

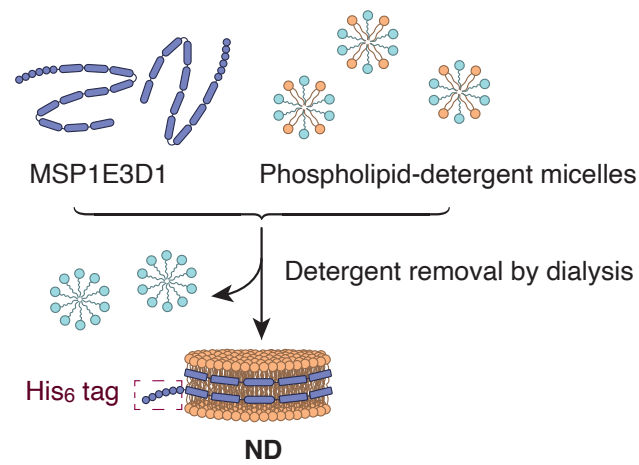
Supplementary Information

Supplementary Methods

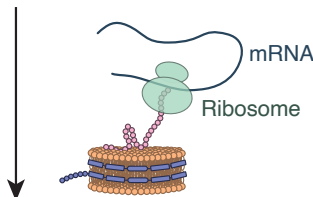
Cell-free protein synthesis with wheat-germ extract. Template mRNAs for cell-free protein synthesis were produced by in vitro transcription with SP6 RNA polymerase. Nucleotide sequences for the recombinant proteins were optimized for the wheat-germ cell-free system and cloned into the pYT08 vector. Plasmids were amplified in *E. coli*, isolated with the use of a Plasmid Maxi Kit (Qiagen), and then subjected to in vitro transcription in a transcription mixture of 100 μ l containing 10 μ g of plasmid as template DNA, 40 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM dithiothreitol, 0.01% BSA, 3 mM of each nucleoside triphosphate, RNase inhibitor (0.8 U/ μ l) (Promega), and SP6 RNA polymerase (0.8 U/ μ l) (Promega). The transcription mixture was gently mixed by pipetting and incubated at 37°C for 3 h in a water bath. The mixture was then centrifuged at 20,000 $\times g$ for 1 min, and the resulting supernatant was transferred to a new microtube, mixed with 13 μ l of 7.5 M ammonium acetate and 250 μ l of 100% ethanol, and incubated at –20°C for 15 min. The synthesized mRNAs were isolated by centrifugation at 20,000 $\times g$ for 20 min at 4°C, and the resulting pellet was washed with 800 μ l of 70% ethanol, isolated again by centrifugation, allowed to dry in air, and then dissolved in 21 μ l of deionized water.

The translation mixture (total volume of 50 μ l) contained 30 mM HEPES-KOH (pH 7.8), 100 mM potassium acetate, 2.7 mM magnesium acetate, 0.4 mM spermidine, 2.5 mM dithiothreitol, 0.3 mM of each amino acid, 1.2 mM ATP, 0.25 mM GTP, 16 mM creatine phosphate, creatine kinase (0.4 mg/ml), RNase inhibitor (0.8 U/ μ l), 15 μ M asolectin nanodiscs, 15 μ l of wheat-germ extract (CellFree Sciences), and 100 μ g of mRNA (in the case of co-expression of two different proteins, 50 μ g of each mRNA were added). The reaction mixture was placed in a dialysis cup with a molecular size cutoff of 12 kDa (Biotech International) and dialyzed against 1 ml of substrate supply solution containing 30 mM HEPES-KOH (pH 7.8), 100 mM potassium acetate, 2.7 mM magnesium acetate, 0.4 mM spermidine, 2.5 mM dithiothreitol, 0.3 mM of each amino acid, 1.2 mM ATP, 0.25 mM GTP, 16 mM creatine phosphate, and 0.005% NaN_3 . The mixture was then incubated at 16°C for 48 h.

