Viral evasion of the integrated stress response through antagonism of eIF2-P binding to eIF2B

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Viral infection triggers activation of the integrated stress response (ISR). In response to viral double-stranded RNA (dsRNA), RNA-activated protein kinase (PKR) phosphorylates the translation initiation factor eIF2, converting it from a translation initiator into a potent translation inhibitor and this restricts the synthesis of viral proteins. Phosphorylated eIF2 (eIF2-P) inhibits translation by binding to eIF2’s dedicated, heterodecameric nucleotide exchange factor eIF2B and conformationally inactivating it. We show that the NSs protein of Sandfly Fever Sicilian virus (SFSV) allows the virus to evade the ISR. Mechanistically, NSs tightly binds to eIF2B (KD = 30 nM), blocks eIF2-P binding, and rescues eIF2B GEF activity. Cryo-EM structures demonstrate that SFSV NSs and eIF2-P directly compete, with the primary NSs contacts to eIF2Bα mediated by five ‘aromatic fingers’. NSs binding preserves eIF2B activity by maintaining eIF2B’s conformation in its active A-State.
The integrated stress response (ISR) is a conserved eukaryotic stress response network that, upon activation by a diverse set of stressors, profoundly reprograms translation. It is coordinated by at least four stress-responsive kinases: PERK (responsive to protein misfolding in the endoplasmic reticulum), PKR (responsive to viral infection), HRI (responsive to heme deficiency and oxidative and mitochondrial stresses), and GCN2 (responsive to nutrient deprivation)1–4. All four known ISR kinases converge on the phosphorylation of a single serine (S51) of the α-subunit of the general translation initiation factor eIF2. Under non-stress conditions, eIF2 forms a ternary complex (TC) with methionyl initiator tRNA (Met-tRNA) and GTP. This complex performs the critical task of delivering the first amino acid to ribosomes at AUG initiation codons. Upon S51 phosphorylation, eIF2 is converted from a substrate to an inhibitor of its dedicated nucleotide exchange factor (GEF) eIF2B. GEF inhibition results from binding of eIF2-P in a new, inhibitory binding orientation on eIF2B, where it elicits allostatic changes to antagonize eIF2 binding and additionally compromise eIF2B’s intrinsic enzymatic activity5,6.

eIF2B is a twofold symmetric heterodecamer composed of two copies each of α, β, δ, γ, and ε subunits7–10. eIF2B can exist in a range of stable subcomplexes (eIF2Bβδγε tetromers and eIF2Bα2 dimers) if the concentrations of its constituent subunits are altered5,8,9,11. While earlier models suggested eIF2B assembly to be rate-limiting and a potential regulatory step, recent work by us and others show that eIF2B in cells primarily exists in its fully assembled decameric, enzymatically active state5,6. Cryo-EM studies of various eIF2B complexes elucidated the mechanisms of nucleotide exchange and ISR inhibition through eIF2-P binding5,6,12–15. Under non-stress conditions, eIF2 engages eIF2B through multiple interfaces along a path spanning the heterodecamer. In this arrangement, eIF2α binding to eIF2B critically positions the GTPase domain in eIF2γ’s γ-subunit, allowing for efficient catalysis of nucleotide exchange12,14. eIF2B’s catalytically active configuration (“A-State”) becomes switched to an inactive conformation upon eIF2-P binding (Inhibited or “I-State”), which displays altered substrate-binding interfaces5,6. I-State eIF2Bβδγε manifests enzymatic activity and substrate engagement akin to the tetrameric eIF2βδγε subcomplex; hence, eIF2-P inhibition of eIF2B converts the decamer into conjoined tetramers, which reduces its GEF activity, lowers the cell’s TC concentration, and results in ISR-dependent translational reprogramming5,6.

Viruses hijack the host cell’s protein synthesis machinery to produce viral proteins and package new viral particles. Numerous host countermeasures have evolved. In the context of the ISR, double-stranded RNA (dsRNA), a by-product of viral replication, triggers dimerization and autophosphorylation of PKR5,6,16. In this activated state PKR phosphorylates eIF2, which then binds to and inhibits eIF2B. As such, cells downregulate mRNA translation as a strategy to slow the production of virions. Viruses, in turn, enact strategies of evasion. Indeed, viral evasion strategies acting at each step of ISR activation have been observed. Influenza virus, for example, masks its dsRNA17,18. Rift Valley Fever virus (RVFV) encodes an effector protein that degrades PKR19. Hepatitis C virus blocks PKR dimerization20. Vaccinia virus encodes a pseudosubstrate as a PKR decoy21. Herpes simplex virus can dephosphorylate eIF2-P22. And some coronaviruses and picornavirus proteins appear to block the eIF2B–eIF2-P interaction23. This evolutionary arms race between host and pathogen can provide invaluable tools and insights into the critical mechanisms of the ISR, as well as other cellular stress responses.

Here, we investigated the previously unknown mechanism by which Sandfly Fever Sicilian virus (SFSV) evades the ISR. SFSV and RVFV are both members of the genus Phlebovirus (order Bunyavirales) which encode an evolutionarily related non-structural protein (NSs)24–26. Across the phleboviruses, NSs serves to counteract the antiviral interferon response, but NSs proteins perform other functions as well27,28. Unlike the RVFV NSs which degrades PKR, SFSV NSs does not impact the levels or phosphorylation status of PKR or eIF29,29. Instead, it binds to eIF2B, inhibiting the ISR. The mechanistic basis of this inhibition was previously unclear. We here provide cellular, biochemical, and structural insight into this question, showing that the SFSV NSs evades all branches of the ISR by binding to eIF2B and selectively blocking eIF2-P binding, thereby maintaining eIF2B in its active A-State.

