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Supplementary Materials for

Structure and mechanism of bactericidal mammalian perforin-2, an ancient agent of innate immunity

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Movie S1 (.mov format). Pre-pore PFN2 oligomers on mica.

Movie S2 (.mov format). Pre-pore PFN2 oligomers on a supported lipid bilayer.

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Fig. S1. Structure determination of mPFN2 pre-pore. (A) Representative cryo-EM micrograph of mPFN2 pre-pore oligomer, arrowheads indicate the pre-pore side views. Bar = 50 nm. (B) Two-dimensional class averages of the mPFN2 pre-pore in multiple orientations indicating also the presence of arcs of subunits as well as full rings. Bar = 10 nm. (C) Global resolution assessment of the pre-pore map measured using the 'Gold-standard' Fourier shell correlation (FSC) curves from two half-reconstructions refined separately in RELION. (D) Local resolution evaluation of the mPFN2 protomer by RELION. (E) Map-to-Model FSC curves showing the correlations between the final atomic model and the final cryo-EM map. (F) Representative regions of cryo-EM densities (mesh) superposed with our atomic model and colored as in Fig. 1.



Fig. S2. Structure-based phylogeny of known structures of MACPF domains from MACPF and CDC proteins. In addition to PFN2 we show astrotactin-2 (ASTN2), the *Bacillus thetaiotaomicron* MACPF domain (*Bth*), stonefish stonustoxin (Stx), gasdermin A (GSDM), pleurotolysin B (PlyB), *Photorhabdus luminescens* MACPF (*Plu*), perforin-1 (PFN1), complement proteins C6, C8α and C8β, *Toxoplasma gondii* perforin-like protein-1 (TgPLP1) and the CDCs pneumolysin (Ply), suilysin (Sly), perfringolysin O (PFO; structure shown), anthrolysin (Aly), streptolysin O (SLO), listeriolysin (LLO; structure shown) and intermedilysin (Ily).



Fig. S3. Characterization of the mPFN2 P2 domain. (A) Structure topology of mPFN2 P2 domain showing three repeated modules. (B) Superposition of the three tandem repeats showing the conservation of the three strands and a disulfide bond from each repeat (RMSD= 0.756-1.001 Å). The three repeats were colored in blue, pink and green respectively with the conserved disulfide bond (in yellow) indicated by the dashed boxes. The enlarged box represents the residues (TLKIF) forming the tip of the first repeat. (C) electrostatic surface potential for the P2 domain crystal structure and (D) for the pre-pore showing marked concentrations of positive and negative potential, with the positively-charged regions in places which will interact with negatively-charged lipid headgroups on membrane binding. (E) Ultracentrifugation-based liposome-binding assays of mPFN2 ectodomain and ectodomain mutant with CTT $\Delta 606-652aa$ truncation.

