The most frequent fetal birth defect associated with prenatal Zika virus (ZIKV) infection is brain calcification, which in turn may potentially affect neurological development in infants. Understanding the mechanism could inform the development of potential therapies against prenatal ZIKV brain calcification. In perivascular cells, bone morphogenetic protein (BMP) is an osteogenic factor that undergoes maturation to activate osteogenesis and calcification. Here, we show that ZIKV infection of cultivated primary human brain pericytes pericytes triggers BMP2 maturation, leading to osteogenic gene expression and calcification. We observed extensive calcification near ZIKV+ pericytes of fetal human brain specimens and in vertically transmitted ZIKV− human signal transducer and activator of transcription 2-knockin mouse pup brains. ZIKV infection of primary pericytes stimulated BMP2 maturation, inducing osteogenic gene expression and calcification that were completely blocked by anti-BMP2/4 neutralizing antibody. Not only did ZIKV NS3 expression alone induce BMP2 maturation, osteogenic gene expression and calcification, but purified NS3 protease also effectively cleaved pro-BMP2 in vitro to generate biologically active mature BMP2. These findings highlight ZIKV-induced calcification where the NS3 protease subverts the BMP2-mediated osteogenic signalling pathway to trigger brain calcification.

Zika virus NS3 protease induces bone morphogenetic protein-dependent brain calcification in human fetuses

Weiqiang Chen1, Suan-Sin Foo1, Eunjin Hong2, Christine Wu2, Wai-Suet Lee2, Shin-Ae Lee3, Denis Evseenko2, Maria Elisabeth Lopes Moreira3, Adolfo García-Sastre4,5,6,7, Genhong Cheng8, Karin Nielsen-Saines9, Patricia Brasil10, Elyzabeth Avvm-Portari11 and Jae U. Jung1*10

The most frequent fetal birth defect associated with prenatal Zika virus (ZIKV) infection is brain calcification, which in turn may potentially affect neurological development in infants. Understanding the mechanism could inform the development of potential therapies against prenatal ZIKV brain calcification. In perivascular cells, bone morphogenetic protein (BMP) is an osteogenic factor that undergoes maturation to activate osteogenesis and calcification. Here, we show that ZIKV infection of cultivated primary human brain pericytes pericytes triggers BMP2 maturation, leading to osteogenic gene expression and calcification. We observed extensive calcification near ZIKV+ pericytes of fetal human brain specimens and in vertically transmitted ZIKV− human signal transducer and activator of transcription 2-knockin mouse pup brains. ZIKV infection of primary pericytes stimulated BMP2 maturation, inducing osteogenic gene expression and calcification that were completely blocked by anti-BMP2/4 neutralizing antibody. Not only did ZIKV NS3 expression alone induce BMP2 maturation, osteogenic gene expression and calcification, but purified NS3 protease also effectively cleaved pro-BMP2 in vitro to generate biologically active mature BMP2. These findings highlight ZIKV-induced calcification where the NS3 protease subverts the BMP2-mediated osteogenic signalling pathway to trigger brain calcification.
deposition of calcium phosphate crystals, resulting in sequential neurological defects. In fact, approximately 30% of Brazilian cohorts of ZIKV-exposed children exhibiting brain calcifications showed delayed childhood neurodevelopment13,14. Despite serious repercussions in fetal development, to our knowledge, no studies have been carried out to understand the molecular detail of ZIKV-induced brain calcification.

Calcification is a process of abnormal calcium phosphate deposition in soft tissues. The bone morphogenetic protein (BMP), one branch of the transforming growth factor-β superfamily, is the major morphogen that promotes vascular and basal ganglia calcifications as well as physiological bone and cartilage formation. In fact, BMP2 has been associated with the osteoblastic transdifferentiation of mesenchymal stem cells in blood vessels15. Moreover, a number of other cell types, including CD146+/platelet-derived growth factor receptor beta-positive (PDGFR-β⁺) pericytes, utilize the BMP2 signal pathway to induce calcification. On cleavage of full-length BMP2 by furin-type proteases16, mature BMP2 is secreted and initiates BMP receptor I/II-mediated signal transduction, leading to the phosphorylation of SMAD1/5/9. The phosphorylated SMAD1/5/9 and SMAD4 complexes subsequently translocate to the nucleus where they induce expressions of a master osteogenic transcription factor, Runx-related transcription factor 2 (RUNX2) and its downstream osteogenic gene, SP7/OSX, as well as important effector genes, such as bone-type tissue non-specific alkaline phosphatase (ALPL/TNAP), dentin matrix acidic phosphoprotein 1 (DMP1) and podoplanin (PDLP/Gp38) (ref. 17). Thus, abnormal activation of the BMP signalling pathway results in aberrant vascular calcification that is associated with several diseases.

In this study, we addressed the molecular mechanism of ZIKV-induced brain calcification. Using human ZIKV⁺ fetal brain specimens and an immunocompetent human STAT2 knockin mouse model, we showed that ZIKV infection of PDGFR-β⁺ perivascular cells induced osteogenic gene expression and calcification. The ZIKV NS3 protease effectively cleaved pro-BMP2/4 to generate biologically active mature BMP2 that induced osteogenic gene expression and calcification. This study discovered a previously unknown viral regulation of osteogenesis where the ZIKV NS3 protease targets the BMP2/4-mediated osteogenic signalling pathway, which ultimately contributes to pathological calcification in the fetal brain.

Results

Perivascular cells are in close proximity to calcium deposits in ZIKV⁺ fetal brain specimens. Computerized tomography neuroimaging revealed scattered parenchymal calcifications in Brazilian ZIKV⁺ infants with severe ventriculomegaly and microcephaly (Fig. 1a). Von Kossa staining of ZIKV⁺ fetal brain specimens from Rio de Janeiro, Brazil also showed extensive calcium depositions that were partially overlapped with haematoxylin and eosin (H&E) staining, suggesting the presence of a cytopathic effect as a result of ZIKV infection (Fig. 1b). Acellular microvascular calcium deposits were also observed in these ZIKV⁺ fetal brains (Extended Data Fig. 1a). Although there is a clear clinical association between ZIKV infection and brain calcification, how ZIKV infection induces pathological calcifications is unclear. To determine which cell types were responsible for calcification in ZIKV⁺ human fetal brains, we performed in situ hybridization of ZIKV RNAs and immunohistochemistry of key cellular biomarkers such as neuronal nuclear antigen (NeuN) for neurons, glial fibrillary acidic protein (GFAP) for astrocytes18 and PDGFR-β for pericytes19,20. Serial sectioning of fetal human brains showed that calcification lesions were surrounded by PDGFR-β⁺ pericytes lining the blood vessels (Fig. 1c and Extended Data Fig. 1b,c). Strikingly, PDGFRβ⁺ perivascular cells near Von Kossa⁺ calcification lesions were strongly positive for ZIKV RNAs, further suggesting that ZIKV-infected perivascular cells are, at least in part, associated with brain calcification (Fig. 1d).

