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**Structure of the Wnt signaling enhancer LYPD6 and its interactions with the Wnt co-receptor LRP6**

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LYPD6 is a Wnt signaling enhancer that promotes phosphorylation of the Wnt co-receptor LRP6 (low-density lipoprotein receptor-related protein 6). It also binds the nicotinic acetylcholine receptor (nAChR). We report here the 1.25 Å resolution structure of the LYPD6 extracellular Ly6/uPAR (LU) domain and map its interaction with LRP6 by mutagenesis and surface plasmon resonance (SPR). The LYPD6<sub>LU</sub> structure reveals a “tri-fingered protein domain” fold with the middle fingertip of the bearing a “Nxl” motif, a tripeptide motif associated with LRP5/6 binding by Wnt inhibitors. Of the Ly6 protein family members, only LYPD6 has an Nxl motif. Since mutations in the LYPD6 Nxl motif abolish or severely reduce interaction with LRP6, our results indicate its key role in the interaction of LYPD6 with LRP6.

**Keywords:** LYPD6; LRP6 binding; Nxl motif;

**Abbreviations:**

Ly6/uPAR, lymphocyte antigen-6 (Ly6) or urokinase-type plasminogen activator receptor (uPAR); TFPD, tri-fingered protein domain; LRP6, low-density lipoprotein receptor-related protein 6; nAChR, nicotinic acetylcholine receptor; SPR, surface plasmon resonance

## Introduction

The Wnt signaling pathway is fundamental for animal embryonic development as well as adult tissue homeostasis and regeneration, conversely mis-regulation of Wnt signaling may contribute to diseases such as cancer or Alzheimer's disease<sup>1-3</sup>. To ensure Wnt signaling is finely tuned to the correct level at the right time and context, animals use a complex set of Wnt ligands, receptors, conductors, inhibitors and enhancers<sup>4</sup>. On the animal cell surface, the core canonical Wnt signaling complex consists of Wnt ligands and receptors of the Frizzled (FZD) and Low-density lipoprotein receptor-related protein 5/6 (LRP5/6) families. Many regulators modulate Wnt signaling through interaction with the core signaling ligands and receptors. Notum and Tiki enzymes modify the Wnt ligands<sup>5, 6</sup>; the secreted frizzled related proteins (SFRPs) and Wnt-inhibitory factor 1(WIF-1) prevent FZD receptors from binding to Wnts<sup>4, 7</sup>; the secreted Dickkopf proteins (DKKs), sclerostin (SOST) or sclerostin domain-containing 1 (SOSTDC1/WISE) antagonize Wnt signaling by binding to the LRP5/6 extracellular domains<sup>8</sup>. For the LRP5/6 binding Wnt inhibitors, a tripeptide asparagine-x-isoleucine motif, the Nxl motif, has been functionally implicated in receptor binding and inhibition<sup>8, 9</sup>. Structures of the LRP6<sub>PE1</sub> (the first beta propeller plus epidermal growth factor (EGF)-like module of LRP6) in complex with Nxl motif containing 7-mer peptide from DKK1 (PDB code 3SOQ) or SOST (PDB code 3SOV) have been determined<sup>8</sup>.

Recently, a lymphocyte antigen-6 and urokinase-type plasminogen activator receptor family member, Ly6/uPAR (LU) domain containing 6 (LYPD6), has been identified as a Wnt/ $\beta$ catenin signaling enhancer that acts by extracellular binding to LRP6

promoting LRP6 phosphorylation<sup>10</sup>. LYPD6, and a closely related LU domain protein, LYPD6B (also named LYPD7) have also been reported to act as modulators of the nicotinic acetylcholine receptor (nAChR)<sup>11 12</sup>. The LYPD6<sub>LU</sub> is tethered to the cell surface by a GPI anchor. The ectodomain of LRP6 comprises four repeats of  $\beta$  propeller domain plus epidermal growth factor (EGF)-like module (PE1-PE4), followed by three LDLR type A domains leading to the single transmembrane helix and cytoplasmic region. Here we report details of the molecular interaction between LYPD6 and LRP6 revealed by the crystal structure of LYPD6<sub>LU</sub> at 1.25 Å resolution and structure based mutagenesis combined with *in vitro* binding studies of LYPD6<sub>LU</sub> to the LRP6<sub>PE1PE2</sub>, LRP6<sub>PE3PE4</sub> segments of the ectodomain of LRP6 (LRP6<sub>ECD</sub>).