Mouse	1 10	2.0	30	α1 202 40	$TT \xrightarrow{\beta_1} 50$	β^2	7.0
Mouse Human bat Zebra_fish coelacanth Ophha turtle chicken Platypus Guinea_pig	MNSFMALVIIWM MNNFRATILFWA NNSAMALCLASLLA MKSRAFHLIMLC MSPSTAIFLSLW MKMLLGALLSWG MVWVLRVVLLARA MNNGLV.VVLLWA MNTYRVALLLWT	ITACA EADKP.L AAWA.KSGKP.S LISAGQGSSM CFISV.CNLH.P FITWCCAREPPE LVAWVKGAEKP.P LATAVAEVERP.Q AAAEADRADGALE VATCA.GEDDLLL	GETGTTGFOIG GEMDEVGVOKG GNQEISAIOC LIRPNNGLRLG GKDLTGGFOC WHPPATGFOKG GPPAVMGLOE EPPPSVGFOC QKPARAGLOC EPPPNETGFOKG	KNALKLPVL SV KNALKLPVL SV KOALNVSVLVA KINSSLTALSV KKIYNLSVL SV KKALKLSSLSV KRALKLPSLSV KRALKLPSLSV KRALKLPVLSV RDALKLPVLSV	, P G G W DNLRN , P G G G W DNLRN , P G G G W DNLRN , P G G W DNLRN , P G R G W DNLRN , P G G G W DNLRN , P G G G W DNLRN , P G G W DNLRN , P G G W DNLRN , P G G W DNLRN	VDM G RV MD L TYT VDM G RV ME L TYS VELGLV L G RSYS IDM G RV MN L SYS ADM G TV MN L NFS LDM G RV MN L NFS LDM G RV I S L G YS LDM G RV I D L SYS VDM G RV L D L G YS IDM G RV L D L G YS	NCK FTE OCRTTE OCRTTE CRTTE CRTTE CRTTE LCKTTK QCKTTE LCKTTE HCRTE HCRTE
	SP	MACPI	=				
Mouse	$\mathbf{TT} \xrightarrow{\beta 3} \mathbf{TT}$	$\beta 4 \qquad \beta 5 \qquad \rightarrow 100$	110	120	130	$\alpha^2 \qquad \beta^6$	→ -
Mouse Human bat Zebra_fish coelacanth Ophha turtle chicken Platypus Guinea_pig	DGOYIIPDEVYTIPQK DGOYIIPDEVFTIPQK DGEVLIPDEVFVIPQK DGVLIPDEVFVIPQK DGVLIPDEVFVIPQK DGAYIIPDEVFTIPQK DGSVLIPDEVFTIPRK DGSYLIPDEVFTIPRK DGFYIIPDQVFTIPLK DGQVIIPNEVFTIPQK	ESNLEMNSEVLES SNLEMNSEILES SVETRAELIDE VSGVETRAELIDE VSGVETNSEILMS SLEMNSEITES SNLEINSELIES SNLEINSEILES SNLEMNSEILES SNLEMNSEILDS	WMNYQSTTSIS WANYQSSTSYS WLNYTDTWAFS WLEQKSSTSSS WLEQKSSTSSS WMDYQCTTAVS WRDYQSATAAS WKDYQSATAAS WKDYQSATAAS WKDYQSATASS WMDYKSSTSSS	INTELSLISSEV INTELSLISSEV INTELSVLPIL NADVSFISVL INTEASFISVL INTEASFISVL NGEVSLISSE INTELSLISSE INTELSLISSE INTELSLISSE INTELSLISSE	GK 7 ST EFQRM GK 7 ST EFQRM GK 7 ST EFQRV AK 7 ST ENQRM GK 7 SA DFQOV GR 7 S S DFRRA GK 7 SS DFHRM GK 7 SA EFQHV GK 7 SA EFQRV GK 7 SA EFQRM	KTLOVKDOAŬTT KTLOVKDOAŬTT RKVŠLEVETVTA KTHOVKESSVT XIHOVKDSSVT XIHOVKDKSVST KTHOVKDQAVTT KTHOVKDQAVTT KTHOVKDRSVTT KTLOVKDQSVTT	R VQ V R N R VQ V R N R VQ L R H R VQ A R Y K VQ A R Y K VQ A R Y K VQ V R N R VQ V R N
