GGTase3 is a newly identified geranylgeranyltransferase targeting a ubiquitin ligase

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Protein prenylation is believed to be catalyzed by three heterodimeric enzymes: FTase, GGTase1 and GGTase2. Here we report the identification of a previously unknown human prenyltransferase complex consisting of an orphan prenyltransferase α-subunit, PTAR1, and the catalytic β-subunit of GGTase2, RabGGTB. This enzyme, which we named GGTase3, geranylgeranylates FBXL2 to allow its localization at cell membranes, where this ubiquitin ligase mediates the polyubiquitylation of membrane-anchored proteins. In cells, FBXL2 is specifically recognized by GGTase3 despite having a typical carboxy-terminal CaaX prenylation motif that is predicted to be recognized by GGTase1. Our crystal structure analysis of the full-length GGTase3–FBXL2–SKP1 complex reveals an extensive multivalent interface specifically formed between the leucine-rich repeat domain of FBXL2 and PTAR1, which unmasks the structural basis of the substrate-enzyme specificity. By uncovering a missing prenyltransferase and its unique mode of substrate recognition, our findings call for a revision of the ‘prenylation code’.

A ssociation with cellular membranes is a prerequisite for the function of many regulatory proteins, which can be either embedded in the lipid bilayer or located at its surface (integral versus peripheral membrane proteins). Many peripheral proteins are targeted to biological membranes as a consequence of posttranslational modification with lipids1. Two isoprenoid lipids derived from intermediates in the cholesterol biosynthetic pathway are utilized by eukaryotic cells for such modification: the 15-carbon farnesyl lipid and the 20-carbon geranylgeranyl lipid2–4. Covalent posttranslational modification with lipids1. Two isoprenoid lipids derived from intermediates in the cholesterol biosynthetic pathway are utilized by eukaryotic cells for such modification: the 15-carbon farnesyl lipid and the 20-carbon geranylgeranyl lipid2–4. Covalent modification of the target proteins by these lipids at a carboxy-terminal cysteine residue, generally referred to as prenylation, is catalyzed by a group of enzymes known as prenyltransferases. In the human proteome, about 300 proteins, many involved in fundamental cellular functions, such as membrane trafficking and signal transduction, are modified by prenyltransferases5. Importantly, some oncogenic proteins, such as the activating mutant forms of H-, N- and K-RAS, require prenylation for their transforming activities4. Inhibition of prenylation, therefore, has been proposed as a therapeutic approach for treating the ~30% of human cancers that are driven by RAS-activating mutations4–8.

Three prenyltransferases have been identified in mammals, farnesyltransferase (FTase), geranylgeranyltransferase type 1 (GGTase1) and geranylgeranyltransferase type 2 (GGTase2)9–12. All three prenyltransferases are heterodimeric enzyme complexes, each consisting of one α and one β subunit. FTase and GGTase1 share a common α-subunit, FTNTA (also known as PTAR2), but contain distinct β-subunits, which are encoded by FNTB and PGGTB, respectively (Fig. 1a). The substrate specificity of FTase and GGTase1 are thought to be determined by a C-terminal CaaX sequence (C: cysteine; a: aliphatic; X: any amino acid), which constitutes the site of lipid modification. Depending on the nature of the utmost X residue, a substrate CaaX motif is recognized by either FTase for farnesylation or GGTase1 for geranylgeranylation. The third prenyltransferase, GGTase2, is formed by RabGGTA (the α-subunit, also known as PTAR3) and RabGGTB (the β-subunit) (Fig. 1a). GGTase2 prenylates the substrate cysteine(s) in less defined C termini, including XXCC, XCCX, CCXX, CCXXX and XCCX. Unlike FTase and GGTase1, GGTase2 requires an accessory protein designated RAB escort protein that provides substrate recognition9–12. Although distinct groups of substrates have been identified for FTase (for example, RAS GTPases, pre-Lamin A and Lamin B), GGTase1 (for example, RHO–RAC GTPases and RAP1B) and GGTase2 (for example, RAB GTPases), several cases of cross-prenylation have been described in the literature16–20. The molecular details of cross-prenylation remain unclear, underscoring the fact that we do not completely understand how these enzymes work.

F-box proteins are the substrate receptor subunits of SCF (Skp1, Cul1, F-box protein) ubiquitin ligase complexes21,22. In humans, there are 69 F-box proteins, each forming a different SCF ligase and promoting the polyubiquitylation of specific substrates. Distinct from most F-box proteins, FBXL2 and its close paralog FBXL20 (the former being ubiquitous and the latter being specifically expressed in neurons23–25) terminate with a prototypical CaaX motif (CVIL), which is strictly conserved across species. FBXL2 has been shown to be geranylgeranylated26 and, on the basis of the sequence of its CaaX motif, is predicted to be a GGTase1 substrate. We have previously shown that the integrity of the CaaX motif is necessary for FBXL2 to assemble into an active SCF ubiquitin ligase complex and interact with two substrates localized at cellular membranes, p85β, a regulatory subunit of the PI3-kinase (PI3K) and IP3 (inositol 1,4,5-
trisphosphate) receptor type 3 (IP3R3)\textsuperscript{27–29}. Other independent studies have also reported membrane-localized substrates for both FBXL2 and FBXL20 (refs.\textsuperscript{23,30–32}). By promoting the degradation of M-RAS and R-RAS, or a distinct F-box protein used as control (FBXL15) interacted with PTAR1 (Fig. 1b, Supplementary Fig. 1b and data not shown). As expected, RAS GTPases and RAP1B, but not FBXL2 or RAB GTPases, were able to co-immunoprecipitate the short and long isoforms of endogenous FNTA (Fig. 1b and Supplementary Fig. 1c). The specificity of the interaction between PTAR1 and FBXL2 was observed even when PTAR1 was co-expressed with substrates of FTase and GGtases and when FBXL2 was expressed at lower levels than the other substrates (Supplementary Fig. 1d,e). Moreover, when the three human prenyltransferase \(\alpha\)-subunits were expressed in HEK-293T cells, we found that only PTAR1, but not FNTA or RabGGTA, co-immunoprecipitated with endogenous FBXL2 (Fig. 1c).