## Materials and methods

### Protein production and crystallization

Human LYPD6 (UniProtKB/Swiss-Prot Q86Y78), residues G46–A141 was PCR amplified from the cDNA (Source Bioscience, Nottingham UK; clone ID 5171270) and cloned into a stable cell line vector pURD<sup>13</sup> with 3C and monoVenus fusion tag ended with 6xHis. The construct results in processed protein of sequence ET-G<sup>46</sup>...(LYPD6<sub>LU</sub>)...A<sup>141</sup>-GTLEVLFQ (the underlined sequences are from the cloning vector). HEK293S GnTI(–) cells<sup>14</sup> were co-transfected with a pURD-LYPD6<sub>LU</sub> and a PhiC31 integrase expression vector (pCB92/pgk- $\Phi$ C31)<sup>15</sup>. The polyclonal population cells from puromycin (2  $\mu$ g·mL<sup>–1</sup>) selection were cultured in Hyperflasks (Corning) in a Compact automated cell culture system<sup>16</sup>. The secreted proteins were captured by Talon Co<sup>2+</sup> affinity resin (Takara Bio Europe SAS) after the conditional media was

dialyzed against PBS. The protein bound Talon beads were washed with 20 mM imidazole and eluted with 300 mM imidazole. The eluted protein was treated with 3C protease to remove the monoVenus-His tag and then deglycosylated using EndoF1 (HEK293S GnTI<sup>-</sup> cells generate mannose-rich glycans at N-linked glycosylation sites which are cleavable by EndoF1 to leave one NAG covalently attached to the asparagine). After reverse tag capture with Talon beads, the protein was subjected to Superdex 200 16/60 column size exclusion chromatography and concentrated to 5 mg·mL<sup>-1</sup> in 10 mM Hepes pH 7.4, 150 mM NaCl buffer. Crystallization screening was carried out using the sitting-drop vapour diffusion method in 96-well plates at 22°C<sup>17</sup>. Octahedron shaped crystals with size approximately 80 µm grew in 0.1 M Tris-HCl, pH8.5 and 2.0 M Ammonium Sulphate.

The LRP6<sub>PE1PE2</sub> (UniProtKB/Swiss-Prot O75581, residues 1-630) and LRP6<sub>PE3PE4</sub> (residues 629-1244)<sup>18</sup> segments of the LRP6 ectodomain were cloned into the pNeo-sec vector<sup>19</sup> and the proteins were produced similarly from G418 (1 mg·mL<sup>-1</sup>) selected polyclonal stable cell lines.

### **Data collection and structure determination**

Crystals were flash frozen by immersion in a reservoir solution supplemented with 25% (v/v) glycerol followed by transfer to liquid nitrogen. Data sets were recorded from crystals at 100K at Diamond Light Source with Pilatus 6M detector (Didcot UK, beamline I04 for Sulphur-SAD and I03 for native) and processed with either HKL2000 or Xia2 (ref<sup>20,21</sup>). Sulphur-SAD datasets were collected at wavelength 1.70

Å using inverse beam mode and merged with Xia2. HYSS was used to locate the sulphur sites which were then input to PHENIX AUTOSOL<sup>22</sup> for structure determination. The protein model was initially built with PHENIX AutoBuild<sup>23</sup>, further manually built with COOT<sup>24</sup> and finally refined with REFMAC<sup>25</sup>. The PyMOL Molecular Graphics System (Schrödinger, LLC.) was used to prepare the figures.

### SPR equilibrium binding studies

The LYPD6<sub>LU</sub> (G46-A141) and its mutants (generated from two-step overlapping PCR) were cloned in a pHL-Avi3 vector<sup>26</sup>. The resulting protein has C-terminal Avi-His tag of sequence GTGGSGGSGGLNDIFEAQIEWHEGRTKHHHHHH where the underlined sequence is the biotin ligase recognition site. This puts the expected LRP6 interaction site (Nxl loop) at the opposite side of the attachment site so that no or little steric hindrance is expected. To create a LYPD6<sub>LU</sub> chimera with SOST Nxl loop, LYPD6<sub>LU</sub> residues 85-VTGNSI-90 were replaced with SOST residues 114-LLPNAI-119. Human LYPD6B (UniProtKB/Swiss-Prot Q8NI32, LYPD6B<sub>LU</sub> residues K40-S164) was cloned into the pHL-Avi3 vector. The LYPD6B<sub>LU</sub> mutant, LYPD6B<sub>Nxl</sub> contains mutations R101N and T103I. To produce biotinylated proteins, the pHL-avi3 tagged plasmids were co-transfected with the pDisplay\_BirA-ER plasmid<sup>27</sup> into HEK293T cells with the media supplemented with 20 µM biotin. The procedure allows *in vivo* biotinylation to occur<sup>27</sup>, and the dialyzed conditional media was directly used for immobilization of ligands. The affinity between LYPD6<sub>LU</sub> or its mutants or LYPD6B<sub>LU</sub> and LRP6<sub>PE1PE2</sub> or LRP6<sub>PE3PE4</sub> was measured at 25 °C in 10 mM HEPES, pH 7.4, 150 mM NaCl and 0.005% Tween20 using a Biacore T100 machine (GE Healthcare). The biotinylated ligands were coupled to a streptavidin-