Mouse	<u>β7</u>	α3 000000000000000000000000000000000000	α4 <u>000000</u>	. 000000	<u>β8</u> β	$ \xrightarrow{\beta 10} \alpha 5 $	2 22
Mouse Human bat Zebra_fish coelacanth Ophha turtle chicken Platypus Guinea_pig	160 170 RIYTVKTNPTSELSLG UYTVKINPTLELSS NIYSVKASEAPGFHPD HLYTVKAYPDFTLDSR HYSVKSKPYFSLDPG LWYTXKDLTTKLDEG UYTAKFDPAAALDKG UYTAKIDPGAGLDKG VYTAKINPSSVLDRE VIYTVKINPASELSWE	180 TKALMDIC DQLE SRKELDIS DRLE LOHLIIS SRLE KRELVEIAN HLE HQOLVTIANQLM HQOLVTIANQLM KKQLITIASHLE RKQLMDIS DRLE RKELMDIS DRLE	190 KNOTKMATYLA NNQTRATYLA NNQTREAQYLA NNQTRHANYLS NNQTRHANYLS NNQTRHANYLA NNQSRMADYLA NNQSRMADYLA NNQTRMADFLS NNQTRMANFLA NNQTRMANFLA	Z C C L L L L N G TH V J S L V L N G TH V J	210 TSVDAGAALT THVDAGALT THVDAGATLV TSVDAGATLV TSVDAGASLV TGVDVGASLV TSVDAGASLV TSVDAGASLV TSVDAGASLV TSVDAGASLV TSVDAGASLV	220 2 E DH VR S SFLL 2 E DH VR S SFLL 2 E DQ VK R DF VK 2 E DQ VK R DF VK 2 E DQ VK TF VK 2 E DQ IKA TF VK 2 E DQ IKA TF VK 2 E DQ IKA TF VK 2 E DL KA TF VK 2 E DH LK STF VQ N 2 E DH LK STF VQ N	NONSON SOSSELE.S SOSDKS NISKKS GRSMRS SMSTRS SWAMRS SWAMRS SORNAK
Mouse	α6 <u>22202020202020</u> 240 250	<u>ک</u> 260	α7 000000 270	β11 → τι 280	α8 <u>000000</u> 290	FTβ12 3003	TTT 10
Mouse Human bat Zebra_fish coelacanth Ophha turtle chicken Platypus Guinea_pig	TVTASAGIAFLNIVNF AVTASAGLAFONTVNF HITLAASGLFYHTVNV SVSASAGANFFDKVKF AVTAASLNFFNKVNL AITAAAGISFHNINFF ATASAGASFHSILDF SITASAAVAFHSIVNV' SITTSAAVSFHSLIDA SVTASAGAVFSKIVNA	KVETDY ISQTSLT KFEENYTSQNVLT EAAASWQVQNRFI DIGGNTSQGSSQS GFMMNTGTESEIA NMGVSVGTEDNLT NIGGSLSTQDAFT KTKDSEGTSSAFT KFGEALHLEGGFN KVDIGYSTEDSLI	KDYLSNRTNSR KSYLSNRTNSR LIYMSNMVASK SSYQGNITYSL KMYVENTVSSR KQYQGNRTGSR KQYLANRTNSR KQYLENRTNSR QNYISNRTSSR KGYLSN <u>RTNS</u> R	VQSFGCVPFYPQ VQSFGCVPFYPQ IRSHGCALFYPQ IQSHGCALFYPQ VESFGCVPFYPQ VESFGCVPFYPQ VESIGCVPFYPQ VESIGCVPFYPQ VQSIGCLAFYPQ VQSIGCLAFYPQ VLSLGGIPFHPQ	ITLETWOKGI ITLOAWOQGI ITLOKWOQGI ITLOKWOQGI ITLOKWOCGI ITLKWOCGI ITLKWOCGI ITLKTWOCGI ITLKTWOCGI ITLKWOCGI	T N H L V A I D RA 31 T N H L VA I D RS 35 S N L VA I S RS 31 L N N L A A I D RS 31 P N L VA I T KS 31 T N Q L VA L D RS 31 S N Q L VA L D RS 31 S N Q L VA I D RS 31 T N H L VA M D RT 31	PLHFFI PLPMLL PLPMLL PLHYFL PLFFF PLFFFI PLYFFI PLYFFI PLYFFI PLYFFI
