

Supporting Information for

Definition of a saxitoxin (STX) binding code enables discovery and characterization of the anuran saxiphilin family

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This PDF file includes:

Supplementary Materials and Methods Figures S1 to S12 Tables S1 to S2 Legends for Movies S1 to S4 SI References

Other supporting materials for this manuscript include the following:

Movies S1 to S4

Supplementary Materials and Methods

Expression and purification of Sxphs and mutants

R. catesbeiana Sxph (*Rc*Sxph) and mutants were expressed using a previously described RcSxph baculovirus expression system in which RcSxph carries in series, a C-terminal 3C protease cleavage site, green fluorescent protein (GFP), and a His₁₀ tag (1). The gene encoding Nanorana parkeri Sxph (NpSxph) including its N-terminal secretory sequence (GenBank: XM 018555331.1) was synthesized and subcloned into a pFastBac1 vector using Notl and Xhol restriction enzymes by GenScript and bears the same C-terminal tags as RcSxph. RcSxph and NpSxph mutants were generated using the QuikChange site-directed mutagenesis kit (Stratagene). All constructs were sequenced completely. RcSxph, RcSxph mutants, NpSxph, NpSxph I559Y, MaSxph, OsSxph, and RiSxph were expressed in Spodoptera frugiperda (Sf9) cells using a baculovirus expression system as described previously for RcSxph (1) and purified using a final size exclusion chromatography (SEC) run in 150 mM NaCl, 10 mM HEPES, pH 7.4. Protein concentrations were determined by measuring UV absorbance at 280 nm using the coefficients calculated usina ExPASY following extinction the server (https://web.expasy.org/protparam/): RcSxph Y558 mutants, 94,875 M⁻¹ cm⁻¹; RcSxph F784C 96.490 M⁻¹ cm⁻¹: *Rc*Sxph F784Y 97,855 M⁻¹ cm⁻¹, *Rc*Sxph and all other *Rc*Sxph mutants 96,365 M⁻¹ cm⁻¹; *Np*Sxph 108,980 M⁻¹ cm⁻¹; *Np*Sxph I559Y 110,470 M⁻¹ cm⁻¹; *Ma*Sxph 93,175 M⁻¹ cm⁻¹; *Os*Sxph 100,625 M⁻¹ cm⁻¹; and *Ri*Sxph 103 605 M⁻¹ cm⁻¹.

Thermofluor (TF) assay of toxin binding

Thermofluor assays for STX and TTX binding were developed as outlined (2). TTX was purchased from Abcam (Catalog # ab120054). 20 µL samples containing 1.1 µM *Rc*Sxph, *Np*Sxph, *Ma*Sxph, *Os*Sxph, *Ri*Sxph, or mutants thereof, 5x SYPRO Orange dye (Sigma-Aldrich, S5692, stock concentration 5000x), 0-20 µM STX or TTX, 150 mM NaCl, 10 mM HEPES, pH 7.4 were set up in 96-well PCR plates (Bio-Rad), sealed with a microseal B adhesive sealer (Bio-Rad) and centrifuged (1 min, 230xg) prior to thermal denaturation. The real-time measurement of fluorescence using the HEX channel (excitation 515-535 nm, emission 560-580 nm) was performed in CFX Connect Thermal Cycler (Bio-Rad). Samples were heated from 25°C to 95°C at 0.2°C min⁻¹. Melting temperature (Tm) was calculated by fitting the denaturation curves using a Boltzmann sigmoidal function and GraphPad Prism: $F=F_{min}+(F_{max}-F_{min})/(1+exp((Tm-T)/C))$, where F is the fluorescence intensity at temperature T, F_{min} and F_{max} are the fluorescence intensities before and after the denaturation transition, respectively, Tm is the midpoint temperature of the thermal unfolding transition, and C is the slope at Tm (2). Δ Tm=Tm_{Sxph+20µM toxin}-Tm_{Sxph}.

Fluorescence polarization assay

Fluorescence polarization assays were performed as described (3). 100 µL samples containing 1 nM fluorescein labeled STX (F-STX), 150 mM NaCl, 10 mM HEPES, pH 7.4, and Sxph variants at the following concentration ranges (RcSxph and RcSxph T563A, 1782A, F784Y, D785N, Q787A, Q787E, K789A, and Y795A, 0-75 nM; RcSxph Y558A and I782A/Y558A, 0-24 nM; RcSxph Y558I, 0-37.5 nM; RcSxph Y558F, 0-100 nM; RcSxph 1782F 0-150 nM; RcSxph F561A, 0-300 nM; RcSxph F784L, 0-500 nM; RcSxph E540D, P727A, and D785A, 0-600 nM; RcSxph F784A, 0-4.8 µM; RcSxph E540A and F784C, 0-10 µM; RcSxph D794A and D794E, 0-12.5 µM; RcSxph F784S, 0-17 µM; RcSxph D794N, 0-20 μM; RcSxph E540Q, 0-25 μM; NpSxph; NpSxph I559Y, 0-75 nM; MaSxph, 0-75 nM; OsSxph, 0-75 nM; and RiSxph, 0-75 nM) were prepared in 96-well black flat-bottomed polystyrene microplates (Greiner Bio-One) and sealed with an aluminum foil sealing film (AlumaSeal II), and incubated at room temperature for 0.5 h before measurement. Measurements were performed at 25°C on a Synergy H1 microplate reader (BioTek) using the polarization filter setting (excitation 485 nm, emission 528 nm). Binding curves for representative high affinity (RcSxph, NpSxph, and RcSxph-Y558I) and low affinity (RcSxph-E540D) proteins were compared at 0.5 h, 1.5 h, 4.5 h, and 24 h, post mixing and indicated that equilibrium was reached by 0.5 h for all samples. The dissociation constants were calculated using GraphPad Prism by fitting fluorescence millipolarization $(mP=P\cdot 10^{-3})$, where P is polarization) as a function of Sxph concentration using the equation: P={(P_{bound}-P_{free}) [Sxph]/(K_d+[Sxph])}+P_{free}, where P is the polarization measured at a given Sxph concentration, Pfree is the polarization of Sxph in the absence of F-STX, and P_{bound} is the maximum polarization of Sxph bound by F-STX (3, 4).

Isothermal titration calorimetry (ITC)

ITC measurements were performed at 25°C using a MicroCal PEAQ-ITC calorimeter (Malvern Panalytical). *Rc*Sxph, *Rc*Sxph mutants, *Np*Sxph, and *Np*Sxph I559Y were purified using a final size exclusion chromatography step in 150 mM NaCl, 10 mM HEPES, pH 7.4. 1 mM STX stock solution was prepared by dissolving STX powder in MilliQ water. This STX stock was diluted with the SEC buffer to prepare 100 μ M or 300 μ M STX solutions having a final buffer composition of 135 mM NaCl, 9 mM HEPES, pH 7.4. To match buffers between the Sxph and STX solutions, the purified protein samples were

diluted with MilliQ water to reach a buffer concentration of 135 mM NaCl, 9 mM HEPES, pH 7.4. (30 μ M for *Rc*Sxph D794E;10 μ M for *Rc*Sxph, other *Rc*Sxph mutants, *Np*Sxph, and *Np*Sxph I559Y) Protein samples were filtered through a 0.22 μ m spin filter (Millipore) before loading into the sample cell and titrated with STX (300 μ M STX for *Rc*Sxph D794 and 100 μ M STX for *Rc*Sxph, other *Rc*Sxph mutants, *Np*Sxph, and *Np*Sxph I559Y) using a schedule of 0.4 μ L titrant injection followed by 35 injections of 1 μ L for the strong binders (*Rc*Sxph, *Rc*Sxph Y558I, *Rc*Sxph Y558A, *Rc*Sxph F561A, *Np*Sxph, and *Np*Sxph I559Y) and a schedule of 0.4 μ L titrant injection followed by 18 injections of 2 μ L for the weak binders (*Rc*Sxph P727A, *Rc*Sxph E540D, and *Rc*Sxph D794E). The calorimetric experiment settings were: reference power, 5 μ cal/s; spacing between injections, 150 s; stir speed 750 rpm; and feedback mode, high. Data were analyzed using MicroCal PEAQ-ITC Analysis Software (Malvern Panalytical) using a single binding site model. The heat of dilution from titrations of 100 μ M STX in 135 mM NaCl, 9 mM HEPES, pH 7.4 into 135 mM NaCl, 9 mM HEPES, pH 7.4 was subtracted from each experiment to correct the baseline.

Crystallization, structure determination, and refinement

*Rc*Sxph mutants were crystallized at 4°C as previously described for *Rc*Sxph (1). Briefly, purified protein was exchanged into a buffer of 10 mM NaCl, 10 mM HEPES, pH 7.4 and concentrated to 65 mg ml⁻¹ using a 50-kDa cutoff Amicon Ultra centrifugal filter unit (Millipore). Crystallization was set up by hanging drop vapor diffusion using a 24-well VDX plate with sealant (Hampton Research) using 3 μ L drops having a 2:1 (v:v) ratio of protein:precipitant. For co-crystallization with STX, STX and the target *Rc*Sxph mutants were mixed in a molar ratio of 1.1:1 STX:Sxph and incubated on ice for 1 hour before setting up crystallization. *Rc*Sxph-Y558I and *Rc*Sxph-Y558I:STX were crystallized from solutions containing 27% (v/v) 2-methyl-2,4-pentanediol, 5% (w/v) PEG 8000, 0.08-0.2 M sodium cacodylate, pH 6.5. *Rc*Sxph-Y558A and *Rc*Sxph-Y558A:STX were crystallized from solutions containing 33% (v/v) 2-methyl-2,4-pentanediol, 5% (w/v) PEG 8000, 0.08-0.2 M sodium cacodylate, pH 6.5. To obtain crystals of the *Rc*Sxph:F-STX complex, *Rc*Sxph was crystallized from solutions containing 33% (v/v) 2-methyl-2,4-pentanediol, 5% (w/v) PEG 8000, 0.11-0.2 M sodium cacodylate, pH 6.5 and then soaked with F-STX (final concentration, 1 mM) for 5 hours before freezing.