Results

The SFSV NSs is a pan-ISR inhibitor. To dissect the role of the SFSV NSs (henceforth referred to as NSs) in ISR modulation, we engineered cells stably expressing either an empty vector, a functional NSs (NSs::FLAG), or a non-functional NSs (FLAG::NSs) (Supplementary Fig. 1). As previously reported, the NSs with a C-terminal FLAG tag (NSs::FLAG) should retain its PKR-evading properties while tagging at the N-terminus (FLAG::NSs) blocks this functionality29. These constructs were genomically integrated into our previously generated ISR reporter system, in which both changes in ATF4 translation and general translation can be monitored5. Both NSs::FLAG and FLAG::NSs were stably expressed in these cells without impacting the levels of key ISR components (eIF2B, eIF2, PKR, PERK) (Fig. 1a). The apparent differences in band intensity between NSs::FLAG and FLAG::NSs may reflect differences in protein stability or, perhaps more likely, differences in antibody affinity for the FLAG epitope at the respective C- and N-terminal tagging locations.

To ask whether NSs is a pan-ISR inhibitor capable of dampening ISR activation irrespective of any particular ISR activating kinase, we chemically activated PERK, HRI, and GCN2 with thapsigargin, oligomycin, and glutamine deprivation/synthetase inhibition through L-methionine sulfoximine, respectively. NSs::FLAG expression dampened the increases in ATF4 translation brought about by activation of any of the kinases (Fig. 1b–d). NSs::FLAG also maintained general translation levels in the thapsigargin and oligomycin treated cells (Fig. 1b, c). Notably, in the context of GCN2 activation, general translation comparably decreased at the highest levels of stress regardless of NSs status (Fig. 1d). This observation likely reflects the additional stress responses that react to reduced amino acid levels, as well as the fact that while the ISR controls translation initiation, ribosome-engaged mRNAs still need sufficient levels of amino acids to be successfully translated. On the whole, these data therefore show that the NSs is a pan-ISR inhibitor akin to the small-molecule ISRIB, which binds to eIF2B and counteracts the ISR by allosterically blocking eIF2-P binding and promoting the eIF2B complex assembly when eIF2B’s decameric state is compromised5,6,30.

NSs binds decameric eIF2B exclusively. To explain the mechanism by which NSs inhibits the ISR, we purified NSs expressed in mammalian cells (Fig. 2a, b). We next validated that NSs binds to eIF2B in vitro by immobilizing distinct eIF2B complexes on agarose beads and incubating them with an excess of NSs (Fig. 2c). As expected, NSs binds to the fully assembled eIF2B(αβδγε)2 decamers (Lane 4). Notably, it did not bind to eIF2Bβδγε tetramers (Lane 5) or to eIF2Bα2 dimers (Lane 6). The NSs interaction with eIF2B thus either spans multiple interfaces that are only completed in the fully assembled complex or...
**Fig. 1** The SFSV NSs is a pan-ISR inhibitor. *a* Western blot of K562 cell extracts. Loading of all lanes was normalized to total protein. *b–d* ATF4 and General Translation reporter levels as monitored by flow cytometry. Trimethoprim, which is necessary to stabilize the ecDHFR::mScarlet-i and ecDHFR::mNeonGreen translation reporters, was at 20 μM for all conditions. *b* Samples after 3 h of thapsigargin and trimethoprim treatment. *c* Samples after 3 h of oligomycin and trimethoprim treatment. *d* Samples after 4 h of glutamine deprivation, L-methionine sulfoximine, and trimethoprim treatment. For *a*, PERK and GAPDH, PKR and eIF2α, and elf2Be and NSs (FLAG) are from the same gels, respectively. elf2Bde is from its own gel. For *b–d*, biological replicates: *n = 3*. All error bars represent s.e.m. Source data are provided as a Source Data file.

**Fig. 2** NSs specifically binds to elf2Be(αβδγε)2 decamers. *a* Size exclusion chromatogram (Superdex 200 Increase 10/300 GL) during NSs purification from Expi293 cells. *b* Coomassie Blue staining of purified NSs. *c* Western blot of purified protein recovered after incubation with elf2B(αβδγε)2, elf2Bβγδε, or elf2Bα2 immobilized on Anti-protein G antibody-conjugated resin. For elf2B(αβδγε)2 and elf2Bα2, elf2Bαx was protein C tagged. elf2Bβγδε was protein C tagged for elf2Bβγδε. For elf2Bβγδε. *d–f* SPR of immobilized d elf2B(αβδγε)2, e elf2Bβγδε, and f elf2Bα2 binding to NSs. For elf2B(αβδγε)2 and elf2Bβγδε, elf2Bβγδε was Avi-tagged and biotinylated. For elf2Bα2, elf2Bαx was Avi-tagged and biotinylated. For d, concentration series: (250–15.625 nM) For e, f, concentration series: (125–15.625 nM). For e, elf2Bβ and elf2Bαx, and elf2Bαx and NSs (6xHis) are from the same gels, respectively. elf2Bde is from its own gel. For b–f, a single biological replicate. Source data are provided as a Source Data file.

interacts with a region of elf2B that undergoes a conformational change when in the fully assembled state.

To quantitatively assess NSs binding to elf2B, we employed surface plasmon resonance (SPR) experiments to determine the affinity of NSs for the various elf2B complexes (Fig. 2d–f). The NSs interaction with decameric elf2B could be modeled using one-phase association and dissociation kinetics. NSs binds to decameric elf2B with a Kd of 30 nM (koff = 3.0 × 107 M−1 s−1, kon = 8.9 × 10−3 s−1) (Fig. 2d). This affinity is comparable to the low nanomolar affinity of ISRIB for decameric elf2B.
NSs rescues eIF2B activity by blocking eIF2-P binding. We next sought to explain the mechanism of NSs inhibition of the ISR using our established in vitro systems for studying eIF2B. As is the case with the small-molecule ISRIB, NSs did not impact the intrinsic nucleotide exchange activity of eIF2B as monitored by a fluorescent BODIPY-FL-GDP loading assay (Supplementary Fig. 2). To mimic the conditions during ISR activation, we repeated our nucleotide exchange assay in the presence of the inhibitory eIF2α-P (Fig. 3a). As expected, eIF2α-P inhibited eIF2B GEF activity \( t_{1/2} = 13.4 \text{ min, s.e.m.} = 1.5 \text{ min} \), but increasing concentrations of NSs \( (25 \text{ nM: } t_{1/2} = 9.2 \text{ min, s.e.m.} = 1.2 \text{ min; } 100 \text{ nM: } t_{1/2} = 6.2 \text{ min, s.e.m.} = 0.5 \text{ min} \) overcame the inhibitory effects of eIF2α-P and fully rescued eIF2B GEF activity (uninhibited \( t_{1/2} = 6.3 \text{ min, s.e.m.} = 0.6 \text{ min} \)).

