Supplementary Information

Structure of the peroxisomal Pex1/Pex6 ATPase complex bound to a substrate

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	Class 3	Class 4
	Single-'seam' conformation	Twin-'seam' conformation
Local anisotropic	phenix.local_aniso_sharpen	phenix.local_aniso_sharpen
half-map sharpening	run_half1_class001_unfil.mrc	run_half1_class001_unfil.mrc
	run_half2_class001_unfil.mrc	run_half2_class001_unfil.mrc
	resolution=4.14	resolution=4.7 local_sharpen=True
	local_sharpen=True	sharpened_map_file_1=.\Output_comman
	sharpened_map_file_1=.\Out	d\half1_local_sharp.ccp4
	put_command\half1_local_sh	sharpened_map_file_2=.\Output_comman
	arp.ccp4	d\half2_local_sharp.ccp4
	sharpened_map_file_2=.\Out	box_size_grid_units=384
	put_command\half2_local_sh	sharpened_map_file=.\Output_command\f
	arp.ccp4	ull_local_sharp.ccp4
	box_size_grid_units=384	
	sharpened_map_file=.\Output	
	_command\full_local_sharp.c	
	cp4	
Density modification	phenix.resolve_cryo_em	phenix.resolve_cryo_em
	half1_local_sharp_aniso.ccp4	half1_local_sharp.ccp4
	half2_local_sharp_aniso.ccp4	half2_local_sharp.ccp4
	seq_file=.\Input\Pex1_Pex6_y	seq_file=.\Input\Pex1_Pex6_yeast.fasta
	east.fasta resolution=4.14	resolution=4.14 blur_by_resolution=True
	blur_by_resolution=True	blur_by_resolution_factor=20
	blur_by_resolution_factor=20	box_cushion=15 b_iso=90
	box_cushion=15 b_iso=90	dm_resolution=3.8
	dm_resolution=3.6	

Supplementary Table 1: Phenix density modification command prompts.

	Data o	collection										
Microscope		Titan Krios with Cs-corrector and XFEG electron										
		source										
Voltage (kV)		300										
Nominal magnification		105,000 x										
Electron Dose (e ⁻ / Å ²)		60										
Number of frames		60										
Detector		Gatan K3										
Pixel size (Å)		0.34 (super res	solution mode)									
Defocus range (µm)		-0.6 to -2.4										
Micrographs		16,763										
	Recor	struction										
Software		SPHIRE 1.4,	RELION 3.1.4, Phenix 1.20									
		Cryosparc 4										
Total extracted particles		1,259,079										
Structure	Clas	ss 3	Class 4									
Pex1/Pex6E832Q	Single-'seam' c	onformation	Twin-'seam' conformation									
Particles	164	,610	128,983									
Symmetry	C1		C1									
Final average resolution, gold	4.1 Å		4.7 Å									
standard FSC=0.143												
Final average resolution after	3.9 Å		4.3 Å									
density modification FSC=0.5												
	Model	refinement	1									
Peptide chains	7		7									
Residues	5564		5564									
Ligands	ATP: 10, ADP:	2, Mg2+: 10	ATP: 10, ADP: 2, Mg2+: 10									
RMSD Bond length (Å)	0.003		0.004									
RMSD Bond angles (°)	0.751		0.881									
Ramachandran outliers (%)	0.04		0.00									
Ramachandran allowed (%)	7.26		9.8									
Ramachandran favored (%)	92.68		90.2									
Rotamer outliers (%)	0.06		0.00									
MolProbity score	1.81		1.89									
Clash score	6.27		6.28									
EMRinger score	1.53		0.99									
B-factors mean (Protein)	132.26		131.73									
B-factors mean (Ligand)	92.38	89.13										

Supplementary Table 2: Cryo-EM data collection and refinement statistics

Supplementary Figures



Supplementary Figure 1: Time-dependent decrease in ATP/ ADP ratio analyzed by HPLC. Representative chromatograms are shown (a-c). Control experiments was performed with ATP alone. The ATP/ADP ratios in the presence of Pex1/Pex6_WB were obtained by quantification of the results from HPLC analyses at different timepoints upon addition of ATP to the protein sample (d-e). Source data are provided as a Source Data file.