		α9				ß13	
Mouse	320 <u>330</u> 330	<u> </u>	22 350	360	ТТ 370	380	390
Mouse Human bat Zebra_fish coelacanth Ophha turtle chicken Platypus Guinea_pig	KPDKLPGLPGPLVKKL NPNMLPDLPGPLVKKV EFEALPELPAPAVHRV NPSTFPDLPFPTVNKL TPNNLPGLPSPILKKL TPSAPEL SPSTLPELPSPILKKL NPETLPELPSPVKKL KPNLLPELPRPLVKQV	SKTVETAVRHYTSKTVETAVKRYYTAAAVHSAINRYYEASTVRKAAERYYKATTVKKTIMLYYASKTIESAIDRYYTATRRYYTAKRVEMAIRRYYTAKRVEMAIRRYYTAKTVEVAVRRYFGQTVEDAVRRYF	FNTHPGCTNUD FNTYPGCTDLN VNAHPGCLRRG VNTIPGCVNUD INTYPGCVNUD FNTYPGCVNLA FNTYPGCTDAA FNTYPGCTDAA FNTYPGCTDAA FNTYPGCTDAA FNTYPGCTDAA FNTYPGCTDAA FNTYPGCTDAA FNTYPGCTDAS	S P N F N F Q A N Y D S P N F N F Q A N Y D S P N F N F Q A N Y D S P N F N F Q A N Y D S P N F N F Y A N Y D S P N F N F Y A N Y D S P N F N F Y A N A D S P N F N F Y A N A D S P N F N F Y A N A D	DSCDAK.VTN GSCEGK.MTN GSCSGCSGNPAN ASCEGP.ITN GSCCGCP.ITN GSCCGCA.GTN GSCTGGT.ATN GSCVGT.MTN GSCVGT.MTN GSCNGK.THN	PTFGGVY2EGTE FSFGGVY2EGTC VSFGVY2EGTC LSFGGIY2KCFP FTFGGVY2EGP FTFGGVY2EGAP FTFGGVY2EGAP FTFGGVY2EGC FTFGGVY2EGC FTFGGVY2EGC STFGGVY2EGC 3	LSGD SGNRD VSGMDA LTP.DG LSGSDA LSGDA LEGPDA LAGPDT LSCGE NTG.KD
			EGF		P2		
Mouse	α10 2222 400	410 TT	β14	}	440	β15	<u>60</u>
Mouse Human bat Zebra_fish coelacanth Ophha turtle chicken Platypus Guinea_pig	.VICQNLEQKNLLTGD VLLCQKLEQKNPLTGD DELCQNYSTPNPLTGH NICDETAQKNPLTGG OVLCQLEQKNPLTGT DVLCQLEQKNPLTGT NELCRVLQQKNPLTGA FLLCQSLEQKNPLTGG LALCPKLQQKNPLTGG	FSCPBGYSPVHLL FSCPSGYSPVHLL LSCPPNYKASVLN FSCPGHYNTTLLH FSCPTUYTPVLLH FSCPGYGYTSIKLL FSCPAGYSVLS FSCPAGYSPVLLG FSCPAGYSPVLLG FSCPSGYTPIRL 4	SQTHEEGKSRLI SQIHEEGKSRLI SELKTSSKIHS SEVVEKGENHI NEERTDDVSRII SQHEEGFSHLI TQEREEGKSHLI SQTTEEGYSRLI SQTTEEGYSRLI SQTTEEGYSRLI	ECKKKCTL 2 HRKCTL 2 CHRKCTL 2 CHRCKSCFI 3 CYPQCHTCWIF 0 HQDCFL 2 RNSCVL 3 CNSCVL 5 KKCTL 5 KKCTL 5 KKCTL 5 KKCTL		SDVFRVAKAEFR SDVFQVAKAFF SDVFQVAKAFF SDSYHVRRAKI SDSYHVRRAKI RNVYHTRKAKIN NDIFTLSKVQFS SDVFWLSRVQFR SDVFWLSRVQFR SDVFQVARAFFR SDVFQVARAFFR	AYWCVA ASWCAP TLWCSS AYWCAA SHWCAA SHWCAA ASWCVA AYWCVA ASWCVA ASWCVA A