To confirm the binding between RabGGTA and FBXL2, we expressed either FLAG-tagged FBXL2 or GFP-tagged FBXL2 in HEK-293T cells and found that endogenous RabGGTA co-immunoprecipitated with FBXL2, but not with FLAG-tagged empty vector or GFP-tagged K-RAS4B (Fig. 1d and Supplementary Fig. 1f). In line with a systematic study of the human interactome\textsuperscript{8}, FLAG-tagged PTAR1 was also able to co-immunoprecipitate endogenous RabGGTB (Fig. 1d). By co-expressing GFP-tagged FBXL2 and FLAG-tagged PTAR1 in HEK-293T cells and performing sequential immunoprecipitations, we found that FLAG-tagged PTAR1 co-immunopurified endogenous RabGGTB as well as GFP-tagged PTAR1 in HEK-293T cells and performing sequential immunoprecipitations, we found that FLAG-tagged PTAR1 co-immunopurified endogenous RabGGTB as well as GFP-tagged FBXL2 as indicated. Immunoprecipitations were carried out sequentially using first an anti-FLAG antibody and then an anti-GFP antibody as described in the Methods. The first elution was done with a FLAG peptide and the second with 1% SDS. The two eluates were then immunoblotted as indicated. All experiments were repeated at least three times. Uncropped blot/gel images are shown in Supplementary Data Set 1.

Results

**FBXL2 binds a previously unrecognized GGtase.** The mammalian genome contains a single paralog of FNTA and RABGGTA encoding PTAR1 (prenyltransferase \(\alpha\)-subunit repeat-containing protein 1), an orphan protein with unknown functions. Peptides corresponding to PTAR1 and RabGGTB were specifically identified in most FBXL2 immunopurifications that we and others analyzed using mass spectrometry\textsuperscript{23,33,34}. Notably, PTAR1 and RabGGTB peptides have never been found, either in the 411 experiments reported by the contaminant repository for affinity purification-mass spectrometry data (www.crapome.org)\textsuperscript{35} or in some 300 purifications of 26 distinct F-box proteins carried out in our laboratory, supporting the specificity of the interaction with FBXL2.

We sought to validate the binding of FBXL2 with PTAR1 and confirm its specificity by screening a panel of 15 proteins containing typical C-terminal prenylation motifs (that is, CXXX, CXXC and XXCC) for their ability to bind PTAR1 in expression in HEK-293T cells. Endogenous PTAR1 interacted specifically with FBXL2 (Fig. 1b and Supplementary Fig. 1a). In contrast, none of the other prenyltransferase substrates (H-RAS, N-RAS, K-RAS4A, K-RAS4B, RAB23, RAB1B, RAB1a, RAB7a, RAB11, RAB23, RAB34, RAB35, M-RAS and R-RAS,) or a distinct F-box protein used as control (FBXL15) interacted with PTAR1 (Fig. 1b, Supplementary Fig. 1b and data not shown). As expected, RAS GTPases and RAP1B, but not FBXL2 or RAB GTPases, were able to co-immunoprecipitate the short and long isoforms of endogenous FNTA (Fig. 1b and Supplementary Fig. 1c). The specificity of the interaction between PTAR1 and FBXL2 was observed even when PTAR1 was co-expressed with substrates of FTase and GGtases and when FBXL2 was expressed at lower levels than the other substrates (Supplementary Fig. 1d,e). Moreover, when the three human prenyltransferase \(\alpha\)-subunits were expressed in HEK-293T cells, we found that only PTAR1, but not FNTA or RabGGTA, co-immunoprecipitated with endogenous FBXL2 (Fig. 1c).
Previous studies have shown that FBXL2 is geranylgeranylated by a previously unrecognized mammalian GGTase, which we named GGTase3 but not RabGGTB. These data strongly support the identification of GGTase3 as the prenyltransferase that acts with endogenous RabGGTB, but not FNTB and PGGT1B. To explore this possibility, we expressed the three human prenyltransferase α-subunits in HEK-293T cells and examined their binding to the three endogenous β-subunits. Similar to RabGGTA, we found that PTAR1 interacted with endogenous RabGGT, but not FNTB and PGGT1B (Fig. 1c). In contrast, FNTA interacted with FNTB and PGGT1B, but not RabGGT. These data strongly support the identification of a previously unrecognized mammalian GGTase, which we named GGTase3, comprising PTAR1 and RabGGT as α- and β-subunits, respectively (Fig. 1a). GGTase3 geranylgeranylates FBXL2 and targets it to cellular membranes. Previous studies have shown that FBXL2 is geranylgeranylated\(^{12}\). We found that bacterially purified FBXL2 served as a substrate of GGTase3 in vitro (Fig. 2a,b). Notably, GGTase3 was able to in vitro geranylgeranylate FBXL2, but not K-RAS4B and FBXW7 (Fig. 2b). We obtained identical results using two bacterial purified GGTase3 preparations that were made independently in our two laboratories using different constructs (Fig. 2a,b and Supplementary Fig. 2a). GGTase1 was also able to prenylate FBXL2 in vitro (Supplementary Fig. 2b), which is expected given the presence of the typical CaaX signature motif of GGTase1 substrates in FBXL2. To investigate which of the two enzymes is responsible for FBXL2 geranylgeranylation in vivo, we incubated RPE1-HTERT1 transfection cells with geranylgeranyl azide for 16 h, collected, lysed and azide selective ligation reaction with sDIBO-Biotin was performed for 1 h to label geranylgeranylated proteins via a copper-free ‘Click-IT’ reaction. After immunoprecipitation with an anti-HA antibody, immunoblots were carried out as described in the Methods. The experiment was repeated three times. A representative result is shown. Uncropped blot/gel images are shown in Supplementary Data Set 1.