Supplementary Figure 2: Expression and purification of recombinant Pex1/Pex6_WB. a) Size exclusion chromatogram (SEC), showing one major peak corresponding to a complex of Pex1 (star) and Pex6_WB (arrow), as confirmed by SDS-PAGE (inset). The red bar indicates fractions, which were pooled for subsequent cryo-EM studies. **b)** Representative micrograph of negatively stained Pex1/Pex6_WB complex after SEC. Scalebar 100 nm. **c)** Representative cryo-EM micrograph of Pex1/Pex6_WB low-pass filtered to 5 Å. Scale bar 20 nm. Boxes represent particles identified by crYOLO. **d)** Representative reference-free 2D class averages of Pex1/Pex6_WB complex. Scale bar 10nm.



Supplementary Figure 3: Single-particle cryo-EM processing workflow. The final maps of class 3 and class 4 from RELION and Phenix have been used for model building. Electron density maps are shown at (class3, single-seam state) 4.42 sigma (2.26 sigma for density-modified) and at (class4, twin-seam state) 4.72 sigma (2.11 sigma for density-modified), respectively.



Supplementary Figure 4: Cryo-EM structure of the single 'seam' state (class 3). a-b) Different surface views and cross-section of the cryo-EM density map colored according to the local resolution, upon post-processing in RELION at 4.42 sigma (a) and after local anisotropic half map sharpening and density modification using Phenix at 2.26 sigma (b). c) Angular distribution for the final round of the refinement. d) 3D FSC curve. e) Half-map FSC curves and f) model-map FSC curves before (gray) and after (red) density modification using phenix. g) Representative areas of the density map superimposed with the molecular model. Densitymodified map is shown at 2.26 sigma.