β-hairpin

	β16	β17	β18 α11	η1 β19	
Mouse	470 480 490	TTTT.	T <u>→→→</u> 2000 510 5	20 530	тт 540
Mouse Human bat Zebra_fish coelacanth Ophha turtle chicken Platypus Guinea_pig	AGQVP.DNSGLLFGGVFTDKTINPMTNAQSC SSQVP.ENSGLLFGGLFSKSINPMTNAQSC TQASPPATIDFLGGLYSNHPNPLTRGWTC THKTP.ENSGYLFGGLFGPGIQNPLTKSSSC THFVP.QGSGYLFGGLYSASSENPLTNTHAC RGPVS.ETSAFLFGGLFSKSVNPMTNAQSC SCPVP.ENSGYLFGGLFSSRSVNPMTNAQSC SCPVP.PDSGYLFGGLFSSRSVNPLTGAQSC TCQVS.SHSGFLFGGLFSSRSVNPLTGAQSC TCQVS.SHSGFLFGGLFSSRSINPVTNAQSC 3	PAGYIPINIFE PAGYIPINIFE SYFYPITIFG SYFYPITIFG SSFYPIKLFD SSFYPIKLFD SSYFPIKLFD SSYFPIKLFD PASYTINIFE	SLKV CVSLDYBLG NLKVCVSQDYBLG DLRVCVSSDPBMC GMMICMSNDYBIG QLKVCVSSDPBTC QLLVCVSSDPBTC QLQLCVSRDYBTC DLRLCVSQDYBC SLRVCVSQDYBC NLKVCVTQDYBLG	FKFSVPFGFFSCIM SRFAVPFGFFSCV TRSVFFGFFSCV TRFSVFFGFFSCQS LQYSVFFGFFSCQ RHSVFFGFFSCQI FRHSVFFGFFSCQI SRYAVFFGFFSCT SRYAVFFGFFSCTA XFSVFFGFFSCTA 8	GNPLVNSDT GNPLACLLK GNPLACLLK GNPLGVLK GNPLGVLK GNPLGCLLN GNPLACAHQ GNPLACACH GNPLASSGQ GNPLVNAAL
	β20	β21			
Mouse	550 560	→TT 570	→ 580	590 600	-
Mouse Human bat Zebra_fish coelacanth Ophha turtle chicken Platypus Guinea_pig	A.KDVRAPSLKKCPGGFSQHL S.RDLGAPSLKKCPGGFSQHP DSPTAYPMKCPKGYNQHQ OSRCPPQFSQHL DQSPGILKDFFYPNSLTNYPMKCPSGYSQHL .GTENDSYLKRCPAGFSQHL .GTAEDPYAKRCPGGFSQHL PGGHPDAPFLKRCPGGFSQHL S.GDHQKKCPGGFSQHL	AVISDGCQVSYC ALISDGCQVSYC AYISDGCQILYC ALISDGCQULYC ALISDGCQVLYC ALISDGCQVDYC ALISDGCQVDYC ALISDGCQVSYC ALISDGCQVSYC ALISDGCQVSYC	VKAGLFTGGSLLF VKSGLFTGGSLPF LRAGTLLDLEQVA VOSGVFSGGHLKF IKAGELFARKLLF VQSGFTDKSLPC VQSGLFTGGALPF VKAGLFTSQSLPF VKAGLFTSQSLPF	VRLPPTKPPLMSQV ARLPPFTPPLSLNN VRLPPFTPPLSLNN VKLPPFLHAAFNLS ARLPPTHFAFSISFL ARLPPTHFAFSISFL ARLPPTTFFPANLP. VRLPFTRPPANLP. VRLPPFSRPPLMSQA	ATNTVI SSCSORLSV ATNTVI SSCSORLSV ATNTVA SAR.AL ATDIIL ATDIIL AVDTVL STNTVL ATNTVI