For *Np*Sxph crystallization, protein was purified as described for *Rc*Sxph, except that the final size exclusion chromatography was done using 30 mM NaCl, 10 mM HEPES, pH

7.4. Protein was concentrated to 30-40 mg ml⁻¹ using a 50-kDa cutoff Amicon Ultra centrifugal filter unit (Millipore). *Np*Sxph crystals were obtained by hanging drop vapor diffusion at 4°C using 1:1 v/v ratio of protein and precipitant. *Np*Sxph crystals were obtained from 400 nl drops set with Mosquito crystal (Sptlabtech) using 20-25% (v/v) PEG 400, 4-5% (w/v) PGA-LM, 100-200 mM sodium acetate, pH 5.0. For STX co-crystallization, *Np*Sxph and STX (5 mM stock solution prepared in MilliQ water) were mixed in a molar ratio of 1.2:1 STX:*Np*Sxph and incubated on ice for 1 hour before setting up the crystallization trays. For F-STX soaking, *Np*Sxph crystals were soaked with F-STX (final concentration, 1 mM) for 5 hours before freezing. Crystals of the *Np*Sxph:STX complex were grown in the same crystallization solution as *Np*Sxph. *Np*Sxph, *Np*Sxph:STX, and *Np*Sxph:F-STX crystals were harvested and flash-frozen in liquid nitrogen without additional cryoprotectant.

X-ray datasets for *Rc*Sxph mutants, *Rc*Sxph mutant:STX complexes, *Rc*Sxph: F-STX, *Np*Sxph, and *Np*Sxph:STX were collected at 100K at the Advanced Photon Source (APS) beamline 23 ID B of Argonne National Laboratory (Lemont, IL), processed with XDS (5) and scaled and merged with Aimless(6). *Rc*Sxph structures were determined by molecular replacement of *Rc*Sxph chain B from (PDB: 600F) using Phaser from PHENIX (7). The resulting electron density map was thereafter improved by rigid body refinement using phenix.refine. The electron density map obtained from rigid body refinement was manually checked and rebuilt in COOT (*8*) and subsequent refinement was performed using phenix.refine.

The *Np*Sxph structure was solved by molecular replacement using the MoRDa pipeline implemented in the Auto-Rikshaw, automated crystal structure determination platform (9). The scaled X-ray data and amino-acid sequence of *Np*Sxph were provided as inputs. The molecular replacement search model was identified using the MoRDa domain database derived from the Protein Data Bank (PDB). The MR solution was refined with REFMAC5 (*10*), density modification was performed using PIRATE(*11, 12*), and was followed by the automated model building in BUCCANEER (*13, 14*). The partial model was further refined using REFMAC5 and phenix.refine. Dual fragment phasing was performed using OASIS-2006 (*12*) based on the automatically refined model, and the resulting phases were further improved in PIRATE. The next round of model building was continued in ARP/wARP (*15*) and the resulting structure was refined in REFMAC5. The final model generated in Auto-Rikshaw (720 out of 825 residues built, and 625 residues automatically docked) was

further used as a MR search model in Phaser from PHENIX (7). The quality of the electron density maps allowed an unambiguous assignment of most of the amino acid residues with the exception of the loop regions and the C2 subdomain showing poor electron density. The apo-*Np*Sxph structure was completed by manual model building in COOT (8) and multiple rounds of refinement in phenix.refine. The *Np*Sxph:STX: structure was solved by molecular replacement using the *Np*Sxph structure as a search model in Phaser from PHENIX (7). After multiple cycles of manual model rebuilding in COOT (8), iterative refinement was performed using phenix.refine. The quality of all models was assessed using MolProbity (*16*) and refinement statistics.

RNA sequencing of *O. sylvatica*, *D. tinctorius*, *R. imitator*, *E. tricolor*, *A. femoralis*, and *M. aurantiaca* Sxphs

Nearly all poison frog species were bred in the O'Connell Lab or purchased from the pet trade (Josh's Frogs) except for O. sylvatica, which was field collected as described in (17). De novo transcriptomes for O. sylvatica, D. tinctorius, R. imitator, E. tricolor, A. femoralis, and *M. aurantiaca* were constructed using different tissue combinations depending on the species. RNA extraction from tissues was performed using TRIzol™ Reagent (Thermo Fisher Scientific). Poly-adenylated RNA was isolated using the NEXTflex PolyA Bead kit (Bioo Scientific, Austin, USA) following manufacturer's instructions. RNA guality and lack of ribosomal RNA was confirmed using an Agilent 2100 Bioanalyzer or Tapestation (Agilent Technologies, Santa Clara, USA). Each RNA sequencing library was prepared using the NEXTflex Rapid RNAseq kit (Bioo Scientific). Libraries were quantified with quantitative PCR (NEBnext Library quantification kit, New England Biolabs, Ipswich, USA) and an Agilent Bioanalyzer High Sensitivity DNA chip, according to manufacturer's instructions. All libraries were pooled at equimolar amounts and were sequenced on four lanes of an Illumina HiSeq 4000 machine to obtain 150 bp paired-end reads. De novo transcriptomes were assembled using Trinity and once assembled were used to create a BLAST nucleotide database using the BLAST+ command line utilities. The amino acid Sxph sequence of *R. catesbeiana* was used as a query to tBLASTN against the reference transcriptome databases. The Sxph sequence for O. sylvatica was lacking the 5' and 3' ends, whose sequence was obtained using RACE as described above. After obtaining a full-length sequence, the top BLAST hits from each poison frog transcriptome were manually inspected and aligned to the O. sylvatica nucleotide sequence to find full sequences with high similarity. Either a single Sxph sequence from each transcriptome was found to be the best match, or there were multiple transcripts that aligned well, in which case a consensus alignment was created. The largest ORF from each species sequence was translated to create an amino acid sequence for alignment. For the *D. tinctorius*, *R. imitator*, and *A. femoralis* sequences, regions covering the STX binding site and transferrin-related iron-binding sites were confirmed by PCR and sanger sequencing.

Identification of P. terribilis, R. marina, B. bufo, and B. gargarizans Sxphs

All P. terribilis frogs were captive bred in the O'Connell lab poison frog colony. All were sexually mature individuals housed in 18x18x18-inch glass terraria, brought up on a diet of Drosophila melanogaster without additional toxins. Frogs were euthanized according to the laboratory collection protocol detailed by (18) and tissues were stored in RNALater. Eye tissue was rinsed in PBS before being placed into the beadbug tubes (Sigma-Aldrich, Z763756) prefilled with 1 mL TRIzol (Thermo Fisher Scientific, 15596018) and then RNA was extracted following manufacturer instructions. RNA was reverse transcribed into cDNA following the protocol outlined in Invitrogen's SuperScript IV Control Reactions First-Strand cDNA Synthesis reaction (Pub. no. MAN0013442, 16 Rev. B). After reverse transcription, cDNA concentration was checked via NanoDrop (Thermo Scientific, ND-ONE-W), and then aliguoted and stored at -20°C until used for PCR. Saxiphilin was amplified from cDNA from P. terribilis in 50 µL polymerase chain reactions following the New England Biolabs protocol for Phusion® High-Fidelity PCR Master Mix with HF Buffer (30) (included DMSO). Each reaction was performed with 1 µL of cDNA. PCR primers were designed based on a O. sylvatica saxiphilin cDNA sequence previously generated by the O'Connell lab. PCR products were cleaned up using the Thermo Scientific GeneJET Gel Extraction and DNA Cleanup Micro Kit (Catalog number K0832) dimer removal protocol, and then sent out for Sanger Sequencing via the GeneWiz "Premix" service. The segments from sequencing were aligned and assembled but found that the 5' and 3' ends of the Sxph sequence for *P. terribilis* were missing, thus the 5' and 3' end sequences were subsequently obtained using RACE. 5' and 3'-RACE-Ready cDNA templates were synthesized using a SMARTer® RACE 5'/3' Kit (Takara Bio, USA) and subsequently used to amplify 5' and 3' end sequences of P. terribilis Sxph using internal gene specific primers.

Initial Sxph sequence for *R. marina* was obtained from the genome by searching the draft Cane Toad genome (19) with tBLASTN using the *R. catesbeiana* Sxph amino acid sequence as a query. Matching segments from the genome were pieced together to produce an amino acid sequence, however, this sequence was missing part of the 3' end. To obtain the 3' residues, the nucleotide sequences from the genome were used to design primers for 3' Rapid Amplification of cDNA Ends (RACE). One *R. marina* individual from a lab-housed colony was thus euthanized in accordance with UCSF IACUC protocol AN136799, and a portion of the liver was harvested for total RNA extraction using TRIzolTM Reagent (Thermo Fisher Scientific). Total RNA integrity was assessed on a denaturing formaldehyde agarose gel. 3'-RACE-Ready cDNA template was synthesized using a SMARTer® RACE 5'/3' Kit (Takara Bio, USA) and subsequently used to amplify 3' end sequences of *R. marina* Sxph using internal gene specific primers designed from *R. marina* genomic sequences. 3' end sequences of *R. marina* Sxph were determined by gel extraction using QIAquick Gel Extraction Kit (QIAGEN) and verified by sanger sequencing.