coated sensor chip, and the purified LRP6<sub>PE1PE2</sub> or LRP6<sub>PE3PE4</sub> proteins in 2-fold serial dilution (from 10  $\mu$ M to 5 nM) were used as analytes to flow through different ligand immobilized flow cells. The response was plotted versus the concentration of the analytes and fitted by nonlinear regression to a one-site saturation binding model with equation  $f = B_{max} \cdot \text{abs}(x) / (K_d + \text{abs}(x))$  (Sigma Plot, Systat software, Inc.)

## Results and Discussion

### The LYPD6<sub>LU</sub> domain shows a tri-fingered protein fold

Human LYPD6 contains amino acid residues A23-L171 (residues M1-A22 constitute the signal peptide that is cleaved after maturation) and comprises only one LU domain and a C-terminal signal sequence for GPI anchorage (Fig.1A). To produce soluble protein suitable for crystallization, several constructs were tested and the construct of LYPD6 residues G46-A141 (LYPD6<sub>LU</sub>) was found to yield 0.5 mg·L<sup>-1</sup> of secreted, stable protein from HEK293S(GNTI-) cells. The purified deglycosylated LYPD6<sub>LU</sub> protein was crystallized in a solution containing ammonium sulphate. The best crystal diffracted to high resolution (1.25 Å). The structure was determined using the sulfur SAD method and has been refined to an R-factor and stereochemistry consistent with the high resolution of the data (Table1). There is one molecule in an asymmetric unit. The final structure contains all residues G46-A141 (Fig.1A) from the construct as well as amino acids “ET” from the vector N-terminal cloning site and “GT” from the C-terminal cloning site and “LEVL” from the 3C protease cleavage site. All residues at the termini and in the loops are visible in the electron density. The overall flat hand shaped LYPD6<sub>LU</sub> domain broadly conforms to

the classical tri-fingered protein domain (TFPD) fold (Fig.1A). However, unlike all the known snake toxin TFPD fold containing proteins (as listed in ref<sup>28</sup>), the first finger of LYPD6<sub>LU</sub> is composed of an  $\alpha$ helix ( $\alpha 1$ ) and a  $\beta$ -strand ( $\beta 1$ ), instead of two  $\beta$ strands. Finger 2 comprises a pair of anti-parallel  $\beta$ strands ( $\beta 2$ ,  $\beta 3$ ) and finger 3 is composed of a short  $\alpha$ helix ( $\alpha 2$ ) and a pair of anti-parallel  $\beta$ strands ( $\beta 4$ ,  $\beta 5$ ). In addition to the basal 3 fingers, residues E123 to A141 form a long loop which shelters one side of the “palm”. There are six disulfide bonds in total. Four of them (C49-C77, C70-C96, C102-C121 and C122-C127) form the tight network at the base of the palm commonly found in the conventional neurotoxin type TFPD fold<sup>29, 30</sup>. One additional disulfide bond (C51-C61) links  $\alpha 1$  and  $\beta 1$  of finger 1 in LYPD6<sub>LU</sub>; this bond is only seen linking the secondary structure elements (two  $\beta$  strands) of finger 1 in non-conventional toxins (for example weak toxin and cardiotoxin) and the Ly6 family of proteins, such as CD59, lynx1 and SLURP1<sup>29, 30</sup>. The remaining disulfide bond in LYPD6<sub>LU</sub> (C107-C118), which links the two  $\beta$ strands ( $\beta 4$ ,  $\beta 5$ ) of finger 3, is distinctive; sequence analysis indicates it exists only in LYPD6, LYPD6B and LYPD1<sup>30</sup>.

LYPD6 is a glycoprotein with two predicted N-linked glycosylation sites at N134 and N147. N147 was not included in our construct. The protein was produced in HEK293S(GNTI-) cells and treated with Endo F1; as expected from the Endo F1 cleavage reaction, only one GlcNAc is seen attached on N134 in the crystal structure (Fig.1A) with weak but unambiguous density.



