Structural basis of heterotetrameric assembly and disease mutations in the human cis-prenyltransferase complex

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The human cis-prenyltransferase (hcis-PT) is an enzymatic complex essential for protein N-glycosylation. Synthesizing the precursor of the glycosyl carrier dolichol-phosphate, mutations in hcis-PT cause severe human diseases. Here, we reveal that hcis-PT exhibits a heterotetrameric assembly in solution, consisting of two catalytic dehydrodolichyl diphosphate synthase (DHDDS) and inactive Nogo-B receptor (NgBR) heterodimers. Importantly, the 2.3 Å crystal structure reveals that the tetramer assembles via the DHDDS C-termini as a dimer-of-heterodimers. Moreover, the distal C-terminus of NgBR transverses across the interface with DHDDS, directly participating in active-site formation and the functional coupling between the subunits. Finally, we explored the functional consequences of disease mutations clustered around the active-site, and in combination with molecular dynamics simulations, we propose a mechanism for hcis-PT dysfunction in retinitis pigmentosa. Together, our structure of the hcis-PT complex unveils the dolichol synthesis mechanism and its perturbation in disease.

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Prenyltransferases are essential enzymes that synthesize isoprenoids, an enormous group of chemically diverse compounds participating in a myriad of cellular processes in all living cells. With chain lengths varying from C_{10} (geranyl diphosphate) to >C_{10,000} (natural rubber), isoprenoids are synthesized by chain elongation of an allylic diphosphate primer via a variable number of condensation reactions with isopentenyl pyrophosphate (IPP, C_5)\textsubscript{2-3}. Prenyltransferases are classified as cis-prenyltransferase or trans-prenyltransferase according to the double bonds they form during the condensation reaction.\textsuperscript{3} Cis-prenyltransferases are further classified according to their product chain length into short-chain (C_{15}), medium-chain (C_{50-55}), long-chain (C_{70-120}), and rubber synthases.\textsuperscript{1} Importantly, while short- and medium-chain cis-prenyltransferase complexes are homodimeric, long-chain cis-prenyltransferases and rubber synthases are formed by a heteromeric subunit assembly of unknown stoichiometry.\textsuperscript{1,4,5} To date, only homodimeric enzymes were structurally characterized.\textsuperscript{6-9} Therefore, our understanding of the mechanisms allowing long-chain isoprenoid formation by heteromeric enzymes remains limited.

The human cis-prenyltransferase (hcis-PT) complex catalyzes the formation of dehydrodolichyl diphosphate (DHDDS, C_{85-100}), a long-chain isoprenoid, by chain elongation of farnesyl diphosphate (FPP, C_{15}) via multiple condensations with IPP (Fig. 1a).\textsuperscript{10} DHDD is the precursor for dolichol-phosphate, the lipidic glycosyl carrier crucial for N-linked protein glycosylation (Fig. 1a).\textsuperscript{11} Localized to the endoplasmic reticulum, hcis-PT is composed of two structurally and functionally distinct subunit types. These include the catalytically active DHDD synthase (DHDDS) and the quiescent Nogo-B receptor (NgBR) subunits.\textsuperscript{10} Importantly, while DHDDS subunits are cytosolic, NgBR can be subdivided into an N-terminal transmembrane domain and a C-terminal pseudo cis-prenyltransferase domain (Supplementary Fig. 1), which lacks detectable catalytic activity and directly interacts with DHDDS.\textsuperscript{10,11}

In line with the crucial significance of N-linked glycosylation for proper cellular function, mutations in both hcis-PT subunits were associated with human diseases. Specifically, DHDDS missense mutations were shown to result in phenotypes ranging from autosomal recessive retinitis pigmentosa (arRP)\textsuperscript{12,13}, through developmental epileptic encephalopathies\textsuperscript{14}, to a case of fatal congenital disorder of glycosylation reported in a patient heterozygous for both a splice site and a nonsense mutation.\textsuperscript{15} Moreover, a missense mutation in the conserved C-terminal RxG

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Fig. 1 Complex stoichiometry and functional analysis of shcis-PT. a Dehydrodolichyl diphosphate synthesis reaction scheme. The IPP moiety is colored red. The hcis-PT subunits DHDDS and NgBR are schematically drawn and colored blue and yellow, respectively. b Representative SEC-MALS analysis of the purified shcis-PT. The black and red curves indicate UV absorption and molecular mass, respectively. The experiment was performed twice using two different protein batches. Inset: SDS-PAGE analysis of the purified complex. Left lane: molecular weight marker, right lane: purified shcis-PT. Molecular weights (kDa) are indicated. Source data are provided as a Source Data file. c Native ESI-MS spectrum obtained using low activation conditions. The main distribution corresponds to the heterotetramer (charge states 21-26). Inset: SDS-PAGE analysis of the complex following glutaraldehyde cross-linking. Left lane: molecular weight marker, middle lane: purified shcis-PT without glutaraldehyde, and right lane: purified shcis-PT with glutaraldehyde. Molecular weights (kDa) are indicated. The experiment was performed twice using two different protein batches. The oligomeric state represented by each state is illustrated using the schematic presentation from panel (a). Source data are provided as a Source Data file. d In vitro activity of purified shcis-PT assessed as IPP incorporation. Experiments were performed as described in the “Methods” section. Data are presented as mean ± SEM (n = 3 independent experiments). Source data are provided as a Source Data file.
motif of NgBR was shown to cause a congenital glycosylation disorder with refractory epilepsy, visual and neurological impairments, congenital scoliosis, and hearing deficit. Recently, missense mutations in NgBR were shown to contribute to the etiology of Parkinson’s disease. Intriguingly, the different pathogenic mutations in hcis-PT seem to have diverse effects on cellular glycosylation. For example, while the missense mutation in the RxG motif of NgBR led to reduced glycosylation in patients fibroblasts, and the patient suffering from the fatal glycosylation disorder displayed hypoglycosylation of serum glycoproteins, the arRP mutation in DHDDS does not seem to have any significant effect on glycosylation in a knock-in mouse model, and some patients with DHDDS-related developmental epileptic encephalopathy display normal glycosylation assay results. Thus, the interplay between the genotype and cellular phenotype may be more complex than originally thought and awaits further exploration.

