Viral and metazoan poxins are cGAMP-specific nucleases that restrict cGAS–STING signalling

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Cytosolic DNA triggers innate immune responses through the activation of cyclic GMP–AMP synthase (cGAS) and production of the cyclic dinucleotide second messenger 2′,3′-cyclic GMP–AMP (cGAMP)1–4. 2′,3′-cGAMP is a potent inducer of immune signalling; however, no intracellular nucleases are known to cleave 2′,3′-cGAMP and prevent the activation of the receptor stimulator of interferon genes (STING)5–7. Here we develop a biochemical screen to analyse 24 mammalian viruses, and identify poxvirus immune nucleases (poxins) as a family of 2′,3′-cGAMP-degrading enzymes. Poxins cleave 2′,3′-cGAMP to restrict STING-dependent signalling and deletion of the poxin gene (B2R) attenuates vaccinia virus replication in vivo. Crystal structures of vaccinia virus poxin in pre- and post-reactive states define the mechanism of selective 2′,3′-cGAMP degradation through metal-independent cleavage of the 3′–5′ bond, converting 2′,3′-cGAMP into linear Gp[2′-5′]Ap[3′]7. Poxins are conserved in mammalian poxviruses. In addition, we identify functional poxin homologues in the genomes of moths and butterflies and the baculoviruses that infect these insects. Baculovirus and insect host poxin homologues retain selective 2′,3′-cGAMP degradation activity, suggesting an ancient role for poxins in cGAS–STING regulation. Our results define poxins as a family of 2′,3′-cGAMP-specific nucleases and demonstrate a mechanism for how viruses evade innate immunity.

The enzyme cGAS recognizes cytosolic DNA and synthesizes 2′,3′-cGAMP to activate STING-dependent interferon and NF-κB immune responses2. 2′,3′-cGAMP is highly stable in the cytosol of human cells, it activates STING at low nanomolar concentrations and can spread through cellular gap junctions to activate immune responses in adjacent cells8,9. The extracellular ATP-metabolizing enzyme ENPP1 is capable of degrading 2′,3′-cGAMP4,6, but no cytosolic nucleases have been identified that are able to selectively target 2′,3′-cGAMP and restrict cGAS–STING signalling. Using cytosolic extracts from human monocytes and kidney cells, we confirmed that 2′,3′-cGAMP is highly stable with no degradation detected after incubation for 20 h (Extended Data Fig. 1a). 2′,3′-cGAMP activates STING to initiate antiviral responses and can be packaged into viral particles during egress8,9. We therefore hypothesized that strong selective pressure would exist for viruses to specifically destabilize 2′,3′-cGAMP and prevent induction and spread of cGAS–STING immune responses. To test for virus-induced degradation of 2′,3′-cGAMP, we designed a biochemical screen and analysed 24 different mammalian viruses representing 13 viral families (Fig. 1a, b and Supplementary Table 1). Radiolabelled 2′,3′-cGAMP was completely degraded when exposed to lysate from cells infected with vaccinia virus (VACV), a member of the family Poxviridae (Fig. 1b). VACV-infected cell lysates rapidly convert 2′,3′-cGAMP into an intermediate product that is then resolved into a second, faster-migrating species on thin-layer chromatography (Fig. 1c). The activity of 2′,3′-cGAMP degradation was associated with VACV infection rather than host cell tissue or species origin (Extended Data Fig. 1b, c), suggesting that this activity is derived from a viral product. We therefore named the viral factor responsible for 2′,3′-cGAMP degradation poxvirus immune nuclease or poxin.

Poxviruses are large DNA viruses that replicate exclusively in the cytoplasm of infected cells and encode factors dedicated to evasion of host immune signalling10. To identify the VACV poxin-encoding gene, we used activity-guided fractionation to specifically enrich poxin from infected cell lysates (Fig. 1d and Extended Data Fig. 2). Mass-spectrometry analysis of fractions enriched using two independent purification methods revealed that only the protein product of the VACV gene B2R (NCBI YP_233066.1) was highly enriched (Fig. 1e). Recombinant protein robustly degraded 2′,3′-cGAMP in vitro (Fig. 1f and Extended Data Fig. 3), using the same two-step reaction and broad pH optimum observed with VACV-infected cell lysates, confirming that the VACV B2R gene encodes poxin.

The activity of VACV poxin is specific for 2′,3′-cGAMP; no degradation was observed using chemically related 3′–5′-linked cyclic dinucleotides (Fig. 2a). This specificity suggests that poxin is specialized for evasion of DNA sensing by cGAS–STING through degradation of the second messenger 2′,3′-cGAMP. Consistent with this hypothesis, poxin cleavage renders 2′,3′-cGAMP unrecognizable to the receptor STING (Fig. 2b) and expression of VACV poxin in human cells was sufficient to restrict cGAS-dependent STING activation (Fig. 2c and Extended Data Fig. 4a, b). We constructed a mutant virus in which poxin was deleted (VACV ΔPoxin) and confirmed that poxin is necessary for infected cell lysates to degrade 2′,3′-cGAMP (Extended Data Fig. 4g). cGAS has previously been shown to be a critical factor for restriction of poxvirus replication11–14. To assess the effect of poxin deletion on VACV fitness in vivo, we next infected mice by scarification and measured viral loads in the resulting skin lesions. Although poxin is dispensable for replication in interferon-deficient cells (Extended Data Fig. 4h), VACV ΔPoxin replication in mice is more than 40-fold attenuated compared to wild-type VACV (Fig. 2d), demonstrating the importance of poxin function in vivo. No increase in interferon-β (IFNβ) signalling was observed during VACV ΔPoxin infection in an interferon-competent cell culture model of replication (Extended Data Fig. 4i), indicating that the key function of poxin in vivo may relate to spread of 2′,3′-cGAMP rather than prevention of interferon signalling in the primary infected cell. Together, these data describe a mechanism in which poxin-mediated cleavage of 2′,3′-cGAMP allows viral evasion of cGAS–STING immunity.

To define the molecular mechanism of poxin activity, we determined a series of X-ray crystal structures of VACV poxin that correspond to the apo (1.7 Å), pre-reactive (2.6 Å) and post-reactive (2.1 Å) states of 2′,3′-cGAMP rather than prevention of interferon signalling in the primary infected cell. Together, these data describe a mechanism in which poxin-mediated cleavage of 2′,3′-cGAMP allows viral evasion of cGAS–STING immunity.