ZIKV infection of primary pericytes induces canonical osteogenic signalling and calcification in vitro. To evaluate the role of pericytes in ZIKV-induced calcifications, human CD146⁺/CD31⁻/CD45⁻ fetal pericytes were infected with ZIKV in vitro (Fig. 2a). This showed that pericytes were effectively infected with ZIKV (African strain MR766 or Asian strain H/PF/2013 or PRVABC59) (Fig. 2b,c). High levels of viral RNAs and infectious viral particles were detected in CD146⁺/CD31⁻/CD45⁻ fetal pericytes within 3 d post-infection with all three ZIKV strains (Fig. 2b,c). Notably, the Asian ZIKV strains continuously replicated at high levels at 8 d post-infection and infectious viruses were detected even at 14 d post-infection (Fig. 2c). While ZIKV-MR766 replicated faster than ZIKV-H/PF/2013 and ZIKV-PRVABC59, ZIKV-MR766 RNAs were not detected after 3 d post-infection and infectious virus titres rapidly declined over time (Fig. 2b,c). Consistently, African ZIKV strain-infected (MR766 or IbH30656) pericytes showed high levels of cell death, whereas Asian ZIKV strain-infected (H/PF/2013 or PRVABC59) pericytes showed minimal cell death (Extended Data Fig. 2a). Overall, these data show that human CD146⁺/CD31⁻/CD45⁻ fetal pericytes are highly susceptible to ZIKV replication. The African ZIKV strain rapidly replicated in fetal pericytes causing high cell death, whereas the Asian ZIKV strain persistently replicated in fetal pericytes with low cell death.

The BMP-mediated osteogenic signal pathway has been shown to contribute to calcifications17–20. Infection of fetal pericytes with ZIKV-H/PF/2013 led to an increase of secreted mature BMP2 in the supernatants, but not in that of mock- or ZIKV-MR766-infected pericytes (Fig. 2d). In addition, infection of fetal pericytes with ZIKV-H/PF/2013 induced substantially high expression of BMP2 compared to BMP4, BMP6, BMP7 and BMP9 as well as the BMP antagonist, NOG, although their activities were not measured (Fig. 2e and Extended Data Fig. 2b). Consistently, expression of the central osteogenic transcription factor genes RUNX2 and SP7/OSX, as well as the downstream mineralization genes ALPL/TNAP, DMP1 and PDPN were markedly induced on ZIKV-H/PF/2013 infection (Fig. 2e). In contrast, African ZIKV-MR766 or ZIKV-IbH30656 infection, which showed massive cell death by 3 d post-infection, exhibited little or no change of their expression (Fig. 2e, Extended Data Fig. 2a,c). Correspondingly, Alizarin Red S staining found aberrant calcification in fetal pericytes infected with ZIKV-H/PF/2013 or ZIKV-PRVABC59 but not with mock or ZIKV-MR766 (Fig. 2fg and Extended Data Fig. 2d). Interestingly, infection of the neuroblastoma or astrocytoma cell line with either ZIKV-MR766 or ZIKV-H/PF/2013 induced extensive cell death without calcification (Extended Data Fig. 2e,f). In addition, ZIKV infection induced BMP2, SP7/OSX, DMP1 and PDPN expression in primary fetal brain-derived pericytes but not in primary fetal brain-derived astrocytes (Extended Data Fig. 2g). RNA sequencing (RNA-seq) analysis of ZIKV-infected peripheral neurons also showed no increase of BMP2, SP7/OSX, DMP1 and PDPN expression (Extended Data Fig. 2h)21. These results suggest that ZIKV infection of fetal pericytes, but not fetal astrocytes or neurons, results in the induction of osteogenic gene expression and calcification.

ZIKV-induced brain calcifications in immunocompetent human STAT2 knockin ZIKV mice. Immunocompetent transgenic human STAT2 knockin mice that carry the replacement of mouse Stat2 with human STAT2 were developed to closely recapitulate human ZIKV pathogenesis15. To investigate ZIKV-induced brain calcification in vivo, three-week-old human STAT2 knockin mice were infected subcutaneously with ZIKV-MR766 or ZIKV-PRVABC59 near the sagittal suture areas and their brain tissues were collected either at 4, 8 or 11 d
Fig. 1 | Calcification in brain specimens from ZIKV-infected fetuses. **a.** Computed tomography scan of calcifications (red arrows) in the subcortical white matter in a nine-month-old infant infected with ZIKV during the second trimester of pregnancy. **b.** Brain tissue sections (10 μm) from ZIKV+ human fetuses derived from stillbirths were stained with Von Kossa (top) and H&E (bottom) stains. **c.** Serial brain sections from ZIKV+ human fetal brain tissue were stained with Von Kossa (calcium, black), anti-PDGFR-β (pericytes), anti-GFAP (astrocytes) or anti-NeuN (neurons). Images were taken at 10x (top panel) and 20x magnification (bottom panel). **d.** Von Kossa and RNAscope duplex in situ hybridization with ZIKV RNA and PDGFRB mRNA (pericytes) in serially sectioned ZIKV+ human fetal brain tissues. Brightness and contrast were adjusted in the magnified inset. The red arrows indicate PDGFRB/ZIKV+ pericytes. The data are representative images from five ZIKV+ fetal donors.
Fig. 2 | Osteogenic gene expression and calcification in ZIKV+ fetal pericytes. **a**, Fetal pericytes (CD146+/CD31−) were infected with African strain ZIKV-MR766, Asian strains ZIKV-H/PF/2013 and PRVABC59 at a multiplicity of infection (MOI) of 0.5 or treated with medium without virus (mock). The blue cell population is the unstained control; the red cell population is stained with CD146 and CD31 antibodies. See Supplementary Fig. 1 for the gating strategy employed. **b,** At 1–4 and 8 d post-infection, cells were collected for viral RNA analysis (n = 4). The infectious virus titre was quantified in supernatants (n = 6 per group) collected from 1–4, 8 and 14 d post-infection. **c,** At 3, 4 and 8 d post-infection, mature BMP2 protein from the supernatants of mock- and ZIKV-infected pericytes was measured (n = 8). **d,** Osteogenic gene expression in fetal pericytes was normalized to GAPDH and presented as fold changes relative to mock controls (n = 6 per group). **f**–**g,** Alizarin Red S staining (f) and Alizarin Red S concentration (g) (mM) of ZIKV-infected fetal pericytes 14 d post-infection (n = 4). Data are presented as the mean ± s.e.m. using a two-way ANOVA followed by Sidak’s multiple comparisons test (**e**) or one-way (**g**) ANOVA with Tukey’s post-test. *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001. **e**–**g,** Data are presented as the mean ± s.e.m. in the box plots showing the upper (75%) and lower (25%) quartiles, with the horizontal line as the median and the whiskers as the maximum and minimum values observed. The exact P values in **g** were obtained by comparing the mock and ZIKV-H/PF/2013 (P = 0.0046) and mock and PRVABC59 (P = 0.0030) groups. Data are representative of three independent experiments.