As NSs’ ability to affect eIF2B activity markedly manifests in the presence of eIF2α-P, we wondered whether NSs blocks eIF2α-P binding to eIF2B. To test this notion, we utilized a fluorescent ISRIB analog (FAM-ISRIB) that emits light with a higher degree of polarization when bound to eIF2B, compared to being free in solution (Fig. 3b, black and red dots on the Y-axis, respectively). It has been previously shown that eIF2α-P binding to eIF2B antagonizes FAM-ISRIB binding by shifting eIF2B into a conformation incapable of binding ISRIB or its analogs (Fig. 3b, blue dot on the Y-axis)\(^5,6\). A titration of NSs into this reaction recovered FAM-ISRIB polarization \( \text{EC}_{50} = 72 \text{ nM, s.e.m.} = 9 \text{ nM} \), indicating that NSs engages eIF2B and disrupts eIF2α-P’s inhibitory binding. To directly show this antagonism, we immobilized eIF2B decamers on agarose beads and incubated with combinations of NSs and eIF2α-P (Fig. 3c). While individually, both eIF2α-P and NSs bound to eIF2B (Fig. 3c, lanes 4 and 5, respectively), in the presence of saturating NSs, eIF2α-P no longer bound eIF2B (Fig. 3c, lane 6). We next sought to analyze the impact of NSs binding on full-length substrate (eIF2) and inhibitor (eIF2-P) binding through SPR experiments. In this assay we first flowed one analyte over immobilized eIF2B (to saturate the binding site) immediately followed by a mixture of both analytes (to assess whether the second analyte could co-bind elsewhere). Consistent with the nucleotide exchange assay in Fig. 3a, eIF2 and NSs co-bound eIF2B (Fig. 3d, f, increases in RU at 60 s). However, as with the phosphorylated eIF2αubain alone, the full phosphorylated heterotrimer (eIF2-P) and NSs did not co-bind (Fig. 3e, g, no increases in RU at 60 s). Together, these results demonstrate that the NSs is a potent inhibitor of eIF2-P binding while preserving eIF2 binding.

NSs binds to eIF2B at the eIF2α-P-binding site and keeps eIF2B in the active A-State. Having established that the NSs blocks eIF2α-P binding to eIF2B, we next assessed whether NSs is an allosteric regulator of eIF2-P binding (as is the case with ISRIB) or, alternatively, whether it directly competes with eIF2-P binding. To answer this question and to rigorously determine NSs’ interactions with eIF2B, we turned to cryo-EM. To obtain a homogeneous sample suitable for structural studies, we mixed full-length NSs with decameric eIF2B at a 3:1 molar ratio. We then prepared the sample for cryo-EM imaging and determined the structure of the eIF2B–NSs complex.

Three-dimensional classification with no symmetry assumptions yielded a distinct class of 137,093 particles. Refinement of this class resulted in a map with an average resolution of 2.6 Å (Supplementary Fig. 4). After docking the individual eIF2B subunits into the recorded density, we observed significant extra

Fig. 3 NSs grants ISR evasion by antagonizing eIF2α-P binding to eIF2B. a Gef activity of eIF2B as assessed by BODIPY-FL-GDP exchange. eIF2B(αβδγε)_2 at 10 nM throughout. \( t_{1/2} = 6.3 \text{ min (No eIF2α-P), 6.2 \text{ min (2 μM eIF2α-P + 100 nM NSs), 9.2 \text{ min (2 μM eIF2α-P + 25 nM NSs), and 13.4 \text{ min (2 μM eIF2α-P). b Plot of fluorescence polarization signal before (red) and after incubation of FAM-ISRIB (2.5 nM) with 100 nM eIF2B(αβδγε)_2 (black) or 100 nM eIF2B(αβδγε)_2 + 5.6 μM eIF2α-P (blue) and varying concentrations of NSs. c Western blot of purified protein recovered after incubation with eIF2B(αβδγε)_2 immobilized on Anti-protein C antibody-conjugated resin. eIF2Bα was protein C tagged. d-g SPR of immobilized eIF2B(αβδγε)_2 binding to saturating d, e 500 nM NSs, f 125 nM eIF2, or g 125 nM eIF2-P followed by d 125 nM eIF2, e 125 nM eIF2-P, or f, g 500 nM NSs. eIF2Bα was Avi-tagged and biotinylated. For e, eIF2e and eIF2α-P, eIF2βf and eIF2γg, and eIF2β and NSs (6xHis) are from the same gels, respectively. For a, b, biological replicates: \( n = 3 \). For c–g, a single biological replicate. All error bars represent s.e.m. Source data are provided as a Source Data file.
density next to both eIF2Ba subunits, indicating that two copies of NSs are bound to each eIF2B decamer (Fig. 4a and Supplementary Fig. 4). The local resolution of the NSs ranges from 2.5 Å (regions close to eIF2B) to >4.0 Å (periphery), with most of the side chain densities clearly visible (Supplementary Fig. 4). To build the molecular model for NSs, we split the protein into two domains. The C-terminal domain was built using the crystal structure of the C-terminal domain of the RVFV NSs (PDB ID: 5OOO) as a homology model (43.8% sequence similarity with the C-terminal domain of the SFSV NSs (residues 85–261)) (Supplementary Fig. 5)\(^3\). The N-terminal domain of the NSs (residues 1–84) was built de novo (Supplementary Table 1). The high-resolution map allowed us to build a model for the majority of NSs. The map quality of both NSs molecules are comparable, and their molecular models are nearly identical (root mean square deviation (RMSD) \(\approx 0.2\) Å). We henceforth focus our analysis on one of them (chain K).