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of poxin that were grown in the presence of 2′,3′-cGAMP yielded a post-reactive structure and clear density for the final product Gp\(^{3′-5′}\) (Extended Data Fig. 6). Notably, metal ions were not observed in these structures and poxin activity is resistant to metal chelation (Extended Data Fig. 7c). Once bound to poxin, the adenine base in 2′,3′-cGAMP is rotated 60° compared to the in-solution or STING-bound conformation, resulting in a distorted 2′,3′-cGAMP conformation that repositions the 2′-OH to serve as the reactive nucleophile (Fig. 3c). VACV poxin residues H17, Y138 and K142 are located proximal to the scissile 3′–5′ bond, and positioning in the active site suggests that H17 and K142 function as the general acid and base of the catalytic mechanism. Analyses of poxin mutants indicated that the His17 and Lys142 residues are required for catalysis. H17A was inactive, whereas H17N and the double mutant H17N/K142R were partially active, suggesting that Arg142 is involved in general base catalysis and水电解作用.

Fig. 2 | VACV poxin is critical for evasion of cGAS-STING immunity. a, VACV poxin is specific for 2′,3′-cGAMP and fails to degrade 3′,3′-cGAMP or other natural cyclic dinucleotide (CDN) species. b, Human (h)STING electrophoretic mobility shift assay showing that poxin degradation of 2′,3′-cGAMP blocks the interaction with STING that is required for downstream immune activation. a, b, The ‘−’ refers to a buffer-only control. c, Cells that express VACV poxin have a reduced response to cGAS-dependent activation of an interferon-β (IFNβ) reporter compared with cells that express a catalytically inactive poxin mutant (poxin(H17A), see Methods). Data are mean ± s.e.m. of three technical replicates and are representative of three independent experiments. d, Mice were infected via scarification with wild-type (WT) VACV or a poxin knockout strain (ΔPoxin). Clearance of VACV ΔPoxin in the skin is increased more than 40-fold compared to wild-type VACV. Two-tailed Student’s t-test, \( *P = 4.01 \times 10^{-8} \). Black dots represent individual mice and grey bars indicate the mean values of each group (n = 15). All data are representative of at least two independent experiments.
In summary, these data reveal the mechanism by which poxin, a metal-independent nuclease, cleaves the canonical bond of 2′,3′-cGAMP to inhibit cGAS–STING signalling.

VACV poxin does not exhibit any detectable structural homology with previously described nuclease or phosphodiesterase enzymes. Poxin is conserved in most viruses in the genus Orthopoxvirus, including the human pathogens monkeypox virus and cowpox virus (Fig. 4a and Extended Data Figs. 8a, 9a), and is sometimes fused to an additional C-terminal domain previously noted to have homology with human schlafen proteins21. We cloned four divergent poxin genes from members of the Poxviridae and each retained specific recognition of 2′,3′-cGAMP and hydrolysis activity (Fig. 4b and Extended Data Fig. 9c). We next used the structure of VACV poxin and conservation of active-site residues to guide a bioinformatics search for divergent poxin family members outside of the Poxviridae. Notably, this search revealed poxin homologues in the Alphabaculovirus genus of insect DNA viruses (p26 proteins, for example, AcNPV p26 (NCBI NP_054166.1)) and poxin genes in insects of the order Lepidoptera, which serve as exclusive hosts of alphabaculoviruses (HDD13 proteins, for example, Bombyx mori HDD13 (NCBI XP_021205460.1)) (Fig. 4c). Insect cellular and viral poxin homologues are enzymatically active, retain specificity for 2′,3′-cGAMP degradation, and insect host poxin activity can be detected in lysates of the insect cell lines S21 and Hi5 (Fig. 4d, e and Extended Data Fig. 9c). In support of a conserved role of insect poxins in immune regulation, the STING signalling pathway is functional in insects18,22,23 and the lepidopteran poxin gene is induced upon pathogen infection24. Poxviruses and baculoviruses can share overlapping host tropisms and readily acquire genes through homologous recombination25,26, potentially explaining how poxins evolved and spread between insects, insect viruses and mammalian pathogens (Fig. 4f).

Through discovery and characterization of a 2′,3′-cGAMP-specific nuclease in VACV-infected cell lysates, our data reveal a potent viral strategy for cGAS–STING pathway evasion. Poxin activity provides a mechanistic explanation for recent findings that have shown that virulent poxviruses inhibit cGAS–STING signalling during infection at a step downstream of cGAS activation13. Two notable exceptions to conservation of poxin activity are Variola major virus, the causative
agent of smallpox disease, and the modified vaccinia Ankara (MVA) vaccine strain, which both show inactivation of poxin (Fig. 4a and Extended Data Fig. 8b). Inactivation of the poxin gene in Variola major virus further suggests that multiple strategies may exist in poxviruses to subvert cGAS–STING immunity.27 Poxvirus vectors are widely used in human and animal vaccination, gene therapy and as cancer oncolytics.28,29 Loss of poxin resulted in significant attenuation of VACV in a mouse model of replication, indicating that modulation of cGAS–STING signalling through poxin deletion may be an important consideration in the design of poxvirus-based vaccines and therapeutics. The ability of 2′,3′-cGAMP to cross cellular gap junctions5 and infiltrate budding viral particles8,9 provides the host an opportunity to counteract pathogen factors that inhibit cGAS–STING activation but that do not completely eliminates all 2′,3′-cGAMP production.30 Through direct targeting of 2′,3′-cGAMP, poxin activity is a viral adaptation to prevent both activation and cellular spread of STING signalling. Conservation of poxins between mammalian viruses and insects reaffirms the ancestral roots of 2′,3′-cGAMP-dependent signalling in animals18, and underscores the wide range of host–virus conflicts that drive new mechanisms of innate immune surveillance and evasion.

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METHODS

Cell lines and viruses. A549, BSC-40, BSR-T7 and Vero cells, as well as VACV (strain western reserve vTF7-3) stocks were gifts from S. Whelan (Harvard Medical School). THP-1 cells and HEK293T cells were purchased from ATCC. All cell lines were maintained in Dulbecco’s modified Eagle medium (DMEM) (VWR) supplemented with 10% fetal bovine serum (FBS) (VWR, Seradigm), with the exception of THP-1 cells, which were maintained in RPMI-1640 medium (Life Technologies) supplemented with 10% FBS and 50 mM 3-mercaptoproethanol. Cell lines used were not authenticated and were not tested for mycoplasma contamination. Infections with VACV were performed for 1 h in 1× PBS supplemented with 1 mM CaCl₂ and 1 mM MgCl₂. After 1 h, excess virus was aspirated, and cells were either collected (at 1 h after infection) or the infection was allowed to continue in DMEM supplemented with 2.5% FBS and 50 mM HEPES-NaOH pH 7.5 until collection at stated time points. Infected cells were washed once in 1× PBS, scraped into 1× PBS, collected by centrifugation and lysed in 1% NP-40, 20 mM HEPES-NaOH pH 7.5, 150 mM NaCl, 1 mM TCEP-KOH to prepare virus-infected cell lysates. Infections with other viruses were performed according to details in Supplementary Table 1. S21 and HeLa H5 rice cell were infected with T. Ross and S. Harrison (Harvard Medical School). Spinner cultures of insect cells were maintained in TC100 medium (Gibco) supplemented with 10% FBS, and cells were collected by centrifugation and lysed with the same lysis buffer as above. VACV was propagated in Vero cells and plaque assays were performed in Vero cells according to published protocols.

