or NS4A-M7A. Data are mean $\pm$ SEM (**d**, **e**). Statistical significance was assessed using one-way ANOVA with Tukey's multiple-comparison test. *** $p < 0.001$, **** $p < 0.0001$. NS, non-significant. Data are representative of at least three independent experiments.

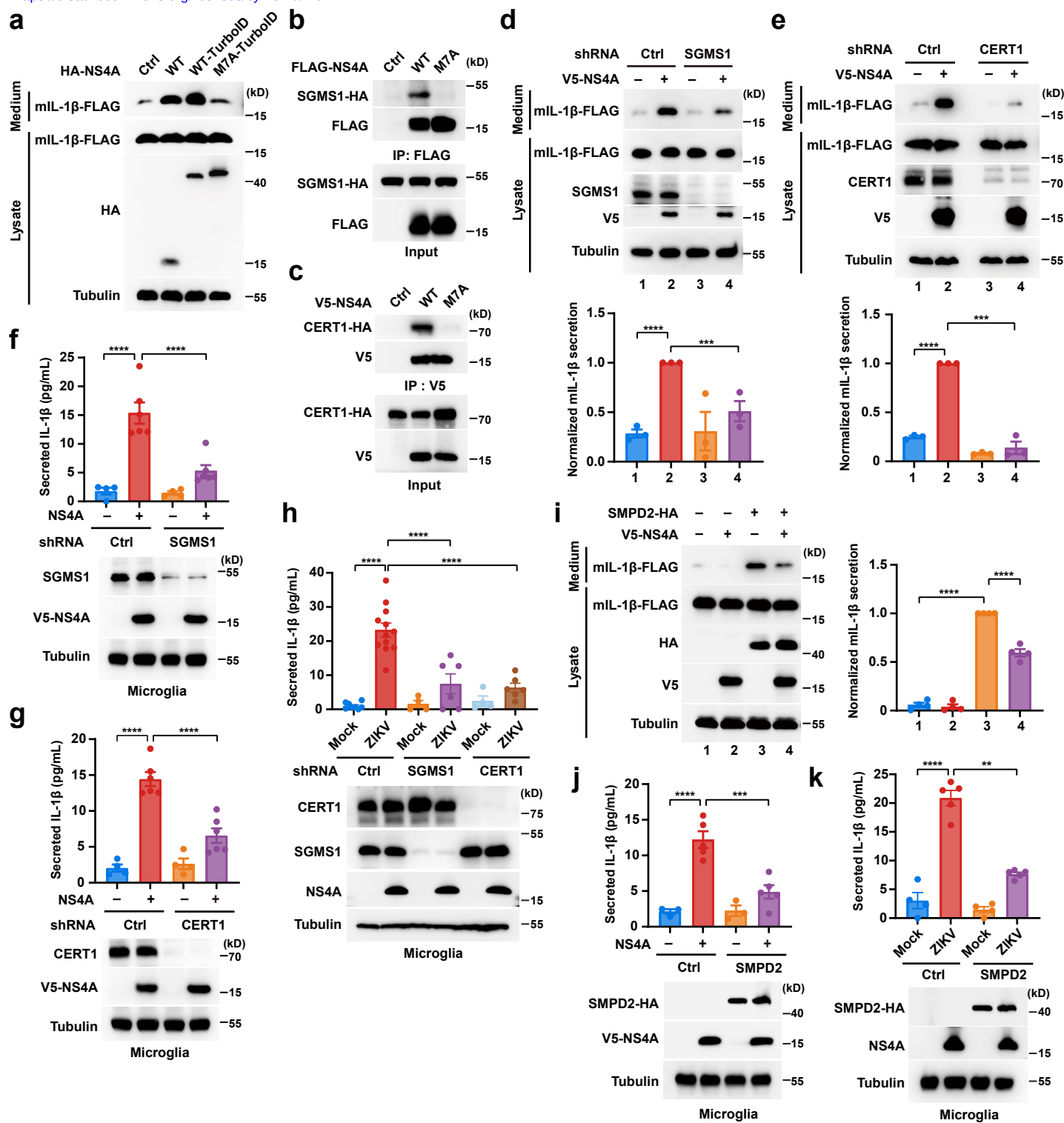


Figure S5

Fig. S5 | SGMS1 and CERT1 are required for NS4A-regulated secretion. **a**, Secretion of mIL-1 β in mIL-1 β -FLAG-expressing HEK293T cells transfected with empty vector (Ctrl) or indicated NS4A constructs. **b**, **c**, Co-IP from HEK293T cells transfected with indicated SGMS1 (**b**), CERT1 (**c**) constructs and NS4A-WT or NS4A-M7A. **d**, **e**, Secretion of mIL-1 β in mIL-1 β -FLAG-expressing HEK293T cells after shRNA-mediated knockdown of SGMS1 (**d**) or CERT1 (**e**) with or without V5-NS4A expression. **f**, **g**, IL-1 β secretion measured by ELISA in microglia cells from E16.5 fetal brains of WT mice. Cells were transduced with lentiviruses expressing V5-NS4A or Ctrl together with shRNA targeting SGMS1 (**f**) or CERT1 (**g**) or a Ctrl shRNA. **h**, IL-1 β secretion measured by ELISA in microglia cells from E16.5 fetal brains of WT mice. Cells were infected with mock or ZIKV together with shRNA targeting SGMS1 or CERT1 or a Ctrl shRNA. **i**, Secretion of mIL-1 β in mIL-1 β -FLAG-expressing HEK293T cells transfected with Ctrl, NS4A, SMPD2 or both NS4A and SMPD2. **j**, IL-1 β secretion measured by ELISA in microglia cells from E16.5 fetal brains of WT mice. Cells were transduced with lentiviruses expressing Ctrl, NS4A, SMPD2, or both NS4A and SMPD2. **k**, IL-1 β secretion measured by ELISA in microglia cells from E16.5 fetal brains of WT mice. Cells were infected with mock or ZIKV together with lentiviruses expressing Ctrl or SMPD2. **d**, **e**, **i**, mIL-1 β secretion was calculated and normalized. Data are mean $\pm$ SEM (**d-k**). Statistical significance was assessed using one-way ANOVA with Tukey's multiple-comparison test (**d-k**). ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. NS, non-significant. All data are representative of at least three independent experiments.