Fig. 2 | GGTase3 geranylgeranylates FBXL2 and is required for its localization to cellular membranes. a. Purified recombinant GGTase3 geranylgeranylates purified FBXL2. Indicated amounts of purified FBXL2 were incubated with 100 ng of purified GGTase3 (either tagged (T) or untagged (UT) versions) to carry out in vitro geranylgeranylation assay using saturating concentrations of tritiated \( ^{14} \)HGGPP as described in the Methods. Each data point represents mean ± s.d. of three biological replicates. Michaelis–Menten kinetics was used to generate an apparent \( K_m \) value of 1.2 μM using Prism Graphpad software. b. In vitro geranylgeranylation assay was carried out and measured as in a using 10 μM of purified FBXL2, FBXW7 or K-RAS4B and 100 ng of purified GGTase3. Bar graphs represent mean ± s.d. from three biological replicates. Source data for panels a and b are available with the paper online. c. HEK-293T cells were cotransfected with the indicated plasmids and processed for the detection of geranylgeranylated FBXL2 using a ‘Click-IT’ assay, as described in the Methods. The experiment was repeated three times. A representative result is shown. Uncropped blot/gel images are shown in Supplementary Data Set 1. d, HeLa cells were transfected with the indicated siRNA oligonucleotides and cDNAs. Twenty-four hours post-transfection cells were incubated with geranylgeranyl azide for 16 h, collected, lysed and azide selective ligation reaction with sDIBO-Biotin was performed for 1 h to label geranylgeranylated proteins via a copper-free ‘Click-IT’ reaction. After immunoprecipitation with an anti-HA antibody, immunoblots were carried out. The experiment was repeated four times. A representative result is shown. Uncropped blot/gel images are shown in Supplementary Data Set 1. e, HeLa cells were transfected first with the indicated siRNA oligonucleotides and then with the indicated GFP-tagged proteins. Live-cell confocal imaging was carried out as described in the Methods. Images show representative frames of three independent experiments. NS, non-silencing. Scale bar, 10 μm.
Fig. 3 | Overall structure of GGTase3–FBXL2–SKP1 complex. a, GGTase3 binding to FBXL2–SKP1 monitored by Octet biolayer interferometry analysis in the absence of GGPP or its analog. Sensogram traces (black lines) of the interactions of His-FBXL2–SKP1 with GGTase3 at three concentrations (300 nM, 100 nM and 33.3 nM) overlaid with curve fits (red lines). The global fit yields a calculated $K_D$ of 69.0 nM ± 1.1 nM. b, Size exclusion chromatography analysis of the purified GGTase3–FBXL2–SKP1 protein complex with sodium dodecylsulfate–polyacrylamide gel electrophoresis analysis of the central fraction containing the protein complex with all four subunits in equal stoichiometry. c, Two orthogonal views of the tetrameric complex containing PTAR1 (orange), RabGGTB (slate), FBXL2 (magenta) and SKP1 (green). The zinc ion at the active site of RabGGTB is shown as a dark yellow sphere. The N and C termini of different proteins are labeled N and C in the corresponding colors.

DNA, which was short interfering RNA (siRNA)-insensitive since the two oligonucleotides used targeted the 5′ untranslated region (UTR). This experiment demonstrated that the decrease in FBXL2 prenylation is not due to an off-target effect of the oligonucleotide to PTAR1. Silencing of FNTA or PGGT1B had no effect on FBXL2 prenylation (Fig. 2d). However, when both PTAR1 and PGGT1B were silenced, the prenylation of FBXL2 became virtually undetectable. This result suggests that GGTase1 is able to compensate for GGTase3 in its absence. In agreement with this possibility, when PTAR1 was depleted, FBXL2 acquired the ability to bind endogenous FNTA (Supplementary Fig. 1f).

Prenylation of proteins is required for their proper subcellular trafficking and localization. Therefore, we investigated the localization of GFP-tagged FBXL2 with and without geranylgeranylation. Using live-cell imaging confocal microscopy, we observed that GFP-FBXL2 decorated cellular membranes, including the plasma membrane and perinuclear vesicles (Fig. 2e). In contrast, GFP-FBXL2(C420S) was observed in a homogeneous pattern in the cytoplasm (with negatively imaged organelles) and the nucleoplasm (Supplementary Fig. 2c). Thus, the CVIL sequence in FBXL2 is necessary for the association with membranes through a different and/or a compensatory mechanism. When both PTAR1 and FNTA were silenced, GFP-FBXL2 lost its residual vesicular localization and became homogenously distributed in the cytoplasm and the nucleus (Fig. 2e). This is in agreement with the notion that, in the absence of PTAR1, FBXL2 becomes a ‘neo-substrate’ of GGTase1, as suggested by its binding to FNTA (Supplementary Fig. 1f) and prenylation in vitro (Supplementary Fig. 2b).