Sequences for *Bufo bufo* (CaucascianToad) and *Bufo gargarizans* (Asiatic toad) Sxphs were identified as sequence searches (tBLASTN) using the *Rm*Sph sequence as a query.

Two-electrode voltage clamp electrophysiology

Two-electrode voltage-clamp (TEVC) recordings were performed on defolliculated stage V–VI *Xenopus laevis* oocytes harvested under UCSF-IACUC protocol AN178461. Capped mRNA for *P. terribilis (Pt)* Na_V1.4 (GenBank: MZ545381.1) expressed in a pCDNA3.1 vector (*20*) was made using the mMACHINE[™] T7 Transcription Kit (Invitrogen). *Xenopus* oocytes were injected with 3–6 ng of *Pt* Na_V1.4 and TEVC experiments were performed 1–2 days post-injection. Data were acquired using a GeneClamp 500B amplifier (MDS Analytical Technologies) controlled by pClamp software (Molecular Devices), and digitized at 1 kHz using Digidata 1332A digitizer (MDS Analytical Technologies).

Oocytes were impaled with borosilicate recording microelectrodes (0.3–3.0 M Ω resistance) backfilled with 3 M KCI. Sodium currents were recorded using a bath solution containing the following, in millimolar: 96, NaCI; 1, CaCI₂; 1, MgCI₂; 2, KCI; and 5, HEPES (pH 7.5 with NaOH), supplemented with antibiotics (50 µg ml⁻¹ gentamycin, 100 IU ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin) and 2.5 mM sodium pyruvate. Sxph responses were measured using Sxph or Sxph mutants purified as described above. Following recording of channel behavior in the absence of toxin, 100 nM STX was applied to achieve ~90% block. Sxph was then added directly to a 1 mL recording chamber containing the toxin to the desired concentration. For all [Sxph]:[STX] ratios, the concentration of the

stock Sxph solution added to the chamber was adjusted so that the volume of the added Sxph solution was less than 1% of the total volume of the recording chamber. All toxin effects were assessed with 60-ms depolarization steps from -120 to 0 mV with a holding potential of -120 mV and a sweep-to-sweep duration of 10 s. Recordings were conducted at room temperature (23 \pm 2 °C). Leak currents were subtracted using a P/4 protocol during data acquisition. Data Analysis was performed using Clampfit 10.6 (Axon Instruments) and SigmaPlot (Systat Software).

F-STX synthesis

All reagents were obtained commercially unless otherwise noted. *N*,*N*-Dimethylformamide (DMF) was passed through two columns of activated alumina prior to use. High-performance liquid chromatography-grade CH₃CN and H₂O were obtained from commercial suppliers. Semi-preparative high-performance liquid chromatography (HPLC) was performed on a Varian ProStar model 210. A high-resolution mass spectrum of F-STX was obtained from the Vincent Coates Foundation Mass Spectrometry Laboratory at Stanford University. The sample was analyzed with HESI-MS by direct injection onto Waters Acquity UPLC and a Thermo Fisher Orbitrap ExplorisTM 240 mass spectrometer scanning m/z 100–1000. F-STX was quantified by ¹H NMR spectroscopy on a Varian Inova 600 MHz NMR instrument using distilled DMF as an internal standard. A relaxation delay (d1) of 20 s and an acquisition time (at) of 10 s were used for spectral acquisition. The concentration of F-STX was determined by integration of ¹H signals corresponding to F-STX and a fixed concentration of the DMF standard.

To an ice-cold solution of saxitoxin-N21-hexylamine (1.4 µmol) in 140 µL of pH 9.5 aqueous bicarbonate buffer (0.2 M aqueous NaHCO₃, adjusted to pH 9.5 with 1 M aqueous NaOH) was added a solution of fluorescein NHS-ester, 6-isomer (2.0 mg, 4.2 µmol, 3.0 equiv, Lumiprobe) in 140 µL of DMSO. The reaction flask was stoppered, wrapped in foil, and placed in a sonication bath for 30 s. The reaction mixture was then stirred at room temperature for 4 h. Following this time, the reaction was quenched by the addition of 0.3 mL of 1% aqueous CF₃CO₂H. The reaction mixture was diluted with 1.1 mL of 10 mM aqueous CF₃CO₂H and 0.3 mL of DMSO and filtered through a VWR 0.22 µm PTFE filter. The product was purified by reverse-phase HPLC (Silicycle SiliaChrom dt C18, 5 µm, 10 x 250 mm column, eluting with a gradient flow of 10 \rightarrow 40% CH₃CN in 10 mM aqueous CF₃CO₂H over 40 min, 214 nm UV detection). At a flow rate of 4 mL/min, F-STX

had retention time of 31.00 min and was isolated as a dark yellow powder following lyophilization (1.08 µmol, 77%, ¹H NMR quantitation).

¹H NMR (600 MHz, D₂O) δ 8.05 (d, *J* = 8.1 Hz, 1H), 7.94 (d, *J* = 8.9 Hz, 1H), 7.48 (s, 1H), 6.95 (d, *J* = 9.0 Hz, 2H), 6.79 (s, 2H), 6.67 (dt, *J* = 9.1, 2.2 Hz, 2H), 4.60 (d, *J* = 1.2 Hz, 1H), 4.09–4.05 (m, 1H), 3.89 (dd, *J* = 11.6, 5.2 Hz, 1 H), 3.70 (dt, *J* = 10.1, 5.5 Hz, 1H), 3.64 (dd, *J* = 8.7, 5.4 Hz, 1H), 3.47–3.42 (m, 1H), 3.27 (t, *J* = 6.6 Hz, 2 H), 2.97–2.89 (m, 2H), 2.36–2.33 (m 1H), 2.30–2.24 (m, 1H), 1.48–1.45 (m, 2H), 1.32–1.29 (m, 2H), 1.25–1.21 (m, 4H) ppm. HRMS (ESI⁺) calcd for C₃₇H₄₁N₈O₁₀, 757.2940; found 757.2918 (M⁺).



Fig. S1. *RcSxph* thermofluor (TF) assay. **A**, Exemplar thermofluor (TF) assay results for *RcSxph* in the presence of the indicated concentrations of STX. Curves for *RcSxph*, E540A, P727A, Y558A, F561A, and T563A are identical to those shown in Figs. 1A and 1B. Δ Tm values are indicated. **B**, Baseline Tm values for *RcSxph* and the indicated mutants. C, Plot of Tm vs. Δ Tm for the proteins

in 'B'. Colored boxes in 'A' and bars in 'B' correspond to $\Delta\!\Delta G$ classifications in Table 1. Error bars are S.E.M.



Fig. S2. F-STX NMR spectrum. ¹H NMR (600 MHz, D₂O) δ 8.05 (d, J = 8.1 Hz, 1H), 7.94 (d, J = 8.9 Hz, 1H), 7.48 (s, 1H), 6.95 (d, J = 9.0 Hz, 2H), 6.79 (s, 2H), 6.67 (dt, J = 9.1, 2.2 Hz, 2H), 4.60 (d, J = 1.2 Hz, 1H), 4.09–4.05 (m, 1H), 3.89 (dd, J = 11.6, 5.2 Hz, 1 H), 3.70 (dt, J = 10.1, 5.5 Hz, 1H), 3.64 (dd, J = 8.7, 5.4 Hz, 1H), 3.47–3.42 (m, 1H), 3.27 (t, J = 6.6 Hz, 2 H), 2.97–2.89 (m, 2H), 2.36–2.33 (m 1H), 2.30–2.24 (m, 1H), 1.48–1.45 (m, 2H), 1.32–1.29 (m, 2H), 1.25–1.21 (m, 4H) ppm.



Fig. S3. Structure of the *RcSxph*:F-STX complex. **A**, Exemplar electron density (1σ) for *RcSxph* (deep teal) and F-STX (yellow). **B**, *RcSxph*:F-STX B-factors for the F-STX ligand and select binding site residues. **C**, Superposition of the STX binding sites of the *RcSxph*:F-STX and *RcSxph*:STX (PDB:600F) (blue) (1) complexes.

Figure S4



Fig. S4. *RcSxph* fluorescence polarization (FP) assay. Exemplar FP binding curves and Kds for *RcSxph* and the indicated mutants. Curves for *RcSxph*, E540A, P727A, Y558A, F561A, and T563A are identical to those shown in Fig. 1D. Colored boxes and lines in correspond to $\Delta\Delta G$ classifications in Table 1. Error bars are S.E.M.