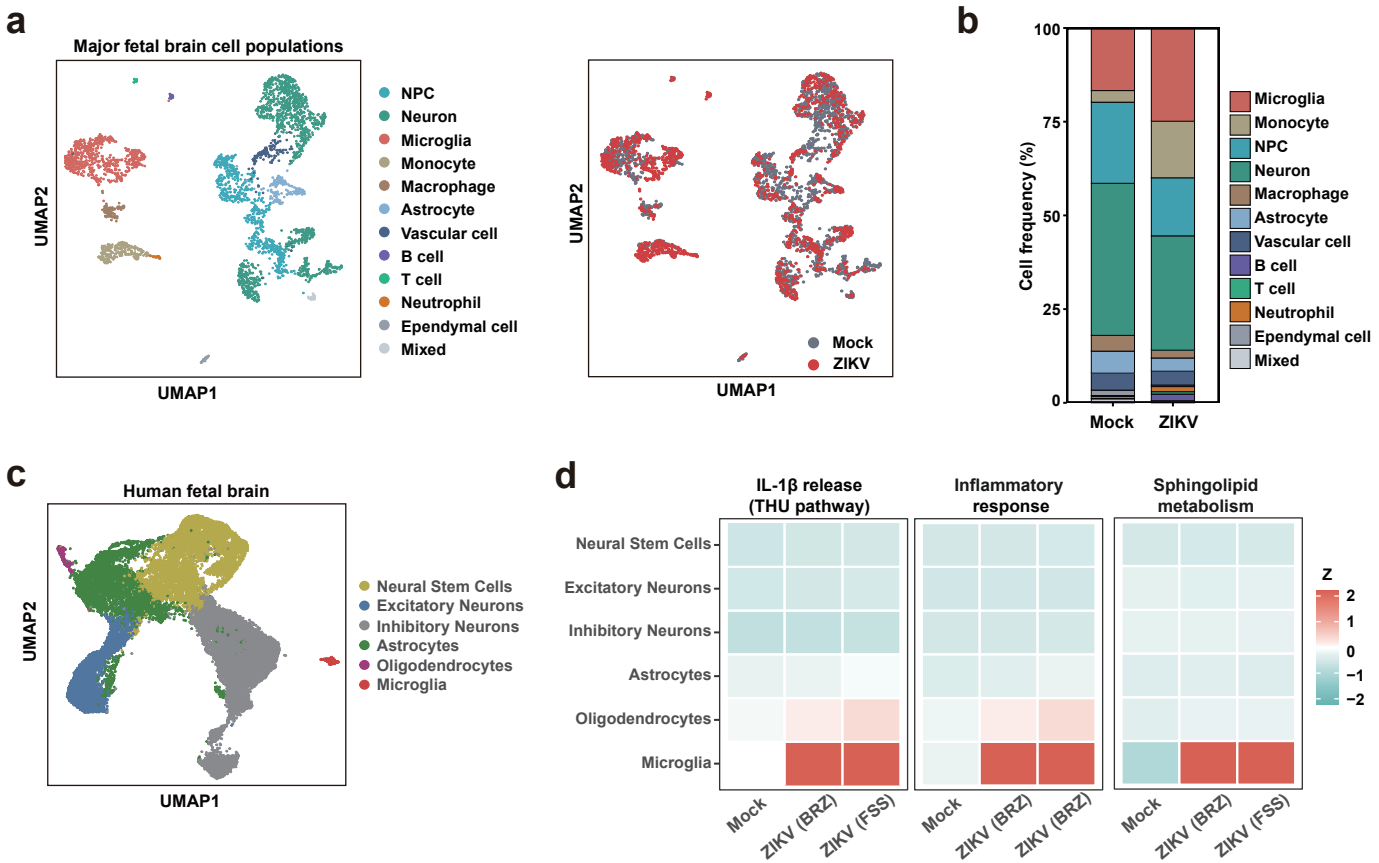


Figure S6

Fig. S6 | ScRNA-seq reanalysis supports a ZIKV-associated THU-sphingolipid inflammatory signature. **a**, UMAP visualization of mouse fetal brain cells with mock or ZIKV infection, colored by major cell type. **b**, Relative abundance of major fetal brain cell populations in mock- or ZIKV-infected mouse samples. **c**, UMAP visualization of human fetal neural cells, colored by major cell type. **d**, Heat maps showing IL-1 β release (THU pathway), inflammatory response and sphingolipid metabolism scores across the indicated major cell types in human fetal brain explant tissue infected with mock, ZIKV-BRZ (Brazilian strain) or ZIKV-FSS (FSS13025 strain). Values are shown as Z-scores.