Overall structure of GGTase3–FBXL2–SKP1. Previous studies have shown that high-affinity binding of substrates to Ftsase and GGTase1 requires preloading of the enzymes with the farnesyl and geranylgeranyl diphosphate (FPP or GGPP) substrate, respectively. Substrate recognition by GGTase2 also depends on an accessory protein that bridges RAB GTPases and the enzyme. Unexpectedly, we detected robust high-affinity (~70 nM) interaction between GGTase3 and FBXL2–SKP1, both purified from Escherichia coli, without the addition of GGPP or a GGPP analog (Fig. 3a). To further validate their strong interaction, we co-expressed the heterodimeric GGTase3 with FBXL2–SKP1 and successfully isolated GGTase3–FBXL2–SKP1 as a tetrameric complex in equal stoichiometry (Fig. 3b). To understand how GGTase3 assembles and recognizes FBXL2, we crystallized the GGTase3–FBXL2–SKP1 complex and determined its structure at 2.5 Å resolution (Table 1).
elongated crescent, mainly consisting of linearly packed α-helical hairpins. It closely wraps around the globular RabGGTB subunit, which is characterized by a zinc ion at its active site. The FBXL2 protein possesses a typical amino (N)-terminal helical F-box motif that interacts with the SCF adapter subunit SKP1 (refs. 40,41). Its C-terminal domain is constructed by leucine-rich-repeats (LRRs) in a remarkably regular curved solenoid structure encompassing half a circle. GGTase3 recruits its substrate FBXL2 predominantly via a unique N-terminal extension (NTE) of PTAR1, which folds into an α/β subdomain and anchors itself onto the concave surface of the FBXL2 LRR domain. Interestingly, no electron density was found for the utmost CaaX motif-containing FBXL2 C-terminal tail, which is most likely attributable to this large intermolecular interface.

GGTase3 contains a unique NTE in PTAR1. PTAR1, the α-subunit of the newly identified GGTase, harbors a series of right-handed anti-parallel coiled-coils that fold into six successively packed α-helical hairpins, which are highly comparable to those of FNTA (PTAR2) and RabGGTA (PTAR3), the α-subunits of known prenyltransferases (Fig. 4a,b)15,39. Despite missing an additional α-helical hairpin at the C terminus, PTAR1 adopts the same crescent-shaped fold that envelops the RabGGTB catalytic subunit through an extensive and conserved interface found in all three previously characterized heterodimeric prenyltransferases. In complex with PTAR1, RabGGTB displays the identical α–α barrel fold as found in GGTase2, consisting of a core of six parallel helices and six peripheral helices15. At a funnel-shaped cavity in the center of α–α barrel, the active site of the enzyme is lined with conserved hydrophobic residues with an intrinsic zinc ion bound at the top. Overall, the architecture of GGTase3 is very similar to the known prenyltransferase complexes.

A striking structural feature that distinguishes PTAR1 from the other two prenyltransferase α-subunits is its unique NTE, which is highly conserved in animals (Supplementary Note 2). Unlike the NTEs of the other two prenyltransferase α-subunits that are...
largely disordered, the PTAR1 NTE is unexpectedly well structured. The PTAR1 NTE adopts an α/β-fold starting with the α1-helix followed by a β-sheet comprising three β-stands (β1–β3) (Fig. 4a,b). It terminates with the α2-helix, which packs orthogonally against α1 and couples NTE to the helical hairpin domain. Stabilized by a hydrophobic core, the PTAR1 NTE is highlighted by a ten amino acid loop, which is flanked by the β1- and β2-strands (hereafter referred to as β1–β2 loop). As described below, this structural loop, together with the rest of the PTAR1 NTE, plays a pivotal role in FBXL2 recruitment.

The LRR domain of FBXL2 contains an unusual pocket. The FBXL2 LRR domain contains 13 complete LRRs (LRR1–LRR13) and an additional β-strand followed by a disordered C-terminal tail with the CaaX motif (Fig. 4c). The FBXL2 LRRs pack in tandem and produce a curved solenoid architecture resembling a semicircular interface.

Fig. 5 | Multivalent FBXL2–GGTase3 interface. a, A top view of the overall FBXL2–GGTase3 interface formed by PTAR1 (orange ribbons) and FBXL2 (magenta surface). b, c, Close-up views of the PTAR1–FBXL2 interface made by the C-terminal and N-terminal FBXL2 LRRs. Selected interface residues are shown as sticks. Dashed yellow lines represent a network of hydrogen bonds and polar interactions. The disordered C-terminal tail of FBXL2 is shown as a dashed magenta line. d, HEK-293T cells were cotransfected with GFP-tagged FBXL2 and either wild-type (WT) FLAG-tagged PTAR1 or the indicated mutants. Twenty-four hours post-transfection, cells were collected for immunoprecipitation and immunoblotting. The experiment was repeated three times. A representative result is shown. Uncropped blot/gel images are shown in Supplementary Data Set 1. e, HEK-293T cells were transfected with either GFP-tagged WT FBXL2 or the indicated GFP-tagged mutants. Twenty-four hours post-transfection, cells were collected for immunoprecipitation and immunoblotting. The experiment was repeated three times. A representative result is shown. Uncropped blot/gel images are shown in Supplementary Data Set 1. f, A model of the FBXL2 C-terminal CVIL motif at the active site of GGTase3 in the presence of a GGPP analog. The zinc ion is shown as a yellow sphere and is buried in the catalytic pocket of RabGGT3 (slate surface). The disordered FBXL2 C tail is shown as a magenta dashed line with the modeled CVIL motif as magenta sticks. A GGPP analog, 3-azaGGPP, is shown as sticks with the geranylgeranyl group in yellow and the pyrophosphate in orange. The residues in the α1- and α2-helices of PTAR1 NTE forming the hydrophobic patch possibly interacting with the FBXL2 tail are shown as orange sticks.
The concave surface of FBXL2 LRRs is formed by the interior parallel β-strands, while its convex surface is constructed by the exterior array of α-helices. In comparison to the LRR domains of other LRR-type F-box proteins40,42,43, the topology of FBXL2 LRRs displays a remarkable regularity with no atypical repeat, loop insert, abrupt kink in curvature or repeat offset. The peculiar feature of the FBXL2 LRRs preceding LRR13 (Fig. 5b). In sequence, this additional β-strand and the short loop following it are two amino acids shorter than the corresponding parts of all LRRs (Fig. 4d). By packing against the last LRR (LRR13), it creates an unusual surface pocket, which we named the ‘LRR13 pocket’, on the apical ridge of the LRR domain (Fig. 4e). As shown in the next section, this FBXL2 pocket serves as the key docking site for GGTase3 binding.