Synthesis of cyclic dinucleotides. 2′,3′-cGAMP for downstream degradation assays was produced using recombinant mouse cGAS. Recombinant human SUMO2-tagged mouse cGAS was purified from E. coli using Ni-NTA affinity, digested with human SENP2 protease and further purified with heparin ion-exchange and 75% size-exclusion chromatography as previously described. Mouse cGAS (14 μM) was incubated for 2 h at 37°C with either 200 μM ATP and 200 μM GTP (high substrate) or 25 μM each (low substrate) in the presence of 1 μM 45-bp stimulatory dsDNA in 20 μl buffer composed of 50 mM HEPES-KOH pH 7.5, 5 mM Mg(OAc)₂, 60 mM KCl and 1 mM DTT. Reactions were trace labelled with [α-32P]GTP (Perkin Elmer), except for experiments in Extended Data Fig. 7a, for which the indicated reactions were labelled with [α-32P]ATP. After completion, reactions were treated with Antarctic Phosphatase (NEB) for 20 min to digest the remaining labelled nucleoside triphosphates and heat-inactivated at 65°C for 20 min before direct use in downstream poxin activity assays. 3′-5′-linked cyclic dinucleotides were enzymatically synthesized in a similar manner using recombinant Vibrio cholerae DncV incubated with either ATP and GTP (3′,5′-cGAMP synthesis), GTP alone (c-di-GMP synthesis), or ATP alone (c-di-AMP synthesis) as previously described. All 3′-5′-linked cyclic dinucleotides were prepared with 200 μM of each required nucleoside triphosphate, except for experiments in Extended Data Fig. 9c, for which synthesis was performed with 25 μM of each nucleoside triphosphate.

2′,3′-cGAMP used for crystallography was enzymatically synthesized by 24 h incubation at 37°C of 100 μM recombinant mouse cGAS with 500 μM each ATP and GTP in the presence of 50 μg ml⁻¹ salmon sperm DNA in 500 μl buffer composed of 10 mM Tris-HCl pH 7.5, 12.5 mM KCl, 10 mM MgCl₂ and 1 mM DTT. Reactions were trace labelled with [α-32P]GTP (Perkin Elmer), except for experiments in Extended Data Fig. 7a, for which the indicated reactions were labelled with [α-32P]ATP. After completion, reactions were treated with Antarctic Phosphatase (NEB) for 20 min to digest the remaining labelled nucleoside triphosphates and heat-inactivated at 65°C for 20 min before direct use in downstream poxin activity assays. 3′-5′-linked cyclic dinucleotides were enzymatically synthesized in a similar manner using recombinant Vibrio cholerae DncV incubated with either ATP and GTP (3′,5′-cGAMP synthesis), GTP alone (c-di-GMP synthesis), or ATP alone (c-di-AMP synthesis) as previously described. All 3′-5′-linked cyclic dinucleotides were prepared with 200 μM of each required nucleoside triphosphate, except for experiments in Extended Data Fig. 9c, for which synthesis was performed with 25 μM of each nucleoside triphosphate.

Poxin biochemical fractionation and identification. Poxin was enriched and identified directly from infected cell lysates. In brief, 16 T150 flasks of BSC-40 cells were infected at an MOI of 5. After 6 h, cells were scraped into 1× PBS, collected by centrifugation and stored at –80°C. The resulting infected material was split into two portions, lysed and fractionated with either IEX- or a HIC-based purification methods. Cells were lysed in 1% NP-40, 20 mM HEPES-KOH pH 7.5, 150 mM NaCl, 1 mM DTT and cytosolic lysates were collected from the supernatant fractions following centrifugation at 22,000g for 10 min. Resulting cytoplasmic extracts were fractionated by IEX using a 1-ml HiTrap Q HP column and a gradient from 0.05 to 1 M NaCl. Fractions with poxin activity were pooled and further purified on a 10/300 5200 size-exclusion column (GE). Alternatively, cytoplasmic extracts were subjected to a 30% (NH₄)₂SO₄ cut at 4°C for 1 h with gentle spinning, and centrifuged at 50,000g for 15 min. Clarified supernatant was loaded on a 1-ml HiTrap Phenyl HIC column (GE), and eluted using a gradient of 1.2–0 M (NH₄)₂SO₄ in a buffer containing constant 150 mM NaCl. Enriched fractions with poxin activity were then further fractionated on a 10/300 755 size exclusion column (GE). After each purification step, 1 μl of each fraction was tested for poxin activity as described above, and active fractions were pooled for additional purification. After IEX and size exclusion, the single fraction with peak activity and inactive background fractions that eluted before and after poxin activity was collected by mass spectrometry. Additionally, a single active fraction was selected for peak activity for HIC and size exclusion. Mass spectrometry protein analysis and label-free quantification of the IEX samples were performed at the Harvard Medical School Taplin Facility.