We have previously shown that DHDDS can form functional homodimers, but these complexes exhibit poor catalytic activity compared to homodimeric orthologs or the heteromeric hcis-PT. Accordingly, previous studies suggested that NgBR can allosterically modulate the activity of the catalytic DHDDS subunit. Indeed, overexpression of NgBR was shown to significantly enhance hcis-PT activity in cells, supporting an NgBR-mediated allosteric modulation of DHDDS activity. This effect was suggested to involve a conserved RxG motif, localized to the NgBR C-terminal tail. Recently, the structure of Nus1, the yeast homolog of NgBR, was determined. The structure, devoid of the N-terminal transmembrane domain, revealed a mass of 39.1 kDa and two sNgBR subunits. In order to determine the stoichiometry of the complex with a molecular weight of 119.7 ± 0.4 kDa (Fig 1b). Within the experimental error of SEC-MALS, this mass may correspond to a stable heterotetramer composed of either two DHDDS (monomer molecular weight = 39.1 kDa) and two sNgBR (monomer molecular weight = 25.2 kDa) subunits or one DHDDS and three sNgBR subunits. In order to determine the stoichiometry of the complex, we used native electrospray ionization (ESI) mass spectrometry (MS) (Fig 1c). Importantly, native ESI spectra, obtained at low activation conditions to preserve the tertiary structure, revealed a mass of 130.1 ± 0.2 kDa, confirming the heterotetrameric organization of the complex with a stoichiometry of two DHDDS and two sNgBR subunits (Fig 1c). However, under these conditions, the deconvoluted mass was higher than predicted due to the presence of sodium cations and low-molecular-weight adducts. Indeed, along with partial complex disintegration upon stepwise activation, a shift of the heterotetrameric population toward lower mass (128.7 ± 0.02 kDa) was observed, and an agreement between theoretical and calculated masses was achieved (Supplementary Fig 2). Finally, we used a cross-linking approach that enables the identification of subunit interactions within the heterotetramer. Treatment with glutaraldehyde, a homobifunctional amine-reactive cross-linker, resulted in the emergence of high-order oligomers, culminating in a heterotetrameric complex with a mass of ~125 kDa (Fig 1c). In addition, although the cross-linking treatment can theoretically yield three types of dimers (DHDDS homodimer, sNgBR homodimer, and DHDDS–sNgBR heterodimer), we could clearly detect only two bands, corresponding to DHDDS homodimers and DHDDS–sNgBR heterodimers, suggesting that the sNgBR subunits exhibit spatial separation in the context of shcis-PT. Together, the biophysical and biochemical characterizations of shcis-PT suggest a dimer-of-heterodimers arrangement.

Next, in order to validate that the purified complex is catalytically viable, we tested its activity in vitro using a radioligand-based assay (Fig 1d). The purified shcis-PT exhibited a catalytic constant of 0.74 ± 0.02 s⁻¹ and a Michaelis constant of 6.23 ± 1.50 and 0.11 ± 0.01 μM for IPP and FPP, respectively. The kcat value is similar to that previously reported for the intact hcis-PT and ~400-fold higher compared to that of homodimeric DHDDS. These results demonstrate that shcis-PT recapitulates the function of the intact hcis-PT complex. The purification and in vitro activity characterization of the soluble hcis-PT complex. Previous sequence and biochemical analyses of NgBR revealed that it interacts with DHDDS via its cytosolic C-terminal pseudo cis-prenyltransferase homology domain (Supplementary Fig 1). Thus, we generated an NgBR construct solely encompassing its cytosolic domain (sNgBR, residues 73–293), where the asterisks designate NgBR residues. Importantly, previous studies using yeast complementation showed that truncation up to position 85 did not affect the ability of NgBR to support cell growth following co-transformation with DHDDS, indicating that the catalytic function of the complex is preserved in the absence of the transmembrane domain. Next, we coexpressed the full-length human DHDDS (residues 1–333) and sNgBR in E. coli. Following purification, we obtained a homogenous population of heteromeric soluble shcis-PT (shcis-PT) (Fig 1b).