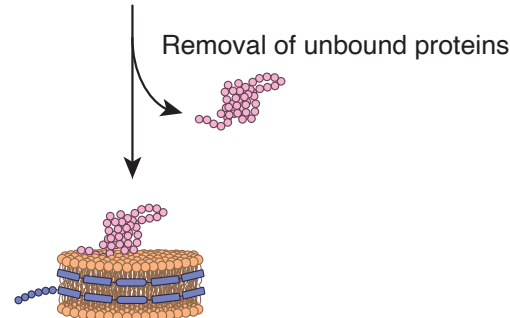
Assembly of empty nanodiscs (NDs)



Cell-free protein expression in the presence of NDs

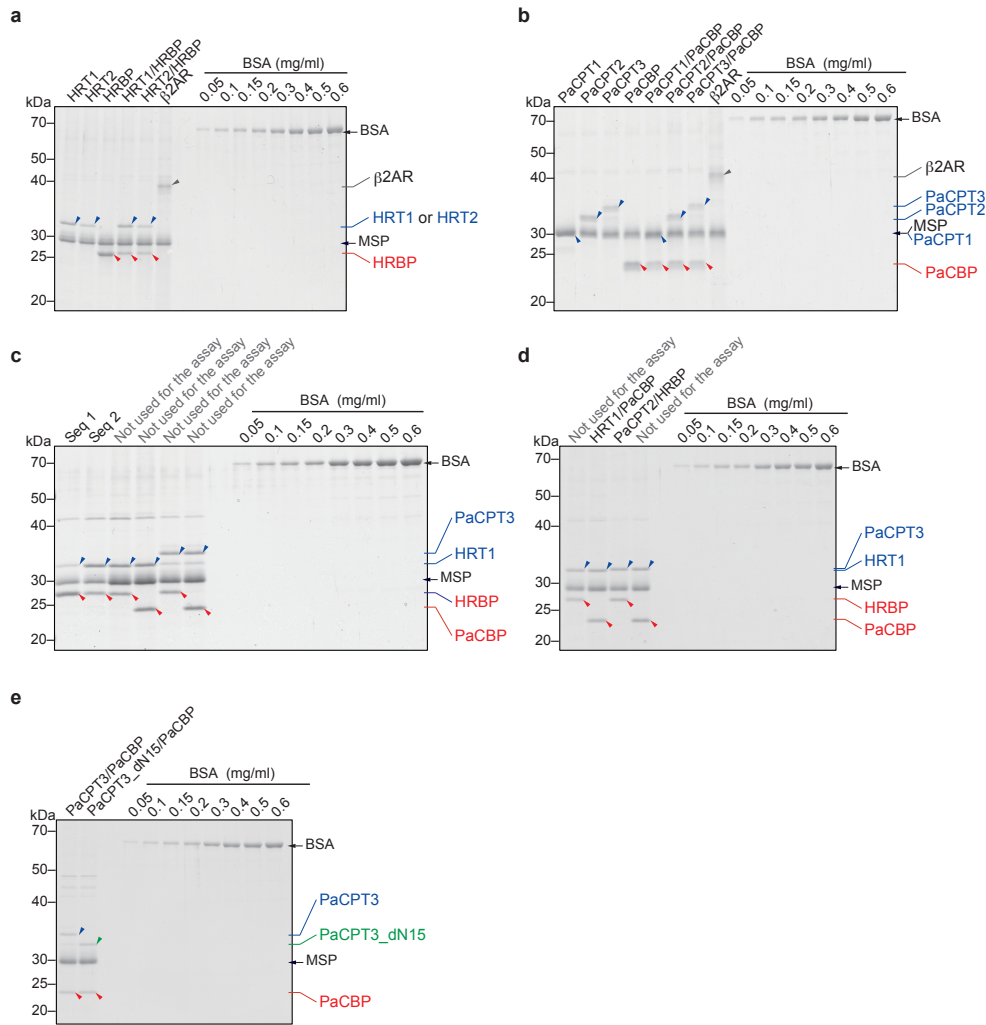


Purification of protein-ND complexes

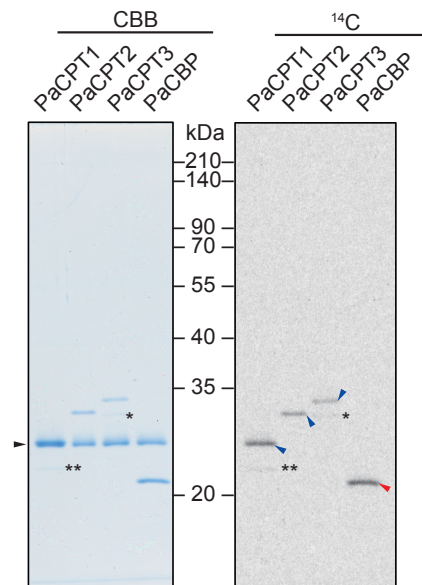


Protein-ND complex

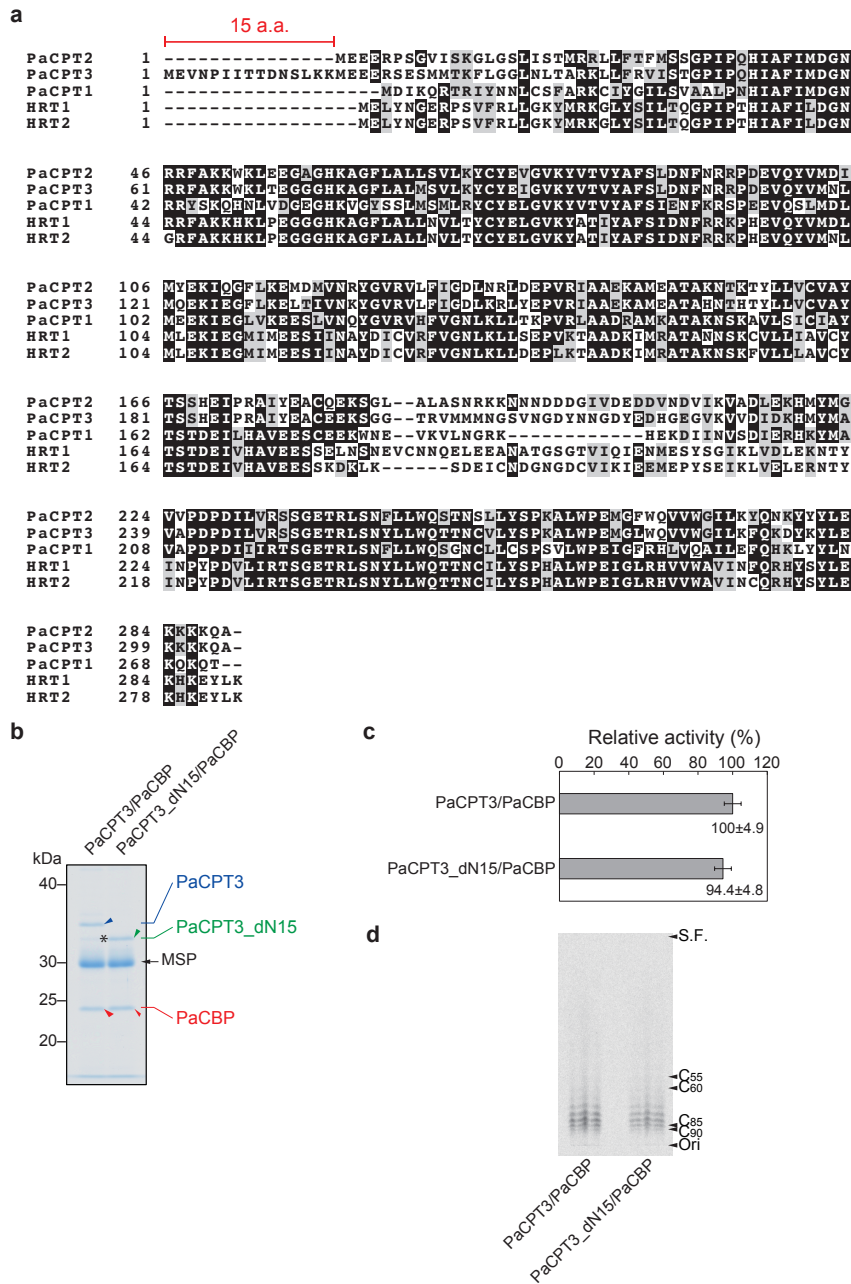
Supplementary Figure S1. Procedure for preparation of protein-nanodisc complexes. Empty nanodiscs (NDs) were prepared from purified membrane scaffold protein (MSP1E3D1) and asolectin, and were then purified by IMAC in a manner dependent on the NH₂-terminal His₆ tag of MSP. The purified nanodiscs were then added to a cell-free translation system, and co-translationally assembled (or bound) proteins were co-purified with the nanodiscs by IMAC.