FBXL2 and GGTase3 form a multivalent interface. Recognition of FBXL2 by GGTase3 is exclusively mediated by the α-subunit PTAR1, which occupies the entire concave side of the F-box protein. A top view of the PTAR1–FBXL2 interface reveals that many FBXL2 LRRs from both the N- and C-terminal halves of the protein participate in PTAR1 binding, leaving only a small gap between the two proteins near the middle region of the FBXL2 solenoid (Fig. 5a).

At the C-terminal half of the FBXL2 LRR domain, the PTAR1 NTE forms a continuous and highly complementary interface with the F-box protein. The hallmark of this interface is the interlocking engagement between the LRR13 pocket of FBXL2 and the α1-helix and the β1–β2 loop of the PTAR1 NTE (Fig. 5a,b). The tip of the PTAR1 β1–β2 loop harbors an asparagine residue, Asn 43, which is strictly conserved among all PTAR1 orthologs (Supplementary Note 2). Acting as a claw, the PTAR1 β1–β2 loop hooks to the FBXL2 pocket with its Asn 43 residue reaching to the bottom of the pocket and forming two hydrogen bonds with FBXL2 backbone groups (Fig. 5b). In doing so, the PTAR1 β1–β2 loop and its nearby α1-helix clamps down the short FBXL2 loop terminating the extra C-terminal β-strand, creating an interdigitated molecular interface. Similar to the tip of the PTAR1 β1–β2 loop, the short loop at the end of the FBXL2 LRR domain, which consists of three residues, Ala 398, Tyr 399 and Phe 400, also has an amino acid sequence invariant among different FBXL2 orthologs (Supplementary Note 1). Phe 400, which is the last traceable residue of FBXL2 in the crystal structure, encloses the LRR13 pocket and secures the docking of PTAR1 Asn 43 inside. Remarkably, this portion of the FBXL2–PTAR1 interface is further reinforced by a network of polar interactions taking place between the PTAR1 α1-helix and the three FBXL2 LRRs preceding LRR13 (Fig. 5b). Together, these features of the interface strongly suggest a critical role in mediating FBXL2 binding to PTAR1.

In addition to its C-terminal LRRs, FBXL2 N-terminal LRRs are also involved in cradling PTAR1 through a relatively flat interface. The majority of the intermolecular contacts in this region are made between the apical ridge of the first five LRRs in FBXL2 and the PTAR1 loop connecting α2- and α3-helices (Fig. 5c). Distinct from the C-terminal interface, this N-terminal interface is characterized by two pairs of reciprocal Trp-Arg interactions, in which the aliphatic side chain of an arginine residue (Arg 86 of FBXL2 and Arg 84 of PTAR1) packs against the indole ring of a tryptophan residue (Trp 81 of PTAR1 and Trp 165 of FBXL2). These interactions are further stabilized by a salt bridge formed between FBXL2 Arg 190 and PTAR1 Asp 85.

Molecular determinants of FBXL2-GGTase3 interaction. To map the important structural elements supporting FBXL2-GGTase3 interaction, we designed and tested the binding activities of a series of PTAR1 and FBXL2 mutants by co-immunoprecipitation from HEK-293T cells. Single- or double-point mutations designed to disrupt the two Trp-Arg pairs located at the N-terminal interface only modestly compromised complex association (Fig. 5d, lanes 4 and 5 and Fig. 5e, lanes 3–8), indicating that FBXL2-PTAR1 interactions at this region only play an accessory role in supporting FBXL2 recruitment to GGTase3. By contrast, removing the side chain of Asn 43 or deleting two amino acids at the tip of the PTAR1 β1–β2 loop severely impaired the binding of FBXL2 to PTAR1 (Fig. 5d, lanes 2 and 3). This effect can also be achieved, to a more complete degree, by both a triple-point mutation of the short FBXL2 loop terminating the extra C-terminal β-strand, or a truncation mutation of the F-box protein with the C-terminal β-strand entirely eliminated (Fig. 5e, lanes 9 and 10). Together, these results definitively establish the interface at the C-terminal end of the FBXL2 LRR domain as a critical ‘hotspot’ for FBXL2 recognition by GGTase3.