Intriguingly, during the final size-exclusion purification step, we noticed that the elution volume of shcis-PT corresponds to a higher-than-expected molecular weight range. Size-exclusion chromatography multivariate light-scattering (SEC-MALS) analysis of the purified shcis-PT revealed a monodispersal population with a molecular weight of 119.7 ± 0.4 kDa (Fig 1b). Within the experimental error of SEC-MALS, this mass may correspond to a stable heterotetramer composed of either two DHDDS (monomer molecular weight = 39.1 kDa) and two sNgBR (monomer molecular weight = 25.2 kDa) subunits or one DHDDS and three sNgBR subunits. In order to determine the stoichiometry of the complex, we used native electrospray ionization (ESI) mass spectrometry (MS) (Fig 1c). Importantly, native ESI spectra, obtained at low activation conditions to preserve the tertiary structure, revealed a mass of 130.1 ± 0.2 kDa, confirming the heterotetrameric organization of the complex with a stoichiometry of two DHDDS and two sNgBR subunits (Fig 1c). However, under these conditions, the deconvoluted mass was higher than predicted due to the presence of sodium cations and low-molecular-weight adducts. Indeed, along with partial complex disintegration upon stepwise activation, a shift of the heterotetrameric population toward lower mass (128.7 ± 0.02 kDa) was observed, and an agreement between theoretical and calculated masses was achieved (Supplementary Fig 2). Finally, we used a cross-linking approach that enables the identification of subunit interactions within the heterotetramer. Treatment with glutaraldehyde, a homobifunctional amine-reactive cross-linker, resulted in the emergence of high-order oligomers, culminating in a heterotetrameric complex with a mass of ~125 kDa (Fig 1c). In addition, although the cross-linking treatment can theoretically yield three types of dimers (DHDDS homodimer, sNgBR homodimer, and DHDDS–sNgBR heterodimer), we could clearly detect only two bands, corresponding to DHDDS homodimers and DHDDS–sNgBR heterodimers, suggesting that the sNgBR subunits exhibit spatial separation in the context of shcis-PT. Together, the biophysical and biochemical characterizations of shcis-PT suggest a dimer-of-heterodimers arrangement.

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complex, further reinforcing that the absence of the N-terminus of NgBR does not impair the catalytic activity of the complex. Moreover, the increased activity of the heteromeric complex compared with homodimeric DHDDS points toward an inter-subunit communication mode, since NgBR lacks catalytic activity.

Structure overview of the shcis-PT subunits. In order to unveil the structural basis of the functional coupling observed in the context of the shcis-PT, we sought to determine its structure using X-ray crystallography. However, as initial crystallization attempts were unsuccessful, we removed residues 167-175 from NgBR (sNgBRΔ167-175), corresponding to an unresolved loop in the structure of the yeast homolog Nus1, assuming that this region is highly flexible and precludes crystallization. Indeed, this construct (termed hereafter shcis-PT) resulted in well-diffracting crystals while demonstrating unperturbed catalytic activity (Supplementary Fig. 3), allowing us to determine its structure in complex with Mg\(^{2+}\) and FPP at 2.3 Å resolution (Supplementary Fig. 4). The asymmetric unit (ASU) contains a single heterodimer (Fig. 2a), with Mg\(^{2+}\)-FPP and an additional phosphate moiety bound only at the DHDDS active-site.

DHDDS can be subdivided into three domains: (i) an N-terminal domain (NTD, residues 1-26), (ii) a canonical catalytic cis-prenyltransferase homology domain (residues 27-250), and (iii) a C-terminal domain (CTD, residues 251-333) (Supplementary Fig. 1). The catalytic cis-prenyltransferase homology domain, which forms the central region of DHDDS and serves for heterodimerization with NgBR (Fig. 2a, right), is composed of 7 α-helices and 6 β-strands (Supplementary Fig. 1), engulfing an elongated active-site cavity. This domain shares high homology with undecaprenyl diphosphate synthase (UPPS), a bacterial medium-chain cis-prenyltransferase homolog, with root-mean-square deviation (RMSD) = 0.76 Å (Supplementary Figs. 5 and 6, Supplementary Data 1). The NTD and CTD flank the catalytic domain. The NTD is wrapped around the catalytic domain (Fig. 2a, left), with its single α-helix, αN, directly packed against helix α7 via a network of hydrophobic interactions. Last, the CTD, absent from short- or medium-chain cis-prenyltransferases, features a "helix-turn-helix" motif composed of two consecutive helices situated immediately downstream of α7 (Fig. 2a, Supplementary Fig. 1). The first helix, αC1, is kinked by 30° relative to the preceding α7 from the catalytic domain. The second helix, αC2, is stabilized against αC1 by a salt bridge between D273 and N of R306, and numerous electrostatic (E168–R321, D182–R309, and R196–E318) and hydrophobic interactions with the heterodimerization interface between DHDDS and NgBR (Fig. 2a).

Similar to DHDDS, the remnant N-terminal domain of sNgBR (NTD, residues 79-100) also encompasses a single α-helix, αN,
likely serving as a structural link between the transmembrane and cytosolic domains in the intact protein. However, while NgBR was previously thought to share the canonical cis-prenyltransferase fold, similar to DHDDS, the structure reveals that the two subunits share low structural similarity (RMSD = 2.07 Å, residues 100–293 of sNgBR and 27–250 of DHDDS). Indeed, the pseudo cis-prenyltransferase homology domain of sNgBR encompasses only six α-helices and five β-strands (Fig. 2a, Supplementary Fig. 1), in contrast to the seven α-helices and six β-strands found in the other cis-prenyltransferases.