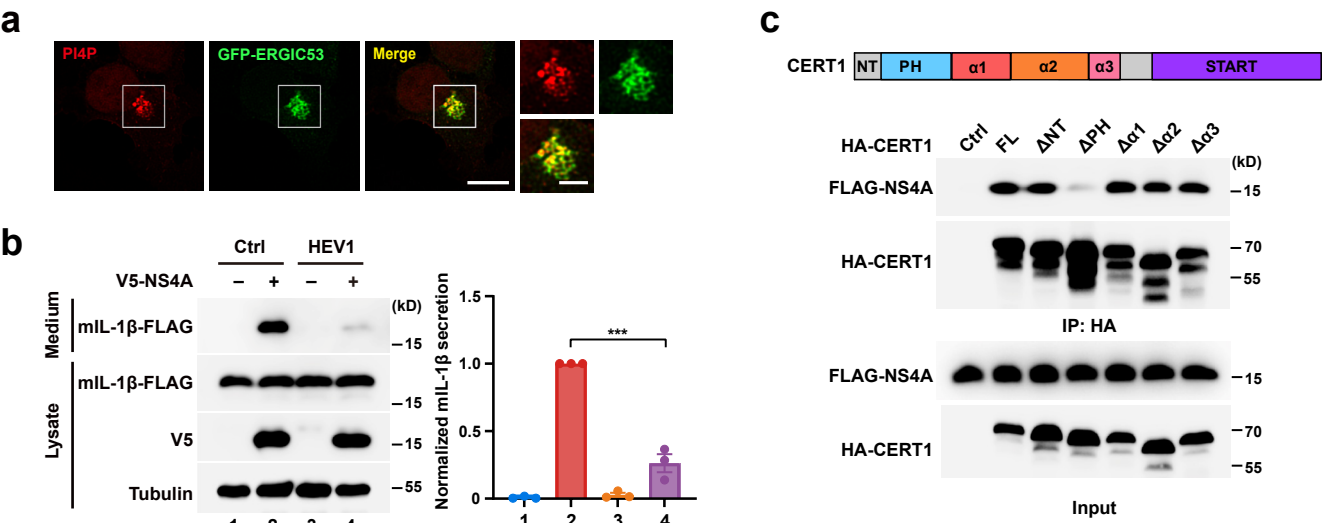


Figure S7

Fig. S7 | NS4A recruits CERT1 to the ERGIC via its PH domain and PI4P to promote IL-1 β secretion. **a**, Fluorescence imaging of PI4P (labeled with mScarlet-OSBP-PH) and GFP-ERGIC53 in COS-7 cells. Scale bars, 10 μ m (overview) and 2 μ m (zoom, magnified views). **b**, Secretion of mIL-1 β in mIL-1 β -FLAG-expressing HEK293T cells transfected with empty vector or V5-tagged NS4A in the presence or absence of HEV1 (20 μ M, overnight). mIL-1 β secretion was calculated and normalized. Data are mean $\pm$ SEM. Statistical significance was assessed using one-way ANOVA with Tukey's multiple-comparison test. *** $p < 0.001$. **c**, Schematic of HA-CERT1 truncation constructs and Co-IP with FLAG-NS4A from HEK293T cells. **a-c**, Data are representative of three independent experiments.