Recombinant protein purification. The VACV poxin gene B2R was PCR-amplified from purified viral DNA and cloned into a custom pET vector designed to express a 6× His-tagged N-terminal human SUMO2 fusion. Poxin homologues were cloned using synthetic DNA fragments (IDT) and HiFi Gibson assembly (NEB). Recombinant poxin proteins were expressed in E. coli BL21 RIL bacteria (Agilent) using 50 μl starter cultures grown in MDG medium and two 1-litre induction cultures grown in M9ZB medium as previously described. Alternatively, MPXV poxin starter and induction cultures were grown with 2YT medium as previously described. VACV poxin with a selenomethionine (SeMet) substitution was grown in overnight MDG starter cultures, and in two 1-litre induction cultures of altered M9ZB medium containing 40% glucose as a sole carbon source, supplemented with 1 μg ml⁻¹ thiamine. SeMet M9ZB cultures were grown to an optical density at 600 nm (OD₆₀₀nm) of around 0.6 before addition of the following amino acids for SeMet labelling and suppression of methionine biosynthesis: 200 μM and 10 μM of acetylserine and 20 μM of α-selenoalanine. Lysines and threonine (VWR) 75 μg ml⁻¹ selenomethionine (Acros Organics). Cultures were allowed to grow for an additional 20 min at 37°C with shaking at 230 r.p.m. and were then transferred to an ice bath for 20 min. Cultures were then supplemented with 0.5 mM IPTG and incubated at 16°C with shaking at 230 r.p.m. for overnight growth. Cultured cells were disrupted by sonication in lysis buffer (20 mM HEPES-KOH pH 7.5, 400 mM NaCl, 30 mM imidazole, 1% glycerol and 1 mM DTT), and poxin proteins were purified from clarified lysates at 4°C using Ni-NTA resin (Qiagen) and gravity chromatography. Ni-NTA resin was washed with lysis buffer adjusted to a higher 1 M NaCl concentration and eluted with lysis buffer adjusted to a higher 300 mM imidazole concentration. The elution fraction was supplemented with approximately 250 μg of human SENP2 protease (fragment D364–L589 with M497A mutation) and dialysed overnight at 4°C in dialysis buffer (20 mM HEPES-KOH pH 7.5, 300 mM NaCl and 1 mM DTT). Untagged poxin was further purified using 16/600 755 size-exclusion chromatography (GE) in 20 mM HEPES-KOH pH 7.5, 250 mM KCl and 1 mM TCEP-KOH. Final protein was concentrated to around 10–35 mg ml⁻¹, flash-frozen in liquid nitrogen and stored at −80°C for crystallography. Alternatively, proteins for biochemistry analysis were supplemented with 10% glycerol before −80°C storage. Poxin mutants tested in Fig. 3 and poxin homologues tested in Extended Data Fig. 4 and Extended Data Fig. 9 were purified as SUMO2 fusions, and dialysed directly into storage buffer without SENP2 digestion.

STING electrophoretic mobility shift assay. Human STING–cyclic dinucleotide electrophoretic mobility shift assays were performed as previously described.

In brief, around 500 nM of 2′,3′-cGAMP or poxin-treated 2′,3′-cGAMP was incubated in a 10–μl reaction with around 20 μM human STING N154–V341 (wild-type R232) in 1× reaction buffer (50 mM Tris-HCl pH 7.5, 5 mM MgCl₂, 1 mM TCEP-KOH) for 30 min at 25°C. Reactions were supplemented with loading dye (6×: 40% sacrose and 0.05% xylene cyanol) and separated on a 6% non-denaturing polyacrylamide gel (7.2 cm tall, prepared with 29:1 acrylamide:bis-acrylamide and 0.5× TBE buffer) run at 100 V
for 45 min with 0.5 x TBE running buffer. The gel was incubated in fixing reagent (10% acetic acid and 40% methanol) for 30 min and then dried at 80 °C before exposure to a phosphor screen and imaging on a Typhoon phosphor-imager (GE).

**Poxin stable cell lines and cell-based IFN-β reporter assays.** Haemagglutinin-tagged wild-type VACV poxin and a catalytically inactive VACV poxin mutant H17A were cloned into the lentiviral transduction vector pRN3. Lentivirus was prepared as previously described and lentivirally transduced HEK293T cells were selected with 1 μg/ml puromycin. Transduced cells were grown in 10% FBS DMEM without selection and DNA was purified with the DNeasy Mini kit (Qiagen) according to the manufacturer’s protocol. Luciferase assays were performed as previously described. In brief, HEK293T cells expressing poxin(WT) or poxin(H17A) were transfected with Lipofectamine 2000 in 96-well plates with: a control pRL plasmid encoding Renilla luciferase under a constitutive promoter (2 ng), a pIFN3 Fluc reporter plasmid encoding firefly luciferase under the IFN-β promoter (20 ng), a pCDNA4 plasmid encoding human STING wild-type allele R232 (10 ng), and either an empty vector or a pCDNA4 plasmid encoding full length human cGAS (1 ng). Luciferase activity was quantified after 18 h and firefly luciferase activity was normalized to the Renilla luciferase control. Fold reporter induction was calculated by dividing the luciferase activity observed with human cGAS by background activity observed with the empty vector transfection.

**Selection and validation of poxin knockout virus.** Selection of poxin knockout virus was performed as previously described. A cassette encoding the super-folding GFP (sfGFP) gene39 flanked by 250-bp homology arms identical to the VACV genome upstream and downstream of B2R was cloned into a pCDNA4 vector. The cassette was amplified by PCR to create linear homologous recombination template DNA. BSR-T7 cells in six-well dishes were infected at MOI 0.05 with VACV (strain Western Reserve)Δ-Poxin. The virus was collected and DNA was amplified and replaced with DMEM supplemented with 2.5% FBS, before immediate transfection with 2 μg of linear homologous recombination template. After 48 h, cells were scrapped into medium, collected by centrifugation, resuspended in DMEM with 2.5% FBS and lysed by three freeze–thaw cycles, vortexing and sonication. The resulting stock was plaque-puriﬁed on Vero cells by cocryogen sfGFP-positive plaques and transferring them into DMEM with 2.5% FBS. VACV ΔPoxin virus was puriﬁed through three iterative rounds of sfGFP-positive plaque puriﬁcation, before viral stocks were prepared for use in downstream experiments. Viral stocks were used as PCR template as previously described and, ampliﬁcations encompassing the entire poxin B2R locus extending through homologus recombination junctions were veriﬁed by Sanger sequencing (Extended Data Fig. 4e).