post-infection to determine osteogenic gene expression, or at 21 d post-infection for Alizarin Red S calcium staining. While higher levels of ZIKV RNA were detected in ZIKV-MR766-infected brains than in ZIKV-PRVABC59-infected brains (Extended Data Fig. 3a), ZIKV-PRVABC59 infection, not ZIKV-MR766 infection, led to the significant increase of early osteogenic genes (BMP2, RUNX2, SP7/OSX and ALPL/TNAPBMP2) at 4 and 8 d post-infection and late osteogenic genes (DMP1 and PDPN) at 11 d post-infection (Extended Data Fig. 3b). Correspondingly, ZIKV-PRVABC59-infected mice showed aberrant brain calcifications (Extended Data Fig. 3c). In contrast, severe tissue damage was observed in the brains of ZIKV-MR766-infected mice with little or no calcium deposition as shown with Alizarin Red S staining (Extended Data Fig. 3c). These results demonstrate that Asian ZIKV infection can trigger activated osteogenic signalling and brain calcifications in an immunocompetent mouse model.

A recent clinical follow-up study of severe cases of 68 ZIKV-exposed children who exhibited fetal abnormalities identified brain calcifications in 99% of these children13. To further demonstrate in vivo ZIKV-induced calcification through maternal–fetal vertical transmission, we infected immunocompetent human STAT2 knockin time-mated pregnant dams with ZIKV-PRVABC59 intraperitoneally at mouse embryonic day 13.5 (E13.5) and collected the pups’ brains at post-delivery day 28 (Fig. 3a). ZIKV-exposed pups’ brains showed high osteogenic gene expression compared to PBS-exposed control mice (Fig. 3b). Strikingly, ZIKV protein and aberrant brain calcium deposits
were readily detected in the midbrain regions of pups’ brains at post-delivery day 28 (Fig. 3c,d). This suggests that ZIKV is vertically transmitted from pregnant dams to their pups, leading to fetal brain calcifications.

ZIKV-induced calcifications through the BMP2-mediated osteogenic signalling pathway. Since soft tissue calcification is elicited by cellular processes that closely resemble physiological bone formation\(^7\), ZIKV-susceptible human bone-derived U2OS cells were
used to dissect the molecular mechanism of ZIKV-induced calcification\textsuperscript{24,26}. Like primary fetal pericytes (Fig. 2b,c), U2OS cells highly supported ZIKV-MR766 or ZIKV-H/PF/2013 infection and replication (Fig. 4a,b). ZIKV-MR766 infection, but not ZIKV-H/PF/2013 infection, led to extensive cell death (Fig. 4c). Interestingly, both ZIKV-MR766 and ZIKV-H/PF/2013 infection of U2OS cells immediately increased mature BMP2 expression in the supernatants at 2 and 3 d post-infection (Fig. 4d). These results suggest that while both ZIKV-MR766 and ZIKV-H/PF/2013 infection increased mature BMP2 in supernatants, only ZIKV-H/PF/2013 infection continuously increased mature BMP2 due to its weaker cytopathic activity.

BMP2 transduces signals by binding to the complexes of type I and II serine/threonine kinase receptors, which induce the phosphorylation of SMAD1/5/9 to activate canonical signaling, ultimately leading to the expression of the osteogenic genes RUNX2, SP7/OSX, DMP1, PDNP and BMP2 (ref. 20). Indeed, ZIKV-H/PF/2013 infection apparently induced a high level of SMAD1/5/9 phosphorylation and increased the expression of RUNX2, SP7/OSX, ALPL/TNAP, DMP1, PDNP and BMP2, whereas ZIKV-MR766 infection did not (Fig. 4e and Extended Data Fig. 4a,b,c). Strikingly, treatment of anti-BMP2/4 neutralizing antibody not only drastically suppressed ZIKV-H/PF/2013-induced expression of the osteogenic genes RUNX2, SP7/OSX, DMP1, PDNP and BMP2 (Fig. 4f) but also completely abrogated ZIKV-H/PF/2013-induced calcium deposition (Fig. 4g,h) without notable effect on the virus load (Extended Data Fig. 4d). In addition, anti-BMP2/4 neutralizing antibody treatment of human fetal brain pericytes also showed suppression (Fig. 4i) without notable effect on the virus load (Extended Data Fig. 4d). These findings strongly indicate that the Asian ZIKV strain infection triggers BMP2-driven osteogenic signals, resulting in calcifications.

ZIKV NS3 protease cleaves pro-BMP2 into its mature forms, promoting osteogenic signal. ZIKV encodes a polyprotein that is cotranslationally and post-translationally processed into three structural proteins—Cpr, prM, Env—and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5). To identify which viral proteins contributed to triggering osteogenic signaling on ZIKV infection, individual ZIKV protein was expressed in fetal primary pericytes by recombinant lentivirus infection\textsuperscript{20}. Interestingly, ZIKV NS3 expression alone was sufficient to markedly increase BMP2, RUNX2, SP7/OSX, DMP1 and PDNP osteogenic gene expression (Extended Data Fig. 4e). These findings strongly indicate that the Asian ZIKV strain infection triggers BMP2-driven osteogenic signals, resulting in calcifications.