Fig. S5. *RcSxph* and *NpSxph* Isothermal titration calorimetry. Exemplar ITC isotherms for **A**, 100 μ M STX into 10 μ M *RcSxph* Y558A, **B**, 100 μ M STX into 10 μ M *RcSxph* Y558I, C, 100 μ M STX into 10 μ M *RcSxph* Y558I, C, 100 μ M STX into 10 μ M *RcSxph* Y558I, C, 100 μ M STX into 10 μ M *RcSxph* Y558I, C, 100 μ M STX into 10 μ M *RcSxph* Y558I, C, 100 μ M STX into 10 μ M *RcSxph* Y558I, C, 100 μ M STX into 10 μ M *RcSxph* Y558I, C, 100 μ M STX into 10 μ M *RcSxph* Y558I, C, 100 μ M stx into 7.9 μ M *NpSxph* I559Y. **F**, Comparison of Δ G_{ITC} for STX and Δ G_{FP} for F-STX for *RcSxph*, *NpSxph*, and indicated mutants. Purple box highlights region of good correlation. Orange box indicates region outside of the ITC dynamic range. *RcSxph* data are identical to Fig. 1G.

Figure S6



Fig. S6. *Rc***Sxph Y558A and** *Rc***Sxph-Y558I structures and STX complexes.** Exemplar electron density (1.5 σ) for **A**, *Rc*Sxph Y558A (purple), **B**, *Rc*Sxph-Y558A:STX (light blue), **C**, *Rc*Sxph-Y558I (pale yellow), and **D**, *Rc*Sxph-Y558I:STX (splitpea). Select residues and STX are indicated.