Human FBXL2 is 423 amino acids long. The last 23 residues following Phe 400 are in a disordered conformation with no clear electron density in the crystal. On the basis of previous structural studies of FTase, GGTase1 and GGTase2 15,37,44,45, we model the C-terminal CVIL motif of FBXL2 into the catalytic pocket of GGTase3 together with a GGP analog (Fig. 5f). The Cα atoms of Phe 400 and the cysteine residue in the CVIL motif are separated by a distance of ~36 Å, which can be comfortably bridged by the missing 19 residues in an extended conformation. Consistent with the role of the FBXL2 LRR domain in mediating PTAR1 binding, the CVIL motif fused to GFP showed little binding to GGTase3.

| Table 1 | Data collection and refinement statistics |
|-----------------|-----------------|
| **GGTase3–FBXL2-SKPI (PDB 6O60)** |
| **Data collection** |
| Space group | P2_1,2_1 |
| Cell dimensions |
| a, b, c (Å) | 86.8, 99.3, 151.2 |
| α, β, γ (°) | 90.0, 90.0, 90.0 |
| Resolution (Å) | 50.00-2.50 (2.54-2.50) |
| Rmerge | 0.098 (0.890) |
| Rfree | 0.109 (0.986) |
| I/σ(I) | 24.3 (2.3) |
| CC(1/2) | 0.991 (0.754) |
| Completeness (%) | 90.5 (93.6) |
| Redundancy | 4.8 (4.7) |
| **Refinement** |
| Resolution (Å) | 49.5-2.5 |
| No. reflections | 41,320 |
| Rwork / Rfree | 0.194 / 0.247 |
| No. atoms | 9,299 |
| Protein | 9,228 |
| Zn ion | 1 |
| Water | 70 |
| B factors |
| Protein | 55 |
| Zn ion | 41 |
| Water | 51 |
| R.m.s. deviations |
| Bond lengths (Å) | 0.008 |
| Bond angles (°) | 1.025 |

*A single crystal was used for diffraction data collection and structure determination. *Values in parentheses are for highest-resolution shell.
PTAR1 NTE present a hydrophobic patch in close vicinity to Reveals an unexpected multivalency in FBXL2-GGTase3 interaction, which spans the entire LRR domain and the C-terminal tail of the F-box protein.

Discussion

Since the 1980s, the biomedical literature has stated that mammals possess three prenyltransferases. In this study, we have identified and characterized a fourth family member, GGTase3, which consists of PTAR1, an orphan α-subunit and the β-subunit RabGGTB. Prenyltransferases have been previously shown to share α-subunits. Our studies reveal that this important enzyme family has also evolutionarily expanded by sharing β-subunits. Notably, our structural and biochemical analyses unveil that the CaaX motif of FBXL2 contributes to, but is not sufficient for, the formation of a stable complex with GGTase3. PTAR1, instead, plays a critical role in FBXL2 recruitment through its uniquely extensive interaction with the LRR domain of the F-box protein. Therefore, in contrast to classical prenyltransferases, our results indicate that additional multivalent structural elements outside the CaaX motif contribute to the FBXL2 specificity of GGTase3. Moreover, our study suggests that GGTase1 can prenylate FBXL2, albeit with less efficiency, when GGTase3 is absent in the cell. This is yet another example that prenyltransferases are able to cross-prenylate substrates, enlightening functional compensation under specific conditions.

By establishing FBXL2 as a substrate of GGTase3, we have also uncovered the first prenyltransferase that physically interacts with the LRR domain of the F-box protein. Therefore, in contrast to classical prenyltransferases, our results indicate that additional multivalent structural elements outside the CaaX motif contribute to the FBXL2 specificity of GGTase3. Moreover, our study suggests that GGTase1 can prenylate FBXL2, albeit with less efficiency, when GGTase3 is absent in the cell. This is yet another example that prenyltransferases are able to cross-prenylate substrates, enlightening functional compensation under specific conditions.

PTAR1 has long been neglected until its identification as a gene product required for hepatitis C virus RNA replication. However, our studies reveal that this important enzyme family has also evolutionarily expanded by sharing β-subunits. Notably, our structural and biochemical analyses unveil that the CaaX motif of FBXL2 contributes to, but is not sufficient for, the formation of a stable complex with GGTase3. PTAR1, instead, plays a critical role in FBXL2 recruitment through its uniquely extensive interaction with the LRR domain of the F-box protein. Therefore, in contrast to classical prenyltransferases, our results indicate that additional multivalent structural elements outside the CaaX motif contribute to the FBXL2 specificity of GGTase3. Moreover, our study suggests that GGTase1 can prenylate FBXL2, albeit with less efficiency, when GGTase3 is absent in the cell. This is yet another example that prenyltransferases are able to cross-prenylate substrates, enlightening functional compensation under specific conditions.

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**Author contributions**

S.K. and M.P. conceived the project. S.K. designed and performed most biochemical, molecular biology and cell biology experiments. H.W. and N.Z. conceived and performed most protein purifications and all crystallization experiments. A.M., K.J. and H.H. performed some of the biochemical experiments. N.F. and M.R.P. helped with the initial microscopy experiments. M.P. and N.Z. directed and coordinated the study and oversaw the results with S.K. and H.W. All authors discussed the results and commented on the manuscript.

**Competing interests**

M.P. is a consultant for BeyondSpring Pharmaceuticals and a member of the scientific advisory boards of CullGen, Inc. and Kymera Therapeutics. N.Z. is a member of the scientific advisory board of Kymera Therapeutics. The authors declare no other competing interests.

