Structure and functional properties of Norrin mimic Wnt for signalling with Frizzled4, Lrp5/6, and proteoglycan

Tao-Hsin Chang, Fu-Lien Hsieh†, Matthias Zebisch‡, Karl Harlos, Jonathan Elegheert, E Yvonne Jones*

Division of Structural Biology, Wellcome Trust Centre for Human Genetics, University of Oxford, Oxford, United Kingdom

Abstract

Wnt signalling regulates multiple processes including angiogenesis, inflammation, and tumorigenesis. Norrin (Norrie Disease Protein) is a cystine-knot like growth factor. Although unrelated to Wnt, Norrin activates the Wnt/β-catenin pathway. Signal complex formation involves Frizzled4 (Fz4), low-density lipoprotein receptor related protein 5/6 (Lrp5/6), Tetraspanin-12 and glycosaminoglycans (GAGs). Here, we report crystallographic and small-angle X-ray scattering analyses of Norrin in complex with Fz4 cysteine-rich domain (Fz4CRD), of this complex bound with GAG analogues, and of unliganded Norrin and Fz4CRD. Our structural, biophysical and cellular data, map Fz4 and putative Lrp5/6 binding sites to distinct patches on Norrin, and reveal a GAG binding site spanning Norrin and Fz4CRD. These results explain numerous disease-associated mutations. Comparison with the *Xenopus* Wnt8–mouse Fz8CRD complex reveals Norrin mimics Wnt for Frizzled recognition. The production and characterization of wild-type and mutant Norrins reported here open new avenues for the development of therapeutics to combat abnormal Norrin/Wnt signalling.

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Introduction

Wnt morphogens, secreted cysteine-rich palmitoleoylated glycoproteins, play critical roles in cell-fate determination, tissue homeostasis and embryonic development (Clevers and Nusse, 2012; Malinauskas and Jones, 2014). Aberrant Wnt signalling leads to cancer, osteoporosis and degenerative illnesses (Anastas and Moon, 2013). Norrie Disease Protein (NDP) gene encodes Norrin (Berger et al., 1992; Chen et al., 1992), a secreted cystine-knot like growth factor, distinct from the lipid-modified Wnt (Willert et al., 2003). Norrin activates the canonical Wnt/β-catenin pathway by interaction with Wnt receptor Frizzled4 cysteine-rich domain (Fz4CRD), and co-receptor low density lipoprotein receptor related protein 5/6 ectodomain (Lrp5/6ECD), plus the auxiliary four-pass transmembrane protein Tetraspanin-12 (Tspan-12) and glycosaminoglycans (GAGs) of heparan sulfate proteoglycans (HSPGs) (Xu et al., 2004; Junge et al., 2009; Ke et al., 2013).

The Norrin mediated pathway maintains the blood-retina and blood-brain barriers (Wang et al., 2012) and regulates angiogenesis in the cochlea and uterus (Rehm et al., 2002; Ye et al., 2011) as well as neuroprotective effects on retinal neurons (Ohlmann et al., 2010; Seitz et al., 2010). Mutations in the NDP gene and the receptor genes, FZ4, LRP5, and TSPAN-12, have been identified for vitreoretinal diseases including Norrie Disease, Familial Exudative Vitreoretinopathy, and Coats’ Disease (Nikopoulos et al., 2010; Ye et al., 2010; Ohlmann and Tamm, 2012). NDP, FZ4, LRP5, and TSPAN-12 knock-out mice experiments further support the notion that dysfunctional Norrin signalling results in impaired retinal angiogenesis (Richter et al., 1998; Kato et al., 2002; Robitaille et al., 2002; Xu et al., 2004; Junge et al., 2009). Unlike Wnts which have promiscuous interactions with Fz receptors, Norrin specifically binds to Fz4CRD, but not to the 14 other CRDs of Fz and secreted...
Frizzled-related protein (sFRP) family members (Hsieh et al., 1999; Smallwood et al., 2007). Similar to Wnt, Norrin (1) binds to Lrp5/6 ECD (Ke et al., 2013); (2) interacts with HSPGs and shows limited spatial diffusion (Perez-Vilar and Hill, 1997; Xu et al., 2004; Smallwood et al., 2007; Ohlmann et al., 2010). As well as being a potential target for therapeutic interventions, an understanding of Norrin mediated signalling will also provide insights into the fundamental features required to trigger canonical Wnt/β-catenin signalling. Structural analyses of the extracellular components and interactions mediating Norrin signalling were considered to be challenging because of the difficulties of generating recombinant Norrin (Perez-Vilar and Hill, 1997; Shastry and Trese, 2003; Ohlmann et al., 2010). Ke et al. (2013) reported a refolding method (from Escherichia coli inclusion bodies) to produce active recombinant Norrin fused with a N-terminal maltose binding protein (MBP-Norrin), an advance that enabled them to determine the crystal structure of MBP-Norrin. Here, we develop an efficient mammalian cell expression method to produce active untagged recombinant Norrin and detail the structural and functional properties of this potential therapeutic agent. Our crystallographic and solution studies further reveal that dimeric Norrin forms a complex with two copies of monomeric Fz4CRD. Our molecular level analysis of the Norrin–Fz4CRD complex bound with GAG analogue, in combination with structure-guided biophysical and cell-based studies, defines the basis for ligand recognition. Structural comparison with the Xenopus Wnt8 in complex with mouse Fz8CRD (Janda et al., 2012) shows that Norrin uses its β-strands to mimic a finger-like loop in Wnt for binding to the Fz receptor CRD. Finally, we note that engineered Norrin mutants resulting from our analyses may be of use as agents for blocking Wnt receptor activation.