## Structural comparison of LYPD6<sub>LU</sub> to other members of the Ly family

The LYPD6<sub>LU</sub> structure reveals a three fingered protein fold which is topologically similar to some snake venom neurotoxins, for example Irditoxin from *Boiga irregularis* (PDB code: 2H7Z, 25% sequence identity, 67 C $\alpha$  aligned at rmsd 3.1 Å)<sup>31</sup>, weak toxin (WTX) from *Naja kaouthia* (PDB code: 2MJ0, 16% sequence identity, 64 C $\alpha$  aligned at rmsd 2.5 Å) and  $\beta$  Cardiotoxin from *Ophiophagus hannah* (PDB code: 3PLC, 26% sequence identity, 58 C $\alpha$  aligned at rmsd 2.4 Å). This proto-toxin (inactive, non-toxic precursor toxin) sub-group of tri-fingered proteins exists in a variety of organisms, from *Drosophila* to human. A Dali server<sup>32</sup> based search for related structures identified Bucandin (PDB code:1F94), a presynaptic snake neurotoxin from *Bungarus candidus* (Malayan krait)<sup>33</sup>, as closely related to LYPD6<sub>LU</sub> (rmsd of 2.0 Å for 59 equivalent C $\alpha$  atoms with sequence identity of 10.7%). The structural superposition of LYPD6<sub>LU</sub> with Bucandin is shown in Fig.1C.

In the human genome, at least 45 gene products contain the Tri-fingered protein domain<sup>34</sup> and among these 36 belong to the ly6/uPAR family<sup>29</sup>. Structure alignment with PDBeFold<sup>35</sup> identified that the transforming growth factor beta type II receptor (T $\beta$ RII) ligand binding domain (PDB code 1KS6)<sup>36</sup> is the closest structural homolog of LYPD6<sub>LU</sub>. Although the sequence identity is only 14%, the two structures can be superimposed with 76 equivalent C $\alpha$  atoms and a rmsd of 2.2 Å (Fig.1D). And the T $\beta$ RII, together with Act-RIIA and BMP-RAI (PDB code 1BTE and 1REW respectively) have an  $\alpha$ helix in their finger1. Both DALI and PDBeFold found similar hits, and 1F94 and 1KS6 are listed as the top hits in the two search engines.

## The tip of LYPD6<sub>LU</sub> finger 2 presents an exposed NxI motif

The crystal structure of LYPD6<sub>LU</sub> reveals the distinctive, tri-peptide, NxI motif exposed at the tip of finger 2 of the TFPD fold (Fig.1B). The NxI motif has been identified as a LRP5/6 (specifically LRP5/6<sub>PE1PE2</sub>) binding motif required for function in the Wnt secreted inhibitors, SOST, WISE, and DKK<sup>8, 9</sup>. The LYPD6<sub>LU</sub> NxI bearing surface area is a negatively charged stripe surrounded by positively charged patches (Fig. 2A). The potential binding surface of LRP6<sub>PE1</sub> for the NxI motif comprises a deep hydrophobic pocket for the isoleucine and a shallower, slightly positively charged pocket accommodating the asparagine. The two pockets are surrounded by an extended region of very strong negative charges on one side, and a stripe of positive charges on the other side (Fig. 4A). LYPD6 exists in many species of vertebrate including humans, but not in the invertebrates *Drosophila* or *Caenorhabditis elegans*. The NxI motif is conserved for mammalian LYPD6 sequences (Fig. 2B, 2C), but not for amphibians. In the zebrafish, the NxI motif isoleucine is replaced by a similar hydrophobic residue, valine. The zebrafish NxV motif may still be able to bind the LRP6<sub>PE1PE2</sub>, as an anti LRP6<sub>PE1</sub> antibody (YW210.09) bearing a NxV motif has been reported to bind at the same LRP6<sub>PE1</sub> site as the canonical NxI motif (PDB code: 3SOB)<sup>8</sup>. Consistent with this hypothesis zebrafish LYPD6 has been reported to immunoprecipitate with LRP6 and function as a Wnt signaling enhancer not only in zebrafish, but also in *Xenopus*<sup>10</sup>. Bourhis *et al* reported that mutation of the asparagine to alanine in the NxI motif abolishes binding<sup>8</sup>, however, it remains to be seen if the *Xenopus* LYPD6 (which contains an ExV motif) can also bind to LRP6 and function as a Wnt signaling enhancer.

## The LYPD6 Nxl motif is essential for binding to LRP6<sub>PE1PE2</sub>

We investigated the Nxl motif's role in the interaction of LYPD6<sub>LU</sub> and LRP6<sub>PE1PE2</sub> or LRP6<sub>PE3PE4</sub> by using SPR to assay for binding. Biotinylated LYPD6<sub>LU</sub> domain protein immobilized onto a streptavidin coated CM5 chip showed no measurable interaction with LRP6<sub>PE3PE4</sub> analyte (Fig. 3A). In contrast, LRP6<sub>PE1PE2</sub> bound to LYPD6<sub>LU</sub> with a  $K_d$  of 0.79  $\mu$ M (Fig. 3A). LYPD6 belongs to a large family of LU domain proteins including LYPD 1-11, however, only LYPD6 has a Nxl motif. LYPD6B has 55% sequence identity and 62% similarity to LYPD6, both are reported to modulate nAChR function<sup>11 12</sup>, but similar to the other family members, LYPD6B lacks the Nxl motif. Instead, LYPD6B has the sequence "RxT", of which the arginine, in particular, is sterically incompatible with Nxl-type binding. Indeed we found no measureable binding between immobilized LYPD6B<sub>LU</sub> and LRP6<sub>PE1PE2</sub> analyte (Fig. 3A). However, a mutated LYPD6B<sub>LU</sub> (LYPD6B<sub>Nxl</sub>), in which the "RxT" is replaced by the Nxl motif (Table 2), gained some binding to LRP6<sub>PE1PE2</sub>, albeit weakly with a  $K_d$  of 31  $\mu$ M (Fig. 3A).