Moreover, NgBR and its yeast homolog, Nus1, do not share high structural similarity (RMSD = 1.33 Å), with the α3 of NgBR occupying the position of the antiparallel βC–βC in Nus1 (Supplementary Fig. 5). The described fold of NgBR prevents interactions with substrates and hinders the catalytic activity of NgBR, as specified below.

**Mechanism of tetramerization via a dimer-of-heterodimers assembly.** The DHDDS–sNgBR heterodimer is formed through a large interface, with a buried surface area of 1938.0 Å² (Fig. 2a, right). This interface is mainly formed by helices α5, α6, and the βE–βF linker of DHDDS and helices α4, α5, and the βD–βE linker of NgBR, with an architecture reminiscent to that observed in homodimeric cis-prenyltransferases. However, the heterodimeric interface also features contacts between the NgBR C-terminus and the active-site of DHDDS. Specifically, the structure reveals that the NgBR C-terminus, which encompasses the RxG motif and was previously suggested to play a critical role in active-site organization, extends across the dimerization interface (Fig. 2a, left). This transverse interaction results in its direct involvement in the organization of the active-site of DHDDS and provides a structural framework for intersubunit communication.

While crystal packing analysis using the protein interfaces, surfaces and assemblies server suggested several possible assemblies, ranging from heterodimers to dodecamers, only one biological tetrameric assembly consistent with the oligomeric state in solution was detected. Importantly, this tetramer is formed by both homotypic interactions between DHDDS “helix-turn-helix” motifs and heterotypic interactions of the “turn” region with sNgBR from adjacent ASUs, burying a total surface area of 793.3 Å² (Fig. 2b). Specifically, this interface is stabilized by two polar networks: (i) a salt-bridge network, centered at the αC1 helix of the adjacent heterodimer and E264 and E271 from the αC1 helix of the adjacent heterodimer, and (ii) a hydrogen-bond network, originating from the interaction of D34, localized to the “turn” between αC1 and αC2, and Q268 from αC1, with S192 of the adjacent heterodimer. Notably, this dimer-of-heterodimer arrangement is consistent with our cross-linking analysis (Fig. 1c).

**Active-site organization.** Although xcis-PT was crystallized in the absence of IPP, a phosphate molecule occupying the IPP pyrophosphate group, as observed in other cis-prenyltransferases, is present at the S1 site and is stabilized through a concerted coordination by the conserved R205, R211, and S213 (Fig. 3c, Supplementary Figs. 4 and 6, Supplementary Data 1). The R211Q mutation was also associated with developmental epileptic encephalopathy (Fig. 3e). As expected, similar to R37H and R38H, R211Q exhibits an ~5-fold reduction in catalytic activity (0.20 ± 0.04 µmol/h/mg protein; n = 5, P < 0.001) (Fig. 3f, Supplementary Figs. 4 and 7). Together, our structure reveals the spatial clustering and functional convergence of disease mutations at the active-site, and provides a plausible explanation for the high conservation of the RxG motif due to its role in active-site organization.

**Hydrophobic interactions in the active-site support isopenoid chain elongation.** According to the current model of chain
elongation by cis-prenyltransferases, the pyrophosphate head-groups are bound at the superficial polar region, while the carbon chains point toward the deep hydrophobic tunnel. During the catalytic cycle, the pyrophosphate group of the initiatory substrate at the S1 site (FPP, C15) is hydrolyzed, followed by condensation of the remaining carbons with the IPP (C5) from the S2 site, yielding a 20-carbon polymer. Then, the elongated product translocates to the S1 site, where the growing carbon chain permeates deeper into the hydrophobic tunnel of the active-site. Finally, a new IPP molecule binds to the S2 site, and the cycle repeats until the active-site can no longer accommodate the long-chain isoprenoid.

The structure reveals that the hydrophobic tunnel of DHDDS is formed by 2 α-helices (α2 and α3) and 4 β-strands (βA, βB, βE, and βF), similar to other cis-prenyltransferases (Fig. 4a, b). It was previously suggested that the opening between α2 and α3
may be larger in DHDDS compared to short- and medium-chain cis-prenyltransferases, leading to a larger diameter enabling the accommodation of longer products. Indeed, in DHDDS, the distance between α2 and α3, measured between the Cα atoms of W64 and L104, is 19.5 Å (Fig. 4b). In contrast, the distance between the corresponding positions in UPPS (F56 and E96), which synthesizes a 55-carbon isoprenoid, is only 15.3 Å.