**Results**

**Production of biologically active Norrin**

To address the challenge of producing Norrin in large quantities, we screened conditions and constructs for Norrin expression (Figure 1A). We found that fusion of Norrin to the C-terminus of small ubiquitin-like...
modifier (SUMO) (Peroutka et al., 2008), in combination with addition of valproic acid (Backliwal et al., 2008), a putative histone deacetylase inhibitor, substantially boosted expression of the secreted protein in human embryonic kidney (HEK) 293T cells (Figure 1B,C). After removal of the SUMO fusion tag, the recombinant Norrin shows a monodispersed state in size-exclusion chromatography (SEC; Figure 1D) and is biologically active in a cell-based luciferase reporter assay (Figure 1E).

The crystal structure of Norrin and its oligomeric state in solution
We determined three crystal structures of Norrin (Figure 2A and Table 1), using selenomethionine-labeled protein for phasing (Figure 2—figure supplement 1). The Norrin protein fold is identical to that of the previously reported MBP-Norrin crystal structure (Ke et al., 2013). Each Norrin monomer comprises three β-hairpins (β1-β2, β3-β4 and β5-β6), a β7 strand at the C-terminus, and four...
intramolecular disulphide bonds (Figure 2—figure supplement 2). The two monomers assemble as an elongated, head-to-tail, dimer (Figure 2A) stabilized by three intermolecular disulphide bridges (Cys93-Cys95, Cys95-Cys93, and Cys131-Cys131), in agreement with small-angle X-ray scattering (SAXS) measurements which showed Norrin dimer in solution (Figure 2—figure supplement 3A and Table 2). The dimer interface is further stabilized by extensive hydrogen bonds and hydrophobic interactions (Figure 2—figure supplement 3B,C). Superposition of all molecules in the asymmetric units from our three crystal forms with the MBP-Norrin structure (Ke et al., 2013) showed an average root-mean-square (r.m.s.) deviation of 1.5 Å over 190 equivalent Cα atoms (Figure 2—figure supplement 3D). On inspection the superpositions revealed a high degree of conformational plasticity in the β1-β2, β3-β4 and β5-β6 loops (Figure 2B). The flexibility inherent in these regions is consistent with the relatively high crystallographic B factor values (Figure 2C). Conversely, the structural comparisons underscore the conserved nature of the interface at the dimer core. It has previously been noted that disruption of the dimer by either Cysteine-to-Alanine mutations of
Table 1. Data collection, phasing and refinement statistics