**Discussion**

Brain calcifications are a frequently reported neurological finding in ZIKV-infected babies born during or in the aftermath of recent outbreaks\textsuperscript{24,25,27-29}. These brain calcifications are linked to severe developmental defects such as motor disorders, cognitive disability, eye abnormalities, hearing deficits and seizures\textsuperscript{30-33}. A follow-up brain imaging in children with congenital ZIKV syndrome after one year showed no further increase in the number or size of calcifications but these children still presented neurological repercussions such as epilepsy and feeding disorders, demonstrating the potential pathological consequences of ZIKV-induced brain calcifications during early childhood development\textsuperscript{44,46}. Yet to date, no studies on the molecular mechanisms of ZIKV-induced brain calcifications have taken place.
Although human neural lineage cells such as neural progenitor cells, astrocytes, oligodendrocytes and neurons have been well studied for ZIKV infection\(^4\), perivascular cells have not yet been studied for ZIKV infection and calcification\(^4,5\). Since pericytes are essential in the development and maintenance of the neurovascular unit, the degeneration of these pericytes has been strongly implicated in neuropsychiatric diseases such as primary familial brain calcification\(^5\). Interestingly, pericytes have also been shown to exhibit stem cell potential similar to mesenchymal stem cells in vitro and in vivo\(^6,7\). For instance, microvascular pericytes express the early osteogenic marker RUNX2 and form bone-line mineralized matrix in vivo, suggesting that pericytes function has osteogenic precursors. In this study, we found that ZIKV\(^\text{+}\) brain tissues from post-mortem human fetuses in the 2015–2016 Brazil outbreak...
induce high inflammatory responses and cell death compared to the African strain counterpart, which causes extensive cell death, leading to less cytotoxicity and infectious virion production. Consistent with previous reports, Asian ZIKV tends to infect neural progenitor cells showed increased BMP2, BMP4, BMP6, BMP7 and BMP9 expression, but not other BMPs. In addition, ZIKV NS3 protease upregulates osteogenic signalling in fetal pericytes. In vitro ZIKV infection of primary human fetal-derived pericytes robustly induced osteogenic gene expression and calcification even in the absence of osteogenic media, suggesting that ZIKV infection alone is sufficient to trigger osteogenic differentiation of perivascular cells. Interestingly, ZIKV-induced calcification is lineage-specific to Asian ZIKV but not to African ZIKV, which is likely due to the persistent replication and low cytopathic activity of Asian ZIKV. Consistent with previous reports, Asian ZIKV tends to infect poorly, leading to less cytotoxicity and infectious virion production compared to the African strain counterpart, which causes extensive cell death. Furthermore, African ZIKV has been demonstrated to induce high inflammatory responses and cell death compared to Asian ZIKV. Thus, these intrinsic differences may explain why Asian ZIKV infection, but not African ZIKV infection, leads to the calcification of perivascular cells.

Calcification is a biological process that may utilize the canonical BMP–SMAD signal pathway, mimicking bone formation. In early embryogenesis, the localized concentration gradients of BMPs are essential for the development of skeletal and nervous systems. Mature BMPs bind to their receptor to induce SMAD1/5/9 phosphorylation, leading to the expression of the master osteogenic regulators RUNX2 and SP7/OSX, and effector proteins ALPL/TNAP, DMP1 and PDPN to trigger calcification. In fact, the processed pro-domain of BMPs such as BMP2 also showed bone-inducing properties. To date, there are over 20 types of BMPs, but only BMP2, BMP4, BMP6, BMP7 and BMP9 have strong osteogenic properties. Interestingly, RNA-seq analysis of ZIKV-infected human neural progenitor cells showed increased BMP2/4 expression, but not other BMPs. Consistently, we also found that ZIKV-infected pericytes showed increased expression of BMP2 and BMP4, but not the other BMPs. In addition, we showed that ZIKV NS3 protease effectively processed full-length BMP2 to generate biologically active secreted mature BMP2 that leads to calcification, which was drastically suppressed by anti-BMP2/4 neutralizing antibody. In addition, BMP2/4 have been shown not only to repress neurogenesis and oligodendrogiogenesis during late embryogenesis and postnatal neural development, but also to strongly induce osteogenesis in cells of mesenchymal origins. In human ZIKV+ brains, most calcification lesions were primarily associated with pericytes, suggesting that the BMP2 released from infected pericytes activates...
osteogenic programmes in an autocrine mode in vivo rather than a paracrine mode. Hence, this study suggests that the ZIKV NS3 protease subverts the BMP2-mediated osteogenic developmental signalling pathway to trigger aberrant brain development and calcifications (Fig. 6f).

Full-length BMP2, similar to full-length BMP4, are assumed to be cleaved by a furin-type convertase at its dibasic site K/R to generate two glycosylated 18–22 kDa mature forms that are secreted to the extracellular compartment. Since the ZIKV NS3 protease domain is a trypsin-like serine protease with high specificity for substrate with

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Fig. 6 | Purified ZIKV NS3 serine protease processes pro-BMP2 to its biologically active mature forms to induce osteogenic signalling. a, At 3 d after transfection with FLAG-tagged WT or mutant NS3, the supernatants and lysates of U2OS cells were subjected to immunoblotting analysis or Ponceau S staining. b, U2OS cells stably expressing WT or mutant NS3 were cultured and stained with Alizarin Red S. c, Schematic of predicted BMP2 cleavage products. d, In vitro cleavage assay of carboxyl terminal HA-tagged purified human BMP2 and purified ZIKV NS3 protease was performed at 37 °C for 3 h, followed by immunoblot analysis. e, Cleaved BMP2 products from the in vitro reaction were added to U2OS cells for 0 or 15 min and cells were collected for immunoblot analysis. Data are representative of two independent experiments. f, Schematic model of ZIKV NS3 protease-induced BMP2 maturation and osteogenic gene expression for fetal brain calcification. ZIKV NS3 protease efficiently cleaved pro-BMP2 to generate secreted mature BMP2 that induces its receptor-mediated osteogenic gene expression and calcification.
dibasic P1 and P2 (ref. 4), it efficiently cleaved pro-BMP2/4 to generate two mature forms of BMP2/4, suggesting that BMP2 and BMP4 could play critical roles in ZIKV-induced osteogenic signalling. Previous studies have shown that ZIKV NS3 protease targets STING and Jak1 to impair the host IFN signalling pathway and apoptotic cell death, respectively4,5. Thus, this study has identified pro-BMP2/4 as a previously undescribed NS3 substrate to induce BMP2/4-mediated osteogenic signal transduction for brain calcification.

Historically, because brain calcifications have often been interpreted as dead brain tissues that calcify after injury, to our knowledge, the molecular mechanism behind brain calcifications has not been explored until now. Thus, this study demonstrates the molecular mechanism of how Asian ZIKV has evolved to exploit BMP2-mediated osteogenic signalling mechanisms, which ultimately contributes to pathological calcification in the fetal brain. This study may also shed light into the pathophysiological process generating calcifications in other congenital infections.

Methods

Human fetal brain tissues. Paraffin blocks containing pathology brain tissue specimens from five cases (one stillbirth and four neonatal deaths due to in utero infection with ZIKV after maternal infection in pregnancy) and three healthy brain tissue specimens from neonatal deaths unrelated to ZIKV infection were obtained from the southeastern state of Rio de Janeiro, Brazil. Brain tissues were formalin-fixed and paraffin-embedded before sectioning and immunohistochemical staining. The infants were all born at term except for two who were born prematurely at 32 weeks. Deceased infants were confirmed positive for ZIKV by either PCR or in situ hybridization in the liver and spleen. All pregnant mothers had reported typical symptoms of ZIKV infection between the 4th and 16th gestational weeks. ZIKV RNA was detected in maternal blood, amniotic fluid and/or samples of post-mortem neonatal liver and spleen. In all five cases, Dengue and Chikungunya virus infection were excluded by enzyme-linked immunosorbent assay (ELISA) and PCR. All cases tested negative for TORCH-like conditions (Toxoplasmosis, Other (syphilis, varicella-zoster, parvovirus B19), Rubella, Cytomegalovirus and Herpes infections).