Supplementary Figure S2. Estimation of protein concentration for protein-nanodisc complexes by SDS-PAGE and CBB staining and comparison with a BSA standard. *Hevea brasiliensis* (HRT1, HRT2, and HRBP) and *P. argentatum* (PaCPT1, PaCPT2, PaCPT3, PaCPT3_dN15, and PaCBP) proteins as well as human β 2AR were assembled with asolectin nanodiscs by cell-free protein synthesis with a wheat-germ translation system. The resulting protein-nanodisc complexes were purified by Ni-NTA column chromatography and then subjected to SDS-PAGE and CBB staining together with BSA standard solutions. The protein concentration of each sample was estimated from band intensity with ImageJ software. The samples shown here include those used for prenyltransferase activity assays in Figure 1 (a), Figure 3 (b), Figure 2 (c), Figure 4 (d), and Supplementary Figure S4 (e). Some of the samples were not used for the enzyme assay.

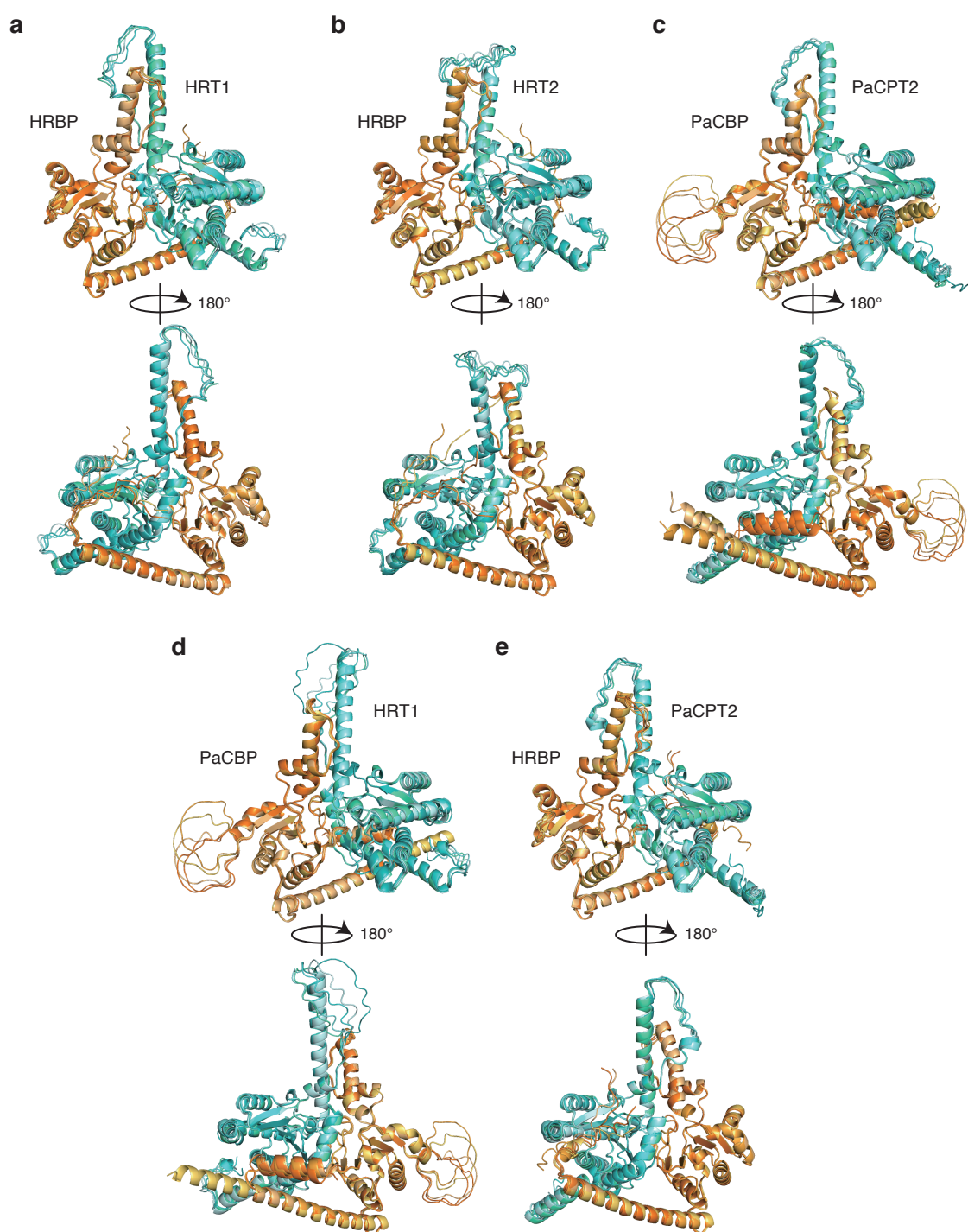


Supplementary Figure S3. Analysis of additional products in cell-free translation reactions. PaCPT1, PaCPT2, and PaCPT3 were synthesized in a cell-free system in the presence of nanodiscs and [^{14}C]leucine. The purified protein-nanodisc complexes were then subjected to SDS-PAGE, and the gel was stained with CBB (left panel) and subjected to autoradiography (right panel). Single and double asterisks indicate minor products in the PaCPT3 and PaCPT1 reaction mixtures, respectively. The black arrowhead indicates the band for MSP of the nanodiscs, which overlaps with that for PaCPT1. Blue and red arrowheads indicate the major ^{14}C -labeled proteins, whose apparent molecular sizes reflect their predicted molecular masses of 31.3 kDa for PaCPT1, 33.2 kDa for PaCPT2, and 34.8 kDa for PaCPT3 and of 28.7 kDa for PaCBP, respectively.