| Crystal form | Norrin–Fz4CRD–SOS | Methylated Norrin–Fz4CRD | Norrin Se-Met | Methylated Norrin |
|--------------|-------------------|--------------------------|---------------|------------------|
| Data collection |                   |                          |               |                  |
| Space group  | $P_6_1$22         | $P_4_1$22                 | $P_2_1$,2$1$ | $P_2_1$,2$1$     |
| Cell dimensions | 119.1, 119.1, 119.2 | 98.9, 98.9, 120.4     | 46.4, 79.1, 243.3 | 45.8, 78.8, 232.8 |
| $α$, $β$, $γ$ (˚) | 90, 90, 90 | 90, 90, 120 | 90, 90, 90 | 90, 90, 90 |
| Wavelength (Å) | 0.9200 | 0.9795 | 0.9686 | 0.9795 |
| Resolution (Å) | 47.3–3.00 (3.18–3.00) | 49.46–2.30 (2.38–2.30) | 65.56–2.40 (2.49–2.40) | 116.39–3.18 (3.26–3.18) |
| R$_{free}$ (%) | 3.1 (54.8) | 4.5 (56.1) | 6.1 (42.3) | 2.8 (23.4) |
| Completeness (%) | 99.9 (100) | 98.9 (97.2) | 99.9 (100) | 99.9 (99.9) |
| Redundancy | 19.6 (20.6) | 6.0 (5.6) | 5.6 (5.7) | 33.3 (9.9) |
| No. reflections | 10,503 (1648) | 26,816 (2514) | 34,722 (3384) | 36,272 (2635) |
| R$_{work}$/R$_{free}$ | 21.5/26.7 | 19.7/22.1 | 21.6/26.2 | 23.3/24.8 |
| No. atoms | Protein 1759 | 2557 | 4930 | 3187 |
|           | Ligand/ion 83 | 39 | 101 | 10 |
|           | Water 0 | 115 | 164 | 122 |
| B-factors | Protein 113 | 63 | 70 | 57 |
|           | Ligand/ion 133 | 71 | 92 | 73 |
|           | Water 0 | 57 | 55 | 51 |
| R.m.s deviations | Bond lengths (Å) 0.005 | 0.004 | 0.009 | 0.005 |
|           | Bond angles (˚) 1.18 | 0.93 | 1.08 | 1.07 |
| Ramachandran plot | Favored (%) 95.5 | 97.0 | 96.7 | 97.2 |
|           | Allowed (%) 4.5 | 3.0 | 3.3 | 2.8 |
| PDB code | 5BQC | 5BQE | 5BPU | 5BQ8 |

Table 1. Continued on next page
intermolecular disulphide bonds or mutations of hydrophobic residues at the dimer interface results in a loss of Norrin-mediated signalling (Smallwood et al., 2007; Ke et al., 2013).

The crystal structure of Fz4CRD

We determined two crystal structures of Fz4CRD (Figure 3 and Table 1). Similar to mouse Fz8CRD (Dann et al., 2001) the Fz4CRD fold comprises four α helices (Figure 3A and Figure 3—figure supplement 1A) stabilized by five disulphide bridges (Cys45–Cys106, Cys53–Cys99, Cys90–Cys128, Cys117–Cys158, Cys121–Cys145). The N-acetylglucosamines on two N-linked glycosylation sites at Asn59 and Asn144 are visible in the electron density map (Figure 3A). Superposition of all Fz4CRD molecules in the asymmetric units from two crystal forms revealed a well-ordered protein fold (Figure 3—figure supplement 1B). The conserved disulphide bonds in FzCRD superfamily members are essential for functional activity. Familial Exudative Vitreoretinopathy disease mutant C45Y results in misfolded protein retained in the endoplasmic reticulum, similar to the effects of Cysteine-to-Alanine mutations in the related CRD of Drosophila Smoothened (Smo) (Zhang et al., 2011; Rana et al., 2013).

Structural comparison showed Fz4CRD closely resembles the CRDs of mouse Fz8 and secreted Frizzled-related protein 3 (sFRP3) with an average r.m.s. deviation of 1.2 Å over 115 equivalent Cα atoms (Figure 3—figure supplement 1C–E) and approximate sequence identity of 35%. Comparisons with the CRDs of muscle-specific kinase (MuSk) and Smo showed more substantial structural differences with an average r.m.s. deviation of 2.3 Å over 86 equivalent Cα atoms (Figure 3—figure supplement 1F–H).
The proteins were produced from HEK293T cells with full glycosylation.

The measured molecular weight (MWMeasured) is obtained by dividing the Volume Porod (Vp [nm³]) by 1.66 (Rambo and Tainer, 2011).

The measured molecular weight (MWMeasured) is calculated from forward scattering of sample (I0) by comparison with reference bovine serum albumin (BSA).

The proteins were produced from HEK293T cells in the presence of kifunensine with limited glycosylation and treated with endoglycosidase-F1.