Intriguingly, the structure shows that the unique NTD of DHDDS can snake into the binding site, interacting with the bound FPP molecule (Fig. 4b). Moreover, the conserved N-terminal W3 (Supplementary Fig. 6, Supplementary Data 1) interacts with F55, F101, and V152, thereby occluding the outlet of the hydrophobic tunnel of the active-site. This results in a surprisingly small active-site volume of 318 Å³. Indeed,
compared with the 371 Å³ in the active-site of the medium-chain UPPS²⁷, the xcis-PT site is seemingly inadequate for accommodating long-chain products. However, the high B factors of the NTD (Supplementary Fig. 10) suggest that it is mobile, possibly assuming different orientations relative to the active-site under physiological conditions. Indeed, it was suggested that hydrophobic residues (W12, F15, and I19) from helix aN interact with the membrane, resulting in increased catalytic activity in the presence of phospholipids²¹. Thus, we suggest that the NTD may shield the hydrophobic tunnel in the apo or FPP-bound states, while being expelled from the tunnel and interacting with the membrane upon chain elongation.

In addition to the active-site diameter, the composition of its terminal region also plays a key role in determination of product length. In UPPS, L137, localized to the N-terminus of βD, was shown to be vital for determining chain length, with the L137A mutant increasing the product length from C₅₅ to C₇₅.²⁵ Our structure reveals that the corresponding position in DHDDS is replaced by C148, a hydrophilic and less bulky residue. Thus, C148 cannot occlude the hydrophobic tunnel outlet as efficiently as L137, similar to the L137A mutant, possibly contributing to long-chain product formation.

NgBR has been long known to lack catalytic activity of its own.¹⁰ Nevertheless, the structural basis for this observation remained obscure. The structure clearly reveals that, in sharp contrast with DHDDS, sNgBR does not contain substrate-binding sites (Fig. 4c). Indeed, while the heterodimerization interface is structurally conserved (Fig. 2a), the NgBR region corresponding to the active-site in cis-prenyltransferases displays a significantly different structural arrangement (Fig. 4d). Specifically, strands βA, βB, and βD and helices a1 and a6 are tightly packed via hydrophobic interactions, leaving this region without a detectable substrate-binding cavity and completely devoid of water molecules. This arrangement provides a structural explanation for the absence of NgBR catalytic activity, due to its incapacity to bind FPP and IPP. Thus, the only active-site of the complex is situated within the cis-prenyltransferase homology domain of DHDDS (Fig. 2).

The molecular mechanism of hcis-PT dysfunction in arRP.

Disease mutations in DHDDS are clustered around the pyrophosphate-binding regions of the S₁ and S₂ sites (Fig. 3). Based on our structure, the functional perturbation caused by most disease-related positions (R37, R38, and R211) is straight-forward, due to their direct involvement in substrate binding. However, the effect of K42E (0.83 ± 0.14 μmol/h/mg protein; n = 7, P < 0.01) (Figs. 3e and 5a, Supplementary Fig. 7), a mutation leading to isolated retinitis pigmentosa¹²,¹³, is not as obvious. We previously suggested mutual stabilizing effect resulting from NgBR and DHDDS co-expression in cells.¹⁰ Interestingly, a deletion mutant of DHDDS lacking the CTD does not support formation between the CTD in DHDDS (Fig. 2).

Discussion

Here, we provide the structure of a heteromeric cis-prenyltransferase. Our biochemical and structural analyses of hcis-PT reveal a heterotetrameric assembly, formed via a dimer-of-heterodimers mechanism, mainly through homotypic interface formation between the CTD in DHDDS (Fig. 2). Furthermore, the structure elucidates the molecular determinants governing substrate binding, the contribution of the RxG motif of NgBR to active-site formation by transverse interactions with DHDDS, and the effect of active-site resident disease mutations (Fig. 3). In addition, the structure unveils how the architecture of NgBR precludes its endogenous catalytic activity while enabling inter-subunit communication, and highlights the molecular determinants supporting formation of long-chain isoprenoid (Fig. 4). Finally, using MD simulations, we unveil the molecular mechanisms of a retinitis pigmentosa causing mutation in hcis-PT (Fig. 5).

To date, dimerization is considered as the common tertiary organization of cis-prenyltransferases.²² Additionally, the structurally characterized family members were homodimeric, encompassing an active-site within each subunit, and thus exhibiting an overall functional symmetry. Recently, the presence of a single heterodimer in the crystallographic ASU of hcis-PT in complex with IPP was interpreted as an evidence for its heterodimeric stoichiometry.²³ Nevertheless, the elution profile reported is identical to that we present here, suggesting the presence of a common heterotetrameric assembly (Fig. 1). Moreover, we show here using SEC-MALS, native ESI-MS, and cross-linking (Fig. 1) that shcis-PT exhibits a heterotetrameric organization, achieved via a dimer-of-heterodimers assembly mediated by homotypic interactions of the CTD of DHDDS (Fig. 2). Importantly, intact hcis-PT harboring the transmembrane domain of NgBR, expressed and purified from Exp2193F cells, was previously subjected to size-exclusion analysis. Although its mass was not directly assessed, it also shares a markedly similar elution profile with shcis-PT. This tetrameric assembly mode may contribute to the previously suggested mutual stabilizing effect resulting from NgBR and DHDDS co-expression in cells.²⁴ Interestingly, a deletion mutant of DHDDS lacking the CTD does not support...
cell growth in a yeast complementation assay\textsuperscript{23}, underscoring the functional significance of tetramerization.