Supplementary Figure S4. Analysis of a truncated form of PaCPT3. **(a)** Alignment of the predicted amino acid sequences of HRT1, HRT2, PaCPT1, PaCPT2, and PaCPT3. Hyphens indicate gaps introduced to optimize alignment. Identical or similar residues among the five proteins are indicated by dark and light shading, respectively, and residue numbers are shown on the left of the sequences. PaCPT2 and PaCPT3 are closely related, sharing the highest sequence identity (89%) among the five proteins. PaCPT3 possesses a methionine at position 16 that corresponds to the first methionine of PaCPT2. **(b)** PaCBP as well as either a truncated form of PaCPT3 lacking the NH₂-terminal 15 amino acids (PaCPT3_dN15) or the

wild-type protein were synthesized in a cell-free system in the presence of nanodiscs, and the purified protein-nanodisc complexes were characterized by SDS-PAGE and CBB staining. The asterisk indicates a protein in the PaCPT3 reaction mixture with a mobility similar to that of PaCPT3_dN15. **(c)** Relative prenyltransferase activity of PaCPT3/PaCBP-nanodisc and PaCPT3_dN15/PaCBP-nanodisc complexes. The raw data used to calculate relative activity are $22,683 \pm 1105$ dpm for PaCPT3/PaCBP and $21,406 \pm 1096$ dpm for PaCPT3_dN15/PaCBP (means $\pm$ s.d. from three independent experiments). **(d)** TLC analysis of extracts prepared from enzyme assay mixtures as in **(c)** with 1-butanol. Both the PaCPT3/PaCBP and PaCPT3_dN15/PaCBP complexes showed similar patterns of polyisoprene chain length.