**Additional information**

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In vitro geranylgeranylation assay. Briefly, recombinant human FBXL2, FBXW7, K-RAS4B, CDC42 and targeted GSTase3 (MBP-PTAR1 and GST-RabGGTB) were purified individually and then mixed to allow complex formation were affinity purified from bacterial system using standard protocols. Untagged GSTase3 (a GST–PTAR1–RabGGTB complex) in which the glutathione S-transferase (GST) was cleaved following purification was obtained as described below. Purified rat GSTase1 (GST-His–FNTA and untagged PGGTB1) was from MilliporeSigma (catalog no. 345852). FBXW7 (10 μM), K-RAS4B (10 μM) and various FBXL2 or CDC42 amounts (1 μM, 5 μM, 10 μM or 25 μM) were first equilibrated to 37 °C in a reaction buffer containing 50 mM Tris–HCl (pH 7.5), 1 mM DTT, 20 mM KCl, 100 μM ZnCl2, 5 mM MgCl2, buffer, 0.4 μM of [3H]geranylgeranylation assay. Of the [3H]geranylgeranlypyrophosphate (GGPP) (American Radiolabeled Chemicals no. ART 348). The geranylgeranylation reaction in a 50-μl volume was initiated with the addition of 100 ng of either GGTTase3 or GGTTase1. The reaction proceeded for 1.5 h at 37 °C while shaking at 900 rpm. and was subsequently blot trod onto Whatman P81 ion exchange cellulose chromatography papers (Lab Supply Outlaws, catalog no. 05-717-2C). The two papers were then placed into an Ultrafree-MC Centrifugal Filter (Millipore, catalog no. UFC00H000) and spun at 13,000 rpm for 2 min. The proteins were washed three times with 600 μl of 95% ethanol and 75 mM K2HPO4 and the last wash was performed with PBS. The two chromatography papers were added to 5 ml of Universal-Equilisk Scintillation Cocktail (MP Bio, catalog no. 0188248001) and the activity was measured via a scintillation counter (Beckman 6500 Multi-Purpose Scintillation Counter). The activity was determined in counts per minute (CPM). Geranylgeranylation of the different proteins was measured by determining the transfer of [3H]geranylgeranyphyophosphate onto purified substrates by GGTTase3 or GGTTase1 and plotted as μM/min using CPM counts. Michaelis–Menten kinetics was used to generate Kₐ and vₐ values, from which the dissociation constant Kₐ was calculated.

Recombinant protein expression and purification. The DNA encoding human GGTTase3 heterodimer (PTAR1–RabGGTB) and FBXL2–SKP1 were subcloned into pFastBac vectors for protein co-expression in High Five (Invitrogen) monolayer insect cells. A monolayer of recombinant pFastBac vectors with N-terminal fusion protein and the tetrameric complex of GGTTase3–FBXL2–SKP1 was isolated by glutathione affinity chromatography using buffer containing 20 mM Tris–HCl, pH 8.0, 150 mM NaCl and 1.0 mM DTT. The eluted tetrameric complex was further purified by anion exchange and gel filtration chromatography, after overnight tobacco etch virus protease cleavage in a final buffer containing 20 mM HEPEs pH 7.5, 150 mM NaCl and 2.0 mM DTT. The protein sample was concentrated and flash-frozen for crystalization. For testing the binding activity of FBXL2–SKP1 with GGTTase3 and its geranylgeranylation by GGTTase1, two individual protein complexes of FBXL2–SKP1 and GGTTase3 were expressed in E. coli with the affinity tags fused on PTAR1 and FBXL2, respectively. The proteins were isolated similarly to the tetrameric complex by three steps of affinity, anion exchange and gel filtration chromatography. The affinity tag may be left on the proteins for the purposes of assay.

Crystallization, data collection and structure determination. The crystals of the GGTTase3–FBXL2–SKP1 complex were obtained at room temperature by the hanging-drop vapor-diffusion method, using 1.0 μl protein sample mixed with an equal volume of reservoir solution containing 0.1 M MES pH 6.0 and 20–22% (v/v) PEG 400. Crystals appeared within 1–2 d and would complete growth in approximately 1 week. Conducting microseeding substantially increased the size of the crystals, and the best crystals diffracted to 2.5 Å resolution. Crystals were cryoprotected and the concentration of 20% (v/v) 20% glycerol in 0.1 M MES pH 6.0 and flash-frozen in liquid nitrogen for data collection. All data sets were collected at 100 K with a wavelength of 1.00 Å at the BL8.2.1 and BL8.2.2 beamlines at the Advanced Light Source of Lawrence Berkeley National Laboratory. Diffraction data were indexed, integrated and scaled with the HKL2000 package. The structure was determined by molecular replacement using Phaser in the CCP4 suite with models of RabGGTase (PDB: 3DSS) and SKP1–SKP2 complex (PDB: 1QYV) structures. The initial models were then rebuilt manually using COOT and refined by Refmac5 and PHENIX. The Ramachandran plot analysis of the final model showed that 96.4% and 3.6% of the residues are in favored and allowed regions, respectively. Complete data collection and refinement statistics are summarized in Table 1. All structure figures were rendered in PyMOL.