We proceeded to probe the contribution of the motif residues to binding. Mutations of the signature Nxl motif residues impacted on the ability of LYPD6<sub>LU</sub> to bind LRP6<sub>PE1PE2</sub>; LYPD6<sub>LU</sub> mutation N88A abolishes binding and I90E severely reduces binding to a  $K_d$  of 19  $\mu$ M (Fig. 3B, Table 2). These results are similar to those previously reported for the equivalent mutations of the Nxl motif in DKK1 and SOST<sup>8</sup>. Interestingly, the relative severity of the effect for the two mutations is reversed in LYPD6<sub>LU</sub> compared to DKK1 and SOST, for both of which the isoleucine to glutamic acid substitution had the bigger effect on binding than the mutation of asparagine to

alanine<sup>8</sup>. In contrast, mutation E111R, a prominently exposed residue chosen because it is located outside the Nxl motif on a different finger (finger 3), does not affect the interaction (Fig. 3B). These SPR data suggest that the Nxl motif is crucial for the interaction of LYPD6<sub>LU</sub> with the LRP6<sub>PE1PE2</sub> receptor, specifically the N-terminal LRP6<sub>PE1PE2</sub> segment, a characteristic resembling that of the secreted Wnt inhibitor SOST, which uses its Nxl motif to bind LRP6<sub>PE1PE2</sub>, but not LRP6<sub>PE3PE4</sub> (ref<sup>8,9</sup>).

In addition to the asparagine and isoleucine residues, the sequence surrounding the Nxl motif has also been suggested to contribute to LRP6 binding in Wnt antagonists<sup>8</sup>. This conclusion is consistent with our finding that the creation of a Nxl motif in the LYPD6<sub>LU</sub> mutant R101N\_T103I generates much weaker binding to LRP6<sub>PE1PE2</sub> than that we observe for LYPD6<sub>LU</sub>. We therefore next asked whether the Nxl motif containing loop of LYPD6<sub>LU</sub> could be replaced with the corresponding loop of SOST. In LYPD6<sub>LU</sub>, the finger 2 tip sequence is 84-EVTGNSI-90. We replaced VTGNSI six amino acids with the SOST loop sequence 114-LLPNAI-119 to generate a chimera (Table 2). SPR binding assays showed the chimera is able to interact well with LRP6<sub>PE1PE2</sub>, with  $K_d$  0.89  $\mu$ M, which is close to the level of binding shown by wild type LYPD6 (Fig. 3B, Table 2).

As both LYPD6<sub>LU</sub> and SOST use the Nxl motif to bind LRP6<sub>PE1PE2</sub>, we superposed the Nxl motif of LYPD6<sub>LU</sub> with the equivalent region from the SOST peptide\_LRP6<sub>PE1</sub> complex structure (PDB code 3SOV)<sup>8</sup> and the SOST apo structure (PDB code 2K8P)<sup>37</sup> (Fig. 4A-C). The conformation of the Nxl motif of LYPD6<sub>LU</sub> is similar to that

of the apo SOST which is structurally different to the LRP6 bound SOST peptide. If the C $\alpha$ s of the Nxl of LYPD6<sub>LU</sub> are aligned with those of the bound SOST peptide, the asparagine side chains have a similar orientation, but the orientations of the isoleucine side chains differ by ~100° (note the isoleucine, I90, in our LYPD6<sub>LU</sub> crystal structure is not involved in lattice contacts and has well-ordered electron density). If N88 and I90 of the Nxl motif in our LYPD6<sub>LU</sub> structure are docked into their putative binding pockets in LRP6<sub>PE1</sub>, finger 1 and finger 3 of LYPD6 clash with LRP6<sub>PE1</sub>. Similarly, the SOST apo structure also clashes with the LRP6<sub>PE1</sub> (Fig. 4A, 4B) if N117 and I119 are simply docked into the binding pockets on LRP6<sub>PE1</sub>. The experimentally observed difference in the conformation of the Nxl motif for apo SOST and LRP6 bound SOST peptide, alongside the steric clashes apparent in complexes modelled by simple docking of apo structures, suggest conformational changes of the Nxl motif are required for LRP6 binding. However, detailed analysis of the interactions between LYPD6 (or SOST) and LRP6 will require the structure(s) of the complex(es). It is noteworthy that although LYPD6 and SOST/DKK bind the same site on LRP6, LYPD6 enhances Wnt signaling and SOST/DKK inhibit Wnt signaling (Fig. 4D, 4E). SOST and DKK are secreted proteins, while LYPD6 is GPI anchored and has been reported to be preferentially localized to lipid rafts in the membrane<sup>10</sup>. The differences in the context in which these ligands bind LRP6 may contribute to the divergence in their effects on Wnt signaling.