4																						
<u>β</u>	A	γββ β		β				γβ	A	-	β		b —	β	A .	->-	Η2 β	βΒ			H3	M
TILKNGAIQLLKI	KVILRSTVCKN	DFPKDNLF	VVYISD	GAQLPS	SQKGYAS I	VKCSLR	SKKSD	SDNKSVG	I PSKKIGV	FIKCD	QI PENHI	ALSSHLV	DAFFT	HPMNG.	AKIKLE	FLQM	NQANI	SGRNA	TVNIK	YFGKDVF	TKSGDQY	YSKL
N2 210	215 220	225 230	235	240	245 25	0 255	260	265 2	70 275	280	285 29	0 295	300	305	310	315	320	325	330	335 34	0 345	350
			βββ				-	β		β	He	-	_	β	<u></u>	н					βγ γ	β
LGGSLLTNNLIL	PTEQIIIEIKK	GESEQQLC	NLNEIS	NESVON	KVTQMGK	EEVKDI	ERHLP	KHYHVKE	TGEVSRTS	KDEDD	TTVNSI	KEMVNYI	TSP11	ATPAI	LDGK	G 1GK	TRLLK	ELINEV	EKDHH	I FVKYAI	CETLHET	TSNL
351 355 360	365 370	375 380	385	390	395 40	0 405	410	415 4	20 425	430	435 44 D1	0 445	450	455	460	465	470	475	480	485 49	0 495	500
	<mark>€</mark>	β βββ	γβ	β γββ	вара но	~		ββ β	β E	γſ			e C			PB	β	β	-112 	<u> </u>		
DKTQKL IMEWCS	FCYWYGPSLIV	LDNVEALFO	GKPQAN 525	DGDPSN	INGQMDNA	SKLLNF	FINQVT	K I FNKDN	KRIRVLFS	GKQKT	INPLLFI	KHFVSET	WSLRA	PDKHAI	GIO	YFFS	KNQ IM	(LNRDL	QFSDL:	SLETEGE	SPLDLEI	I FTE
301 303 310	515 520	525 530	555	540	045 55	1110	500	100 1	10 515	560	202 25	U 393	000	005	010	015	020	1122	030	035 04	0 045	0.00
	β Μ4	Se Bl	β γβ	- \				γβ	βγ		<u>i – í</u>			β			β				<u> </u>	-
K1 FYDLQLERDCI	DNVVTRELFSK	SLSAFTPS/	ALRGVK	LTKETN	IKWGD1C	ALANAKI	WLLET	LEWPTKY	EPIFVNCF	VRLRS	HLLYGY	GCGKTLI	ASAVA	QQCGL	FISVE	GPEI	LNKFIC	GASEQN	IRELF	ERAQSVK	PCILFFE	DEFD
651 655 660	665 670	675 680	685 D2	◆ ⁶⁹⁰	695 70	0 705	710	715 7	20 725	730	735 74	0 745	750	755	760	765	770	775	780	785 79	0 795	800
β	β H24	BB I	β F	β	ββ βββ	ββ		ββ		1	ββ β		ββ Η2	6 	H27		~		B	3	ββ	β
STAPKRGHDSTG	VTDRVVNQLLT	QMDGAEGLI	DGVYIL	AATSRP	DLIDSAL	LRPGRLI	KSVIC	NIPTESE	RLDILQAI	VNSKD	DTGQKKI	ALEKNAL	DLKLIA	EKTAG	SGADI	QGLC	YNAYLI	SVHRW	LSAAD	QSEVVPC	NDNIEYE	FSIN
801 805 810	815 820	825 830	835	840	845 85	0 855	860	865 8	70 875	880	885 89	0 895	900	905	910	915	920	925	930	935 94	0 945	950
ββ		β	β		NP ^Y	β			ββ													
EHGRREENRLRLI	KTLLQQDVVHE	TKTSTSAAS	SELTAV	VTINDL	LEACQET	KPSIST	SELVKL	RGTYDRF0	QKDR													
b	905 970	975 980	965	790	995 IO	1005	1010	1012 10	20													
D																						
β	HI	β	β Α	0		A	β	βA	H2		A	H3 Y	В	H		-	H5			ββ C	β ββ	ββ 🖁
-		<u>.</u>	-									-		-	<u>U</u> C	-	-0		-		_	
MKASLTFSLSGT	YAPCS I SRDI Y	LEYGDKKAI	ECLYGT 25	IRL PQY	/GPGCTPG	K1VHCVI	DDSLP	FCSIVVP	SKLFGFMF	PTQPTM	OFCYFEP I	LDNVVPV	/LDSVT	TELINE(ULYSKI 110	MDL P	QEMQQ	125	KYNIN:	SMETVVI 125 Li	ISRDILTS	SGLC
N1 C	10 20 D	20 50		40	45 50	,	00	05 7	0 15	00	0.5	1 22	100	105	110	11.5	120	140	1.50	155 14	0 145	100
βββ	γββ	βΗΟ	β	γβ	3 💾 β	D		110			E HO		E			- E		D		011		
OT INCEDEDOCT	UDETETOLIU	-	,			_		H8	В	βββ	E H9	βγ	βF	ββ	-	E	β	D	β	H10	<u> </u>	
QTENCSPEPQUE	VDFIEIQLILV	MINTEOPT C	ALUXANT	EDEEXA		CAL C LD	-	HS	B	βββ		β	βF		>	- E	β		β			
151 155 160	165 170	NDTEQKLS. 175 180	ALKYAN 185	EDEEYA	ALPKIGTN 195 20	SALS I D	LESLPC 210	H8 TISRDLLI 215 2	B RPAPHINE 20 225	βββ DNS I Y. 230	E H9	βγ LRLDVTS 0 245	β F GSF11 250		VRLVK 260	LFVL 265	β LLPNGI 270	D FKKRT I 275	β <u>YAPPK</u> 280	H10 1 I AS F PE 285 25	CSVVT1S 0 295	SKSN 300