Viruses. African ZIKV strains MR766 (passage 5; Vero cells; Uganda, 1947) and IbH30656 (passage 2; Vero cells; Nigeria, 1968), as well as Asian ZIKV strain PRVABC59 (passage 2; Vero cells; Puerto Rico, 2015; ATCC) virus stocks were purchased from ATCC. The Asian ZIKV strain H/PF/2013 (passage 2; Vero cells; French Polynesia, 2013) was kindly provided by M. Diamond (Washington University School of Medicine). All ZIKV virus stocks were propagated in the Vero cell line (ATCC CCL-81) and the supernatant was collected at 3–5 days post-infection. Viral titres were determined by plaque assays on Vero cells. Vero cells were authenticated by morphology check, growth curve analysis and validated to be Mycoplasma-free with Hoechst staining.

Animals and infection. Immunocompetent human STAT2 knockout mice were bred and maintained within the animal unit of University of Southern California. Twenty-one-day-old male and female mice of equal sex distribution were infected subcutaneously above the sagittal suture with 2 μl of 105 p.f.u. of ZIKV-PRV ABC59 diluted in PBS to a volume of 200 μl at mouse E13.5. Animal studies were carried out in the animal facility managed and maintained by the University of California Animal Resource Center. Facilities were maintained at an acceptable range of 20–26°C at a humidity of 30–70% on a 12 h dark–12 h light cycle. All procedures used in this study complied with federal and institutional guidelines enforced by the University of Southern California Institutional Animal Care and Use Committee and were granted institutional approval after veterinary and committee review.

Bacterial strains, mammalian cell lines and primary cells. Escherichia coli TOP10 were grown in lysogeny broth (Lennox; Sigma-Aldrich) medium for genetic manipulations with ampicillin (100 μg ml−1). HEK293T, U2OS, U251, SK-N-SH and Vero E6 cells were purchased from ATCC and cultured in DMEM (Gibco) supplemented with 10% FCS (Seradigm) and 100 U ml−1 streptomycin and 100 μg ml−1 penicillin (1% penicillin-streptomycin; Gibco). CHO FD11 cells were kindly provided by S. Leppa (National Institute of Allergy and Infectious Diseases, National Institutes of Health). ZIKV NS3 stably expressing cells were generated by transduction with lentivirus generated from pCDH-NS3-3xFLAG-puro construct, followed by selection with puromycin (1 μg ml−1; Gibco). Human fetal bone tissues were obtained from Novogenix Laboratories after informed consent and elective termination; primary fetal cad 1246/1231/1245-NS3 pericytes were isolated from bone marrow as described previously7. Primary fetal human perivascular pericytes (termed pericytes; catalogue no. 1800) were obtained from Sciencell Research Laboratories. All cells were maintained at 37°C with 5% CO2. To inhibit BMP2/4 activity, cells were treated with 2 μg ml−1 of purified mouse IgG1 isotype control (clone 11711, catalogue no. MA9802; R&D Systems) or human BMP2/4 neutralizing antibody (clone 100230, catalogue no. MA3552100; R&D Systems).

Plasmid constructs. The infectious clone of PRVABC59, kindly provided by R. Sun (University of California, Los Angeles) was used as the DNA template for the PCR amplification of individual viral proteins. Amplified PCR products were cloned into lentiviral pCDH-puro vector with a C-terminal 3xFLAG tag. The pCDH-NS3-2223-BMP2 construct was generated by cloning BMP2 into the lentiviral vector pCDH-NS3-2223-BMP2. To generate a full-length recombinant BMP2 proteins, modifications were made to the BMP2 construct by the introduction of a FLAG tag after the signal peptide (amino acid residues 1–22) and was subsequently recombined onto pRES-puro vector with C-terminal haemagglutinin (HA) tag. The bZIP construct was purchased from Addgene. All plasmid sequences were analysed using SnapGene v5.2.3.

Immunohistochemical staining. Fetal human brain and mouse brain tissues were fixed in 4% paraformaldehyde and embedded in paraffin. The, 10-μm sections were dewaxed, rehydrated and stained with H&E, Alizarin Red S, Von Kossa stain and/or haematoxylin. Immunohistochemical staining was performed according to the manufacturer’s instructions. This was followed by immunohistochemical staining with anti-human PDGFR-α (clone 3C11, catalogue no. 91367, Cell Signalling Technology) and anti-human PDGFR-β (clone 2C11, catalogue no. 91368, Cell Signalling Technology). Neun (1:50 dilution; catalogue no. ABN78; Merck Millipore) antibodies and counterstained with haematoxylin.

Flow cytometry. Surface staining of fetal pericytes was performed with CD31 Alexa Fluor 488 (1:100 dilution; clone WM59, catalogue no. 303110; Biologend) and CD45 Alexa Fluor 647 (1:100 dilution; clone PH112, catalogue no. 361014; BioLegend) antibodies. The Zombie Violet fixable viability kit (catalogue no. 423113; BioLegend) was used for fixable live/dead staining according to the manufacturer’s instructions. Fluorescence-activated cell sorting (FACS) acquisition was performed on a BD FACS Canto II (BD Biosciences), using the BD FACS Diva v6.0 software. FACS data were analysed using the FlowJo v10.0 software (FlowJo LLC).

RNA extraction, viral load and gene expression analyses. Total RNA extraction was performed with the RNeasy Mini Kit (QIAGEN) according to the manufacturer’s instructions. RNA concentration was determined by NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). ZIKV RNA was reverse-transcribed with the iScript cDNA Synthesis Kit (Bio-Rad Laboratories) according to the manufacturer’s instructions. Specific ZIKV NS1 primers and probes targeting conserved NS1 region across all four ZIKV strains (MR766, IbH30656, H/PF/2013 and PRV ABC59) were designed for viral load detection. The standard curve was generated from serially diluted plasmid expressing MR766 NS1 protein. Viral load and gene expression quantitative PCR with reverse transcription (RT-qPCR) were performed with 10 ng of complementary DNA per well with SsoAdvanced Universal Probe Supermix (Bio-Rad Laboratories) or SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories), respectively. All RT-qPCR reactions were performed on the CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories) on 96-well plates. All RT-qPCR data were analysed with the CFX Manager v3.1 software. Viral loads in specimens were interpolated from standard curves with Prism v8.4 (GraphPad Software). All viral load RT-qPCR performed in this study included mock controls where no Ct values could be detected. Gene expression fold change was calculated using the ΔΔCt method based on MicroSoft Excel. Briefly, ΔCt studies were calculated using ΔCt(mock control) = ΔCt(mRNA)−ΔCt(housekeeping gene−GAPDH). The fold change for each gene was calculated as 2−ΔΔCt. All primers and probe sequences used in this study are listed in Supplementary Table 1.