The measured molecular weight (MWMeasured) is obtained by dividing the Volume Porod (Vp [nm³]) by comparison with reference bovine serum albumin (BSA).

The theoretical molecular weight (MWTheoretical) is predicated from amino acid sequence plus the molecular weight of N-linked glycans (see ‘Materials and methods’, SEC-MELS analysis for detailed information of calculation).

The proteins were produced from HEK293T cells with full glycosylation.

Table 2. Molecular properties of the proteins determined by SAXS

| Proteins       | N-Glyc state | Rg (nm)* | Dmax (nm)† | Volume porod (Vp [nm³]) | MWTheoretical (kDa)‡ | MWMeasured (KDa)§ | MWMeasured (KDa)# |
|----------------|--------------|----------|------------|-------------------------|----------------------|-------------------|-------------------|
| Fz4CRD deglyc | 1.98         | 6.93     | 33.0       | 17.1 (monomer)          | 15.9                 | 19.9              |
| Fz4CRD glyc   | 2.24         | 7.84     | 41.1       | 21.4 (monomer)          | 23.7                 | 24.7              |
| Norrin         | 2.74         | 9.18     | 37.4       | 27.2 (dimer)            | 33.5                 | 22.5              |
| Norrin–Fz4CRD deglyc | 3.41 | 11.92 | 93.8       | 61.3 (2:2 complex)      | 57.9                 | 56.5              |

*Rg is Radius of gyration, calculated from Guinier plot using AutoRg (Petoukhov et al., 2012).
†Dmax is the maximum dimension of the particle, calculated by GNOM (Svergun, 1992).
‡The theoretical molecular weight (MWTheoretical) is predicated from amino acid sequence plus the molecular weight of N-linked glycans (see ‘Materials and methods’, SEC-MELS analysis for detailed information of calculation).
§The measured molecular weight (MWMeasured) is calculated from forward scattering of sample (I0) by comparison with reference bovine serum albumin (BSA).
#The measured molecular weight (MWMeasured) is obtained by dividing the Volume Porod (Vp [nm³]) by 1.66 (Rambo and Tainer, 2011).
†The proteins were produced from HEK293T cells in the presence of kifunensine with limited glycosylation and treated with endoglycosidase-F1.
‡The proteins were produced from HEK293T cells with full glycosylation.

Assessment of the monomeric states of FzCRD in solution

Fz receptors are members of the GPCR family (Nichols et al., 2013), known for formation of receptor dimers, although it is unclear whether dimerization is mediated by the CRD, transmembrane helices or intracellular domain. In the case of Fz4, β-galactosidase complementation in combination with bioluminescence resonance energy transfer and split-yellow fluorescence protein assays suggest that Fz4 exists as dimer on the cell membrane in the absence of Norrin or Wnts (Kaykas et al., 2004; Ke et al., 2013). However, ligand-independent receptor dimerization of Fz4 is not sufficient to activate signalling (Xu et al., 2004; Ke et al., 2013). Interestingly, we found that our Fz4CRD structures form the same dimeric assembly in two crystal lattices (r.m.s. deviation of 0.7 Å over 238 equivalent Cα atoms from two crystal forms; Figure 3—figure supplement 2A). The dimer interface has an average 1330 Å² buried surface area, in agreement with the characteristics of known protein–protein interfaces (Lawrence and Colman, 1993). However, this Fz4CRD dimer (front-to-front) is distinct from the previously reported crystal structure of mouse Fz8CRD dimer (back-to-back; Figure 3—figure supplement 2B) (Dann et al., 2001). We were therefore curious to assess the dimerization characteristics of the CRDs of Fz receptors. Size-exclusion chromatography coupled to multi-angle light scattering (SEC-MALS) results (Figure 3B and Table 3) showed Fz4CRD, Fz5CRD and Fz8CRD exist as monomers in solution at 50 μM concentration, in agreement with previously reported SEC studies of Fz8CRD and SEC-MALS analyses of MuSKCRD and SmoCRD (Stiegl er et al., 2009; Nachtergaele et al., 2013). SAXS measurements further support the conclusion that Fz4CRD is monomeric in solution at 290 μM concentration (Figure 3C,D). Taken together, our results suggest that the CRDs of Fz receptors exist as monomers and may not be involved in receptor dimerization; multiple GPCRs dimerize through their hepta-helical transmembrane domains (Rios et al., 2001). However, we cannot exclude the possibility that in the environment of the cellular membrane the weak interaction propensities of the CRDs, in combination with the transmembrane domains, are important for the dimerization of Fz receptors.