Two copies of NSs bind to one decameric eIF2B in a symmetric manner (Fig. 4a). An overlay of the NSs-bound eIF2B and the eIF2a-P-bound eIF2B structures (PDB ID: 6O9Z) shows a significant clash between the NSs and eIF2-aP, indicating that, unlike the allosteric regulator ISRIB, NSs binds in direct competition with eIF2a-P (Fig. 4d–f). Interestingly, whereas eIF2a-P forms extensive interactions with both the \(\alpha\) and the \(\delta\) subunits of eIF2B, the NSs mainly interacts with the eIF2Ba subunit. The expansive interactions between eIF2a-P and both eIF2Ba and eIF2B\(\delta\) mediate a shift in eIF2B’s conformation from eIF2B’s enzymatically active A-State to its inhibited I-State\(^5,6\). Thus, despite binding to a region known to influence eIF2B’s conformation, an overlay of the NSs-bound eIF2B and apo eIF2B shows that the overall conformation of eIF2B in the two structures are virtually identical (Fig. 4b). By contrast, the eIF2B–NSs and eIF2B–eIF2a-P overlay shows major conformational differences (Fig. 4c). Together, these structural data, paired with our in vitro assays, show that the NSs grants SFSV evasion of the ISR by directly competing off eIF2-P and restoring eIF2B to its enzymatically active A-State.

**NSs uses a novel protein fold containing aromatic fingers to bind eIF2B.** Next, we sought to interrogate the molecular details of the NSs–eIF2B interaction. As mentioned above, NSs consists of two domains. Its N-terminal domain (amino acids 1–84) consists of six \(\beta\)-strands and interacts directly with eIF2B. A search in the DALI protein structure comparison server did not reveal any hits, suggesting a novel protein fold. \(\beta\)-Strands 1 and 2 and \(\beta\)-strands 3 and 4 form two antiparallel \(\beta\)-sheets and fold on top of the C-terminal domain (Supplementary Fig. 6b). The C-terminal domain (amino acids 85–261) is largely \(\alpha\)-helical and presumably supports the folding of the N-terminal domain, as truncating the C-terminal domain results in the complete loss of NSs activity in terms of ISR evasion (Supplementary Fig. 7). Also, despite the moderate sequence conservation of the C-terminal domain of the SFSV NSs and the RVFV NSs, their structures overlay extensively (RMSD \(\approx 0.2\) Å, Supplementary Fig. 6).

The surface of the N-terminal domain forms a hand shape that grips the alpha helices of eIF2Ba, akin to a koala grabbing a eucalyptus branch (Fig. 5a and Supplementary Fig. 10). In this arrangement, the N-terminal domain extends three loops that sit in a groove between helices \(\alpha3\) and \(\alpha4\) and the third loop just below helix \(\alpha3\), effectively sandwiching helix \(\alpha3\) (Fig. 5b). Together, the three loops extend five aromatic amino acids to contact eIF2Ba. We refer to these aromatic amino acids as “aromatic fingers”. On the top side of helix \(\alpha3\), the side chain of NSs Y5 forms a cation–\(\pi\) interaction with eIF2Ba R74 and its backbone carboxyl forms a hydrogen

![Fig. 4 Overall architecture of the eIF2B–NSs complex.](https://doi.org/10.1038/s41467-021-26164-4)
Indeed, alanine substitutions of the aromatic ISR, likely through reducing the binding affinity of the NSs, allows translation of ATF4 and maintains general translation at rates that did not compromise NSs stability and, as with WT NSs, did not affect the expression of NSs variants in the dual ISR reporter cells. The point mutations (Y5A/F7A, Y79A/F80A, and F33A) and stably expressed these variants, showing reduced ATF4 translation blunted by PKR (Fig. 6a). A similar picture emerged from analysis of the NSs::FLAG expressing WT NSs::FLAG was ATF4 translation blunted by PKR, becoming phosphorylated in all cell lines, but only in cells lacking NSs., generating stable lines with alanine mutations (H36A and D37A) while this mutation compromises NSs binding it does not appear to entirely break the interaction (Fig. 6d). In contrast, NSs::FLAG (D37A)-expressing cells appear unable to resist ISR activation. Although the structure suggests only a mild ionic interaction between NSs D37 and eIF2B δR321, we reason the D37A mutation might not only break the ionic interaction, but also potentially alter the conformation of the loop. As a result, V38 would move, disrupting its stacking with M6, an amino acid next to two aromatic fingers (Y5 and F7) (Supplementary Fig. 8). Thus, changes to D37 and H36 could result in the repositioning of the eIF2Bα-facing aromatic fingers, leading to a complete loss of NSs interaction with eIF2B. Together, these data provide a rationale for NSs’ potent and selective binding to only fully assembled eIF2Bαδβγε δε2 decamers.

**Discussion**

As one of the strategies in the evolutionary arms race between viruses and the host cells they infect, mammalian cells activate the ISR to temporarily shut down translation, thus preventing the synthesis of viral proteins. Viruses, in turn, have evolved ways to evade the ISR, typically by disarming the PKR branch through countermeasures that lead to decreased levels of eIF2-P, thus allowing translation to continue. In this study, we show that SF5V expresses a protein (NSs) that allows it to evade not just PKR-mediated ISR activation, but all four branches of the ISR, through a mechanism that exploits the conformational flexibility of eIF2B. NSs is an antagonist of eIF2B’s inhibitor eIF2-P, deploying an overlapping binding site. Whereas eIF2-P shifts eIF2B to its inactive I-State conformation by closing the angle between the eIF2βα and eIF2βδ subunits, NSs engages the enzyme to opposite effect, binding to an overlapping site with eIF2-P but preserving the angle between eIF2βα and eIF2βδ and locking it into its active A-State conformation (Fig. 7).