151 155 160	165 170 G 000	NDTEQKLS. 175 180 H12	ALKYAN 185	EDEEYA 190	ALPKIGTN 195 20 H o G	SALSID	LESLPC 210	H8 T I SRDLLI 215 2	B RPAPHINE 20 225 HI4	βββ DNS 1 Y. 230	FTDAETI 235 24 G	βγ LRLDVTS 0 245	β F GSF11 250		VRLVK 260	E LFVL 265	β LLPNGI 270 G	D FKKRT I 275	β <u>YAPPK</u> 280	HIO 1 LASEPE 285 25 HI5	CSVVT1S 0 295	SKSN 300
151 155 160	ιό5 170 G βββ	NDTEQKLS. 175 180 H12	ALKYAN 185	EDEEYA 190	ALPKIGTN 195 20 Η β G	SALS 1D 205 N2	LESLPC 210 ββ β	H8 TISRDLLI 215 2	β RPAPHINE 20 225 β γ HI4	βββ DNS I Y. 230 β	E H9 AFTDAETI 235 22 G	βγ LRLDVTS 0 245	β F GSF11 250	ββ 255 β H	VRLVK 260 ββ	E LFVL 265	β LLPNGI 270 G	D FKKRTI 275	β <u>YAPPK</u> 280 βββ	HIO 1 I ASF PE 285 25 HI5	CSVVT18 0 295 7	<u>sksn</u> 300 ββ
	165 170 G βββ F I SRVGGM OS	HI2		ISO	ALPKIGTN 195 20 Η β G		ESLPC 2iο ββ β	H8 TISRDLLI 215 2 Y	B RPAPHINE 20 225 B 7 HI4 DESDDEDE	βββ DNS I Y. 230 β	E H9 FTDAETI 235 22 G SNDSL WW	βγ LRLDVTS 0 245	β GSF11 250 β	ββ 255 β H DNSHF	VRLVK 260 ββ	E LFVL 265	β LLPNGI 270 G TTNIT	D FKKRTI 275	β <u>YAPPK</u> 280 βββ ¹ 1.SRSN	HIO 1 I AS F PE 285 25 HI5 LORYYGH	CSVVT1S 0 295 γ	SKSN 300 ββ
151 155 160 1 β 1GHTD1P1ANQVI 301 305 310	165 170 G βββ F I SRVGGWLQS 315 320	NDTEQKLS. 175 180 H12 <u>QKCFQN111</u> 325 330	ALKYAN I85 LTTLKK 335	EDEEYA 190 FFSESK 340	ALPKIGTN 195 20 H B G C C C C C C C C C C C C C C C C C C C	SALSID 0 205 N2 0 LIPIAFI 0 355	LESLPC 210 ββββ DS SMAD 360	H8 T I SRDLLI 215 2 Y LNI AEEN 365 3	B RPAPHINE 20 225 β γ H14 DESDDEDE 70 375	βββ DNS I Y. 230 β ELGQYYI 380	E H9 AFTDAETI 235 22 G CNDSLVWI 385 36	βγ LRLDVTS 0 245 FVTSAEL 0 395	β F SGSF1T 250 β .DCFSK 400	DNSHF 405	260 ββ 11DPNF 410	E 1 265 CTKL 1 415	β LLPNGI 270 G TTNITT 420	D FKKRT I 275 NRRPLP 425	γAPPK 280 βββ ¹ LSRSNI 430	HI0 1 I AS F PE 285 25 HI5 LQRYYGH 435 42	CSVVT1S 0 295 7 7 AETFYYE 0 445	<u>SKSN</u> 300 ββ DLH1 450
151 155 160 1 β 1GHTD1P1ANQVI 301 305 310 H16	ιό ι†ο G βββ F I SRVGGWLQS 315 320	NDTEQKLS. 175 180 H12 QKCFQN111 325 330 I	ALKYAN I85 LTTLKK 335	EDEEYA 190 FFSESK 340 H17	ALPKIGTN 195 20 H β G CR I LCQND 345 35	SALSID 205 N2 N2 0 1P1AF1 0 355	ESLPC 210 ββ β DSSMAD 360 88	H8 TISRDLLI 215 2 Y LNIAEEN 365 3	B RPAPHINE 20 225 β γ H14 DESDDEDE 70 375 γ H18	βββ 230 β ELGQYYI 380	E H9 CFTDAETI 235 24 G CNDSLVWI 385 39 BBB	βγ LRLDVTS 0 245 FVTSAEL 0 395 1	B SGSF11 250 B LDCFSK 400 B H19	ββ 255 β H 255 DNSHF 405	ββ 110PNF 410	E 265 CTKL 1 415	β 270 G TTN1T 420 0	D FKKRT I 275 NRRPLP 425	γAPPK 280 βββ	HI0 285 25 HI5 LQRYYGH 435 44 D1 R	CSVVT1S 0 295 γ ΑΕΤΓΥΥΕ 0 445	SKSN 300 ββ DLH1 450 8 H2