Our structure hints toward the mechanisms allowing long-chain isoprenoid synthesis by h\textit{cis}-PT. While the deep hydrophobic tunnel, engulfing the elongating product, is walled by $\alpha_2$, $\alpha_3$, $\beta_A$, $\beta_B$, $\beta_E$, and $\beta_F$ (Fig. 4), previous studies pinpointed the length of $\alpha_3$ as a key contributor to chain-length determination\textsuperscript{26}. Indeed, previous studies of UPPS showed that an insertion of 3 residues, corresponding to $^{107}$EKE$^{109}$ in human DHDDS, led to an increase in product length from C$_{55}$ to C$_{70}$. The product length was further increased to C$_{75}$ if a 5-residue insertion, mimicking the yeast ortholog Srt1, was introduced, establishing the correlation between the length of $\alpha_3$ and the product\textsuperscript{26}. Finally, deletion of the $^{107}$EKE$^{109}$ sequence was shown to result in product...

**Fig. 5 Structural basis for h\textit{cis}-PT-related arRP.**

a In vitro activity of purified h\textit{cis}-PT harboring the K42E mutation was measured as IPP incorporation following 1-h incubation in the presence of 0.1 $\mu$M enzyme, 20 $\mu$M FPP, and 100 $\mu$M IPP. Data are presented as mean ± SEM, $n = 7$ independent experiments. The activity of the WT is shown for reference. One-sided student’s t test was performed for data analysis, $^{**}P = 0.0069$. Source data are provided as a Source Data file.

b A representative conformation of the most abundant cluster of the WT protein. R38, K42, and E234 are shown as sticks. R38 points toward the active-site cavity, while K42 and E234 form a stable salt bridge.

c A representative conformation of the most abundant cluster of the complex harboring DHDDS-K42E. R38, E42, and E234 are shown as sticks. R38 points away from the active-site cavity, forming a new salt bridge with the mutant E42, colored orange.

d Distance distribution between positions 42 and 234, measured between the charge centers of E234 and K42 (light blue) or E42 (orange).

e Distance distribution between positions 38 and 42, measured between the charge centers of R38 and K42 (light blue) or E42 (orange).

f Average (± SD) RMSF of the active-site residues within 5 Å of the crystallized substrates for the WT (light blue, $n = 3$) and mutant (orange, $n = 4$) complexes. Source data are provided as a Source Data file. Inset: Active-site residues within 5 Å of the crystallized substrates are colored cyan and shown as sticks.
shortening. In full agreement with these observations, the structure of \( \text{cis-PT} \) reveals that the 107EKE109 results in a kink in a3, leading to an \( \sim 4 \) Å increase in the hydrophobic tunnel diameter (Fig. 4b) compared with UPFS. In addition to the increased tunnel diameter, we propose that the local environment conferred by C148 may allow expulsion of the elongating product directly into the adjacent membrane during catalysis, allowing the formation of products that exceed the active-site volume. Together, the increased length of a3, the composition of the hydrophobic tunnel outlet, and the membrane association of the complex jointly contribute to long-chain isoprenoid production by \( \text{hcis-PT} \).

The organization of the CTD of DHDDS, underlying \( \text{hcis-PT} \) tetramerization, provides a mechanistic explanation for the poor activity exhibited by DHDDS homodimers. The helix-turn-helix motif, following a7 (Fig. 2), is incompatible with formation of the extensive interaction network observed within each \( \text{hcis-PT} \) heterodimer active-site (Fig. 3). In contrast, the assembly of DHDDS with NgBR allows the complementation of the active-site by the transverse interactions with the C-terminal tail of NgBR (Fig. 3). Importantly, such transverse interactions are also observed in homodimeric cis-\( \text{PT} \) transfersases (Supplementary Fig. 9), lacking a C-terminal helix-turn-helix motif, supporting the functional importance of these interactions in coupling active-site organization with enhanced catalytic activity. Consistent with this notion, mutations in the C-terminal tail of NgBR were previously shown to result in reduced catalytic activity. Now, our structure offers a mechanistic understanding of the effects imposed by these mutations. Indeed, introduction of R290H in the context of the intact complex was shown to result in decreased catalytic activity. The soluble construct we used for structural investigation also exhibits decreased activity upon mutation (Fig. 3). Structural comparison to other cis-\( \text{PT} \) transfersases (Supplementary Fig. 9) revealed that the guanidinium group of R290 is necessary and sufficient to substitute the Mg\(^{2+}\) ion within the active-site, interacting with both substrates. This interaction may contribute to the translocation of the product pyrophosphate group from S2 to S1, following the condensation reaction and release of the hydrolyzed pyrophosphate and Mg\(^{2+}\) ion. In contrast, histidine is both shorter and partially charged under physiological pH, making this residue insufficient to functionally and structurally substitute for the conserved arginine. Moreover, further emphasizing the functional significance of the NgBR C-terminus, previous introduction of the G292A mutation increased the K_m for IPP by \( \sim 6 \)-fold while reducing the turnover rate by \( \sim 12 \)-fold. As shown here, G292\(^*\) is directly involved in IPP binding (Fig. 3), and measuring the \( \psi/\gamma \) angles reveals that this position can only be occupied by glycine. Finally, we show that the backbone carboxylate of K293 interacts with the catalytic residues R37 and R85 (Fig. 3c). In accordance with the pivotal role of this interaction network, either deletion of K293 or the addition of a terminal alanine resulted in diminished catalytic activity of the intact complex. Together, the heterodimerization architecture observed here supports the notion that although DHDDS is considered as the catalytically active subunit, both subunits are necessary for efficient dolichol synthesis.