Figure	e S7		SP	Ν1β1Ν1	α1N1	β 2N1	α 2N1	β 3N1	α 3Ν1	β 4 Ν1	T1 α 1T	Ch	en e <i>t al.</i>
RcSxph -19 NpSxph -19 - MaSxph -19 - DtSxph -19 - OSSxph -19 - RiSxph -30 M PtSxph -21 - EtSxph -27 - AfSxph -21 -	RR SS YA ASQ EM M M M M M M VS SR KI	APT FQT A ALT FHT A ALT FQT A RMT PQVL RMT LRVL RMT - LRVL RMT TAFQVL RMT FAFQVL RMT FALLG L	L F F T I I S L L Y F T I VG L L Y F T I I G L L C L C L V L L C L C L W A L L C V C L A L L C V C L A L L C L C L W V L L C L C L V L	S F A A P N AK QV R W C S F A AS D A R H VQ W C S F V T S S A R D V R W C S S A A P N I R NV R W C S S A A P N S R NV R W C S S A A P N S R NV R W C S S A A P N A R NV R W C S S A A P N A R NV R W C S S A A P N S R T V R W C	A I S DLEQ K K CN DL V T I S H LEQK K CN DL V VI S D LEQK K CN DL V T S DT E EQK CN DL V Y T S DA E EQK CN DL V Y T S DA E EHK CN DL V T S DA E EHK CN DL V	G S C NV P D I T L V C V L G S C NV P D I T L A C V Y G S C XV S G I T L V C V H T S C HV D E I L L I C V K T S C HV D E I L L I C V K T S C HV D E I L L I C V K T S C HV D E I L L I C V K N S C HV D I L L K C V K N S C HV D I L L K C V K	R SSTED CMT AI K R SSTEN CMAAI K R SSTEN CMT AI F K SSTED CVR AI S K SSTED CVR AI S	C D GQ AD AM F L D S C D GQ AD AM F L D S C D EQ AD AM F L D S S N G E AD A I S L D S S N G E AD A I S L D S S N G E AD A I S L D S S N G E AD A I S L D S S N G E AD A I S L D S S N G E AD A I S L D S S N G E AD A I S L D S	GE VY EA SK DP GD VY KA S L DH GD VY KA S L DP KD VY KA S L HP KD VY KA S L HP KD VY KA S L DP	Y N LK P I I A EP Y S Y N LK P I I A EP Y S Y N LK P I I A ES Y S F N LK P I MT EA Y S F N LK P I MT EA Y S F N LK P I MT EA Y S F N LK P I MT EG Y S F N LK P I MT EG Y S	SN RD LQ KC LK ER QQ / LH RE LT KC LK HR QE S SH KE CLK KR QE C - ER EHT PCMR HR QS V - ES EHT PCMR HR QS V - ES EHT PCMR HR QS V - EK EHT PCMR HR QS V - EK EHT PCMR HR QS V - ER EHT PCMR HR QS V	AL - A KK MI - G HY I S LGG DK MV KG RY I A LG GAN V L - GQ F V V LG GKN MK I GA F V V LG GK MI I GA F V V LG GK KI K I GA F V V LG GK KI K I GA F V V LG GK KMK I GA F V V LG GK Q LI I GA F V	P QC DEKGNYQ 118 P QC DEKGNYH 120 P QC DEKG YQ 116 P KC DEKGNYV 119 P KC DEKGNYV 119 P KC DEKGNYV 119 P KC DEKGNYV 119 P KC DEKGNYA 146 P KC DEKGNYA 140
ji I	3 1Τ β2 1	-		Тр						Т2	α 2Τ		β 3Τ β 4Τ
RcSxph 138 P NpSxph 121 P MaSxph 117 P OrsSxph 120 P Possph 120 P Prisxph 120 P Ptsxph 120 P Ptsxph 120 P Afsxph 120 P Afsxph 120 P	QQ CH GS TG HCW VQ CH AS TG YCW QQ CY GS TG HCW KQ CH GS TG YCW KQ CH GS TG YCW KQ CH GS TG YCW KQ CH GS TG YCW KQ CH GS SG YCW KQ CH GS SG YCW	C VN AM GEK I C VN AN GEK I C VN AI GEEI C LN EN GEEI	S G T N T P P G E G T N T T P V V G T K T Q P G E G T R T P P G K G T R S R P G	Q TR AT Q T P AT Q T Q A T N S G L T CE NK AN K P F N S G L T CE NK AN K P F N P G L T CE NK AN K P F T K L L T CE D T A D K P T K S L T CE D A A NK P T K V L T CE D A A NK P	CLKERQKLLSAK CLKERQKLLSAK CLKERQKLLSAK CLKERQKLLSAK CLKERQKLLSSK CLKERQKLLSSK CLKERQKLLSSK	P S P A V F V P E C D E K G P S P A V F V P E C D E K G P S P A V F V P E C D E K G P S P A V F V P E C D E K G P A P T V F V P E C D E K G P S P V F V F V P E C D E K G	N Y R PQQS H V N Y R P E Q S H V N Y R P PQ S H V N Y R P E Q S H V N Y R P Q Q C H I N Y R P Q Q C H I N Y R P R Q C H D	Y SWCV DEYGEEV Y SWCV DEYGEEV Y SWCV DEYGEEV Y TWCV DEYGEE Y CWCV DEYGEE Y CWCV DEYGEE Y CWCV DEYGEE	V F GS R N FP GK V L GS RT FP GK V F GS RS FP GK V F GS RN FP GK V L FS RS FP GK I F GS RT FP GK	CP SQ VLTK CP SQ VLTK CE RH DQ TK PP K P CE AS GE TA P F K P CE AS GE TP P F K P CE AS GE TP P F K P CY AS DE PL P F K CE P S GE TL P F K A CE AS GE TL	CL K E RQ VA G G DE KV CL K E RQ E A G G KR I A CL K DRQ KA L AK KM CI K E RNK V I S VA E P L CI K E RNK V I S AA E P L CI K E RNK V I S AA E P L CI K E RNK V I S T E P L CL K Q RNK V I S T E P L CI K E RNK V I S T A E P L	Y L G R F V P Q C D E K G A I G R Y I P Q C D E Q G A I G G F V P Q C D E K G L G A F L P D CD E N C L G A F V P D CD E K C L G A F V P D CD E K C L G A F V P D CD E K C L G A F V P D CD E K C R G A F L P E CD E K C	NY EP QQ FH G ST G Y S 201 NY EP QQ FH G ST G Y C 203 N F K P RQ C H G ST G Y C 207 Y F SP L Q FH G ST G Y S 271 Y F SP L Q FH G ST G Y S 271 Y F SP L Q FH G ST G Y S 271 Y F SP L Q FH G ST G Y S 271 Y F SP L Q FH G ST G Y S 271 Y F SP L Q FH G ST G Y S 273 Y F S F G Y C S S S C S C S C S C S C S C S C S C
			β 5	Ο Ν2 β1Ν2	β 2Ν	12 (α 1Ν2	α	2 N 2			_ α 3 N 2	β 3Ν2
RcSxph 202 W MpSxph 204 W MaSxph 204 W DtSxph 204 W DtSxph 204 W DtSxph 204 W DtSxph 201 W DtSxph 272 W RtSxph 272 W PtSxph 272 W PtSxph 272 W AfSxph 299 W AfSxph 293 W	V NA IGEETAC CV NA IGEKTEC CT NANGEKTAA CV TK NGEETKC CV TK NGEETKC CV TK NGEETKC CV TK NGDETKC CV TENGVETKC CV TENGVETC CV TK NGDETPC CC T TK DGEETEC	T KT P P G K I P T NT P P G N T Q T NT P P Q Q S P T RT GP G Q T P T RT GP G Q T P T RT G P G Q S P T R T G P G Q S R T R T G P G Q S R	ATCQKHDL PTCQSHDW PTCERHDL PTCEVSAP PTCDVPAP PTCEVPAP PTCEVPAP HICEVKAP PTCDIPAP	VT TCHYAVAMVKK D- TCHYAVAVVKN D- SCHYAVAVVKK V- TLHYAVAVVKK A- TLHYAVAVVKK V- TLHYAVAVVKK V- TLHYAVAVVKK V- TLHYAVAVVKK G- TVHYAVAVVKK	S S A F Q F NQ L K G K R S S S T F Q F GQ L K G K R S S S I F Q F Q L K G K R S S S S F Q L DQ L K G K R S S S S F Q L DQ L K G K R S S S F H L DQ L K G K R S S S S F H DQ L K G K R S S S S F Q L D E L K G K R S	CH SC VS KT DCWKAL CH SG LS KT DCWKAP CH SC VS KT DCWKAP CHSAV GEAACWVAP CHSAV GEAACWVAP CHSAV GEAACWVAP CHSAV GEAACWVAP CHSAV GEAACWVAP CHSAV GEACWVAP	VTV LVEKKLLSV VNVFVEKKLLPV VSVLVDKNLLSV LNVLLKKKLLU LNVLLKKKFLL LNVLLKKKLLU LNVLLKKKLLS LNIFLKKNLSS	VDGPAKESIQRA VDGLAKGSIERA VDRAAKESIEKA VEEPEQKSIEKV VEEPEQKSIEKV VEEPEPKTIEKV VGGPEQKSIEKV LEGAEVKSIEKA VEGPEQKSIEKA	M SK F F SV SC I V SK F F SA SC I V SK F F SD SC I A SE I F SA SC A A SE I F SA SC A	P GA TQ TN LC KQ C P GA T E TN LC KQ C P GA T E TN LC KQ C P GA T E TN LC RQ C R GAQE AN LC EQ C P GAQ E ST LC EQ C	K GEEGKNCKNSHDEF I GEEEKKCKSSHDEF MEGGKKCMSSHDEF A GQEDQ-CTRGPGE A GQEDX-CTRGPGE A GQEDK-CTRGPGE A GQEDK-CTRGPGE A GQEDK-CHRGPGE A GQEDK-CHRGPGE A GQEDK-CHRGPGE	P Y Y GN Y G A F R C LK P Y Y GD HG A F R C LC P Y Y GD HG A F R C LK P Y Y GD EG A F R C L K P Y Y GD EG A F R C L R P Y Y GD EG A F R C L R P Y Y GD EG A F R C L R P Y Y GD EG A F R C L R P Y Y GD EG A F R C L K	K EDMG DV AF LR 353 2 DKG DV AF LR 354 5 CKG DV AF LK 354 K DC KG DV AF VE 421 CD KG DV AF VE 440 K D KG DV AF VE 421 K D KG DV AF VE 421 K D KG DV AF VE 421 K D KG DV AF VE 448 K D D KG DV AF VE 448 K D D KG DV AF VE 442
	β	4N2 β5	N 2	β5Ν	1 α4N1	I		α5	N1 α6	N1	<mark>C1</mark> β1C1	α 1C1	β 2C1
RcSxph 354 S MpSxph 355 N MaSxph 349 N DfSxph 422 D OsSxph 441 D RiSxph 422 D PtSxph 422 D PtSxph 422 D AfSxph 449 D AfSxph 443 D	TALSDEHSEV TVLSDTHSEV TVLSDTHSED TALT GQ YSDN TALT GQ YSDN TALT GQ YSDN TALT GQ YSDN TALSGQ YSDN TILSGQ YSDN TILSGQ YSND	E L L CP DNTR E L L CP DNTR	KP LN KY KE KP LN KY KE R PL N KY KE R PL SQ YK I R PL SQ YK I R PL SQ YK I R PL SQ YK I R PL SQ YK I	CN LG T V PA GT V V T CN LG KV PA DAV V T CN LG KV PA DAV V T CN FGR I PR HS V V T CN FGR I PR HAV V T CN FGR I PR HAV V T CN FGR VPR HAV V T CN FGR I PR HAV V T	R K I S D KT ED INNFL R KAGD KT KD INDFL R KS GD K I KD INDFL R ST GD KM KD IT EYL R ST GD KM KD IT EYL R SS GD KM KD IT EYL R SS GD KM KD IT EYL R SS GD KM KD IT EYL	M EAQK RQCK LF S S A L EAQK K KCK LF G S P L EAQK KHCQL F S S A LQAQK KECK LF S S T LQAQK KECK LF S S T VQGQ K KECK LF S S T L EAQK KECK LF S S T L EAQK KECK LF S S T L KAQK KECK LF G S A	H GK DLM F DD ST I H GK DLM F DD ST 1 H GK DLM F DD ST 1 H GK DL F E DT TS H GK DL L F E DT TS H GK DL L F E DT TS H GK DL L F E DT T H GK DL L F E DT T V GK DL L F E DT T	LQ LA L LS S EV DA TH LAP LP S E I D A TQ LS LL PP E V DV SA LI A LP S AM DT SA LI A LP S AM DT TAL IT LP S AM DT TGL I A LP S AM DT TS LI VLP S AM DT	FLYLGV KLFH FFFLGVKWYN FFFLGVQWLN FLFLGPDLFN FLFLGPDLFN FFFLGSELFK FLFLGPELFH FLFLGPELFS	AMKALT GDAHLP AMKALTEDVKLP ITMKTLTADVKPP GMKTLNG-ARPP GMKTLNG-AHPP GMKTLG-TRPP GMKTEG-TRPP GMKTLG-TRPP GMKTLG-AHPP GMKTLG-AHPP	S- K N K V RWCT I N K L S- K N K V R WCT I N K L S- K N K V R WCT V N L R V KQQ I R WCPQSK N R V KQK I R WCPQSK N Q V QQ I R WCPQSK N R V H R E I R WCPQNK N S - SQE I R WCPQSK N	EKMK CD DWSAVSC MMKCKDWAAVSC KMKCDWSAVSC EKKKCDWSSVSC EKKKCDWSSVSC EKKKCDWSSVSC EKKKCDWSSVSC EKKKCDWSSVSC EKKKCDWSSVSC EKKKCDWSSVSC	G A I ACTEASC 504 G A I ACTEASC 505 G A I ECTEASS 499 G A I ECTEASS 499 G A I ECTEPSS 572 G S I ECTEPSS 591 G A I ECTEPSS 572 G A I ECTEPSS 572 G A I ECTEPSS 572 G A I ECTEPSS 598 G A I ECTEPSS 598
	α 2C1	β 3C1	α 3C1	β 4C1	α4C1 C2	β1C2	β 2	C 2	α1C2	•			
RcSxph 505 P Np5xph 506 P MaSxph 500 P Dt5xph 573 G R5xph 573 G Pt5xph 573 G Pt5xph 573 G Pt5xph 593 A Et5xph 599 G Af5xph 593 A	K G CV KQ I L K G E H CV KQ I L K G E H C I KQ I V K G L E C V K I L K D L E C I KM I L K D J E C I KM I L K G J E C I E M I L K G Q K C I E K I L K G L E C I E K I L K G	A DA VK LE VC A DA VT LD VC A DA VT LD VC A DA V N LD TA A DA V N LD A A DA V N LD A A EA V T LD C A EA V T LD C	YMYEALMC YMYMALMC YMYEALKC HTYTALKC HAYTALKC HAYTALKC HAYTALKC	GLLPAVEEYHNKD GLLPAVEEYPNKD GLLPAVEYPNKD GLLPALEYNKD GLLPSLEFRKD GLLPSLEFRKD GLLPSVEYRNKD GLLPSVEYRNKD GLLPSVEYRNKD GLLPSVEYRNKD GLVPSLDEYRNKD	D FG PC KT PG SP TT E D FG PC KT PG ST K E D FG PC KT PG ST K E D LI PCQI PG AD TT E D LI VCQI PG AE Y SE D LI PCQI PG SE Y SE D LI PCQV PG SE Y SE D LI PCQV L EA EY TE D LI PCQV L EA EY TE D LV PCQI PG AE Y SE	F GT LR AV ALVKKSN F GT KR AV ALVKKSN F GAKR AV ALVKKSN F GSYR IV ALVKKSN F GSYR IV ALVKKTD F GSYR IV ALVKKTD F GSYR IV ALVKKTD F GSYR IV AV VKKTD F GSYR IV AV VKKTD	IK D I NWN N IK GK IK D I KWN N LK GK IK D I NWH N LK GK IK D I TWN N LQ GK	KSCHTGVGDIAG KSCHTGVGDQAG KSCHTGVGDQAG KSCHTGVGDMIG KSCHTRAGDMIG KSCHTRAGDMIG KSCHTRVGDMIG KSCHTSVNDMVG KSCHTSVNDMVG	WVIPVSLIRF WVIPAGLISN WIIPVGLISR WNIPVYLISK WNIPVYLISK WNIPIYLISK WNIPIYLIYK WKIPHALLYK WTVPISLIYK	RQ ND NS D I DS FF C RQ ND N I D I ES FF C Q I D N I D I ES FF C C Q I D N CN I D SF FS K TK NC D L GS Y FS K TK NC D L GS Y FS K TK NC D G SF FS K TK SC DF GS Y FS K TK NC D I GS Y FS	E S C AP G S DT K S N L C E S C AP G S DT N S K L C E S C AP G S DT T S N F C Q S C AP G S P I D S N L C Q S C AP G S P I D S N L C Q S C AP G S P I D S N L C Q S C AP G S T I D S N L C K S C AP G S A I D S N L C Q S C AP G S A I D S N L C	K LC IG DP KN SAAI K LC IG DP ENPKAS K LC IG DP SNPMAN K LC IG DP QN TEAN K LC IG DP QN TEAN K LC IG DP QN P EAN K LC IG DP QN TK SN E LC IG DP QN TAN	NT K C S L S D K E A 656 5 T R C S L S D K E A 657 9 T K C S L S D K E A 657 9 T K C S L S D K E A 724 9 T K C S L D K E A 724 9 T K C S L D K E A 724 9 T K C S L D K E A 724 9 T K C S L D K E A 750 9 T K C S L D K E A 750 9 T K C S Q N D K E A 744
	<mark>e</mark> α2C2	β 3C2	α 3C2		β 4C2 β 5C2	β 6C2	<mark>β5</mark> C1	α5C1			β6C1	α6C1	α7C1
RcSxph 657 Y NpSxph 658 Y MaSxph 652 Y DtSxph 725 Y OsSxph 744 Y RiSxph 725 Y PtSxph 725 Y PtSxph 725 Y AfSxph 751 Y	Y GNQG AF R C LV Y GN EG AF R C LV HG NE GA I R C L V Y GN EG AV R C LV Y GN EG AV R C LV Y GH EG AI R C LV Y GN EG AI R C LV Y GN EG AI R C LV Y GN EG AI R C LV	EK GDVA FV F EK GDVA FV F	HTV VF ENT HTV VF ANT HTA VF ENT HTA VF ENT HTA VF ENT HTA IF ENT HTA IF ENT HTA VF ENT	D GK NP A VWA KN LK D GK NP A EWA KD LK D GK NP A EWA KD LK D GK NP A LWA KD LK	SEDFELLCLDGSRA SEDFELLCLDGSRA SEDFELLCPDGSRA STDFELLCPDGSRA STDFELLCPDGSRA STDFELLCPDGSRA STDFELLCPDGSRA STDFELLCLDGSRA STDFELLCLDGSRA	PV SNYK SC KLSG IP PV TNYR GC NLSG LP PV SNYK TC KLSG LP PV SNYK TC KLSG I A PV SDYK KC KLSG I A PV SDYK KC KLSG I A PV SDYK KC KLSG I A PV SDYK C KLSG I A PV SDYK RC KLSG I A	P A I VT R E E S I P R A I VT R E E S V P R A I VT R E E S V NQ VT VT R PE S V NQ VT VT R PE S V NQ I A I T R PE S V NQ VT VT R PE S V	SDV V RI VA NQQS SDV V RI LI NQQS SDV V RI LI NQQS KDV V RI TQ NQQS KDV V RI TQ NQQS KDV V RI IQ NQQS KDV V RI IL NQQS ADV LRI IL NQQS	L Y GR K G FE K L Y GR NG FE K L F GR NG FE K L Y GR TG S L K L Y GR TG S L K L Y GR TG SQ K L Y GR NG FQ K	DMFQLFSSNKGN DMFQMFSSAKGQN DMFQMFSSNKGQN DIFQMFSSSYGQN DIFQMFSSSYGQN DIFQMFSSSYGQN DIFQMFSSSYGQN DIFQLFSSYGQN DIFQLFSSYGQN		RG - PKD IM EDYFK RQ - PKD IM EDYFK RQ - PKD IM EDYFK RM LDRD IM DDYFK RM LDRD IM DDYFK	CK P Y YT T VY GA 807 CV R YY T A VY SA 808 CK P Y NA VY NT 802 GK P F HK HL I R D 876 CK P F HK YV NR D 876 CK P F HK YV RR D 902 CK P F HK TV FR D 896
RcSxph 808 S NpSxph 809 S MaSxph 803 S DtSxph 877 N OSSxph 896 N RiSxph 877 N PtSxph 877 N PtSxph 877 N PtSxph 877 N	α 8 C R S AM S S EL I S A R S AV P S EL I P C V P S EL I S A D C FP I S A L A T D C L P K S A L A T E C L P K S A L A T E C FP C A L T A	CTIKHC CTFKHC CTFKHC CTFKHC SPHH- SCSFHH- SPHH- SFHH- SFHH- SFHH- SFHH- SFHH- SFHH- STHH- STHH- STHH- STHH- STHH- STHH- STHH- STHH- STHH- STHH- STH STHH- STH STH STH STH STH STH STH STH STH STH											