The crystal structure of Norrin in complex with Fz4CRD

We purified Norrin–Fz4CRD complex (Figure 4—figure supplement 1A) and determined the crystal structures of methylated Norrin–Fz4CRD (dimethylated surface-exposed lysine residues; Figure 4—figure supplement 1B,C) and Norrin–Fz4CRD–SOS (complex bound with heparin mimic sucrose octasulfate, SOS; Figure 4A and Figure 4—figure supplement 1D) at 2.3 Å and 3.0 Å resolution, respectively (Table 1). These two complex structures show different stoichiometries: a 2:1 complex for the methylated Norrin–Fz4CRD and a 2:2:2 stoichiometry for the Norrin–Fz4CRD–SOS complex, the architecture of which resembles a butterfly (Figure 4A). The Norrin–Fz4CRD binding interface is conserved between the complex structures (Figure 4—figure supplement 1E). Each Fz4CRD interacts one-to-one with a separate Norrin chain, burying on average 1680 Å² of surface area.
To investigate the preference for complex formation in a 2:1 or 2:2 stoichiometry, we performed SEC-MALS (Figure 4B) and SAXS (Figure 4C) measurements in the absence of SOS. Both methods show Norrin interacts with Fz4 CRD in a 2:2 stoichiometry. Lysine methylation of the Norrin–Fz4 CRD complex was used to facilitate crystal lattice formation (Walter et al., 2006; Malinauskas et al., 2011), and on close inspection of the structure we found Lys102 and Lys104, two residues which contribute to the Norrin–Fz4 CRD interface, (see next section) are dimethylated in the uncomplexed subunit of the Norrin dimer (Figure 4—figure supplement 1C), and contribute instead to a lattice contact. This observation suggests that the 2:1 stoichiometry merely reflects the favourable crystallization characteristics of a sub population of asymmetrically methylated Norrin–Fz4 CRD complexes. Thus although the

Figure 3. Crystal and solution structures of unliganded Fz4 CRD. (A) Schematic domain organization (SP, signal peptide; TM, transmembrane domain; CD, cytoplasmic domain). Crystallization constructs are rainbow coloured. Disulphide bonds are drawn and blue hexagons denote N-linked glycosylation sites. Cartoon representation of Fz4 CRD in rainbow colouring. N-linked N-acetyl-glucosamines (GlcNAc) and disulphide bonds are shown as blue sticks. (B) SEC-MALS experiments. The red line represents the molecular weight (left ordinate axis) and black lines show the differential refractive index (right ordinate axis) as well as SDS-PAGE (Inset). The numbers denote the corresponding molecular weights of each peak. (C and D) SAXS analyses of deglycosylated and glycosylated Fz4 CRD solution structures. The experimental scattering data (black circles) and calculated scattering patterns (coloured lines) are shown and the Fz4 CRD solution structure model is presented. The upper right inset shows the experimental (black circles) and calculated (coloured lines) Guinier region. The dashed lines delimit the range of fitting for Rg analysis (Rg ≤ 1.3). The bottom right inset shows the experimental (black circles) and calculated (coloured lines) pair distance distribution P(r) curve.

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The following figure supplements are available for figure 3:

Figure supplement 1. Multiple sequence alignment and structural analysis of cysteine-rich like domains.
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Figure supplement 2. Distinct dimeric assembly of Fz4 CRD and mouse Fz8 CRD observed from crystal structures.
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To investigate the preference for complex formation in a 2:1 or 2:2 stoichiometry, we performed SEC-MALS (Figure 4B) and SAXS (Figure 4C) measurements in the absence of SOS. Both methods show Norrin interacts with Fz4 CRD in a 2:2 stoichiometry. Lysine methylation of the Norrin–Fz4 CRD complex was used to facilitate crystal lattice formation (Walter et al., 2006; Malinauskas et al., 2011), and on close inspection of the structure we found Lys102 and Lys104, two residues which contribute to the Norrin–Fz4 CRD interface, (see next section) are dimethylated in the uncomplexed subunit of the Norrin dimer (Figure 4—figure supplement 1C), and contribute instead to a lattice contact. This observation suggests that the 2:1 stoichiometry merely reflects the favourable crystallization characteristics of a sub population of asymmetrically methylated Norrin–Fz4 CRD complexes. Thus although the
methylated Norrin–Fz4 CRD structure usefully provides high-resolution information for the ligand–receptor interface (Figure 4—figure supplement 1F), the Norrin–Fz4 CRD–SOS structure defines the overall architecture of the native complex (Figure 4A). The two Fz4 CRD diverge from the Norrin dimer without contacting each other (Figure 4A), and with their C-termini suitably oriented for attachment to the same cell surface.