**Measurement of virus interferon induction by qRT-PCR.** A549 cells were infected at an MOI of 5 with wild-type VACV or VACV ΔPoxin and lysed 5 h after infection. As a positive control, cells were permeabilized for 30 min with digitonin buffer (10 μg/ml digitonin, 50 mM HEPES-KOH pH 7.5, 100 mM KCl, 3 mM MgCl2, 0.1 mM DTT, 85 mM sucrose, 0.2% BSA, 1 mM ATP and 0.1 mM GTP) and stimulated with or without 25 μM 2′,3′-cGAMP as previously described14,15. The cells were lysed directly in DMEM supplemented with 10% FBS, the medium was replaced and cells were incubated for 5 h before lysis. Infected or stimulated cells were lysed directly in TRIZol reagen (Life Technologies) and RNA was extracted according to the manufacturer’s instructions. Reverse transcription was carried out in a buffer composed of 25 mM Tris-HCl pH 8.4, 37.5 mM KCl and 1.5 mM MgCl2 using MMLV-MS reverse transcriptase43. qPCR was performed using LUNA qPCR Master Mix (Applied Biosystems), 500 nM forward primer, 500 nM reverse primer, 150 nM probe and the template DNA. Thermal cycling conditions were 95 °C for 2 min and 95 °C for 10 min for one cycle, followed by 45 cycles of amplification (94 °C for 15 s and 60 °C for 1 min). To calculate the viral load, a standard curve was established from DNA of a VACV stock with previously determined titre. Corresponding Ct values obtained by the qPCR method were plotted on the standard curve to estimate viral load in the skin samples. Comparisons for two groups were calculated using two-tailed Student’s t-test. Blinding was not performed during data collection or analysis, as results were based on quantitative assessment of viral load.

**Crystallization and structure determination.** VACV poxin crystals were grown by hanging-drop vapour diffusion at 18 °C. SeMet-labelled VACV poxin was crystallized in apo form and native VACV poxin crystals were grown in complex with the phosphorothioate non-hydrolysable analogue of 2′,3′-cGAMP (c[G(2′,5′)pS]-R-(3′,5′)pS(S), stereoisomer 1) (Biolog) yielding a pre-reactive structure or in complex with native 2′,3′-cGAMP, yielding a post-reactive structure. SeMet-substituted apo VACV poxin crystals grew in 100 mM HEPES-NaOH pH 7.0, 20–22% PEG-2000 MME and cryoprotected by the addition of 20% ethylene glycol. Native VACV poxin crystals were grown with 300 μM non-hydrolysable 2′,3′-cGAMP or with 3.3 μg ml−1 purified 2′,3′-cGAMP in 100 mM NaOAc pH 4.8, 10–11% PEG-2000 and cryoprotected by the addition of 30% ethylene glycol. X-ray diffraction data were collected at the Advanced Light Source (beamline 8.2.1) and the Advanced Photon Source (beamline 24-ID-C).

X-ray crystallography data were processed with XDS and AIMLESS45 using the SSRL autoxds script (A. Gonzalez, Stanford SRRl). Experimental phase information for VACV poxin was determined using data collected from SeMet-substituted crystals. In total, 35 sites were identiﬁed with Hgs8 in PHENIX and an initial map was produced using SOLVE/RESOLVE and model building was performed using Coot before reﬁnement in PHENIX. The 1.3 A SeMet apo VACV poxin structure was subsequently used as a molecular replacement search model for determination of pre-reactive and post-reactive native VACV poxin structures. Final structures were reﬁned to stereochemistry statistics for Ramachandran plot (favoured/allowed), rotamer outliers and MolProbity score as follows: VACV poxin apo, 97.65%/2.35%, 0.15% and 1.23; VACV poxin pre-reactive state, 96.14%/3.7%, 1.15% and 1.58; VACV poxin post-reactive state 97.93%/2.07%, 0.72% and 1.15.

**Bioinformatics and poxin homologue identiﬁcation.** Position-speciﬁc iterative BLAST (PSI-BLAST) search was used to identify homologues of VACV poxin, using VACV poxin as the query sequence. Using the blastings (Brb BLAST) program, 11 extension (E = 1, conditional compositional score matrix adjustment). All signiﬁcant (E value less than 0.05) sequences that contained the known VACV poxin active site and key structural residues were selected after each PSI-BLAST round (up to 500) for reﬁnement of the substitution matrix in each additional PSI-BLAST round. After one single PSI-BLAST round, poxvirus sequences were identiﬁed. Reﬁnement of the substitution matrix by a further round (2 total rounds) allowed identiﬁcation of baculovirus p26 proteins, and a third round identiﬁed lepidopteran host HD13 proteins as homologues of VACV poxin. Using alignments to VACV poxin and the crystal structure, full-length homologue sequences possessing putative catalytic residues were selected for recombiant production and biochemical poxin activity assays.

**Statistics and reproducibility.** Most biochemistry experiments are representative of at least three independent experiments, with select experiments representative of two independent experiments: virus lysate screen (Fig. 1b), poxin fractionation and mass-spectrometry analysis (Fig. 1d, e), STING gel-shift interactions (Fig. 2b), PCR validation of VACV poxin qRT–PCR experiments (Extended Data Fig. 4d), VACV ΔPoxin growth curves (Extended Data Fig. 4h) and VACV ΔPoxin interferon qRT–PCR experiments (Extended Data Fig. 4i). Mice scarification experiments were performed with a sample size previously determined to ensure sufﬁcient statistical power. All other experimental replicate details are listed in the extended data ﬁgure legend.

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

**Data Availability**

Coordinates and structure factors of the VACV poxin apo and poxin–2′,3′-cGAMP complexes have been deposited in Protein Data Bank (PDB) under accession codes 6E6A, 6E8A and 6E9A. All other data are available in the manuscript or the supplementary materials.
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Extended Data Fig. 1 | VACV-induced 2′,3′-cGAMP degradation is cell-line and tissue-type independent. 

**a.** TLC analysis of the stability of 2′,3′-cGAMP (the 3′–5′ bond is radiolabelled) following incubation in human monocyte (THP-1) or kidney (HEK293T) cytosolic lysates. 2′,3′-cGAMP is highly stable with no degradation detected after >20 h of incubation. 

**b.** TLC analysis of VACV-induced 2′,3′-cGAMP degradation. Lysates were prepared from African green monkey (*Chlorocebus aethiops* (Vero)) and golden hamster (*Mesocricetus auratus* (BSR-T7)) cells. These lysates exhibit 2′,3′-cGAMP degradation activity after infection with VACV, but not after mock infection (M). 