151 155 160 1 β 1GHTD1P1ANQVI 301 305 310 H16	165 170 G βββ F 1 SR VGGWLQS 315 320	NDTEQKLS. 175 180 H12 QKCFQN111 325 330 I	ALKYAN I85 LTTLKK 335 β	FFSESK 340 H17	ALPKIGTN 195 20 H β G CRILCOND 345 35	SALSID	ESLPC 2lo ββ β DS SMAD 360 ββ	H8 T I SRDLLI 215 2 γ 1 LNIAEENI 365 3 β β'	β RPAPHINE 20 225 β γ H14 DESDDEDE 70 375 γ H18	βββ DNS 1 Y, 230 β ELGQYYI 380	E H9 AFTDAETI 235 24 G MDSLVWI 385 36 βββ	βγ LRLDVTS 0 245	β GGSF1T 250 β .DCFSK 400 β H19	ββ 255 β H DNSHF 405	260 ββ 11DPNF 410 β	E 265 CTKL1 415 ββ H20	β LLPNGI 270 G TTNITT 420	D FKKRT1 275 NRRPLP 425	γAPPK 280 βββ ¹ LSRSNI 430 L	HI0 1 LASFPE 285 25 HI5 LQRYYGH 435 44 D1 β	CSVVT1S 0 295 γ ΑΕΤΓΥΥΕ 0 445	SKSN 300 ββ DLH1 450 β H2
151 155 160 1 β 1GHTD1P1ANQVI 301 305 310 H16 FPYVRQLVN1LE	IÓS IŤΟ G βββ FISRVGGWLQS 3IS 320 TSFNCSQRGIT	NDTEQKLS 175 180 H12 OKCFQN111 325 330 I LNASVLLH	ALKYAN I85 LTTLKK 335 β STTNNW	EDEEYA 190 FFSESK 340 H17 GKATMV	ALPKIGTN 195 20 H B G CRILCOND 345 35 RFASKYL	SALSIDI 0 205 N2 N2 LIPIAFI 0 355 GIHLLE	LESLPC 210 ββ β DSSMAD 360 ββ	H8 T I SRDLLI 215 2 γ 1 LNI AEENI 365 3 β β ⁻ TSNSRQLI	B RPAPHINE 20 225 β γ H14 DESDDEDE 70 375 γ H18 0 STSK11C	βββ DDNS I Y. 230 β ELGQYYI 380 SYTRAKO	E H9 VFTDAETI 235 24 G CNDSLWM 385 35 βββ CENVLPY2	βγ LRLDVTS 0 245	β GGSF11 250 β DCFSK 400 β H19 AHLDS	ββ 255 β H 255 255 255 255 255 255 255 255 255 25	CVRLVk 260 ββ LIDPNR 410 β β	E 265 265 CTKL1 415 ββ H20 PEAIK	β LL PNGI 270 G TTNIT 420 D LQKS IN	D FKKRT I 275 NRRPLP 425 NFEMSK	γAPPK 280 βββ LSRSNI 430	HI0 1 1ASFPE 285 25 HI5 LORYYGH 435 44 D1 β TFKFPGT	XCSVVT1S 0 295 7 XAETFYYE 0 445 TFVGSVN	SKSN 300 ββ DLH1 450 β H2 NN1D
151 155 160 1 β 1GHTD1P1ANQV/ 301 305 310 H16 FPYVRQLVN1LE 451 455 460	165 170 G βββ F I SRVGGWLQS 315 320 TSFNCSQRG11 465 470	NDTEQKLS 175 180 H12 <u>OKCFQN111</u> 325 330 T <u>LNASVLLH8</u> 475 480	ALKYAN IŚ5 LTTLKK 335 β STTNNW 485	FFSESK 340 HI7 GKATMV 490	ALPKIGTY 195 20 H B G GRILCONE 345 35 /RFASKYL 495 50	SALSID O 205 N2 N2 O 355 GIHLLE O 505	ESLPC 2lo ββ β DSSMAD 360 ββ IDCLSL 5i0	H8 215 2 γ 1 LNI AEENI 365 3 β β- TSNSRQLI 515 5	B RPAPHINE 20 225 B γ H14 DESDDEDE DESDDEDE 10 375 γ H18 DSTSK11C 20 525	βββ DNS IY, 230 β LGQYYI 380 GY IRAKG 530	E H9 4FTDAETI 235 22 G CNDSLVWI 385 35 βββ ENVLPY/ 535 52	βγ LRLDVTS 0 245 FFVTSAEL 0 395 1 SPAV1FL 0 545	β F 505 F 11 250 β .DCF 5k 400 β H19 AHL DS 550	ββ 255 β H dos H LLLDV1 555	CVRLVk 260 ββ 11DPNF 410 β β 410 β β β sanodif 560	E 265 265 265 415 415 415 415 415 265	β LLPNGI 270 G TTNITI 420 D LQKS IN 570	P FKKRT I 275 VRRPLP 425 VFEMSK 575	β YAPPK 280 Bββ LSRSNI 430 LLDDF 580	HI0 1 I AS FPE 285 25 HI5 LORYYGH 435 42 D1 β FKF PGT 585 55	7 AETFYYE 0 445 TFVGSVN 0 595	SKSN 300 ββ DLH1 450 β H2 NN1D 600
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Supplementary Figure 5. Secondary structure of Pex1(chain c) (a) and Pex6(chain d) (b) computed with PDBsum (http://www.ebi.ac.uk/pdbsum/)¹. α -helices are labeled H1, H2, ...,H41 and β -strands by their sheets as A, B, ...,K. Structural motifs β -turns, γ -turns, and β -hairpins are marked as β , γ , and \supset , respectively. Important conserved motifs are indicated: Walker A (dark purple), Walker B (green), ISS (cyan), pore loops (red loop) and arginine fingers (orange).