By forming the \( \text{hcis-PT} \) complex, DHDDS and NgBR were shown to play a crucial role in cellular dolichol synthesis. Mapping disease mutations in DHDDS onto the structure (Figs. 3 and 5) reveals their clustering around the pyrophosphate-binding regions of the S1 and S2 sites (Fig. 5). Interestingly, these mutations can be subdivided into mutations that directly or indirectly interfere with substrate association. The mutations that directly perturb substrate binding include R37H, R38H, and R211Q (Fig. 3e). These mutations result in a similar reduction in catalytic activity (Fig. 3f) and are clinically associated with developmental epileptic encephalopathies. In contrast, K42E, which leads to isolated arRP, is indirectly involved in substrate coordination (Fig. 3e) and leads to a milder reduction in catalytic activity (Fig. 5a). As the pathogenic effect of K42E was not readily apparent from the structure, we hypothesized that the charge reversal caused by K42E may alter the electrostatic interactions that play a vital role in substrate coordination (Figs. 3e and 5). Remarkably, MD simulations uncovered a novel salt bridge between the mutant E42 and the catalytic residue R38 (Fig. 5b–e), hindering substrate binding, and thus providing a mechanistic explanation for the pathogenicity of this mutation (Fig. 5a). Interestingly, additional to this local structural alteration, we also observed a tendency for global increase in active-site dynamics, including the C-terminus of NgBR (Fig. 5f, Supplementary Fig. 11). Inspection of the active-site residues RMSD along the trajectories of the individual simulations revealed destabilization in three of the four replicates (Supplementary Fig. 11c), and the mean RMSF values of these residues are consistently slightly higher for the mutant compared to the WT complex (Fig. 5f). This allosteric effect, emanating from the charge reversal in position 42 and displacement of R38, aligns well with the observation that patients harboring the K42E mutation display a characteristic shortening of their plasma and urinary dolichols. Specifically, we suggest that the globally enhanced dynamics of the active-site may result in weakening of the association with long-chain products, leading to their premature release. Future studies are needed to determine whether altered substrate interaction, either through direct or indirect mechanisms, converges into a similar enhancement of active-site dynamics, resulting in a common outcome of product-length shortening, along with the reduction in catalytic activity (Figs. 3f and 5a).

Together, with the growing spectrum of diseases related to \( \text{hcis-PT} \) dysfunction, the structure sheds light on the mechanisms of dolichol synthesis and their disruption in disease. Moreover, it establishes a molecular framework that may enable the rational design of specific \( \text{hcis-PT} \) activity modulators for the treatment of arRP and additional congenital glycosylation disorders.

### Methods

**Cloning.** Full-length human DHDDS (residues 1–333, UniProt Q86SQ0) was cloned into pET-32b plasmid and sNgBR (residues 73–293, UniProt Q96E22) or sNgBR(A167→175) (\( \text{hcis-PT} \)) cloned into pETM-11 plasmid as thioredoxin (TRX) fusion proteins. The constructs include a 6xHis-tag (DHDDS) or Strep-tag (followed by an NgBR(A167→175) to facilitate protein purification and a TEV-protease (tobacco etch virus) cleavage site to remove the affinity tags and TRX fusion. Mutations were introduced using the QuickChange method and verified by sequencing. Primers used for mutagenesis are listed in Supplementary Table 1.

**Protein expression and purification.** *Escherichia coli* T7-expressed competent cells were co-transformed with DHDDS and sNgBR (\( \text{hcis-PT} \)) or sNgBR(A167→175) (\( \text{hcis-PT} \)), grown in Terrific Broth medium at 37 °C until reaching OD\(_{600}\)nm = 0.6, and induced at 16°C by adding 0.5 mM isopropyl \( \beta \)-D-1-thiogalactopyranoside (IPTG). Proteins were expressed at 16°C for 16–20 h, harvested by centrifugation (~5700 × g for 15 min), and then resuspended in a lysis buffer containing 20 mM Tris (2-hydroxymethyl)-1-piperazinethanesulfonic acid (HEPES), pH 7.5, 150 mM NaCl, and 1 mM tris(2-carboxyethyl)phosphine (TCEP) and 0.02% (v/v) triton X-100, supplemented with 1 μg/ml DNase I and a protease inhibitor mixture. Resuspended cells were homogenized and disrupted in a microfluidizer. Soluble proteins were recovered by centrifugation at ~ 40,000 × g for 45 min at 4°C. Overexpressed proteins were purified on a HisTrap HP column, followed by purification on a Strep-Tactin column and TEV-protease cleavage of the purification tags and TRX fusions. The reaction mixture was concentrated and loaded onto a Superdex-200 preparative size-exclusion column pre-equilibrated with 20 mM HEPES, pH 7.5, 150 mM NaCl, and 1 mM TCEP. Purified proteins were flash-frozen in liquid nitrogen and stored at −80°C until use. Protein purity was >95%, as judged by SDS-PAGE.