**c.** Time-course analysis of 2′,3′-cGAMP-degradation activity following infection of BSC-40 cells (*C. aethiops*) with VACV. 2′,3′-cGAMP degradation activity is detectable early <1 h after infection and persists beyond 18 h post-infection. In all panels, the ‘−’ refers to a buffer-only control. All data are representative of three independent experiments.
Extended Data Fig. 2 | Biochemical fractionation and mass spectrometry identification of VACV poxin. a, Schematic of purification process developed to enrich VACV poxin from infected cell lysates. Lysates were fractionated using Q IEX and S200 size-exclusion chromatography (purification scheme 1, left) or ammonium sulfate precipitation followed by phenyl hydrophobic interaction and S75 size-exclusion chromatography (purification scheme 2, right). Fractions were tested for 2',3'-cGAMP degradation activity at each stage of purification and active fractions were pooled for subsequent purification steps. Fractions with peak activity after size exclusion were analysed with mass spectrometry. Fold enrichment of proteins in the IEX active fraction compared to two inactive fractions was calculated using label-free mass spectrometry quantification. 

b, List of VACV proteins identified in each purification scheme. VACV poxin is encoded by the B2R gene (green).
Extended Data Fig. 3 | Purification and biochemical characterization of VACV poxin. a, Purification of recombinant VACV poxin from E. coli. VACV poxin was expressed as an N-terminal 6×His-SUMO fusion, the tag was proteolytically removed and VACV poxin was isolated using S75 size-exclusion chromatography. VACV poxin migrates at around 50 kDa, consistent with a homodimeric complex. b, SDS–PAGE Coomassie stain analysis of purified recombinant VACV poxin. c, Reaction time-course analysis of recombinant VACV poxin 2′,3′-cGAMP-degradation activity. VACV poxin rapidly cleaves 2′,3′-cGAMP through a slower-migrating intermediate product, identical to the activity observed in VACV-infected cell lysates. The ‘−’ refers to a buffer-only control incubated for 120 min. Data are representative of three independent experiments. d, e, pH titration of the 2′,3′-cGAMP-degradation activity of recombinant VACV poxin and VACV-infected cell lysate. Recombinant VACV poxin and VACV-infected cell lysates share an alkaline pH optimum of 8.2–10.6. d, The ‘−’ refers to a buffer-only control at pH 7.5. Data are representative of three independent experiments.
Extended Data Fig. 4 | See next page for caption.
Extended Data Fig. 4 | Construction and validation of poxin-expressing cells and poxin knockout virus. a, TLC analysis of lysates from HEK293T cells after transduction with poxin(WT) or the poxin(H17A) catalytically inactive construct, and selection with puromycin. HEK293T poxin(WT) cells but not control cells show degradation of 2′,3′-cGAMP after a 1-h reaction. Data are representative of two independent experiments. The ‘−’ refers to a buffer-only control. b, Western blot analysis of poxin-transduced cell lines demonstrating expression of both VACV poxin(WT) and poxin(H17A) proteins. Data are representative of two independent experiments. Gel source data are available in Supplementary Fig. 1. c, Schematic demonstrating strategy for poxin (B2R) knockout by homologous recombination and replacement with sfGFP. Coloured arrows depict primers used for PCR and sequencing validation of selected viral clones. d, PCR analysis of parental VACV and VACV ΔPoxin confirming removal of B2R and replacement with the sfGFP gene. Data are representative of two independent experiments. e, Sequencing trace confirming replacement of B2R with sfGFP in the genome of VACV ΔPoxin. f, Bright-field and fluorescence microscopy showing Vero cells infected with wild-type VACV poxin or VACV ΔPoxin after 20 h at MOI = 1. VACV ΔPoxin-infected cells express sfGFP. Data are representative of three independent experiments. g, TLC analysis of 2′,3′-cGAMP after incubation with lysates of cells infected with wild-type or ΔPoxin viruses. VACV ΔPoxin-infected cells lack detectable 2′,3′-cGAMP degradation activity. The ‘−’ refers to a buffer-only control; M refers to a mock-infection control. Data are representative of three independent experiments. h, Multiple cycle growth curve (MOI = 0.01) of wild-type and ΔPoxin VACV strains in Vero cells, demonstrating that poxin knockout has no effect on viral growth kinetics in interferon-deficient cells in cell culture (n = 2). Data are mean ± s.e.m. i, qRT–PCR analysis of the transcriptional induction of IFNβ and an interferon-stimulated gene (CXCL10) following infection of A549 cells with wild-type or ΔPoxin VACV after 5 h at MOI = 5 (n = 2). Poxin deletion does not increase IFNβ-dependent signalling in cell culture under these conditions. As a positive control, STING-dependent signalling in A549 cells was stimulated with 2′,3′-cGAMP and digitonin permeabilization.
Extended Data Fig. 5 | Structural analysis of VACV poxin. a, VACV poxin consists of two domains and homodimerizes to form the active complex. The N-terminal domain (green) has structural homology to viral 3C-like proteases (r.m.s.d. of 2.9 Å). The norovirus NS6 protease and Bos taurus trypsin protease are coloured in green and presented in the same orientation for comparison. Z-scores were obtained from the DALI server. The C-terminal domain (cyan) of VACV poxin has no known structural homologues. b, Overlay of the apo (red) and 2′,3′-cGAMP bound pre-reactive (green/cyan/grey) poxin structures. 2′,3′-cGAMP binding induces a 4 Å movement of the clamp helix and repositions the active site for 3′–5′-bond hydrolysis. c, VACV poxin dimerization is mediated by antiparallel β-strand hydrogen bonding between monomers, as well as side-chain interactions within a hydrophobic core composed of M183, M185 and F187.
Extended Data Fig. 6 | Structural analysis of poxin 2′,3′-cGAMP binding. a, Overview of interactions in the VACV poxin–2′,3′-cGAMP complex that mediate substrate specificity. VACV poxin residues make three types of interactions with 2′,3′-cGAMP: sequence-specific contacts with the guanine base (left), hydrogen-bonding interactions with the 2′–5′ bond (middle) and sequence non-specific contacts with the adenine base (right). b, Simulated annealing omit maps showing electron density of 2′,3′-cGAMP before and after poxin cleavage. Base identities can clearly be assigned in both pre- and post-reactive structures. A clear gap exists in the post-reactive structure between the guanine 5′-OH and adenine 3′-phosphate confirming that the poxin product is Gp[2′–5′]Ap[3′]. c, tRNA splicing endoribonucleases are metal-independent enzymes that degrade ribonucleotide substrates through a 2′–3′-cyclic phosphate intermediate. These enzymes share the poxin catalytic triad composed of histidine, tyrosine and lysine, suggesting a related catalytic mechanism, despite the lack of sequence or structural homology.
Extended Data Fig. 7 | VACV poxin degrades 2′,3′-cGAMP through hydrolysis of the 3′–5′ bond. a, TLC analysis of poxin activity using 2′,3′-cGAMP radiolabelled at the 2′–5′-(α32P-A) or 3′–5′-(α32P-G) phosphodiester bonds. Radiolabelled 2′,3′-cGAMP was incubated with recombinant VACV poxin and then treated with phosphatase to remove exposed phosphates from the final product. Following hydrolysis, the guanosine phosphate is exposed for phosphatase removal, confirming the structural findings that VACV poxin specifically cleaves the 3′–5′ linkage of 2′,3′-cGAMP. The ‘−’ refers to a buffer-only control. b, Schematic of VACV poxin induced hydrolysis of 2′,3′-cGAMP. c, TLC analysis of VACV poxin 2′,3′-cGAMP-degradation activity in the presence of 5 mM EDTA metal chelation or divalent cation supplementation. Divalent cations were supplemented at the following concentrations: 5 mM Mg2+, 5 mM Ca2+, 1 mM Mn2+, 1 μM Co2+, 1 μM Ni2+, 1 μM Cu2+ or 1 μM Zn2+. Poxin activity is resistant to EDTA and divalent cations have no effect on the reaction, confirming the structural findings that VACV poxin activity is metal-independent. The ‘−’ refers to a buffer-only control; the ‘+’ refers to treatment with VACV poxin alone without metal addition. d, TLC analysis of mutants of the active site of VACV poxin that were incubated for 20 h with 2′,3′-cGAMP or 3′,3′-cGAMP demonstrates that all active-site mutants retain specificity for 2′,3′-cGAMP. The ‘−’ refers to a buffer-only control. All data are representative of three independent experiments.
Extended Data Fig. 8 | Alignment of poxin proteins conserved in poxvirus representatives. a, The poxin protein is highly conserved in mammalian poxviruses. The alignment is shaded according to conservation of physiochemical amino acid property, and numbered above according to the VACV poxin amino acid sequence. The determined VACV poxin secondary structure is depicted below, active-site residues are indicated with a red dot and boxed in red, and residues that contact 2',3'-cGAMP are boxed in orange. VACV poxin residues 195–219 are not observed in the crystal structure. Sequences depicted in alignment are as follows: VACV WR (vaccinia virus strain Western Reserve, accession YP_233066.1), VACV Cop (vaccinia virus strain Copenhagen, accession P20999.1), VACV Dryvax (vaccinia virus strain Dryvax, accession AEY37376.1), VACV ACAM2000 (vaccinia virus strain ACAM2000, accession AAQ93281.1), VACV NYVAC (vaccinia virus strain NYVAC), RPXV Utr (rabbit poxvirus strain Utrecht, accession AY484669.1), HSPV MNR76 (horsepox virus strain MNR76, accession ABH08291.1), CPXV AUS1999 (cowpox virus strain AUS1999, accession ADZ24189.1), MPXV ZAR (monkeypox virus strain Zaïre-96-1-16, accession NP_536592.1), CMLV CMS (camelpox virus strain CMS, accession AAG37679.1), TATV DAH68 (taterapox virus strain Dahomey 1968, accession YP_717493.1), CPXV GER2002 (cowpox virus strain GER2002, accession ADZ30373.1), CPXV BR (cowpox virus strain Brighton Red, accession NP_619978.1), ECTV MOS (ectromelia virus strain Moscow, accession NP_671672.1), VPXV (volepox virus, accession YP_009281928.1), SKPV (skunkpox virus, accession YP_009282874.1), RCNV (raccoonpox virus, accession YP_009143488.1), YKV (yokapox virus, accession YP_004821513.1), NY_014 (NY_014 virus, accession YP_009408559.1), Murmansk (Murmansk poxvirus, accession YP_009408359.1), EPTV (Eptesipoxivirus (Eptesicus fuscus), accession YP_009408111.1), PTPV (Pteropus scapulatus, accession YP_009268718.1), Melanoplus sanguinipes (M. sanguinipes entomopox virus, accession NP_048308.1).