### **Possible interaction area with nAChRs**

LYPD6 has been shown to be able to bind multiple subtypes of nAChR<sup>38</sup>, including the  $\alpha 7$  subtype, which was previously reported not able to bind LYPD6 (Ref <sup>11</sup>). Binding of LYPD6 to nAChRs results in attenuated nicotine-induced hippocampal

inward currents<sup>38</sup>. The structure of LYPD6, or other TFPDs, in complex with nAChRs is not available. However, the structure of human  $\alpha 7$  AChR chimeric with acetylcholine binding protein (AChBP) in complex with the TFPD family member  $\alpha$  bungarotoxin ( $\alpha$ btx) has been reported (PDB code: 4HQP)<sup>39</sup>.  $\alpha$ btx uses its finger 2 tip to interact with  $\alpha 7$  nAChR, and can compete with LYPD6 for binding<sup>38</sup>. However, unlike  $\alpha$ btx, the LYPD6 finger 2 loop does not form an  $\alpha$ helix, and lacks the phenylalanine and arginine residues which  $\alpha$ btx uses to interact with  $\alpha 7$  nAChR. Since both LYPD6 and LYPD6B interact with nAChRs, and their LU domains have amino acid identity of 45% ( Fig. 5A), we modelled the LYPD6<sub>LU</sub> structure with SWISS-MODEL<sup>40</sup> using LYPD6<sub>LU</sub> as template, and looked for sequence conservation of residues exposed on the three fingers of the LYPD6<sub>LU</sub> structure. While most of the prominent surface residues on finger 2 and finger 3 of LYPD6<sub>LU</sub> are not conserved in LYPD6B<sub>LU</sub> (Fig. 5A), residues D57, Y57, R63 and W64 in the distinctive finger 1 helix of LYPD6 are exposed and conserved in LYPD6B (equivalent residues, D70, Y72, R77, W78, Fig. 5B). We then modelled the  $\alpha 7$  nAChR-LYPD6<sub>LU</sub> complex (Fig.4C), with HADDOCK2.2 webserver<sup>43</sup>. The top HADDOCK scored (-83.3 +/- 8.5, arbitrary units) model is shown in Fig. 5C. The model has Van der Waals energy -41.7 +/- 4.2 kcal/mol and electrostatic energy -104.4 +/- 14.4 kcal/mol. The rmsd from the overall lowest-energy structure is 0.9 +/- 0.6 Å. All 4 residues (Fig. 5B) conserved between LYPD6<sub>LU</sub> and LYPD6B<sub>LU</sub> in finger 1 are involved in interactions in the modelled complex. We therefore speculate that these residues may be involved in the interaction of LYPD6 or LYPD6B with nAChRs.

## Conclusion

The high-resolution crystal structure of the LYPD6<sub>LU</sub> shows a tri-fingered protein fold with a distinctive  $\alpha$ helix in finger 1 and a Nxl motif prominently displayed at the tip of finger 2. Among members of the large Ly6/uPAR family, only LYPD6 possesses the Nxl motif, which is crucial for the LRP6<sub>PE1PE2</sub> binding activity. SPR binding assays reveal LYPD6<sub>LU</sub> can interact with LRP6<sub>PE1PE2</sub> but not LRP6<sub>PE3PE4</sub>. The closely related family member LYPD6B<sub>LU</sub> does not interact with LRP6<sub>PE1PE2</sub>, but on mutation to generate a Nxl motif gains binding capability to LRP6<sub>PE1PE2</sub>. Mutation of the Nxl motif of LYPD6<sub>LU</sub> abolishes or severely reduces the LRP6<sub>PE1PE2</sub> interaction. When the LYPD6<sub>LU</sub> finger 2 loop is replaced by the Nxl containing loop of SOST, the chimera can still interact with LRP6<sub>PE1PE2</sub>. In combination, the results from our structure guided mutagenesis and SPR binding assays reveal that the Nxl motif plays a key role in the interaction of LYPD6 with LRP6. Hereto, Nxl motif mediated binding to the LRP6<sub>PE1PE2</sub> segment of the LRP6 ectodomain has been characteristic of secreted antagonists of Wnt signaling, such as SOST, DKK and WISE. It is notable that Nxl mediated binding by the GPI-anchored LYPD6 receptor results in enhancement in Wnt signaling<sup>10</sup>. This is an intriguing divergence in outcome from a structurally similar interaction. Further investigation is required to provide insight into the factors and mechanisms that determine the balance between the enhancer and antagonist functions of Nxl- mediated binding in Wnt signaling.