			стт		
Mouse	$\begin{array}{cccccccccccccccccccccccccccccccccccc$	0000 0 650	660	670 68	o
Mouse Human bat Zebra_fish coelacanth Ophha turtle chicken Platypus Guinea_pig	VTNSETARSWIKDPQTNQWKIGEPLELRAM VTNSENARSWIKDSQTHQWRIGEPIELRRAM LVDSSDLQVWVQKGCSHWLQANISDKSLPA .VMTEGERSWVRVGETKMWRLAKPGDIKQMQ YVMKPGESSWVKDSQTQMWKIADATDEKKFT VTSSNGDQTWVKDPQTQQWKLANSEEVLHYL VTSVDGARSWVKDAQTGLWRLGPAETRRAA VSSGDGESAWVRDGTGRAWRLAQPIEIQQAA VTNSETEKSWVRDSTHLWKLGEPSEVRRII VTSSEGSSWIRDSTHQ	TVIHGDSNGMSG NVIHGDGGGLSG NVIHGDAGGLSG SILD.ASGPSV SILD.ASEMSG 2LF0.PQGSVSG SLGG.GGGLSG EMVR.GRGLLSG KLVQGSERGLSG NGGLSG	GEAAGITIGUTIA GAAAGUTUGUTTI WSIVGTCLGIIVG GKKAGVAIGIIVI GAAAGISIAUTTI GEAAGISIAUTTI GETAGUTUGUTTG GETAGUTUGUTTG GEVAGUAAAVVVG GEAAGLTTGITTI GAAVGVSVAUTSI	LGIVITLAIYGTRKY LAVVITLAIYGTRKF VVV VALVVAGTVVIMKRR LAALIALAVVGTKRY LATLIGLVVRVCKRY LATLIGLVVRVCKRY LAFLIALAIYGSRRL LVTVLAMVCYGRWRY LAGLVLLAFYGIRKY	KK NRFSSLKLN KK KK K R KS
			тм	Cytosc	lic tail
<i>Mouse</i> Mouse Human bat Zebra_fish	690 700 710 REYQETEEQESL.VGSLATDATVLNGEEDPS KAYQATEERQSL.VPGTAATGDTTYQEQGQS 	PA PA ENPNQQLLS.			
coelacanth Ophha turtle chicken Platypus Guinea_pig	RGYRSLEGSSMVEDQVQYGAIENSDPLAS REFSKTEGEKTCLMSNNPVYGLPEMEEEDT RGYRRVEEGQSL.IPGSAAYGTAAELGESCQ RGYLAMGEGARL.PVESPEDSTTVNMGEGHQ RDYIQKQRLISG.SANYTAGPTHPNETFD KGYQPIDGEQKN.VFVETGTGNIPELQPQRF	EAGNSTA. LQPEQEKQPV PD QEPAGLTE PG PA			