Previously, we and others showed that the GEF activity of eIF2B is modulated conformationally: eIF2B’s substrate (eIF2) binding stabilizes it in the A-State, whereas its inhibitor (eIF2-P) binding induces a hinge motion between the two tetrameric subunits, resulting in a conformation that cannot engage the substrate optimally (I-State). Our structure shows that NSs antagonizes the endogenous inhibitor (eIF2-P) by directly competing it off and stabilizing eIF2B in the active conformation. Owing to the reported single digit nM affinity of eIF2-P for eIF2B,
**Fig. 6** All 5 aromatic fingers are required for NSs evasion of the ISR. 

- **a, c** Western blot of K562 cell extracts 3 h after treatment with 50 nM thapsigargin. Loading of all lanes was normalized to total protein. 
- **b, d** ATF4 and General Translation reporter levels as monitored by flow cytometry after 3 h of thapsigargin and trimethoprim (20 μM) treatment. For **a**, ATF4 and eIF2α, eIF2Bε and NSs (FLAG), and eIF2Bδ and eIF2α-P are from the same gels, respectively. GAPDH is from its own gel. For **c**, ATF4 and GAPDH, eIF2Bε and NSs (FLAG), and eIF2Bδ and eIF2α-P are from the same gels, respectively. eIF2α is from its own gel. For **b**, biological replicates: n = 3. For **d**, biological replicates: n = 4. All error bars represent s.e.m. Source data are provided as a Source Data file.

**Fig. 7** Model for regulation of eIF2B activity. Like the small-molecule ISRIB and the substrate eIF2, NSs binds to and stabilizes the active, “wings up” conformation of eIF2B (A-State). eIF2-P induces the inhibited “wings down” conformation of eIF2B (I-State).
this likely entails a cellular excess of NSs relative to eIF2-P (which should be expected given the high levels at which viral proteins are typically expressed)\textsuperscript{33–35}. While NSs binds to the inhibitor-binding site, it does not induce the conformational change that the inhibitor binding induces. This mechanism is reminiscent of the antagonistic inhibition of GPCRs, such as the β-adrenergic receptors, where binding of an agonist ligand shifts the receptor to its active conformation, whereas binding of an antagonist ligand occupies an overlapping but not identical binding site that lacks contacts required to induce the activating conformational change\textsuperscript{36–39}. NSs, however, is an antagonist of an inhibitor (eIF2-P). Thus, by inhibiting an inhibition, it actually works as an eIF2B activator under conditions where eIF2-P is present and the ISR is induced.

In its ability to modulate eIF2B, NSs is not unique among viral proteins. The beluga whale coronavirus (Bw-CoV) protein AcP\textsubscript{10} likewise allows evasion of the host cell ISR by interacting with eIF2B, as does the picornavirus AIVL protein\textsuperscript{41}. It was suggested that AcP\textsubscript{10} makes contacts with eIF2Ba and eIF2Bδ, akin to NSs, and hence may act through a similar mechanism by allosterically regulating eIF2-P, although no structural information is yet available. By primary sequence comparison, AcP\textsubscript{10}, AIVL, and NSs show no recognizable homology with one another, indicating that viruses have evolved at least three—and likely more—different ways to exploit the eIF2-P binding site on eIF2B to shut off the ISR. Therefore, inhibiting the eIF2B–eIF2-P interaction through the antagonism of eIF2-P binding could also be a general strategy used by many viruses.

Our structure and mutational analysis suggest that the binding of different parts of NSs to eIF2B occurs in a highly synergistic manner. While the amino acids facing eIF2Bδ do not seem to make sufficiently intimate contacts to provide a significant contribution to the enthalpic binding energy, changing them disrupts binding. It is plausible that the contacts of NSs with eIF2Bδ allow the optimal positioning of the aromatic fingers through allosteric communications between the loops and thus license NSs for tight binding.

The structure of the eIF2B–NSs complex reveals a previously unknown site on eIF2B that is potentially drugable. Unlike ISRB, which stabilizes eIF2Bδ’s A-State through binding to a narrow pocket at the center of eIF2B and stapling the two tetrameric halves together at a precise distance and angle, NSs binds to a different interface on the opposite side of the protein. With ISRB-derivatives showing extreme promise to alleviate cognitive impairment, the ability to modulate the optimal positioning of the aromatic fingers through allosteric communications between the loops and thus license NSs for tight binding is of great interest.

Aberant ISR activation underlies many neurological disorders (traumatic brain injury, Down’s syndrome, Alzheimer’s disease, amyotrophic lateral sclerosis), as well as certain cancers (metastatic prostate cancer)\textsuperscript{40–47}. Virotherapy, where viruses are used as a therapeutic agent for particular diseases, has seen the most success in the realm of cancer treatment where the infection either directly attacks cancer cells (oncolytic virotherapy) or serves to activate host defenses which target virus and cancer alike\textsuperscript{48,49}. Indeed, decades of evidence have shown that cancer patients who experience an unrelated viral infection can show signs of improvement, paving the way for the generation of genetically engineered oncolytic viruses that have only just received FDA approval in the last decade\textsuperscript{50,51}. With our ever-growing understanding of diverse host–virus interactions, a whole host of new virotherapies are imaginable that can exploit the evolved functionalities of viral proteins such as the NSs.