Plaque assay. Vero E6 cells were seeded onto 24-well plates at a density of 1 × 105 cells per well and cultured to confluency. Serially diluted samples (10−1 to 10−3) were added to the cell monolayers and incubated for 2 h at 37°C in a 5% CO2 incubator. Cells were then overlaid with DMEM containing 5% FCS and 1% agarose (Sigma-Aldrich) and incubated at 37°C for 24 to 48 h. After incubation, the overlay was removed, cells were fixed with 10% neutral buffered formalin (Sigma-Aldrich) and plates were visualized by staining with 0.1% crystal violet. Viral titres were expressed as p.f.u. per millilitre.

ELISA. The human BMP2 ELISA assay (catalogue no. DY355; R&D Systems) were performed according to the manufacturer’s instructions. Alizarin Red S staining and quantification. Cells were washed twice with PBS, fixed in 4% paraformaldehyde, washed twice with distilled H2O followed by
incubation with Alizarin Red S stain for 30 min at room temperature with gentle shaking. Stained cells were rinsed in PBS for 10 min with gentle agitation 3 times. To quantitatively Alizarin Red S staining, cells were incubated with 800 μl of 10% (v/v) acetic acid for 30 min with gentle agitation. The monolayer was then scraped from the plate and transferred with 10% (v/v) acetic acid to a 1.5 ml microcentrifuge tube. The slurry was vortexed for 30 s, overlaid with 500 μl of mineral oil (Sigma-Aldrich), heated to 85 °C for 10 min and transferred to ice for 5 min. The slurry was centrifuged at 20,000 × g for 15 min and the supernatant removed to a new 1.5 ml microcentrifuge tube. Then 200 μl of 10% (v/v) acetic acid was added to neutralize the acid and absorbance readings were taken at 405 nm with the FilterMax F5 Multi-Mode Microplate Reader (Molecular Devices).

Immunoblotting analysis. Whole-cell lysates were lysed with radioimmunoprecipita
tion assay lysis buffer and measured for protein concentration by Bradford Protein Assay (Thermo Fisher Scientific) to equalize protein loading. Proteins (25–50 μg) were resolved on SDS–polyacrylamide gel electrophoresis gels and transferred to a polyvinylidene difluoride membrane by semi-dry transfer at 25 V for 45 min (Trans-Blot Turbo, Bio-Rad Laboratories). All membranes were blocked in 5% milk in Tris-buffered saline with Tween 20 (TBST, pH 8.0, Sigma-Aldrich) and probed overnight with the indicated antibodies in 5% milk or 5% BSA in TBST. Primary antibodies included: rabbit anti-BMP2 (1:1,500 dilution; EPR20807, catalogue no. ab214821; Abcam); rabbit anti-phospho-SMAD1 (Ser463/465)/SMAD5 (Ser463/465)/SMAD9 (Ser465/467) (1:1,000; clone DB510, catalogue no. 13820S; Cell Signaling Technology); rabbit anti-SMAD2/3 (1:1,000 dilution; catalogue no. 9744S); Cell Signaling Technology); rabbit anti-Smad-1/5/8 (anti-ZIKV NS3 (P<0.05), **P<0.01, ***P<0.001 and ****P<0.0001. For the analyses between two groups, a two-tailed unpaired Student's t-test or Mann–Whitney U-test were used. For comparisons between more than two groups, either a one-way analysis of variance (ANOVA) with Tukey's post-test or Kruskal–Wallis test with Dunn's post-test and a two-way ANOVA with Tukey's or Sidak's post-test were used. All data were assessed for Gaussian distribution using the Shapiro–Wilk test and Log2 transformation was used to achieve normality. Data (mean ± s.e.m.) are presented in box and whisker plots showing the upper (75%) and lower (25%) quartiles, with the horizontal line as the median and the whiskers as the maximum and minimum values observed.

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

Data availability

The gene expression data presented in Extended Data Fig. 2h of this manuscript were reanalysed from RNA-seq data reported by Oh et al.4 and deposited with the Gene Expression Omnibus under accession no. GSE87750. Data supporting the full scope of this study are available upon reasonable request. All RT–qPCR primers and probe sequences used in this study are listed in Supplementary Table 1. Source data are provided with this paper.

Received: 27 January 2020; Accepted: 11 December 2020; Published online: 28 January 2021