EfSxph 903 N EC FPQTAL TNAC LFHH-919 AfSxph 897 N DC LPK SAL AT AC SFHH-913 **Fig. S7. Frog Sxph sequence alignment.** Sxph sequence alignment for *Rc*Sxph, *Np*Sxph, *Ma*Sxph, *Dt*Sxph, *Os*Sxph, *Ri*Sxph, *Pt*Sxph, *Et*Sxph, and *Af*Sxph. Domains and secondary structure are from *Rc*Sxph. N1 (dark green), N2 (light green), Thy1 domains (orange), C1 (marine), C2 (cyan). STX binding site residues are indicated by stars and colored based on the alanine scan results in Table 1. Residues corresponding to transferrin Fe³⁺ and carbonate ligands are indicated by orange and blue hexagons, respectively and highlighted (*1, 21*).

Chen et al.

Figure S8



Fig. S8. Toad Sxph sequence alignment. Sxph sequence alignment for *Rc*Sxph, and toad saxiphilins *Rm*Sxph, *Bb*Sxph (NCBI:XM_040427746.1), and *Bg*Sxph(NCBI:XP_044148290.1). Domains and secondary structure are from *Rc*Sxph. N1 (dark green), N2 (light green), Thy1 domains (orange), C1 (marine), C2 (cyan). STX binding site residues are indicated by stars and colored based on the alanine scan results in Table 1. Residues corresponding to transferrin Fe³⁺ and carbonate ligands (*1, 21*) are indicated by orange and blue hexagons, respectively and highlighted. Only beginning and ends of the Thy1 domains are shown. Total number of Thy1 domains are indicated.