Supplementary Figure 6: Analysis of the nucleotide binding pockets a-b) Nucleotide models and corresponding cryo-EM density of the D1 (upper panels) and D2 (lower panels) ring of the single-seam (a) and twin-seam (b) state. Density-modified map is shown at 2.26 sigma (class3) and 2.11 sigma (class4). A-F indicate Pex subunits. c) Position of nucleotide binding pockets and D2-interfaces respective to the disengaged seam (F) domain for the single-seam (upper-inset) and twin-seam state (lower-inset). Letters (from A-F) define the individual subunit position within the stair-case. Domain A occupies the highest position of the spiral. d) Measurements of the opening of the nucleotide binding pocket for the single- (gray) and twin-seam (red) state. Shown are the distances between the C α of Walker A Thr and the C α of the Arg-finger of the neighboring subunit (Pex1-Pex6 interface: Pex1(T745)-Pex6 (R892); Pex6-Pex1 interface: Pex6(T779)-Pex1(R855). e) Contact area between the large and small AAA+ domains of neighboring D2 domains. The buried surface area was measured between the D2 domains of Pex1 (residues 681-1020) and Pex6 (residues 723-1030).



Supplementary Figure 7: Structural dynamics of the D1 Ring between single- and twinseam state. a) Position of nucleotide binding pockets and D2-interfaces for the single-seam (left inset) and twin-seam state (right inset). **b-c)** Shown are measurements of the opening of the b) nucleotide binding pocket (Pex1 T468 to Pex6 D582 and Pex6 T491 to Pex1 K563) and the c) buried surface between adjacent subunits for the single- (gray) and twin-seam (red) state. The buried surface area was measured between the D2 domains of Pex1 (residues 201-680) and Pex6 (residues 201-722). **d-e)** D1 pore of d) single seam state and e) twin-seam state as surface representation. The blue transparent sphere has a diameter of 15 Å. Note the widening of the pore from single- to twin seam-state. **f)** Single-seam state (gray) superimposed on twin-seam state (colored) with chains D (except pore loop) removed to show the interior. Note that the twin-seam (left protomers, gray) swing in on the opposite side of the pore loops 1 staircase, during transition to single-seam (left protomer, colored).