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Extended Data Fig. 1 | See next page for caption.
Extended Data Fig. 1 | Perinatal ZIKV infection in brain tissues of deceased fetuses exhibits pronounced calcifications. a, Paraffin-embedded brain tissue sections (10 μm) from ZIKV-positive deceased human fetuses were stained with Von Kossa (red arrow). b, Immunohistochemical staining for NeuN (neurons), PDGFRβ (pericytes) or GFAP (astrocytes) were performed using sequential brain sections of ZIKV-positive human fetal brain tissue. Arrows indicate NeuN-, PDGFRβ- or GFAP-positive cells. c) RNAScope duplex in-situ hybridization with ZIKV RNA and PDGFRβ mRNA of healthy human fetal brain tissues. Black arrows indicate PDGFRβ-positive cells lining the blood vessels. Neither non-specific staining ZIKV RNA nor calcium deposit was detected in healthy tissues. Data in a-c are examined from biologically independent human specimens (healthy n = 3; ZIKV n = 5) and are representative of two independent repeats.
Extended Data Fig. 2 | See next page for caption.
**Extended Data Fig. 2 | Asian ZIKV infection, but not African ZIKV elicits in vitro calcification in pericytes.** Human fetal pericytes were infected with different ZIKV strains at MOI of 0.5 or mock (PBS) as control (n=4 biologically independent cells per group). a, At 3 dpi, cell viability was quantified. See Supplementary Data Fig. 1 for gating strategy. b, BMP4, BMP6, BMP7, BMP9 and NOG expression between ZIKV MR766- and ZIKV H/PF/2013-infected pericytes at 1, 3 and 4 dpi (n=6 biologically independent cells per group). Data in a and b are presented as mean ± SEM in box plots showing the upper (75%) and lower (25%) quartiles, with the horizontal line as the median and the whiskers as the maximum and minimum values observed. c, Osteogenic gene expression between ZIKV MR766- and ZIKV IbH30656-infected pericytes was normalized to GAPDH and expressed as fold change relative to mock controls (n=3 biologically independent cells per group). Data are presented as mean ± SEM. d, At 8 and 14 dpi, mock- or ZIKV-infected human fetal pericytes were subjected to Alizarin red staining for calcium deposition. Data are representative of two independent experiments. e-f, Alizarin red staining were performed on mock- or ZIKV-infected SK-N-SH and U251 at 14 and 21 dpi. Data are representative of four independent experiments. g, Human primary fetal pericytes (n=5) and astrocytes (n=6) were infected with PBS (mock) or ZIKV PRVABC59 at MOI of 0.5. At 3 dpi, osteogenic gene expressions were normalized to GAPDH and expressed as fold changes relative to mock controls. Data are presented as mean ± SEM and are representative of two independent experiments. h, RNAseq data generated from Nature Neuroscience; 2017; 20(9); p1209-1212 10.1038/nn.4612 (GEO ID: GSE87750) was reanalyzed for osteogenic gene expression. Human primary peripheral neurons were infected with ZIKV PRVABC59 of MOI 0.4 and harvested at 3 dpi. Statistical analyses were performed using one-way ANOVA with Tukey’s posttest (a and c). ***P<0.001 and ****P<0.0001. Exact P values in a compared between ZIKV MR766 and ZIKV H/PF/2013 group (P=0.0008), ZIKV MR766 and ZIKV PRVABC59 group (P=0.0005), ZIKV IBH30656 and ZIKV H/PF/2013 group (P=0.0001) and ZIKV IBH30656 and ZIKV PRVABC59 group (P=0.0001).
Extended Data Fig. 3 | See next page for caption.
Extended Data Fig. 3 | ZIKV infection of 3-weeks-old human STAT2 knockin mice resulted in brain calcification. Three-week-old hSTAT2KI were s.c. infected with ZIKV. a, At 8 dpi, ZIKV RNA was quantified in the brain (MR766 n = 7; PRVABC59 n = 5). b, Osteogenic gene expression in mock- or ZIKV-infected hSTAT2KI brains (PBS n = 5; MR766 n = 6; PRVABC59 n = 6) were normalized to GAPDH and presented as fold changes relative to mock controls. c, Brain sections were stained with H&E or Alizarin red for calcium deposit. Black dotted line in the magnified insert indicates vasculature and blue dotted line in the magnified insert indicates prominent calcification sites. Data are presented as mean ± SEM, using Mann-Whitney U-test (a) or two-way ANOVA followed by Tukey’s multiple comparisons test (b). *P < 0.05, **P < 0.01 and ***P < 0.001. Exact P values in a compared between ZIKV MR766 and ZIKV PRVABC59 group (P = 0.0051). Data in a-c are representative of three independent experiments.
Extended Data Fig. 4 | See next page for caption.
Extended Data Fig. 4 | ZIKV infection of osteoblastic-like cells increase osteogenic gene expression. a–b, Mock- and ZIKV-infected U2OS cells were harvested at 2 and 4 dpi respectively for osteogenic gene expression. c, Band intensity of pSMAD1/5/9 from U2OS whole cell lysate. d, At day 1–4, IgG or nAb-treated and mock or ZIKV-infected U2OS cells (n = 6) were harvested for RNA extraction and viral load against ZIKV NS1 RNA was quantified using qRT-PCR (N.D.; Not detected) (e) Human primary fetal brain pericytes are infected with ZIKV PRVABC59 or PBS (mock control) that were treated with 2 μg/mL of mouse IgG isotype control or human BMP2/4 neutralizing antibody. At 4 dpi, cells were harvested for osteogenic gene expressions, normalized to GAPDH and presented as fold changes relative to mock controls (n = 4/group). Data in a, b, d and e are presented as mean ± SEM in box plots showing the upper (75%) and lower (25%) quartiles, with the horizontal line as the median and the whiskers as the maximum and minimum values observed. Data are analyzed using Kruskal-Wallis test followed by Dunn’s multiple comparisons (a), Mann-Whitney U-test (b), or two-tailed unpaired Student t-test (e). *P < 0.05, **P < 0.01, ***P < 0.001. Exact P values in b compared between Mock and ZIKV H/PF/2013 (BMP2 P = 0.0002; RUNX2 P = 0.0011; OSX P = 0.0002; ALPL P = 0.0002; DMP1 P = 0.0002 and PDPN P = 0.0002). Exact P values in e compared between IgG-treated and Nab-treated group (BMP2 P = 0.0054; RUNX2 P = 0.0005; OSX P = 0.0085; DMP1 P = 0.0099 and PDPN P = 0.0005). Data are representative of two independent experiments.
Extended Data Fig. 5 | Overexpression of ZIKV NS3 protease induced osteogenic gene expression in osteoblastic-like cells. U2OS cells (n = 6) were transiently transfected with plasmids encoding individual ZIKV genes for 3 days. Transfected cells were harvested for qRT-PCR analysis of osteogenic gene expressions. Gene expressions were normalized to GAPDH and expressed as fold changes relative to vector control. Data are presented as mean ± SEM in box plots showing the upper (75%) and lower (25%) quartiles, with the horizontal line as the median and the whiskers as the maximum and minimum values observed. Data are representative of three independent experiments.
Extended Data Fig. 6 | See next page for caption.
Extended Data Fig. 6 | ZIKV NS3 protease is highly conserved across different lineages. a, Sequence alignment comparison between BMP2 gene in human (homo sapiens; amino acid residues 1-396) and hamster (Cricetulus; amino acid residues 1-399). b, Purity of BL21 strain-derived recombinant ZIKV NS3 protease, CHO-FD11 cells-derived full-length BMP2 and BMP4 were determined by coomassie blue stain. MW: molecular weight. c, Sequence alignment comparison between human BMP2 (amino acid residues 1-396) and BMP4 (amino acid residues 1-408). d, Sequence alignment of NS3 protease domain (amino acid residues 1-177) was compared across two African ZIKV (MR766, IbH30656) and two Asian ZIKV (PRVABC59 and H/PF/2103). The red-colored letters indicate protease catalytic triad. e, In vitro cleavage assay of carboxyl terminal HA-tagged purified human BMP4 and purified ZIKV NS3 protease were performed at 37 °C for 3 h, followed by immunoblot analysis. Data in b and e are representative of three independent repeats.
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For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

| n/a | Confirmed |
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| ☐ X The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement |
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| ☐ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons |
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| ☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings |
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| ☐ Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated |

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

**Software and code**

Policy information about availability of computer code

**Data collection**

All flow cytometry data were acquired using BD FACSDIVA v6.0 software (BD Biosciences). All immunoblotting data were imaged using Chemidoc chemiluminescence system (Bio-Rad). All histology data were imaged using BZ-X710 microscope (Keyence). All qRT-PCR data were acquired using CFX Manager 3.1 software (Bio-Rad).