**Cross-linking.** About 8 μM of \( \text{shcis-PT} \) were cross-linked by incubation with 0.005% glutaraldehyde at room temperature for 15 min. Reactions were quenched by the addition of sodium dodecyl sulfate and \( \beta \)-mercaptoethanol containing...
sample buffer, followed by 10 min of incubation at room temperature. Cross-linked products were analyzed by SDS-PAGE.

**Enzyme kinetics.** The activity of purified xcis-PT was measured using a radioligand-based assay\(^{8,32,33}\). About 0.01–0.1 µM of purified proteins were mixed with FPP and \(^{14}C\)-IPP to initiate the reaction in buffer composed of 25 mM Tris-HCl, pH 7.5, by Zeba Spin columns (0.5 mL, 7-kDa cutoff) and adjusted to 10 µM concentration. The sample was loaded into a homemade quartz-glass ESI tip that was mounted onto a custom-built nESI source interfaced to Waters Synapt G2Si. Analyses at different activation settings were performed. Low activation (trapping collision energy 10 V) was used to obtain native-like conditions, while high-trap collisional energies (up to 110 V) were employed to strip the adducts and obtain more accurate mass. Key instrument parameters were sampling cone voltage 40 V, source offset 20 V, trap gas 4 mL/min and source temperature 20°C, and ESI tip voltage 1.8 kV. Data were analyzed in MassLynx 4.1.

**Table 1 Data collection and refinement statistics.**

| Table 1 Data collection and refinement statistics. |
|-----------------------------------------------|
| **6Z1N: xcis-PT** |
| Data collection | Space group | R32:H |
| Cell dimensions | a, b, c (Å) | 184.1, 184.1, 112.6 |
| α, β, γ (°) | 90, 90, 120 |
| Resolution (Å) | 46.02–2.30 (2.44–2.30)* |
| R<sub>syn</sub> or R<sub>merge</sub> | 0.166 (2.026) |
| I/σ | 16.3 (16) |
| Completeness (%) | 100.0 (100.0) |
| Redundancy | 21.1 (20.7) |
| Reflections | 45.99–2.30 |
| No. of reflections | 32,477 |
| Sample buffer, followed by 10 min of incubation at room temperature. Cross-linked products were analyzed by SDS-PAGE. |

**Conservation analysis of DHDDS.** The Ensemble server\(^{39}\) was used to search for human DHDDS orthologs. These sequences were used to generate a multiple-sequence alignment using Clustal\(^{42}\). The resulting alignment was used as input for the Consurf server\(^{43}\), which outputs a conservation score for each residue.

**Molecular dynamics (MD).** A single human NgBR–DHDDS heterodimer was used. Mutation K42E was modeled using Schrodinger’s Maestro 11.2. All the structures were prepared using the Protein Preparation Wizard (Schrodinger Release 2017-2: Schrodinger Suite 2019-2 Protein Preparation Wizard; Schrodinger, LLC, New York, NY, 2016) as implemented in Schrodinger’s Maestro 11.2 (Schrodinger Release 2017-2: Schrodinger Suite 2019-2 Protein Preparation Wizard; Schrodinger, LLC, New York, NY, 2016). This protocol adds missing hydrogen atoms considering a pH value of 7.0 ± 1.0, optimizes the hydrogen-bond network, and performs restrained minimization. Crystallographic ligands, ions, and water were removed. The MD simulations were performed using GROMACS version 2018.2\(^{44}\), with OPLS forced\(^{45}\). Each protein complex was submerged in TIP3P water model in a triclinic box with 13 Å extension around the protein. Potassium and chloride ions were added to the water phase in order to neutralize the system and to obtain a salt concentration of 0.15 M. The simulations were conducted in periodic boundary conditions with particle-mesh Ewald electrostatics with 10 Å cutoff for long-range interactions\(^{46}\). First, the simulated systems were energy minimized with the steepest descent algorithm and constant pressure of 1 atm under Parrinello–Rahman coupling algorithm. The LINCS algorithm was applied to bond lengths involving hydrogen, allowing an integration time step of 2 fs\(^{44}\). Three and four replicas of MD simulations were conducted for each of the WT and mutant constructs, respectively. The resulting trajectories were visually inspected using VMD 1.9.3 software\(^{47}\). Clustering was performed by the clustering analysis tool of Gromacs (gmx cluster). The GROMOS clustering algorithm with a cutoff of 0.12 nm was used to determine the neighboring structures in the clusters. The stability of the resulting trajectories and the average mobility of the protein residues 5 Å around the crystallographic ligand were tested based on the RMSD of the backbone atoms of the protein from the equilibrated structures and on the RMSF, respectively. RMSD and RMSF were calculated using the rms and rmsf utilities of the GROMACS package, respectively. Distances along the trajectories’ time and distances probabilities were calculated using the distance utility.

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

**Data availability.** Atomic coordinates and structure factors for the structure of xcis-PT in complex with Mg\(^{2+}\) and FPP have been deposited in the Protein Data Bank with accession number 6Z1N. Source data are provided with this article.

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