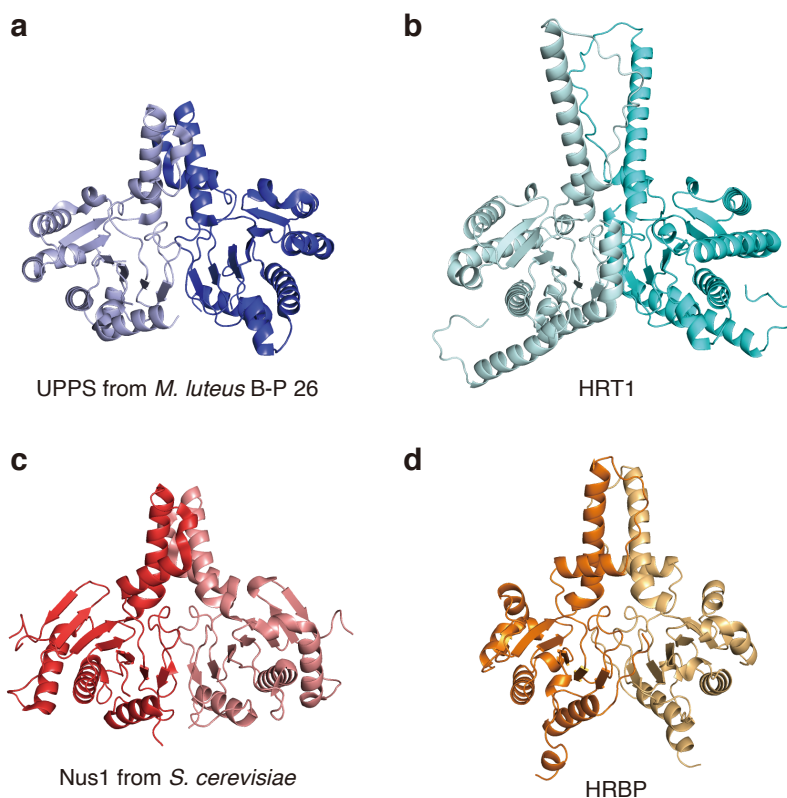


Supplementary Figure S5. Structural models of full-length CPT/CPTL complexes. HRT1/HRBP (a), HRT2/HRBP (b), PaCPT2/PaCBP (c), HRT1/PaCBP (d), and PaCPT2/HRBP (e) complexes are shown, with CPT proteins colored cyan, and CPTL proteins orange. Each pair of images represents views before and after vertical rotation through 180 degrees.

HRT1	--MELYNGERPSVFRLLGKYMRKGLYSILTQGPITHIAFILDGNRRFAKKHKLPEGGGH	1
PaCPT2	MEEEERPSGVTSKGLGSLISTMRLLFTFMSSGPQPHTAFIMDGNRRFAKKWLEEGAGH	60
DHDDS	-----MSWIKEGELSLWERFCANIKAQMPMKHTAFIMDGNRRYAKKCQVERQEGH	51
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HRT1	KAGFALLNLVITYCYELGVKYATIYAFSDNFRRRKPEHVQYVMDLMEKIEGMIMEESII	118
PaCPT2	KAGFALLSVLYKYCYEVGVKYVITYAFSLDNFNRRRPEDEVQYVMDIMYEQIGFLKEMDMV	120
DHDDS	SQKHGVCIAEIRLWCLNLGILEVTYAFSIEFNKRSKSEVDGLMDLARQKFSRLMEEKEKL	111
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HRT1	NAYDICVRVFNGLKLLSEPVKTAADKIMRATANSNCKVLLIAVCYTSTDEIVHAVEESSE	178
PaCPT2	NRYYGRVLFTGDLNRLDEPVRIAAEKAMEATAKNKTYLLNVCVATYSHEIPRAIYEACQ	180
DHDDS	QKHGVCIAEIRLWCLNLGILEVTYAFSIEFNKRSKSEVDGLMDLARQKFSRLMEEKEKL	171
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HRT1	LNSNEVCNNQGLEEANATGSGTVIQIENMESYSIGIKLVDELKNTYINPYPDVLI RTSGET	238
PaCPT2	EKSGLALASNRKKNNDDDG--IVDEDDVDVTKYADLEKHYMGMVGPDPDILVRSGET	238
DHDDS	GVEQGLDPSDISES-----LDLCKLYTNRSPHPDILIRTSGEV	210
	 : : : : :	
HRT1	RLSNYLLWDTTNCILYSYPHALWPEIGLRHVWVAVINCQRHYSYLEKHKEYLK-----	290
PaCPT2	RLSNFLWDTTNSLLYSPKALWPEMGFWGVVGIKYQKYYYLEKKKKQA-----	289
DHDDS	RLSDFLWDTTNSHCLVFPQVLWPEYTFWNLFEAQLQFMNHSMLQKADMYAEERKRQQL	270
	*** : *** : . * : * : * : . : . . : * : . : * : *	
HRT1	-----	
PaCPT2	-----	
DHDDS	ERDQATVTEQLRLEGQLASGDAQLRRLRHLKLSARREERVQQLQALELKRADWLARLGT	330
HRT1	---	
PaCPT2	---	
DHDDS	ASA 333	