Fig. S4. Sequence alignment of PFN2 across species. Sequence alignment of PFN2 based on amino acid sequences taken from Uniprot: mouse (A1L314), human (Q2M385), bat (G1QCG4), zebrafish (Q7SXE0), coelacanth (H3A4Y7), ophha (V8NKD5), turtle (K7FJT2), chicken (R4GL52), platypus (F6W8W1) and guinea pig (H0VL25). Numbers above the sequence alignment correspond to amino acid residue numbers relative to the mouse PFN2 sequence, where residue 1 is the initial methionine. The pre-pore secondary structures determined from mouse PFN2 are marked along the sequence. Different domain sequences are indicated by lines color-coded as in Fig. 1. The dashed box between β 14 and β 15 represents the β -hairpin of the P2 domain. The mutated residues forming the disulfide lock are indicated by gold arrowheads. The conserved N-glycosylation sites are labeled with cyan asterisks below cyan lines.



Fig. S5. pH- and concentration-dependent pore-forming activity of mPFN2. (A) Liposome leakage assay of purified mPFN2 protein incubated with liposomes containing 50% PC, 10% PS and 40% *E.coli* total extract at pH7.5 showed no pore forming activity. The insert shows final leakage of sulforhodamine B as mean \pm s.d. from three technical replicates. (B) Final leakage of sulforhodamine B from liposomes incubated with different concentrations of mPFN2 as shown in Fig. 4A, expressed as mean \pm s.d. from three technical replicates. (C) Final leakage of sulforhodamine B from liposomes pre-formed at different pH values as shown in Fig. 4B, expressed as mean \pm s.d. from three technical replicates. (D) Negative stain micrograph showing a few mPFN2 pores (boxed) formed on liposomes at pH5.5. Bar = 50nm.



Fig. S6. pH-dependent pore-forming activity of mPFN2. (A and B) Representative negativestain micrographs showing that at pH5.5, the amount of mPFN2 pre-pore binding to a liposome containing 30% SM is dramatically reduced (B), compared to that bound to the liposome containing 40% *E.coli* total lipid extract (A). (C) Representative negative-stain micrograph of mPFN2 incubated with liposomes containing 40% *E.coli* total lipid extract at pH4.0, showing the clustering of the liposomes. (D) Representative negative-stain micrograph of mPFN2 incubated with liposomes containing 30% SM at pH4.0, showing more distributed pore oligomers without clustering of the liposomes. Magenta arrowheads show the presence of arcs in (C) and (D). Bar = 100 nm.



Fig. S7. Disulfide locked mPFN2 pre-pores at pH 5.5. (A-C) Close-up views of the TMH2 helix disulfide lock (B) and P2-CTT inter-domain lock (C) as boxed in (A). (**D**, **E**) Representative negative-stain micrograph and 2D class averages of oxidized PFN2^{K251C/G286C} and PFN2^{G568C/T594C} incubated with liposomes at pH5.5. (**F**) Sulforhodamine B release from liposomes incubated with mPFN2^{WT}, PFN2^{K251C/G286C} and PFN2^{G568C/T594C} respectively indicating the blockage of pore formation by the introduced mutation at pH5.5. The insert shows final leakage of sulforhodamine B expressed as mean \pm s.d. from three technical replicates. (**G**) Pre-incubation of PFN2^{K251C/G286C} mutant with 1mM DTT restored the pore forming activity of mPFN2, while 10mM DTT was required for restoring the pore forming activity of the PFN2^{G568C/T594C} mutant. Final leakage of sulforhodamine B represented as mean \pm s.d. from three independent experiments.



Fig. S8. Structure determination of mPFN2 pore. (**A**) Representative negative-stain micrograph and 2D class averages of mPFN2 pore. Representative top view and side view particles are indicated with a green square and a circle, respectively. (**B**) Representative cryo-EM micrograph and 2D class averages of mPFN2 pore oligomers, a green square and circle indicate the top and side views of the pore, respectively. Bars represent 50 nm in the micrograph and 10 nm in the 2D class averages in A and B. (**C**) Global resolution assessment of the pore map measured using the 'Gold-standard' Fourier shell correlation (FSC) curves from two half-reconstructions refined separately in RELION. (**D**) Local resolution evaluation of the mPFN2 pore by RELION.

	Pre-pore dataset (EMDB-10134)	Pore dataset (EMDB-10135)		
Data collection and processing	(PDB 6SB3)	(PDB 65B5)		
Magnification	48077	/0983		
Voltage (kV)	300	200		
Flectron exposure $(e_{-}/Å^{2})$	40	45		
Defocus range (um)	-12 5	-12 5		
Derocus range (μ iii) Pixel size (\mathring{A})	1.04	1 22		
Symmetry imposed	C16	C16		
Initial particle images (no.)	246 755	183 241		
Final particle images (no.)	41 693	24 936		
Man resolution (Å)	3 5	5		
ESC threshold	0.143	0 143		
Man resolution range (Å)	33-55	41-92		
Mup resolution range (11)	5.5 5.5	1.1 9.2		
Refinement				
Initial model used (PDB code)	3NSJ			
Model resolution (Å)				
FSC threshold	0.5			
Model resolution range (Å)	3.8			
Map sharpening <i>B</i> factor ($Å^2$)	-104.6			
Model composition				
Non-hydrogen atoms	75712			
Protein residues	9664			
Ligands	32			
<i>B</i> factors (Å ²)				
Protein	36.62			
Ligand	40.47			
R.m.s. deviations				
Bond lengths (Å)	0.002			
Bond angles (°)	0.552			
Validation				
MolProbity score	1.97			
Clashscore	13.12			
Poor rotamers (%)	0			
Ramachandran plot				
Favored (%)	95.10			
Allowed (%)	4.90			
Disallowed (%)	0			