Supplementary Figure 8: Distinct features of the small D1-ATPase domains of Pex1 and Pex6 determine the "trimer-of-dimers" arrangement in the D1 ring. a) Top view of the Pex1/Pex6 D1 ring. The Pex6/Pex1 dimer indicated by a black line. The D1 small ATPase subdomains ($_{s}D1$) of Pex1 (beige) and Pex6 (blue) are highlighted. b) Structural alignment of Pex1($_{s}D1$) (residues 605-685, beige) and Pex6($_{s}D1$) (residues 621-721, blue transparent). Note the deletion in helix α 13 and α 14 of Pex1($_{s}D1$). c) Contact site of Pex6($_{s}D1$) (orange highlight) binds to a hydrophobic pocket formed by helix α 7, and the loop upstream (residues 447-456) of Pex1($_{L}D1$). The middle panel shows a close-up view of this interface, with the surface of the pocket colored by hydrophobicity. The conserved histidine H592 of Pex1($_{L}D1$) (yellow highlight) binds into a negatively charged pocket formed by the prolonged helices α 26 and α 27 of Pex6($_{s}D1$). A close-up view of this interface is shown in the right panel.

d) Contact site of $Pex1(_{s}D1)$ with $Pex6(_{L}D1)$ (less compact interface between dimers). Due to the deletion in helix $\alpha 13$ of $Pex1(_{s}D1)$, Y654 (orange highlight) binds to the hydrophobic pocket formed by helix $\alpha 16$ and the loop upstream (residues 464-478) of $Pex6(_{L}D1)$. The middle panel shows a close-up view of this interface, with the surface of the pocket colored by hydrophobicity. Interestingly, due to the deletion, the analogous conserved H609 of $Pex6(_{L}D1)$ (yellow highlight, right panel) does not contact $Pex1(_{s}D1)$.



Supplementary Figure 9: Distinct features of the small D2-ATPase domains fine-tune trimer-of-dimer arrangement in the D2-ring. a) Top view of the Pex1/Pex6 D2 ring. The Pex6/Pex1 dimer is indicated by a black line. The D2 small ATPase subdomains ($_{s}D2$) and large ATPase domains ($_{L}D2$) of Pex1 (red) and Pex6 (blue) are highlighted. b) Structural alignment of Pex1($_{s}D2$) (residues 868-1021, beige) and Pex6($_{s}D2$) (residues 900-1030, blue transparent). Note the distinct Pex1($_{\alpha}28$) and Pex6($_{\alpha}38$) protrusion domains highlighted by the dashed line. c) Contact site of Pex6($_{s}D2$) with Pex1($_{L}D2$) (compact Interface within the dimer). Hydrophobic residues (M951, L952, M955 and Y944) mediate interactions within the dimer binding into a hydrophobic groove (right panel) formed by Pex1($_{\alpha}18$) and the loop upstream. d) Contact site of Pex1($_{s}D2$) with Pex6($_{L}D2$) (loose interface between dimers). Two hydrophobic residues (Y918 and Y921) on Pex1($_{\alpha}27$) mediate interactions between dimers via binding into a hydrophobic pocket (right panel) formed by Pex6($_{\alpha}28$) and the loop upstream.