## Figure legends

**Fig. 1.** Cartoon presentation of the human LYPD6 structure and alignments.

(A) Cartoon presentation of human LYPD6<sub>LU</sub> domain, colored as rainbow, from blue (N-terminal) to red (C-terminal). One glycosylation site is shown in grey sticks. Disulfide bonds are shown in yellow sticks. The GPI anchor and membrane are shown diagrammatically. (B) shows Nxl motif residues N88 and I90 (in grey sticks). (C) The alignment of LYPD6<sub>LU</sub> (cyan) with the Bucandin (grey) snake toxin (PDB code:1F94). (D) The alignment of LYPD6<sub>LU</sub> (cyan) with the transforming growth factor beta type II receptor (T $\beta$ RII) ligand binding domain in purple color (PDB code 1KS6).

**Fig. 2.** Surface features of LYPD6<sub>LU</sub>. (A) Electrostatic properties of LYPD6<sub>LU</sub>. The protein surface is colored by electrostatic potential at  $\pm 5$  kT/e (red, acidic; blue, basic). (B) The surface of LYPD6<sub>LU</sub> is colored by residue conservation (conserved, magenta; variable, cyan; surface generated by Consurf<sup>41</sup>). Sequences of 7 members of the LYPD6 family (chosen from representative species which are distributed across the phylogenic tree) were included in the sequence conservation analysis. They are: *Hs*, Human (*Homo sapiens* UniProtKB: Q86Y78); *Pt*, Chimpanzee (*Pan troglodytes*, UniProtKB: H2QIT5); *Mm*, Mouse (*Mus musculus*, UniProtKB:Q8BPP5); *MI*, Little brown bat (*Myotis lucifugus* UniProtKB:G1PID1); *Ac*, American chameleon, green anole (*Anolis carolinensis* UniProtKB:H9G7P9); *Dr*, Zebrafish (*Danio rerio*, UniProtKB:Q66IA6); *Xl*, African clawed frog (*Xenopus laevis*, UniProtKB: T2BD45). (C) The sequence alignment of the LYPD6 finger 2 loop for the above species.



**Fig. 3.** SPR analysis of the LYPD6<sub>LU</sub> and LRP6<sub>PE1PE2</sub> or LRP6<sub>PE3PE4</sub> interaction. (A) Biotinylated constructs of the human LYPD6<sub>LU</sub>, LYPD6B<sub>LU</sub> and LYPD6B<sub>Nxl</sub> were immobilized on individual flow cells of a CM5 chip, respectively. The LRP6<sub>PE1PE2</sub> (LRP6\_12) or LRP6<sub>PE3PE4</sub> (LRP6\_34) was used as analyte respectively. (B) The LYPD6<sub>LU</sub> mutants N88A, I90E, E111R and the LYPD6<sub>LU</sub> finger 2 loop Nxl bearing six amino acids replaced with the corresponding SOST loop sequence (Finger2\_loop\_SOST) were used as ligand. LRP6<sub>PE1PE2</sub> was used as analyte. The SPR sensorgrams are shown at the right panel. ND, not detectable; RU, resonance units.

**Fig. 4.** Predicted interactions between LYPD6 and LRP6. (A) The LYPD6<sub>LU</sub> (cyan ribbon) and apo SOST (grey ribbon, PDB code 2K8P)<sup>37</sup> are docked on the surface of LRP6<sub>PE1</sub> by overlapping C<sub>α</sub> atoms of the Nxl motif with the corresponding atoms of the SOST peptide\_LRP6<sub>PE1</sub> complex (PDB code 3SOV)<sup>8</sup>. LRP6<sub>PE1</sub> is shown as electrostatic surface and the bound SOST peptide as purple sticks. (B) Close up views of the Nxl binding pocket and surrounding area. Black arrows indicate clashes of LYPD6<sub>LU</sub> and the apo SOST with LRP6<sub>PE1</sub>. (C) Overlaid Nxl motifs of the three structures showing the conformational differences. (D), (E) Cartoon diagrams showing proposed binding modes of LRP6-LYPD6 (D) and LRP6-SOST or DKK (E).