b, Schematized alignment of poxvirus genomic DNA showing the poxin–schlafen fusion protein. White boxes indicate predicted open reading frames beginning with the annotated start codons and ending with the first stop codon; deletion and nonsense mutations are shown as orange and red bars. CPXV encodes an intact poxin–schlafen fusion protein, whereas the VACV genome contains a stop codon immediately following the poxin coding region and a frameshift mutation in the schlafen (B3R) gene. Poxin is inactivated in MVA and VARV by serial mutation.
Extended Data Fig. 9 | Conservation of poxin family members and 2′,3′-cGAMP-specific nuclease activity in Poxviridae, Baculoviridae and host Lepidoptera. a, Phylogenetic conservation of poxin family members in Poxviridae, Baculoviridae and host Lepidoptera genomes. Poxin catalytic residues (red) and 2′,3′-cGAMP-interacting residues (black) are indicated on the right, shaded in blue according to conservation, and listed according to VACV poxin amino acid number (Poxviridae, top) or AcNPV poxin amino acid number (Baculoviridae, middle). The metazoan poxin sequences from moth and butterfly genomes (Lepidoptera, bottom) share homology throughout the entire poxin protein and exhibit identical 2′,3′-cGAMP degradation activity, but the alignment with viral poxins does not allow definitive assignment of the catalytic residues. Phylogram schematics are based on previous analyses52–55. b, Coomassie-stained SDS–PAGE analysis of recombinant SUMO2-tagged poxin homologue proteins. c, TLC analysis of recombinant viral and host cellular poxin activity after 20 h incubation with substrates. All viral and metazoan poxin family members are specific 2′,3′-cGAMP nucleases. No activity is detected using the chemically related cyclic dinucleotide 3′,3′-cGAMP. The ‘−’ refers to a buffer-only control. Data are representative of three independent experiments.
### Extended Data Table 1 | Summary of data collection, phasing and refinement statistics