**Data analysis**

Microsoft Office Excel 365, FlowJo v10.0 (Treestar), Graphpad Prism v7.0 and 8.4, SnapGene 5.2.3, Image Lab 5.2.1

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

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Life sciences study design

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

Sample size
Sample sizes were determined following best practices in the field and authors’ experience. Sufficient experimental replicates and independent experiments were performed to ensure that results are reliable and reproducible with the sample numbers used in this study.

Data exclusions
No data were excluded.

Replication
Each experiment was repeated at least twice to ensure reproducibility of findings. Results were consistent in all independent experiments.

Randomization
All data were analyzed, no randomization is needed.

Blinding
Blinding is not relevant for this study as all data were analyzed.

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

Materials & experimental systems

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

Methods

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

Antibodies

Antibodies used

| Antibodies                  |
|-----------------------------|
| 1) anti-human PDGFRβ (clone 28E1, Cell Signaling Technology, 3169S) |
| 2) anti-GFAP (clone G5, Cell Signaling Technology, 3670S) |
| 3) anti-NeuN (clone N/A, Millipore, ABN78) |
| 4) anti-CD31-AF488 (clone WM59, BioLegend, 303110) |
| 5) anti-CD146-AF647 (clone P1H12, BioLegend, 361014) |
| 6) anti-BMP2 (clone EPR20807, Abcam, ab214821) |
| 7) anti-phospho-SMAD1 (Ser463/465)/ SMAD5 (Ser463/465)/ SMAD9 (Ser465/467) (clone D5B10, Cell Signaling, 13820S) |
| 8) anti- SMAD1 (clone N/A, Cell Signaling, 97435) |
| 9) anti-ZIKV NS3 (GeneTex, GTX133309) |
| 10) anti-Flag (clone N/A, Sigma, F7425) |
| 11) anti-HA (clone 16812, BioLegend, 901503) |
| 12) anti-actin (clone C4, Santa Cruz, sc-47778) |
| 13) anti-rabbit IgG, HRP-conjugated (Cell Signaling Technology, 70745) |
| 14) anti-mouse IgG, HRP-conjugated (Cell Signaling Technology, 7076V) |
| 16) Mouse IgG1 Isotype control (clone 11711, R&D Systems, MAB002) |
| 17) Mouse anti-Human BMP2/4 (clone 100230, R&D Systems, MAB3552100) |

Validation

| Antibodies                  |
|-----------------------------|
| 1) anti-human PDGFRβ: PDGF Receptor β (28E1) Rabbit mAb detects endogenous levels of PDGF receptor β protein. The antibody may cross-react with PDGF receptor α when highly overexpressed. Species cross-reactivity is determined by western blot. Recommended Antibody Dilutions: Western blotting 1:1000, Immunoprecipitation 1:50, Immunohistochemistry (Paraffin) 1:100. Antibody has been validated commercially and associated with 181 citations. |
| 2) Mouse anti-GFAP: GFAP (G5) Mouse mAb detects endogenous levels of total GFAP protein. Species Reactivity against Human, Mouse, Rat. Species cross-reactivity is determined by western blot. Application Dilutions for Western Blotting 1:1000, |
Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)  HEK293T, U2OS, U251, SK-N-SH, Vero E6 cells were purchased from ATCC and CHO FD11 cells were kindly provided by Dr. Stephen Leppla (NIAID, NIH). Primary fetal brain vascular pericytes and fetal astrocytes were purchased from ScienCell Research laboratories.

Authentication  Cell line authentication was performed by morphology check and growth curve analysis.

Mycoplasma contamination  Cell lines were tested negative for mycoplasma contamination, validated by Hoechst staining.

Commonly misidentified lines  No commonly misidentified lines were used in this study.

(See ICLAC register)

Animals and other organisms

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

Laboratory animals  Immunocompetent human STAT2 Ki mice. 21-day-old male and female mice, of equal sex distribution and 6 to 8 weeks old pregnant female dams were used in this study. Animal studies were carried out in animal facility managed and maintained by the University of California (USC) Animal Resource Center. Facilities were maintained at an acceptable range of 68–79°F at a humidity of 30–70% on a 12h dark/12h light cycle. All procedures used in this study complied with federal and institutional guidelines enforced by the USC Institutional Animal Care and Use Committee (IACUC) and were granted institutional approval after veterinary and committee review.

Wild animals  No wild animals were used in this study.

Field-collected samples  No field-collected samples were used in this study.

Ethics oversight  University of Southern California (USC) IACUC
Note that full information on the approval of the study protocol must also be provided in the manuscript.

Flow Cytometry

Plots

Confirm that:
- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation

Fixable live/dead staining was performed according to manufacturer’s procedures. Briefly, prior to reconstitution of live/dead stain, spin down the vial of lyophilized reagent in a microcentrifuge to ensure the reagent is at the bottom of the vial. Wash cells with PBS buffer (no Tris buffer and protein free). Dilute Zombie AquaTM dye at 1:100-1000 in PBS. Resuspend 1-10 x 10^6 cells in diluted 100 μl Zombie AquaTM solution. To minimize background staining of live cells, titrate the amount of dye and/or number of cells per 100 μl for optimal performance. Different cell types can have a wide degree of variability in staining based on cell size and degree of cell death. Incubate the cells at room temperature, in the dark, for 15-30 minutes. Wash one time with 2 ml BioLegend’s Cell Staining Buffer (Cat. No. 420201) or equivalent buffer containing serum or BSA. Continue performing antibody staining procedure as desired. Cells can be fixed with paraformaldehyde or methanol prior to permeabilization or can be analyzed without fixation.

Instrument

FACS acquisitions was performed on BD FACSCanto II (BD Biosciences).

Software

FACS acquisitions was performed on BD FACSCanto II (BD Biosciences), using BD FACSDIVA software. All FACS data were analyzed using FlowJo software.

Cell population abundance

Post-sorted sample of CD146+/CD31-/CD45- pericytes was kindly provided by Dr. Denis Evseenko (USC) as described previously (Corselli, M. et al. Perivascular support of human hematopoietic stem/progenitor cells. Blood 121, 2891-2901, doi:10.1182/ blood-2012-08-451864 (2013)). The purity was determined using FACS acquisitions on BD FACSCanto II (BD Biosciences) with anti-CD31 and CD146 antibodies, and analyzed using BD FACSDIVA software. The purity of sorted cells were >90%.

Gating strategy

The preliminary gating for the starting population used forward scatter (FSC) and side scatter (SSC) parameters. Singlets were then gated using FSC-Height by FSC-Area. Live/dead population was gated using fixable Zombie Aqua dye vs SSC-Area. Unstained and secondary controls were used to determine negative boundaries. Single stained controls were used to determine positive boundaries.

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