**Fig. 5.** Structure similarity and modelling. (A) Sequence alignment of LYPD6<sub>LU</sub> and LYPD6B<sub>LU</sub> with the secondary structure of LYPD6<sub>LU</sub> indicated on top and solvent accessibility (acc) at bottom (blue, accessible; cyan, intermediate; white, buried). (B) Comparison of the structures of LYPD6<sub>LU</sub> (cyan) and the modelled LYPD6B<sub>LU</sub>

(magenta). The surface exposed residues in finger 1 common to both proteins are shown as sticks. (C) Superposition of a putative LYPD6- $\alpha 7$ -nAChR complex obtained by docking and the experimentally determined  $\alpha$ Bgtx-  $\alpha 7$ -nAChR complex (PDB code 4HQP<sup>39</sup>).  $\alpha 7$ -nAChR is shown in grey, LYPD6 in cyan and  $\alpha$ Bgtx in yellow.

### Accession codes

Coordinates and structure factors have been deposited in the Protein Data Bank under accession code 6GBI.

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**Table 1 Data collection and refinement statistics**

<b>Data collection</b>		
Data set	S-SAD	Native
Wavelength (Å)	1.70	0.979
Space group	$P4_1 2_1 2$	$P4_1 2_1 2$
Unit Cell dimensions (Å)	$a=61.94, b=61.94, c=68.93; \alpha=\beta=\gamma=90^\circ$	$a=61.98, b=61.98, c=68.55; \alpha=\beta=\gamma=90^\circ$
Resolution (Å)	61.92-1.99 (2.05-1.99)	45.97-1.25 (1.28-1.25)
Unique reflections	8742 (144)	37608 (516)
$R_{\text{merge}}$	0.069 (0.280)	0.023 (0.629)
$\langle I \rangle / \langle \sigma I \rangle$	63.2 (9.5)	31.2 (3.7)
Completeness (%)	99.9 (90.7)	100 (99.9)
Redundancy	71.2 (7.7)	12.3 (10.2)
<b>Phasing</b>		
Resolution (Å)	61.92-2.60	
No. of sites	11	
BAYES-CC (%)	$58 \pm 14$	
FOM	0.49	
<b>Refinement</b>		
Resolution (Å)		45.97-1.25
No. reflections		37544
$R_{\text{work}} / R_{\text{free}}$		0.162/0.165
No. atoms		1913
Average $B$ -factor (Å <sup>2</sup> )		23
<b>R.m.s. deviations</b>		
Bond lengths (Å)		0.005
Bond angles (°)		1.164

Numbers in brackets refer to the highest resolution shell of data

**Table 2 Summary of Nxl motif containing proteins' affinities to LRP6<sub>PE1PE2</sub>**

Name	Sequence	Affinity
<b>LYPD6</b>	<sup>83</sup> MEVTG <b>NS</b> ISVTKRCVP <sup>98</sup>	$K_d=0.79 \mu\text{M}$
<b>LYPD6B</b>	<sup>96</sup> FTSHG <b>RST</b> SITKKCA <sup>110</sup>	Not detectable
<b>LYPD6B<sub>Nxl</sub></b>	<sup>96</sup> FTSHG <b>NS</b> ISITKKCA <sup>110</sup>	$K_d=31 \mu\text{M}$
<b>LYPD6_SOST</b>	<sup>83</sup> FT <b>LLPN</b> AI S I T K R C V P <sup>98</sup>	$K_d=0.89 \mu\text{M}$
<b>LYPD6_E111R</b>	<sup>83</sup> MEVTG <b>NS</b> ISVTKRCVP <sup>98</sup> ... <sup>107</sup> CRDS <b>R</b> HEGH <sup>115</sup>	$K_d=0.46 \mu\text{M}$
<b>LYPD6_I90E</b>	<sup>83</sup> MEVTG <b>NE</b> SVTKRCVP <sup>98</sup>	$K_d=19 \mu\text{M}$
<b>LYPD6_N88A</b>	<sup>83</sup> MEVTG <b>AS</b> ISVTKRCVP <sup>98</sup>	Not detectable
<b>DKK1</b>	<sup>35</sup> SVLNS <b>NA</b> IKNLPPPLG <sup>50</sup>	$K_d=0.06 \mu\text{M}^{42}$ or $\text{IC}_{50}=6.3 \mu\text{M}^8$
<b>SOST</b>	<sup>113</sup> RLLP <b>NA</b> IGRGKWWRP <sup>127</sup>	$\text{IC}_{50}=21 \mu\text{M}^8$
<b>WISE</b>	<sup>108</sup> PVLP <b>NW</b> IGGGYGTKY <sup>122</sup>	$\text{IC}_{50}=32 \mu\text{M}^8$

The mutated residues are indicated in red color. The visible residues in the X-ray complex structures of DKK1 and SOST are underlined.