Table S1. Cryo-EM data collection, refinement, and validation statistics.

	Native 1 (2 copies)	Au-derivative	Native 2 (8 copies)**
Data collection			
Space group	P2 ₁	P2 ₁	P1
Cell dimensions			
a, b, c (Å)	70.53, 31.27, 103.65	71.01, 30.91, 103.31	70.23, 70.05, 128.14
α, β, γ (°)	90, 90.42, 90	90, 91.61, 90	78.5, 79.05, 85.01
Resolution (Å)	58.98-2.05 (2.09-2.05)*	57.44 - 2.44 (2.48 - 2.44)	51.18 - 3.17 (3.28 - 3.17)
$R_{\rm merge}$	0.07 (1.374)	0.126 (1.462)	0.2618 (1.858)
$R_{\rm pim}$	0.03 (0.575)	0.054 (0.858)	0.0769 (0.3183)
ΙσΙ	12.7 (1.3)	9.9 (1.3)	10.41 (1.18)
Completeness (%)	99.4 (99.2)	99.7 (99.8)	95.30 (59.72)
Redundancy	6.5 (6.7)	6.6 (6.6)	10.7 (1.5)
CC-half	0.999 (0.661)	0.997 (0.545)	0.993 (0.778)
Refinement			
Resolution (Å)	51.81 - 2.05 (1.15 - 1.11)		51.18 - 3.17 (3.28 - 3.17)
No. reflections	189385 (33178)		405935 (3449)
Unique reflections	28963 (7980)		37940 (2362)
$R_{\rm work} / R_{\rm free}$	21.21% / 24.49%		18.87% / 22.46%
No. atoms			
Protein	2988		11992
Ligand/ion	110		
B-factors			
Protein	74.24		90.0
Ligand/ion	79.31		
R.m.s. deviations			
Bond lengths (Å)	0.009		0.003
Bond angles (°)	1.1		0.76

Table S2. X-ray crystallographic statistics of P2 domain.

*Values in parentheses are for highest-resolution shell.

** Several datasets from the same crystal were merged together.

Movie S1. Pre-pore PFN2 oligomers on mica. High-speed atomic force microscopy (HS-AFM) image sequence of perforin-2 pre-pore assembly dynamics on mica.

Movie S2. Pre-pore PFN2 oligomers on a supported lipid bilayer. High-speed atomic force microscopy (HS-AFM) image sequence of hexagonal-packed perforin-2 pre-pores freely formed on a supported lipid bilayer.

Movie S3. Mobility of membrane-bound pre-pore PFN2 oligomers. High-speed atomic force microscopy (HS-AFM) movie of a force-sweep experiment in which the tip of the HS-AFM is used to sweep membrane-bound pre-pores from the field of view, and they are allowed to redistribute themselves when the tip force is subsequently minimized.

Movie S4. Real-time pore formation imaged by HS-AFM. High-speed atomic force microscopy (HS-AFM) experiment showing the conformational change occurring when membrane-bound pre-pore PFN2 oligomers are exposed to an acid shock (see Materials and Methods for more details). The pre-pore to pore transition starts at 14 seconds after injection of 0.1M HCl and results in a ~4nm increase in the oligomer height above the surface to which it is attached, and the transition finishes within 3 seconds. The break up of many ring form pre-pores into arcs is probably due to the sudden change in pH caused by addition of 0.1M HCl. See also Main Text Fig. 5.