**Methods**

**Cloning of NSs expression plasmids.** The NSs:6xHis Exp239 expression plasmid for transient transfection was generated using In-Fusion HD cloning. The SFSV NSs sequence\textsuperscript{29} was inserted into the pXSIN vector backbone and a 6xHis tag was added at the C-terminus. The various NSs overexpression plasmids for stable lentiviral integration were generated using In-Fusion HD cloning. The SFSV NSs sequence was inserted into the pDRB vector backbone and a FLAG tag was added at the C-terminus (pMS110, pMS127, pMS128, pMS129, pMS130, pMS131, pMS132, pMS133) or N-terminus (pMS111). The various NSs truncations did not have a FLAG tag (pMS119, pMS120, pMS121, pMS122, pMS123). An empty vector control plasmid with no NSs insertion was also generated (pMS085). An IRES followed by the puromycin resistance gene, a T2A self-cleaving peptide, and the β-galactosidase allows for selection based on antibiotic resistance or GFP signal (what was used in this study) (Supplementary Fig. 1). Full plasmid details are shown in Supplementary Table 2.

**Cloning of tagged human eIF2B expression plasmids.** eIF2B\textsubscript{2} (encoding eIF2Bδ) and eIF2B\textsubscript{δ} (encoding eIF2Bδ) had previously been inserted into sites 1 and 2 of pACYCDuet-1, respectively (pJ707). In-Fusion HD cloning (Takara Bio) was used to edit this plasmid further and insert an Avi tag (GLNDIFEAQKEYHE) or a Protein C tag (EDQVDPRLIDGK) at the N-terminus of eIF2B\textsubscript{2}, immediately following the pre-existing 6xHis tag (pMS001 and pMS003). eIF2B\textsubscript{2} (encoding eIF2B\textsubscript{2}) had previously been inserted into site 1 of pETDuet-1 (pJ707). In-Fusion HD cloning was used to edit this plasmid further and insert an Avi tag at the N-terminus of eIF2B\textsubscript{1}, immediately following the pre-existing 6xHis tag (pMS026). The Avi tag allows selective, single, and complete biotinylation of the tagged protein.

**Generation of stable NSs-expressing cells in an ISR reporter cell line.** Our previously generated dual ISR reporter K562 cells expressing a stably integrated ATF4 reporter (pMS086), general translation reporter (pMS078), and dCas9-KRAB were used as the parental line\textsuperscript{5}. The various NSs overexpression constructs (Supplementary Table 2) were integrated using two lentiviral vectors. Vesicular stomatitis virus (VSV)-G pseudotyped lentivirus was prepared using standard protocols and 293T packaging cells. Viral supernatants were filtered (0.45 μm low protein binding filter unit (EMD Millipore)) and concentrated 10-fold. 100,000 Da molecular mass cutoff). Concentrated supernatants at 0.45 μl/glucose, and 25 mM HEPES supplemented with 10% FBS, 2 mM l-alanyl-l-glutamine (Gibco GlutaMAX), and penicillin/streptomycin, supplemented with polybrene to 8 μg/m, brought to 1.5 ml in a six-well plate, centrifuged for 1000 g, Cells were then allowed to recover and expand for ~1 week before sorting on a Sony SH8000 cytometer to isolate cells that had integrated the reporter. Roughly 100,000 BFP-positive cells (targeting the highest 1–3% of expressers) were then sorted into a final pooled population and allowed to recover and expand. Cells expressing NSs truncations (pMS119–pMS123) were not sorted and instead analyzed as a polyclonal population, gating for BFP-positive cells during data analysis.

**Western blotting.** Western blotting was performed as previously described\textsuperscript{5}. In brief, approximately 1,000,000 cells of the appropriate cell type were digested in individual assays and then pelleted, washed, pelleted again, and resuspended in lysis buffer. Cells were then rotated for 30 min at 4°C and then spun at 12,000 g for 20 min to pellet cell debris. Protein concentration was measured using a bicinchoninic acid (BCA assay) and determined using a Protein Assay Dye Reagent Concentrate (Bio-Rad). Total protein concentration was normalized to the least concentrated sample.

**Equal protein content for each condition (targeting 10 μg)** was run on 10%
Membranes were developed with SuperSignal West Dura (Thermo Fisher Scientific). Developed membranes were imaged on a LI-COR Odyssey gel imager for 0.5–10 min depending on band intensity.

ATF4/general translation reporter assays. ISR reporter cells (at ~500,000/ml) were co-treated with varying combinations of drugs (20 μM trimethoprim plus one of the following: thapsigargin, oligomycin, or glutamine deprivation (and no FBS)) as indicated in Table 3. Membranes were developed with SuperSignal West Dura (Thermo Fisher Scientific). Developed membranes were imaged on a LI-COR Odyssey gel imager for 0.5–10 min depending on band intensity.

Purification of human eIF2B subcomplexes. Human eIF2α (pT707), Avi-tagged eIF2β (pMS026), protein C-tagged eIF2γ (pMS027), eIF2βγγβ (pT703 and pT704 co-expression), Avi-tagged eIF2βγγ (pMS001 and pT704 co-expression), and Protein C-tagged eIF2βγγβ (pMS003 and pT704 co-expression) were purified as previously described with a minor modification for purification of the Avi-tagged species. One Shot BL21 Star (DE3) chemically competent E. coli (Invitrogen) were transformed with the requisite expression plasmids and grown in LB with kanamycin and chloramphenicol (eIF2β tetramer preps) or ampicillin (eIF2βγ preps). At an OD600 of ~0.8 I M IPTG (Gold Biotechnology) was added and the culture was grown overnight at 16 °C. Using the EmulsiFlex-C3 (Avestin), Cells were harvested and lysed through three cycles of high-pressure homogenization in lysis buffer (200 μM HEPES-KOH, pH 7.5, 250 mM KCl, 1 mM dithiothreitol (DTT), 5 mM MgCl2, 15 mM imidazole, and Complete EDTA-free protease inhibitor cocktail (Roche)). For eIF2βγ, preps 200 mM imidazole was used. The lysate was clarified at 30,000g for 30 min at 4 °C. Lysate was then clarified at 30,000g for 60 min at 4 °C.