HRBP	MDLKPAGGQVRNRLVDPISYHFLQLFWRTLHLVSLWYLQVMQMIEGLISSGLVKR	60
PaCBP	--MDLVAESQKFFRRTSQSGLIVLLHHVVHLTISVL YIVREIFRAIESLYITNGYVK	58
NgBR	---MTGLYELVWRVLHALLCLHRTL TSWLRVFRGTWNWIWRCCRCAASAVLAPLGFTLR	57
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HRBP	YG-----ALDIDKVRYLAIVVDSEEA YQISKV	87
PaCBP	YT-----NINLRQVKYLGI VSDSEARNISKV	85
NgBR	KPPAVGNRRHRHPRPGSGCLAAAHHRMRWRADGRSLEKL PVHMGLVITEVEQE PFSFDI	117
	::: *::: : : : :	: : *
HRBP	IQLLKWVEDMGVKHLC LYSKGVLTKNKTTIMESLNN-----AMPFEAEVK	134
PaCBP	VELL EWL SAIGVKKIC L YDREGVLKKSAVIMERFGS-----TETSND SAVA	132
NgBR	ASLVVMCMAGVISYISVYDHQGFKRNNSSLMEIL KQQQELGLDCSKSYSP EFANSNDK	177
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HRBP	DVLLDQKQMTVEFASSDGKEAITRAANVLFMKYLYAKYATGVGKEEPCTED QMDEALKA	194
PaCBP	NPLS-KKRMDFEVSITDGKEA VA KAANLLFKYYVD EDS----EKPF FTEHTL TEALKT	187
NgBR	DDQLNLCHLAVKLSPEDGGADI VRAAQDFQLVAQKQKR----PTDL DVTLSA LSLSS	232
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HRBP	IGYKGPEPDLLIIYGPVRCHLGFSP WRLRYTEMVHMGPLRYMNLGSLKKA IHRFTTVQON	254
PaCBP	LQGVEPDPDLLIIYGPVRCHLGFPA WRLRYTEMVHMGPLRYKKYGL LKAIHRFTVKVQN	247
NgBR	NG--CPDPDLVLKFGPVDS TLGLFWHLRLTESVLSPHLNISYEDFFSALRQYAACEQR	290
	* *	: *
HRBP	YGT	257
PaCBP	YGS	250
NgBR	LKG	293
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Supplementary Figure S7. Structure of the undecaprenyl diphosphate synthase (UPPS) homodimer from *Micrococcus luteus* B-P 26 (PDB code 1F75) (**a**), structural model of an HRT1 homodimer (**b**), structure of Nus1 from *Saccharomyces cerevisiae* (PDB code 6JCN) (**c**), and structural model of an HRBP homodimer (**d**).

Supplementary Table S1. Amino acid sequences used as input for AlphaFold2 modeling.