Figure S9

	SS4/SS7	SS5	SS6/SS8
	α1 *	β 1 ** *β 2	**
RcThyl-1 1A	LQK <mark>C</mark> LKERQQAL-AKKMI-GHYIPQ <mark>C</mark> D	EKGNYQPQQ <mark>C</mark> HGSTGH <mark>C</mark> WC	VNAMGEKISGTNTPPGQTRAT <mark>C</mark> ERH
RcThy1-2 1B	LPK <mark>C</mark> LKERQVALGGDEKVLGRFVPQ <mark>C</mark> D	EKGNYEPQQ <mark>F</mark> HGSTGY <mark>S</mark> W <mark>C</mark>	VNAIGEEIAGTKTPPGKIPAT <mark>C</mark> QKH
NpThy1-1 1A	LTK <mark>C</mark> LKHRQESLGGDKMVKGRYIPQ <mark>C</mark> D	EKGNYHPVQ <mark>C</mark> HASTGY <mark>C</mark> WC	VNANGEKIEGTNTTPVQTPPT <mark>C</mark> PSQ
NpThy1-2 1A	LTKCLKERQEALGGKRIAIGRYIPQCD	EQGNYRPMQ <mark>C</mark> HGSTGY <mark>C</mark> WC	VNAIGEKIEGTNTPPGNTQPTCQSH
Malhyl-1 IA		EKGSYQPQQCYGSTGHCWC	
Mainy1-2 IA			
Dt Hy1-1 IA Dt Thy1-2 IB			
DtThy1-3 1B	ETACIKERNKVLSVAEPLLGAELPDCD	ENGYESPLOFHGSTGYSWC	VTKNGEEIKGTRTGPGOTPPTCEVS
OsThv1-1 1A	OTPCMRHROSVLGGKKMIIGAFVPKCD	EKGNYVPKOCHDSTGFCWC	LNENGEEIEGTRTPPGNSGLTCENK
OsThy1-2 1B	KPPCLKERQKLLSAK-PSPAVFVPECD	EKGNYRPEQSHVYSWC	VDEYGEEVLGSRTFPGKPPKP <mark>C</mark> EAS
<i>Os</i> Thy1-3 1B	ETP <mark>C</mark> IKERNKVLSAAEPLPGAFVPD <mark>C</mark> D	EKGYFSPLQFHGSTGHSW <mark>C</mark>	VTKNGEEIKGTRTRPGQTPPT <mark>C</mark> DVP
<i>Ri</i> Thyl-1 <mark>1A</mark>	HTP <mark>C</mark> MRHRQSVLGGKIMKIGAFVPK <mark>C</mark> D	EKGNYVPKQ <mark>C</mark> HGSTGY <mark>C</mark> WC	LNENGEEIEGTRTPPGNPGLTCENK
<i>Ri</i> Thy1-2 <mark>1B</mark>	KPP <mark>C</mark> LKERQKLLSAK-PSPAVFVPE <mark>C</mark> D	EKGNYRPQQSHVYSW <mark>C</mark>	VDEYGEEVFGSRSFPGKPPKP <mark>C</mark> EAS
RiThy1-3 1B	ETP <mark>C</mark> IKERNKVLSAAEPLLGAFVPD <mark>C</mark> D	EKGYFSPLQSHGSTGYSW <mark>C</mark>	VTKNGDEIKGTRTGPGQSPPT <mark>C</mark> EVP
PtThyl-1 1A	HTPCMRHRQSVLGGKKIKIGAFVPKCD	EKGNYVPKQ <mark>C</mark> HGSTGY <mark>C</mark> WC	
Ptiny1-2 IB			VDEYGEEVFGSRNFPGRPPRPCVAS
Ptiny1-3 1B E+Thy1-1 1A			
EtThy 1-2 1A			
EtThy 1-3 1B		DKGYFTPOOFHGSTGYSWC	VTKNGDETPGTRTGPGOTPHTCEVK
AfThy1-1 1A	HTPCMKHR0SVLGGK0LIIGAFVPKCD	EKGNYAPKO <mark>C</mark> HGSSGYCWC	LNENGEEIKGTRSRPGTKVLTCEDA
AfThy1-2 1A	KPPCLKERQKLLSEN-PSPTVFVPECD	EKGNYRPRO <mark>C</mark> HDYCWC	VDEYGEEIFGSRTFPGKPPKACEAS
AfThy1-3 1A	ETL <mark>C</mark> IKERNKVLSTAEPLRGAFLPE <mark>C</mark> D	ekgyfspmq <mark>c</mark> hsstgh <mark>c</mark> wc	TTKDGEEIEGTRTGPGQSRPT <mark>C</mark> DIP
RmThy1-1 1A	STP <mark>C</mark> LRHRQRVLGARKPQIGEFVPE <mark>C</mark> D	EKGNYFPKQ <mark>C</mark> YGTTGY <mark>C</mark> WC	VDEHGDEIPVGRAKQGKVNIS <mark>C</mark> EYA
RmThy1-2 1A	EKP <mark>C</mark> MKEQRKALSGGQPLRGAFIPN <mark>C</mark> D	EKGNYSPKQ <mark>C</mark> HGSTGY <mark>C</mark> WC	VDENGAEISGSRTPPGQQVPTCGSY
RmThy1-3 1A	GATCIKDRYKVLGAGKPLPGAFVPDCD	EKGDYRPQQ <mark>C</mark> HGSTGH <mark>C</mark> WC	VSKDGVEIQGTRAAPGQSPPTCEDP
BbThy1-1 1A	STPCLRHRQRVLGAKKPHIGEFMPECD		VDENGAERHVGRAKQGKVNISCEYT
BDINY1-2 IA		EEGNYNPKQCHGSIGYCWC	
BbThy1-4 1A		EKGNYSPKOCHOSTOVCWC	VNENCKETSCSPTPPCOOVPTCVAS
BbThy1-5 1A		EKGEYRPKOCHGSTGYCWC	VSKDGKETOGTRAAPGOSPPTCODT
BbThv1-6 1A	ETSCIKEROKVLGAVKPILGAFVPDCD	EKGEYRPKOCHGSTGYCWC	VSKDGKEIOGTRAARGOSPPTCODT
BbThy1-7 1A	ETS <mark>CIKELQKVLGAVKPMVGAFVPDC</mark> D	EKGEYRPKQ <mark>C</mark> HGSTGY <mark>C</mark> WC	VSKDGKEIQGTRVAPGQSPPTCQDT
BbThy1-8 1A	ETS <mark>C</mark> IKERQKVLGAVKPILGAFLPD <mark>C</mark> D	EKGEYRPKQ <mark>C</mark> HGSTGY <mark>C</mark> WC	VSKDGKEIQGTRAAPGQSPPT <mark>C</mark> QDT
<i>Bb</i> Thy1-9 1A	ETS <mark>C</mark> IKERQKVLGAVKPIVGAFVPD <mark>C</mark> D	EKGEYRPKQ <mark>C</mark> HGSTGH <mark>C</mark> WC	VSKDGKEIQGTRVAPGQSPPT <mark>C</mark> QDT
<i>Bb</i> Thy1-10 1A	ETS <mark>C</mark> IKERQKVLGAVKPILGAFLPD <mark>C</mark> D	EKGEYRPKQ <mark>C</mark> HGSTGY <mark>C</mark> WC	VSKDGKEIQGTRAAPGQSPPT <mark>C</mark> QDT
BbThy1-11 1A	ETSCIKERQKVLGAVKPILGAFLPDCD	EKGEYRPKQ <mark>C</mark> HGSTGY <mark>C</mark> WC	VSKDGKEIQGTRAAPGQSPPTCQDT
Bblhyl-12 IA			VSKDGKEIQGIRVAPGQSPPICQDI
BDIRY1-15 1A BbTby1-14 1A			
BbThy1-15 1A		EKGEYRPKOCHGSTGYCWC	VSKDCKETOCTRAAPCOSPETCODT
BbThy1-16 1A	ETSCIKEROKVLGAVKFILGALVEDCD	EKGEYRPKO <mark>C</mark> HGSTGHCWC	VSKDGKETQGTRVAPGOSPPTCODT
BaThv1-1 1A	OGPCOKERORORERGRPLLGAFEPKCD	EKGNYOPKOCHGSTGYCWC	VNEEGKTIDGTKTPPGOKSVTCEDH
BgThy1-2 1A	STPCLRHRQSVLGANKPQIGAFVPDCD	EKGNYSPKQ <mark>C</mark> FGSTGY <mark>C</mark> WC	VDEHGDEIEGVRAKQGKVNITCEYT
BgThy1-3 1A	EKP <mark>C</mark> MKERRKSLSGGQPLPGAFMPD <mark>C</mark> D	EKGNYSPKQ <mark>C</mark> HGSTGY <mark>C</mark> WC	VNENGKEISGSRTPPGQQVPTCGAS
BgThy1-4 1A	ETS <mark>C</mark> IKERQKVLGAATPILGAFVPD <mark>C</mark> D	AKGDYRPKQ <mark>C</mark> HGSTGH <mark>C</mark> WC	VSKDGKEIQGTRTAPGQTPPT <mark>C</mark> EIP
BgThy1-5 1A	EKP <mark>C</mark> MKERRKSLSGGQPLPGAFMPD <mark>C</mark> D	EKGNYSPKQ <mark>C</mark> HGSTGY <mark>C</mark> WC	VNENGKEISGSRTPPGQQVPT <mark>C</mark> GAS
BgThy1-6 1A	ETS <mark>C</mark> IKERQKVLGAEKPILGAFVPD <mark>C</mark> D	EKGDYRPKQ <mark>C</mark> HSSTGH <mark>C</mark> WC	VSKDGKEIQGTRTAPGQTPPT <mark>C</mark> EIP
BgThy1-7 1A		EKGNYSPKQCHGSTGYCWC	VNENGKEISGSRTPPGQQVPTCGAS
Bginy1-8 IA			VSKDGKEIQGIRIARGQSPPICEIP
BgThy1-9 IA BgThy1-10 IA			VSKDCKETOCTPACPCOTPPTCEDK
B_{α} Thv1-11 1A		EKGEYRPKOCHGSTGHCWC	VSKDGKETOGTRAAPROSPPTCETP
BqThv1-12 1A	ETSCIKE00KVRAG-KPILGAFVPDCD	EKGDYRPKOCHGSTGHCWC	VSKDGKEIOGTRAACGOSPPTCEIP
BgThy1-13 1A	ETS <mark>CIKERQKVLGAEKPILGAFVPDCD</mark>	EKGEYRPKQ <mark>C</mark> HGSTGH <mark>C</mark> WC	VSKDGKEIQGTRAGPGQSPPTCEDT
BgThy1-14 1A	ETS <mark>C</mark> IKERQKVLGAATPILGAFVPD <mark>C</mark> D	EKGDYRPKQ <mark>C</mark> HGSTGH <mark>C</mark> WC	VSKDGKEIQGTRAGPGQSPPT <mark>C</mark> EIP
<i>Bg</i> Thy1-15 1A	EKS <mark>C</mark> IKERQKVRSPRKPILGAFVPD <mark>C</mark> D	EKGDYRPKQ <mark>C</mark> HSSTGH <mark>C</mark> WC	VSKDGKEIQGTRAARGQSPPTCEDP
	Loop 1	Loop 2	Loop 3

Fig. S9. Thy1 domain sequence alignment. Thy1 domains from *Rc*Sxph, *Np*Sxph, *Ma*Sxph, *Dt*Sxph, *Os*Sxph, *Ri*Sxph, *Pt*Sxph, *Et*Sxph, *Af*Sxph, *Rm*Sxph, *Bb*Sxph, and *Bg*Sxph and the type (1A or 1B) are shown. Secondary structure from *Rc*Sxph Thy1-1 is shown. Cysteine are highlighted. SS4-SS8 indicate disulfide numbers from *Rc*Sxph. Loop regions are indicated.

Figure S10

Chen et al.



Fig. S10. *Np*Sxph structure and comparisons with *Rc*Sxph. A, and B, Exemplar electron density for A, *Np*Sxph (2Fo-Fc, 1.5 σ , grey) and (Fo-Fc, 3.0 σ , green). B, *Np*Sxph:STX (2Fo-Fc, 1.5 σ , grey). *Np*Sxph (marine), STX (pink), and PEG400 (yellow) are shown. STX (pink) from the *Np*Sxph:STX complex is shown in 'A' to compare with the PEG400 position. Select residues are labelled. C, *Np*Sxph and *Rc*Sxph superposition using the C-lobes. N- and C-lobes are green/light green and marine/light blue for *Np*Sxph and *Rc*Sxph, respectively. Arrow indicate relationships between *Np*Sxph and *Rc*Sxph N-lobes. D, Superposition of *Np*Sxph (green) and *Rc*Sxph (light green) N-lobes. E, Superposition of *Np*Sxph (marine) and *Rc*Sxph (light blue) C-lobes. F, Cartoon diagram of *Np*Sxph and *Rc*Sxph superposition from 'C' showing the change in Thy1 domain

positions. *N*pSxph Thy1 domains (orange) and *Rc*Sxph Thy1 domains (magenta) are indicated. **G**, Cartoon diagram of *N*pSxph and *Rc*Sxph Thy1 domains superposed on Thy1-1. *N*pSxph and *Rc*Sxph Thy1-1 and Thy1-2 are light orange and pink and orange and magenta, respectively. H, Superposition of individual *N*pSxph and *Rc*Sxph Thy1-1 and Thy1-2 domains. Colors are as in 'G'. Disulfide bonds are indicated. Comparisons are made using the STX bound structures *N*pSxph (PDB: 8D6M) and *Rc*Sxph (PDB: 6O0F) (1).