All following purification steps were conducted on the AKTA Pure (GE Healthcare) system at 4 °C. Clarified lysate was loaded onto a 5 ml HisTrap HP column (GE Healthcare). For eIF2β tetramer preps the column was then washed in a buffer containing 20 mM HEPES-KOH, pH 7.5, 200 mM KCl, 1 mM DTT, 5 mM MgCl2, and 15 mM imidazole. For eIF2βγγβ, preps 30 mM KCl and 20 mM imidazole were used. The sample was then eluted with a linear gradient up to 300 mM imidazole. eIF2B containing fractions were collected and applied to a MonoQ HR 10/100 GL column (GE Healthcare) equilibrated in 20 mM HEPES-KOH pH 7.5, 5 mM MgCl2, and 15 mM imidazole, and Complete EDTA-free protease inhibitor cocktail (Roche)). For eIF2βγγβ, preps 200 mM imidazole was used. The lysate was clarified at 30,000 g for 30 min at 4 °C. Lysate was then clarified at 30,000g for 60 min at 4 °C.

For Avi-tagged species, after running samples over a MonoQ HR 10/100 GL column (GE Healthcare) equilibrated in 20 mM HEPES-KOH pH 7.5, 200 mM KCl, 1 mM DTT, and 5 mM MgCl2. For eIF2βγγβ, preps 30 mM KCl was used. The column was washed in the same buffer, and the protein was eluted with a linear gradient up to 500 mM KCl. eIF2β containing fractions were collected and concentrated with an Amicon Ultra-15 concentrator (EMD Millipore) with a 10 kDa molecular mass cutoff and flash frozen in liquid nitrogen.

In vitro NSs/eIF2α-P immunoprecipitation. Varying combinations of purified eIF2α-P, NSs6×His, eIF2βγγβ, eIF2βγγγ, and eIF2βγγγγ were incubated (with gentle rocking) with Anti-protein-C antibody-conjugated resin (generous gift from Ashish Manglik) in Assay Buffer (20 mM HEPES-KOH, pH 7.5, 150 mM KCl, 5 mM MgCl2, 1 mM TCEP, 1 mg/ml bovine serum albumin (BSA), 5 mM CaCl2). After 1.5 h the resin was pelleted by benchtop centrifugation and the supernatant was removed. Resin was washed 3× with 1 ml of ice cold Assay Buffer before resin was resuspended in Elution Buffer (Assay Buffer with 5 mM EDTA and 0.5 mg/ml protein C peptide added) and incubated with gentle rocking for 1 h. The resin was then pelleted and the supernatant was removed. Samples were analyzed by Western Blotting as described above.
100 nM eIF2B(αβγδε)2 + 2.5 nM FAM-ISRIB (Praxis Bioresearch) in FP buffer (20 mM HEPES-KOH pH 7.5, 100 mM KCl, 5 mM MgCl₂, 1 mM TCEP) and measured in 384-well non-stick black plates (Corning 3820) using the ClarionStar PLUS (BMG LabTech) at room temperature. Prior to reaction setup, eIF2B(αβγδε)2 was assembled in FP buffer using eIF2Bβγδε and eIF2βα at ~100 nM on a Biotin CAPture Series S sensor chip (Cytiva Life Sciences) to achieve maximum response (Rmax) of ~100 response units (RU) upon binding. A molar equivalent of each eIF2B species was immobilized. Twofold serial dilutions of purified NsNs were flowed over the captured eIF2B complexes at 30 μL/min for 90 s followed by 600 s of dissociation flow. Following each cycle, the chip surface was regenerated with 3 M guanidine hydrochloride. A running buffer of 20 mM HEPES-KOH pH 7.5, 100 mM KCl, 5 mM MgCl₂, 1 mM TCEP was used throughout. The resulting sensorgrams were fitted over the captured eIF2B complexes at 30 µL/min at 10°C and 30 min prior to measurement of parallel and perpendicular intensities (excitation: 482 nm, emission: 530 nm). Data were plotted in GraphPad Prism 8, and where appropriate, curves were fit to log[inhbitor] vs response function with variable slope.

Affinity determination and competition analysis by SPR. NsNs affinity determination experiments were performed on a Biacore T200 instrument (Cytiva Life Sciences) by capturing the biotinylated eIF2B(αβγδε)2, eIF2Bβγδε, and eIF2βα at ~100 nM on a Biotin CAPture Series S sensor chip (Cytiva Life Sciences) to achieve maximum response (Rmax) of ~100 response units (RU) upon binding. A molar equivalent of each eIF2B species was immobilized. Twofold serial dilutions of purified NsNs were flowed over the captured eIF2B complexes at 30 µL/min for 90 s followed by 600 s of dissociation flow. Following each cycle, the chip surface was regenerated with 3 M guanidine hydrochloride. A running buffer of 20 mM HEPES-KOH pH 7.5, 100 mM KCl, 5 mM MgCl₂, and 1 mM TCEP was used throughout. The resulting sensorgrams were fitted to a 1:1 Langmuir binding model using the association and then dissociation model in GraphPad Prism 8.0. For NsNs and eIF2/eIF2-P competition experiments, eIF2B(αβγδε)2 was immobilized as described above. A solution containing 500 nM NsNs, 125 nM eIF2, or 125 nM eIF2-P was flowed over the captured eIF2B for 60 s at 30 µL/min to achieve saturation. Following this binding reaction, a second injection of 500 nM NsNs and either 125 nM eIF2 or 125 nM eIF2-P was performed.

Sample preparation for cryo-electron microscopy. Decameric eIF2B(αβγδε)2 was prepared by incubating 20 μM eIF2Bβγδε with 11 μM eIF2βα, in a final solution containing 20 mM HEPES-KOH, pH 7.5, 200 mM KCl, 5 mM MgCl₂, and 1 mM TCEP. This 10 μM eIF2B(αβγδε)2 sample was further diluted to 750 nM and incubated with 2.25 μM NsNs::6xHIS on ice for 1 h before plunger freezing. A 3 µl aliquot of the sample was applied onto the Quantifoil R 1/2/1/3/4/0 mesh Gold grid and we waited for 30 s. A 0.5 μl aliquot of 0.1–0.2% Nonidet P-40 substitute was added immediately before blotting. The entire blotting procedure was performed using Vitrobot (FEI) at 10°C and 100% humidity.