|                    | SeMet-VACV Poxin Apo (6EA6) | VACV Poxin Pre-reactive State (6EA8) | VACV Poxin Post-reactive State (6EA9) |
|--------------------|-----------------------------|--------------------------------------|---------------------------------------|
| **Data collection**|                             |                                      |                                       |
| Space group        | P 1 2 1                     | P 1 2 1                              | C 1 2 1                               |
| Cell dimensions    |                             |                                      |                                       |
| a, b, c (Å)        | 55.0, 93.2, 94.1            | 59.4, 92.3, 257.1                    | 215.4, 57.2, 133.1                    |
| α, β, γ (º)        | 90.0, 105.63, 90.0         | 90.0, 93.7, 90.0                     | 90.0, 120.4, 90.0                     |
| Wavelength         | 0.97920 Å                   | 0.97918                              | 0.97918                               |
| Resolution (Å)     | 46.60–1.70 (1.73–1.70)     | 49.48–2.60 (2.65–2.60)               | 38.28–2.10 (2.14–2.10)                |
| R_pim              | 4.9 (64.2)                  | 5.1 (84.4)                           | 3.5 (68.1)                            |
| I/σ(I)             | 20.3 (1.5)                  | 9.3 (1.2)                            | 9.9 (1.1)                             |
| CC_{1/2}           | 93.0 (55.8)                 | 91.9 (34.8)                          | 99.9 (84.7)                           |
| Completeness (%)   | 99.3 (89.6)                 | 99.0 (97.7)                          | 99.2 (97.2)                           |
| Redundancy         | 38.6 (22.6)                 | 3.0 (3.0)                            | 3.5 (3.2)                             |
| **Refinement**     |                             |                                      |                                       |
| Resolution (Å)     | 46.60–1.70                  | 49.48–2.60                           | 38.28–2.1                            |
| No. reflections    |                             |                                      |                                       |
| Total              | 3,858,564                   | 252,224                              | 288,415                               |
| Unique             | 99,918 (4,418)              | 84,690 (4,369)                       | 81,341 (4,327)                       |
| Free (%)           | 2.1                         | 2.3                                  | 2.5                                   |
| R_work / R_free    | 15.99 / 18.22               | 21.77 / 25.23                        | 19.94 / 23.39                        |
| No. atoms          |                             |                                      |                                       |
| Protein            | 6160                        | 15,319                               | 7,762                                 |
| Ligand             | 136                         | 230                                  |                                       |
| Water              | 750                         | 156                                  | 202                                   |
| B factors          |                             |                                      |                                       |
| Protein            | 29.92                       | 96.31                                | 66.28                                 |
| Ligand             | 109.63                      | 59.71                                |                                       |
| Water              | 40.74                       | 59.07                                | 52.00                                 |
| r.m.s deviations   |                             |                                      |                                       |
| Bond lengths (Å)   | 0.012                       | 0.004                                | 0.007                                 |
| Bond angles (º)    | 1.39                        | 0.93                                 | 1.13                                  |

All datasets were collected from individual crystals. Values in parentheses are for the highest resolution shell.
Reporting Summary

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Statistical parameters

When statistical analyses are reported, confirm that the following items are present in the relevant location (e.g. figure legend, table legend, main text, or Methods section).

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

Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

Data collection

All radioactivity-based imaging was collected using Typhoon scanner control 5.0.1
Chromatography traces collected using GE Unicorn 7.1
Protein gel images collected using BioRad ImageLab 2.3.0.07
DNA gel images collected using BioRad Quantity One 4.6.9
Protein homologs identified using NCBI PSI-BLAST (web-based: https://blast.ncbi.nlm.nih.gov/Blast.cgi)
Microscopy images collected using SPOT advanced 5.2
qPCR data were collected using CFX Manager Software 3.0
Data analysis

X-ray crystallography data processing was carried out using XDS (Version January 26, 2018, BUILT=20180409), and CCP4 7.0.057 (Pointless, Aimless, Truncate)

X-ray crystallography experimental phasing, molecular replacement, and refinement carried out using Phenix 1.13-2998 (Includes HySS, SOLVE/RESOLVE, Phaser, Refine)

Protein structure model building was carried out using Coot 0.8.9

Final protein structure model was analyzed and images generated using PyMOL 1.7.4.4

Graphing and statistical analysis was carried out using Prism 7

Protein alignments were generated using Jalview 2.10.1

Sanger sequencing chromatograms were generated using 4Peaks

Data

Policy information about availability of data

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

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

Coordinates and structure factors of the VACV Poxin Apo and Poxin 2’-3’ cGAMP pre-reactive and post-reactive complexes have been deposited in PDB under accession codes 6EA6, 6EA8, and 6EA9. All other data are available in the manuscript or the supplementary materials.

Field-specific reporting

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

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

For a reference copy of the document with all sections, see nature.com/authors/policies/ReportingSummary-flat.pdf

Life sciences study design

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

Sample size

Sample size for the mouse VACV replication assay was based on previously published protocols (Pan et al., 2016 PMID: 28219080).

Data exclusions

No data were excluded from analyses

Replication

All attempts to replicate data proved successful.

Randomization

Mice were randomized into control and experimental groups.

Blinding

Blinding was not performed for data analysis or group allocation, as mice were randomly assigned to each experimental group, and data were collected by unbiased quantitative means, and not qualitative means.

Reporting for specific materials, systems and methods

Materials & experimental systems

Methods

n/a Involved in the study

☑️ Unique biological materials

☑️ Antibodies

☑️ Eukaryotic cell lines

☑️ Palaeontology

☑️ Animals and other organisms

☑️ Human research participants

n/a Involved in the study

☑️ ChIP-seq

☑️ Flow cytometry

☑️ MRI-based neuroimaging
## Unique biological materials

**Policy information about availability of materials**

**Obtaining unique materials** The poxin knockout VACV strain generated by the authors is available upon request.

## Antibodies

| Antibodies used | Sigma rat anti-HA-peroxidase conjugate, clone 3F10 (catalog 12013819001). Bethyl labs rabbit anti-RPS19 antibody (catalog A304-002A) |
|----------------|-------------------------------------------------------------------------------------------------------------------------|
| Validation     | Sigma anti-HA-peroxidase: As noted on the manufacturer’s webpage, this antibody has previously been validated and cited by at least two publications (Lee et al., 2014 PMID: 24782309 and Su et al., 2013 PMID: 23401860). Bethyl labs anti-RPS19 antibody: As noted on the manufacturer’s webpage this antibody has previously been validated and used in publications including Lee et al., 2015 PMID:25849773 and Lee et al., 2016 PMID: 27462815. |

## Eukaryotic cell lines

**Policy information about cell lines**

| Cell line source(s) | AS49, BSRT7, Vero, and BSC-40 cells were a gift from Sean Whelan. THP-1 (catalog ATCC TIB-202) and HEK 293T (catalog ATCC CRL-3216) cells were purchased directly from ATCC. |
|---------------------|-------------------------------------------------------------------------------------------------------------------------|
| Authentication      | THP-1 and HEK 293T cells were validated by ATCC. AS49, BSRT7, Vero, and BSC-40 cells were not authenticated. |
| Mycoplasma contamination | Cell lines were not tested for mycoplasma contamination. |
| Commonly misidentified lines | No misidentified lines were used. |

## Animals and other organisms

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

| Laboratory animals | Mice were obtained directly from Jackson Labs. All mice used were female C57BL/6, 7 weeks of age. |
|--------------------|-------------------------------------------------------------------------------------------------------------------------|
| Wild animals       | This study did not involve wild animals. |
| Field-collected samples | This study did not involve field-collected samples. |