Protein	Amino acid sequence
HRT1	MELYNGERPSVFRLLGKYMRKGLYSILTQGPIPTHIAFILDGNRRFAKKHKLPE GGGHKAGFLALLNVLTICYELGVKYATYAFSIDNFRRKPHEVQYVMDLMLE KIEGMIMEESIINAYDICVRFVGNLKLSEPVKTAADKIMRATANNSKCVLLIA VCYTSTDEIVHAVEESSELNSNEVCNNQELEEANATGSGTVIQIENMESYSYGIK LVDLEKNTYINPYPDVLRITSGETRLSNYLLWQTTNCILYSPHALWPEIGLRHV VWAVINCQRHYSYLEKHKEYLK
HRT2	MELYNGERPSVFRLLGKYMRKGLYSILTQGPIPTHIAFILDGNRRFAKKHKLPE GGGHKAGFLALLNVLTICYELGVKYATYAFSIDNFRRKPHEVQYVMNLMLE KIEGMIMEESIINAYDICVRFVGNLKLDEPLKTAADKIMRATAKNSKFVLLLA VCYTSTDEIVHAVEESSKDKLSDEICNDGNGDCVIKIEEMEPYSEIKLVELER NTYINPYPDVLRITSGETRLSNYLLWQTTNCILYSPHALWPEIGLRHVWVAVIN CQRHYSYLEKHKEYLK
PaCPT2	MEEERPSGVISKGLGSLISTMRRLFTFMSSGPIQHIAFIMDGNRRFAKKWKLE EGAGHKAGFLALLSVLKICYEVGVKYVTYAFSLDNFNRRPDEVQYVMDIM YEKIQGFLKEMDMVNRYGVRVLFIGDLNRLDEPVRIAAEKAMEATAKNTKTY LLVCVAYTSSHEIPRAIYEACQEKSGLALASNRKKNNDDDGIVDEDDVNDVI KVADLEKHYMGVVPDPDILVRSSGETRLSNFLLWQSTNSLLYSPKALWPEMG FWQVVWGILKYQNKYYYLEKKKKQA
HRBP	MDLKPAGGQVRNRLVDPISYHFLQFLWRTLHLLVSLWYLQVSMVQMIEGFL ISSGLVKRYGALDIDKVRylaIVVDSEEAYQISKVIQLLKWVEDMGVKHLCLY DSKGVLTNKKTIMESLNNAMPFEEAVEKDVLDDQKQMTVEFASSSDGKEAI TRAANVLFMKYLKYAKTGVGKEEPCFTEDQMDEALKAIGYKGPEPDLLLIYG PVRCHLGFSPWRLRYTEMVHMGPLRYMNLGSLKKAIHRFTTVQQNYGT
HRBP(55–257)	SGLVKRYGALDIDKVRylaIVVDSEEAYQISKVIQLLKWVEDMGVKHLCLYD SKGVLTNKKTIMESLNNAMPFEEAVEKDVLDDQKQMTVEFASSSDGKEAIT RAANVLFMKYLKYAKTGVGKEEPCFTEDQMDEALKAIGYKGPEPDLLLIYGP VRCHLGFSPWRLRYTEMVHMGPLRYMNLGSLKKAIHRFTTVQQNYGT
PaCBP	MDLVAESQKFFRRTSQSGSIVLFLWHVHVLTVISVLYIVREIFRAIESYLITNGYV KTYTNINLQRVKYLGIIVDSDEARNISKVVELLEWLSAIGVKKICLYDREGVLK KSKAVIMERFGSTETSNDSAVANPLSKKRMDFEFVSITDGKEAVAKAANLLFKK YYVDEDSEKPFFTETHLTEALKTLGQVEPDPELLLIYGPVRCHLGFPAWRLRYT EMVHMGPLKYKKFGLILKAIHRFTKVKQNYGS

PaCBP(53–250)	NGYVKTYTNINLQRVKYLGIVVDSDEARNISKVVELLEWLSAIGVKKICLYDR EGVKKSKAVIMERFGSTETSNDHAVANPLSKRMDFEFVSITDGKEAVAKAAN LLFKKYYVDEDSEKPFFTETHLTEALKTLGQVEPDPLLIYGPVRCHLGFAW RLRYTEMVHMGPLKYKKFGLILKAIHRFTKVKQNYGS
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