Electron microscopy data collection. Cryo-EM data for the eIF2B–NsNs complex were collected on a Titan Krios transmission electron microscope operating at 300 keV, and micrographs were acquired using a Gatan K3 direct electron detector. Cryo-EM data for the eIF2B–NsNs complex were collected on a Titan Krios transmission electron microscope operating at 300 keV, and micrographs were acquired using a Gatan K3 direct electron detector. Cryo-EM data for the eIF2B–NsNs complex were collected on a Titan Krios transmission electron microscope operating at 300 keV, and micrographs were acquired using a Gatan K3 direct electron detector.

Image processing. The micrograph frames were aligned using MotionCorr2. The contrast transfer function (CTF) parameters were estimated with GCTF55. Particles were picked in Cryosparc v2.15 using the eIF2B EMDB: 23209 as a template. Particles were extracted using a 800 pixel box size, and classified in 2D77. Classes that showed clear protein features were selected and extracted for ab initio reconstruction followed by homogeneous and heterogeneous refinement. Particles belonging to the best class were then re-extracted with a pixel size of 2.09 Å, and then subjected to nonuniform refinement, yielding a reconstruction of 4.25 Å. These particles were subjected to another round of heterogeneous refinement followed by nonuniform refinement to generate a consensus reconstruction consisting of the best particles. These particles were re-extracted at a pixel size of 0.835 Å. Then, CTF refinement was performed to correct for the per-particle CTF as well as beam tilt. A final round of 2D classification followed by nonuniform refinement was performed to yield the final structure of 2.6 Å.

Atomic model building, refinement, and visualization. To build models for the eIF2B–Nss complex, the previously determined structures of the human eIF2B in its apo form [PDB 2J70] was used as the starting model for the eIF2B part2. To build the NsNs model, we first ran the structure prediction program RaptorX using the full-length NsNs sequence. The predicted structure is divided into two parts: the C-terminal domain predicted based on the structure of the RSVF NsNs (PDB 5000) and the N-terminal domain is predicted without a known PDB structure as a template.31 The predicted full-length structure was docked into the EM density corresponding to the NsNs in UCSF Chimera, and then subjected to rigid body refinement in phenix40. The models were then manually adjusted in Coot and then refined in phenix.real_space_refine using global minimization, secondary structure restraints, Ramachandran restraints, and local grid search. Then iterative cycles of manual rebuilding in Coot and phenix.real_space_refine were performed. The final model statistics were tabulated using Molprobity62. Distances were calculated from the atomic models using UCSF Chimera. Molecular graphics and analyses were performed with the UCSF Chimera package.59, UCSF Chimera is developed by the Resource for Biocomputing, Visualization, and Informatics and supported by NIH U54 Grant P41-GM103311. The atomic model is deposited in the PDB under accession code 7RLO. The EM map is deposited into EMDB under accession code EMD-24535.

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

Data availability. The data that support this study are available from the corresponding author upon reasonable request. The cryo-EM structure generated in this study has been deposited in the protein data bank under the accession code 7RLO. The corresponding EM map has been deposited in the EM database under the accession code EMD-24535. The structure of the RVFV NsNs used for model building is available in the protein data bank under the accession code 5000. Source data are provided with this paper.

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31. Kaur, K. et al. M.S. and J.Z.C. generated the cell lines, and performed the experiments. M.S., L.W., J.Z.C., and R.E.L. designed the experiments. P.W. supervised the research. M.S., L.W., J.Z.C., and R.E.L. designed the experiments. P.W. is an inventor on U.S. Patent 9708247 held by the Regents of the University of California that describes ISRIB and its analogs. Rights to the invention have been licensed to Calico Life Sciences LLC for a generous gift of purified eIF2 heterotetramer; Z. Yu and D. Bulkley of the UCSF Center for Advanced Cryo-EM facility, which is supported by NIH grants S100OD021741 and S100OD020054; and the Howard Hughes Medical Institute (HMI). We also thank the QB3 shared cluster for computational support. This work was supported by generous support from Calico Life Sciences LLC (to P.W.); a generous gift from The George and Judy Marcus Family Foundation (to P.W.); the Belgian-American Educational Foundation (BAEF) Postdoctoral Fellowship (to M.B.), the Damon Runyon Cancer Research Foundation Postdoctoral Fellowship (to L.W.); and the Jane Coffin Child Foundation Postdoctoral Fellowship (to R.E.L.). A.F. is a HHMI faculty scholar and a Chan Zuckerberg Biohub investigator. P.W. is an investigator of the Howard Hughes Medical Institute.

Author contributions
P.W. supervised the research. M.S., L.W., J.Z.C., and R.E.L. designed the experiments. M.S. performed all cloning. M.S., J.Z.C., R.E.L., and M.B. expressed and purified proteins. M.S. and J.Z.C. generated the cell lines, and performed the flow cytometry experiments. M.S. performed the binding assays (SPR and bead immobilization). M.S., J.Z.C., and R.E.L. performed the nucleotide exchange assays. M.S., J.Z.C., and M.B. performed the FAM-ISRIB-binding assays. M.S. performed all western blotting. L.W. performed cryo-EM sample preparation, data collection, processing, and model building with A.F. providing additional model building input. M.S., L.W., and P.W., prepared the rough manuscript draft, with finalizing input from all authors including J.Z.C., R.E.L., M.B., J.D.W., and A.F.

Competing interests
P.W. is an inventor on U.S. Patent 9708247 held by the Regents of the University of California that describes ISRIB and its analogs. Rights to the invention have been licensed by UCSF to Calico. The remaining authors declare no competing interests.

Additional information
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