Fig. S11. Electrostatic surface potentials for A, *Np*Sxph (PDB: 8D6M) and **B,** *Rc*Sxph (PDB: 6O0F) (1) calculated using APBS (22) for the STX bound conformations in the absence of STX. Insets show STX binding pocket with select residues indicated. STX is shown as sticks (**A**, pink, and **B**, firebrick).

Figure S12

Chen et al.



Fig. S12. Structure of the *NpSxph:F-STX* complex. **A**, Exemplar electron density for *NpSxph:F-STX* (2Fo-Fc, 1.5 σ , grey). *NpSxph* (cyan) and F-STX (orange). **B**, Comparison of *NpSxph:STX* (marine) and *NpSxph:F-STX* STX binding sites. STX from *NpSxph* is pink. F-STX is orange. Select residues are indicated.

Table S1 Crystallographic data collection and refinement statistics						
	RcSxph -Y558A PDB:8D6P	<i>Rc</i> Sxph -Y558A:STX (co-crystal) PDB:8D6S	<i>Rc</i> Sxph -Y558I PDB:8D6Q	<i>Rc</i> Sxph -Y558I:STX (co-crystal) PDB:8D6T		
Data Collection						
Space group	P212121	P212121	P212121	P212121		
Cell dimensions a/b/c (Å)	96.61, 109.05, 254.89	95.98, 107.14, 253.04	96.39, 107.15, 254.79	96.03, 107.81, 253.58		
$\alpha/\beta/\gamma$ (°)	90, 90, 90	90, 90, 90	90, 90, 90	90, 90, 90		
Resolution (Å)	47.81-2.60 (2.65- 2.60)	47.37-2.60 (2.65-2.60)	47.61-2.70 (2.76- 2.70)	47.5-2.15 (2.19-2.15)		
Rmerge (%)	0.108 (4.094)	0.115 (3.964)	0.159 (4.833)	0.089 (1.558)		
Ι/σΙ	12.9 (0.9)	12.5 (0.8)	8.8 (0.6)	16.2 (1.2)		
CC(1/2)	0.998 (0.532)	0.998 (0.458)	0.998 (0.419)	0.999 (0.599)		
Completeness (%)	99.6 (100)	99.9 (100)	99.9 (99.8)	99.5 (93.6)		
Redundancy	13.4 (13.9)	13.4 (14.0)	13.3 (14.0)	12.2 (6.2)		
Total reflections	1116931 (62978)	1085831 (61318)	975607 (62994)	1736282 (40652)		
Unique reflections	83173 (4517)	81054 (4377)	73319 (4504)	142848 (6596)		
Wilson B-factor	83.42	84.83	90.34	44.01		
Wavelength (Å)	1.033	1.033	1.033	1.033		
Refinement						
Rwork / Rfree (%)	22.79/26.37	23.20/26.17	23.42/27.21	20.73/23.37		
No. of chains in AU	2	2	2	2		
No. of protein atoms	12616	12616	12622	12622		
No. of ligand atoms	0	42	0	72		
No. of water atoms	77	60	79	794		
RMSD bond lengths (Å)	0.002	0.002	0.003	0.003		
RMSD angles (°)	0.50	0.49	0.55	0.59		
Ramachandran	94.90/4.91/0.18	94.29/5.47/0.25	93.61/6.08/0.31	95.33/4.30/0.37		
favored/allowed/outliers (%)						

Table S1 Crystallographic data collection and refinement statistics (continued)						
	RcSxph:F-STX (soaked) PDB:8D6U	<i>Np</i> Sxph PDB:8D6G	<i>Np</i> Sxph:STX (co-crystal) PDB:8D6M	NpSxph:F-STX (soaked) PDB:8D6O		
Data Collection						
Space group	P212121	R3	R3	R3		
Cell dimensions a/b/c (Å)	96.44, 109.37, 256.36	229.046, 229.046, 67.428	228.848, 228.848, 67.224	229.186, 229.186, 67.347		
α/β/γ (°)	90, 90, 90	90, 90, 120	90, 90, 120	90, 90, 120		
Resolution (Å)	47.97-2.65 (2.70- 2.65)	43.29-2.2 (2.279-2.2)	42.55-2.0 (2.071-2.0)	43.31-2.2 (2.279-2.2)		
Rmerge (%)	0.112 (4.136)	0.05218 (0.8687)	0.05456 (0.9961)	0.07078 (1.889)		
Ι/σΙ	11.1 (0.8)	12.66 (0.89)	14.11 (1.11)	19.64 (1.35)		
CC(1/2)	0.999 (0.513)	0.999 (0.465)	0.998 (0.637)	0.999 (0.602)		
Completeness (%)	99.9 (99.8)	98.60 (91.37)	99.89 (99.61)	99.90 (99.97)		
Redundancy	10.4 (11.0)	3.3 (2.1)	5.2 (4.8)	10.6 (11.0)		
Total reflections	830262 (49019)	219806 (12669)	459480 (42277)	707304 (73200)		
Unique reflections	79524 (4469)	66067 (6127)	88641 (8856)	66898 (6682)		
Wilson B-factor	83.73	52.73	49.89	55.11		
Wavelength (Å)	1.033	1.033167	1.033167	1.033167		
Refinement						
Rwork / Rfree (%)	23.46/26.51	19.50/23.55	19.25/22.09	19.64/24.26		
No. of chains in AU	2	1	1	1		
No. of protein atoms	12630	6373	6385	6346		
No. of ligand atoms	110	38	59	93		
No. of water atoms	88	229	287	161		
RMSD bond lengths (Å)	0.002	0.004	0.004	0.006		
RMSD angles (°)	0.50	0.60	0.62	0.75		
Ramachandran favored/allowed/outliers (%)	94.23/5.41/0.37	95.71/4.17/0.12	95.85/3.90/0.24	94.19/5.56/0.25		

	Tuble 02 Resultion in and Apound in the internoughanite officing parameters								
			N (sites)	Kd (nM)	ΔH (kcal mol ⁻¹)	ΔS (cal mol ⁻¹ K ⁻¹)	ΔG (kcal mol ⁻¹)	n	
RcSxph		WT	1.02 ± 0.01	1.2 ± 0.8	-16.1 ± 0.2	-12.7 ± 0.9	-12.3 ± 0.5	3	
		Y558A	1.01 ± 0.01	1.2 ± 0.4	-15.3 ± 0.0	-11.1 ± 1.1	-12.2 ± 0.2	2	
		Y558I	1.05 ± 0.03	1.1 ± 0.5	-15.5 ± 0.3	-10.5 ± 0.6	-12.2 ± 0.3	2	
	Sxph	F561A	1.07 ± 0.01	13.4 ± 1.4	-12.7 ± 0.2	-6.6 ± 0.9	-10.8 ± 0.1	3	
	Rc	P727A	0.97 ± 0.03	31.3 ± 11.6	-11.5 ± 0.0	-4.2 ± 0.9	-10.3 ± 0.2	2	
		E540D	0.98 ± 0.02	68.9 ± 10.7	-16.3 ± 1.7	-21.9 ± 5.7	-9.8 ± 0.1	4	
		D794E	0.99 ± 0.01	312.5 ± 2.9	-11.8 ± 0.0	-8.9 ± 0.1	-8.9 ± 0.1	2	
	hq	WT	0.92 ± 0.02	2.5 ± 0.1	-18.7 ± 0.2	-23.2 ± 0.8	-11.8 ± 0.1	2	
NpSxI	I559Y	0.94 ± 0.03	2.5 ± 0.8	-16.8 ± 0.2	-16.9 ± 1.1	-11.8 ± 0.2	4		

Table S2 *Rc*Sxph:STX and *Np*Sxph:STX thermodynamic binding parameters

Movie S1 (separate file). *RcSxph-Y558A conformational changes upon STX binding.* Morph between the apo-*RcSxph-Y558A and RcSxph-Y558A*:STX structures showing the STX binding pocket. Sidechains are shown as sticks. STX is red.

Movie S2 (separate file). *Rc***Sxph-Y558I conformational changes upon STX binding.** Morph between the apo-*Rc***Sxph-Y558I and** *Rc***Sxph-Y558A:STX structures showing the STX binding pocket.** Sidechains are shown as sticks. STX is red.

Movie S3 (separate file). Conformational changes between *RcSxph* and *NpSxph***.** Morph between apo-*RcSxph* (PDB:600D) (*1*) (starting structure) and apo-*NpSxph* (final structure). N-lobe (green), C-lobe (blue), and Thy domains (magenta) are shown. N1, N2, C1, and C2 subdomains and Thy1-1, and Thy1-2 are labeled.

Movie S4 (separate file). *NpSxph* conformational changes upon STX binding. Morph between the apo-*NpSxph* and *NpSxph*:STX structures showing the STX binding pocket. Sidechains are shown as sticks. STX is red.

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