Supplementary Figure 10: Interactions between the D1 and D2 ring. a) Overview of the D1-D2 interactions via the protrusion domains of Pex1(i), Pex6(ii) and flexible linker peptides (yellow double-arrows). A Pex1/Pex6 subunit dimer is shown in surface representation. The clockwise (from top) adjacent Pex6 subunit is shown in ribbon representation. b) The structural elements involved in D1-D2 interactions are shown in ribbon representation and highlighted in color. In particular, the linker peptides (Pex1 (red, aa680-690); Pex6 (blue, residues 722-729)) covalently link the D1 and D2 ATPase cassettes. The protrusion domain of Pex1(D2) (amphipathic $\alpha 28$ helix) is flanked by long flexible loops and establishes an interface between neighboring dimers via anchoring at the adjacent Pex6(N1) domain (green) (i). The protrusion domain of Pex6(D2) (helix α 38) mediates contact between the Pex6(D2) and the Pex1(_sD1) domains within a subunit dimer (ii). c) Close-up view of interface (i) (upper-insets) and ii (lower insets). Please note: The Pex1(D2) protrusion domain (α 28) is not well resolved. To address this, the interface (i) (Pex1(D2) (residues 940-976, red) and Pex6(N1)(1-201, green) (left panel) was predicted using Alphafold. Note that the Pex1 β-strand upstream of α28 enters the β -sheet 5 (β 5) of Pex6. Together they form a hydrophobic groove, to which α 28 (red) docks (right panel). ii This intra-dimer interface is flexible and depends on the nucleotide binding state of Pex6(D2). The Pex6(α 38) protrusion helix at the tip of Pex6(α 37) undergoes large movements (indicated by a dashed arrow) during ATPase cycle (right panel). During the transition from twin- to single seam state, α 38 of the Pex6(D2) domain reengages and binds into a charged groove of Pex1(D1). Scalebar kcal/(mol·e) at 298 K.



Supplementary Figure 11: Density and molecular model of PL1 and substrate of singleseam (class3) (a) and twin-seam state (class4) (b). Density-modified map is shown at 2.26 sigma (a, class3) and 2.11 sigma (b, class4).



Supplementary Figure 12: Cryo-EM structure of the single 'twin-seam' state (class 4). a-b) Different surface views and cross-section of the cryo-EM density map colored according to the local resolution, upon post-processing in RELION at 4.42 sigma (a) and after local anisotropic half map sharpening and density modification using Phenix at 2.11 sigma (b). c) Angular distribution for the final round of the refinement. **d)** 3D FSC curve. **e)** Half-map FSC curves and **f)** model-map FSC curves before (gray) and after (red) density modification using phenix. **g)** Representative areas of the density map superimposed with the molecular model. Density-modified map is shown at 2.11 sigma.



Supplementary Figure 13: Inter-subunit signaling (ISS) motif controls ATP hydrolysis. a) Illustration showing the arrangement of the D2 ring in p97 (closed conformation PDB: 7LN5)². Note that the ISS motif of subunit F (ADP) is retracted from the nucleotide site of the neighboring subunit E and primes this site for hydrolysis. **b-c)** Illustration showing the arrangement of the D2 ring in Pex1/Pex6 in "single-seam" (b) and "twin-seam" state (c). Note that the ISS at the tight dimeric interface (D/E) ("single-seam", b) blocks hydrolysis. Upon release, the dimer (E/F) ("twin-seam", c) is less compact, the ISS is retracted and the dimeric interface is primed for hydrolysis. **d)** Pex6/Pex1 "twin-seam dimer" (chain E/F, colored blue/red) superimposed with staircase engaged Pex6/Pex1 dimer from the single-seam state (chain D/E, chain D removed, chain E transparent red) aligned on the large ATPase subdomain of Pex6 to visualize conformational changes upon dimer-detachment from the staircase. Note large conformational changes of the Pex1(_SD2) subdomain upon detachment from the staircase. **e)** Same as in (**d**) but viewed from top. Pex1(_LD2) is removing the ISS motif from the nucleotide pocket to prime it for hydrolysis.

Source data



Source data 1: Uncropped gel corresponding to Supplementary Figure 2a.

Supplementary References

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