Occt biolayer interferometry measurement. Binding affinity of His-tagged FBXL2–SKP1 with GGTTase3 was measured using the Octet Red 96 (FortBio, Pall Life Sciences) following the manufacturer’s procedures in triplicate. The optical sensor were coated with Anti-Penta-His (HIS1K), loaded with 500 nM His-tagged FBXL2–SKP1 purified protein as ligand and quenched with 2.5 μM His-GST protein before kinetic binding analysis. The reactions were carried out in black 96-well plates maintained at 30 °C with a volume of 200 μl in each well. Binding buffer contained 25 mM HEPES pH 7.7, 150 mM NaCl, 2.0 mM DTT and 0.1% (v/v) BSA. Purified GGTTase3 as the analyte was serially diluted in the binding buffer to acquire complete measurement. No binding of the analyte to the unlaoded protein was observed. Binding kinetics of the analyte at all concentrations were obtained simultaneously using instrumental defaults. All data were analyzed with the Octet data acquisition software. The association and dissociation curves were globally fitted with 1:1 ligand model. The kak and kav values were used to calculate the dissociation constant Kd with direct binding analysis.

Cell lines. All cell lines were purchased from American Type Culture Collection and routinely tested for mycoplasma.

Live-cell imaging. HeLa cells incubated with various siRNA oligonucleotides were plated in dishes with a glass base (ThermoFisher Scientific, catalog no. 150682) in DMEM medium supplemented with 10% FBS overnight before transient transfection of GFPP-tagged cDNAs of indicated genes with Lipofectamine 3000 reagent. At 1-h post-transfection the cells were placed in fresh medium and incubated for 16 h at 37 °C and supplemented with 5% CO2. Live-cell imaging of cells was carried out with Zeiss LSM-510 META confocal microscopes using a 63x oil-immersion objective in the incubation chamber at 37 °C supplemented with 5% CO2. In each experiment, ten independent frames were captured. Images were captured and processed with the ZEN/ ZEN lite imaging software from Zeiss.

Antibodies, plasmids and chemicals. A rabbit polyclonal antibody against the peptide corresponding to amino acids 309 to 359 (CDGGLINSKDSQGFQKFRK) of human PTAR1 primary sequence was custom made by YenZyme Antibodies, LLC. The specificity of the antibody was validated by silencing PTAR1 in primary normal human fibroblasts and HEK-293T cell lines (Supplementary Fig. 1a). Antibodies were from Abcam (FNTA, FBXL2, RPS6K, GSK3B, AKT2, PAPR1, RPS6K), Abnova (PGGTB1), Aviva Biosystems (RabGGTA), Sigma (anti-FLAG M2, anti-FLAG, β-actin), Santa Cruz Biotechnology (SKP1) and Covance (anti-hemagglutinin (HA)). A polyclonal rabbit antibody to FBXL2 was generated using a peptide against FBXL2 as previously described. Human cDNA open reading frames for PTAR1, FNTA and RabGGTA were purchased from OriGene. GFP-tagged cDNA of Rab1a, Rab7a, Rab11, Rab34 and Rab35 were obtained from Addgene. The various tagged genes and mutants were generated in pEGFP and pEGFP1 vectors by subcloning or by site-directed mutagenesis methodologies, as described.

Immunoprecipitation and immunoblotting. RPE1–HTERT, HeLa and HEK-293T cells were transiently transfected using Lipofectamine 3000 reagent. At 16 h after transfection HEK-293T cells were incubated with MG132 for 3 h before collecting. Lysis of cell pellets was carried out with lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, glycerol 10%, 1 mM EDTA, 50 mM Na2, NP-40 0.1%) supplemented with protease and phosphatase inhibitors. Protein extraction in solubilised lysates was carried out with Bio-Rad DC protein assay according to the manufacturer’s protocol (catalog no. 650–080, Bio-Rad). Protein expression was measured by Coomassie blue staining, or with western blotting. Erbb4, GCLM, PTAR1 and RabGGTA were immunoprecipitated by using an anti-GFP antibody and elution with 1% SDS. The percent of the eluate was set aside and the remaining 90% was subjected to a second immunoprecipitation using an anti-FLAG antibody and elution with 1% SDS. The two eluates were then immunoblotted.

Gene silencing. Cells were grown in live-cell imaging compatible dish (ThermoFisher Scientific, catalog no. 150682) and 10-cm dish formats. ON-TARGET siRNA oligonucleotides from Dharmacon Inc. for various genes (PTAR1, PGGT1B, FNTA and Rab6) and the non-target control were used at 5 nM final concentration for 24–72 h using Lipofectamine RNAmax reagent, according to the manufacturer’s instruction (Invitrogen). ON-TARGET plus human PTAR1 siRNA (L-001311-01, LI-001311-01, L-001311-09-0005, L-001311-10-0002): GAAGCUAGGUAUAACCGGA, GAACAGGAGAUGAAUUGA.
CCAUAGUCCGGUUGAAAA, GGAGGAACCCACACAUAGA. ON-TARGETplus human FNTA siRNA (L-008807, LU-008807), ON-TARGETplus human RABGGTA siRNA (L-005097), ON-TARGETplus human PGGT1B (L-008703-00-0005) and ON-TARGET non-targeting siRNA #1 (D-001810-01-05).

Statistics and reproducibility. Data sets were analyzed by GraphPad Prism software with Student’s unpaired t-test and analysis of variance. Error bars represent either s.d. or s.e.m. as indicated in the figure legends. All immunoblot images and immunofluorescence images are representative of at least three independent experiments, except where specified in the figure legends.

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Data availability
Structural coordinates and structural factors have been deposited to the Protein Data Bank (PDB) under accession number 6O60. Source data for Fig. 2a,b and Supplementary Fig. 2b are available with the paper online. All other data are available from the authors upon request.

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#### Antibodies

- Antibodies used: Antibodies (commercially available) used in our investigations are appropriately cited in Methods, section “Antibodies, Plasmids, and Chemicals”.
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|---------------------|--------------------------------------|
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