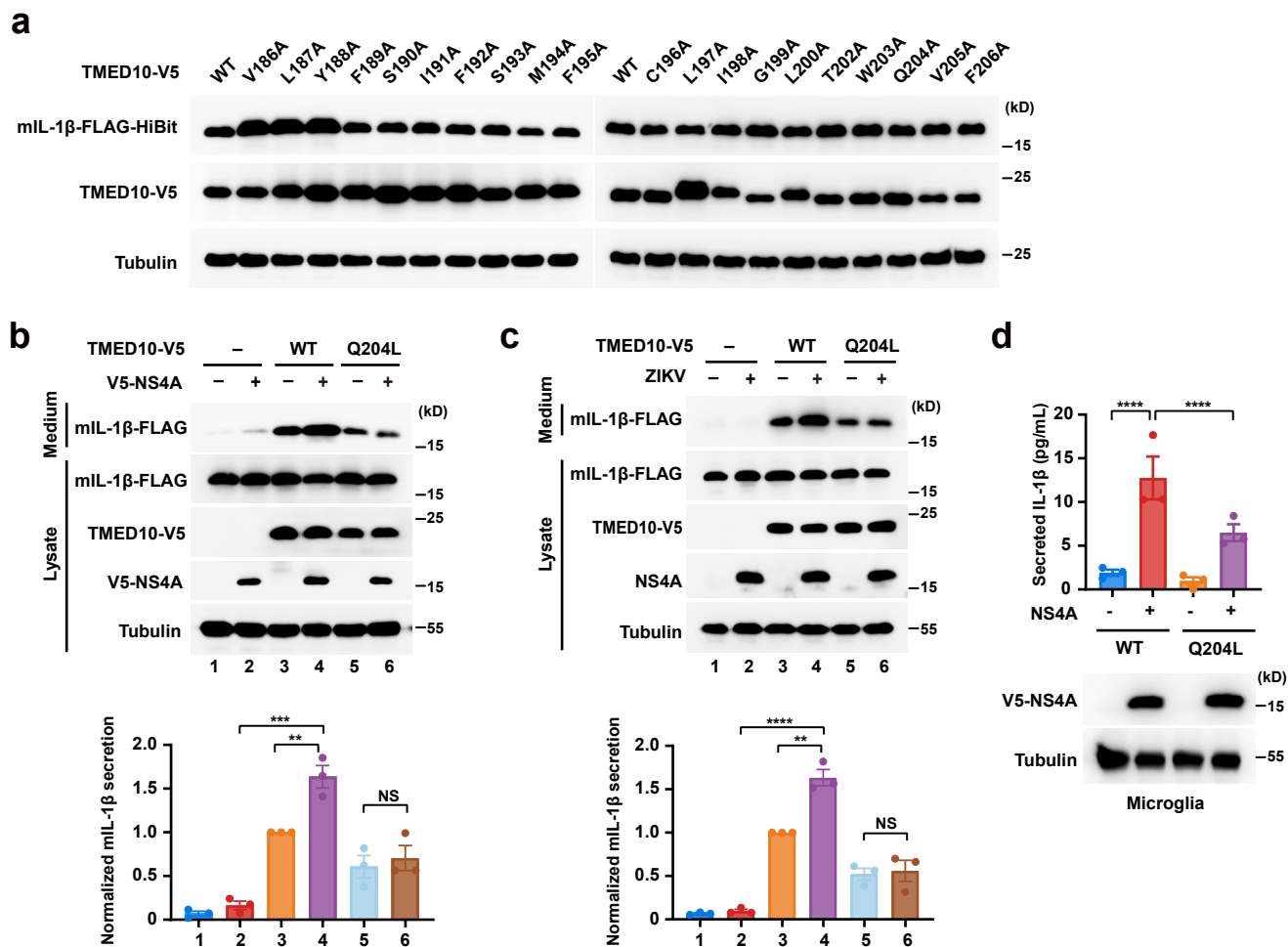


Fig. S8 | TMED10-Q204L failed to support IL-1 β secretion induced by either NS4A expression or ZIKV infection. **a**, Immunoblot showing the expression levels of mIL-1 β -FLAG-HiBiT and V5-tagged TMED10 mutants in cell lysates from the complementary NanoLuc luciferase secretion assay shown in **Fig. 6a**. Secretion of mIL-1 β in mIL-1 β -FLAG-expressing TMED10-KO HEK293T cells co-transfected with V5-tagged NS4A, TMED10-WT or TMED10-Q204L. **c**, Secretion of mIL-1 β in mIL-1 β -FLAG-expressing TMED10-KO HEK293T cells either infected with mock or ZIKV and expressing Ctrl, TMED10-WT or TMED10-Q204L. **d**, IL-1 β secretion measured by ELISA in microglia cells from E16.5 fetal brains of WT or Q204L mice. Cells were transduced with lentiviruses expressing Ctrl or NS4A. **a**, **b**, mIL-1 β secretion was calculated and normalized. Data are mean $\pm$ SEM (**b-d**). Statistical significance was assessed using unpaired two-tailed Student's *t*-test (**b**, **c**) and one-way ANOVA with Tukey's multiple-comparison test (**d**). ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001. NS, non-significant. Data are representative of at least three independent experiments.