The Norrin–Fz4 CRD complex has a novel architecture; the mode of interaction of Norrin is distinct from that of other cystine-knot secreted growth factors (transforming growth factor-β, bone morphogenetic protein, platelet-derived growth factor, and vascular endothelial growth factor) with either their receptors or antagonists (Figure 4—figure supplement 2). Neither Norrin nor Fz4 CRD undergoes large conformational changes upon complex formation, although the flexibility of residues involved in the binding interface is reduced (Figure 4—figure supplement 3). Interestingly, superposition of the Norrin–Fz4 CRD complex and the previously reported MBP-Norrin structure resulted in steric clashes between the Fz4 CRD and the MBP (Figure 4—figure supplement 4). This suggests that MBP hinders Norrin interaction with Fz4 CRD consistent with MBP-Norrin only having half of the signalling activity of untagged Norrin (Ke et al., 2013).

Analyses of binding interfaces
At the ligand–receptor interface (Figure 5A) two β-hairpins in Norrin (β1-β2 and β5-β6) contact three loops in Fz4 CRD (I, II, and III). Fz4 CRD loop I hydrogen bonds to Norrin (Figure 5B). Fz4 CRD loop II makes extensive hydrophobic contacts plus one salt-bridge (Fz4 CRD Lys109 with Norrin Asp46; Figure 5C). Fz4 CRD loop III interacts with Norrin via an extensive hydrogen bond network as well as hydrophobic contacts (Figure 5D). Interactions with SOS involve the positively charged residues of Lys58, Arg107, Arg109, and Arg115 on Norrin, plus His154 and Asn155 on Fz4 CRD loop III (Figure 5E). These residues define a likely binding site for GAGs, in agreement with previous reports of Norrin interactions with extracellular matrix and heparin (Xu et al., 2004; Ohlmann et al., 2010).

Verification of Fz4 binding site
The Norrin–Fz4 interface revealed in our crystal structures (Figure 6A,B) is in excellent agreement with reported disease-associated mutations (Figure 6C) and surface residue conservation (Figure 6D). We performed mutagenesis and functional assays to verify this Fz4 binding site. Surface plasmon resonance (SPR) experiments (Figure 6E and Figure 6—figure supplement 1A) show a micromolar equilibrium dissociation constant between Norrin and Fz4 CRD. Mutations of either H43N/V45T or L61N/A63S, which resulted in the introduction of an N-linked glycosylation site in the Fz4 binding site on Norrin, completely abolish the interaction (Figure 6—figure supplement 1B). Norrin disease-associated mutants V45E and L61P/A63D lose binding affinity for Fz4 CRD (Figure 6—figure supplement 1C). In contrast, Norrin mutants L52N/K54S and M114N/L116S (to introduce an N-linked glycans...
glycosylation site in the β1-β2 loop or β5-β6 loop, respectively), predicted to lie outside the Fz4 binding site (Figure 6A), show the same binding affinity as wild-type (Figure 6—figure supplement 1E). Cell-based Wnt/β-catenin responsive luciferase assays (Figure 6F) further support the significance of

Figure 4. Crystal structure and solution behaviour of Norrin–Fz4CRD complex. (A) Ribbon representation of Norrin (magenta and pink) in a 2:2:2 complex with Fz4CRD (cyan and pale cyan) and SOS (green). (B) SEC-MALS analyses. The profile of molecular weight (left ordinate axis) and differential refractive index (right ordinate axis) are shown as thick and thin lines, respectively. SDS-PAGE (Inset) shows Norrin in complex with Fz4CRD (triplet band for glycosylated Fz4CRD, marked as green circles, represents glycosylation heterogeneity). (C) SAXS experiments. Experimental scattering data (black circles) and calculated scattering patterns (coloured lines) are shown to a maximal momentum transfer of $q = 0.35 \text{ Å}^{-1}$. Individual data: fit pairs are displaced along an arbitrary y axis to allow for better visualization. Bottom curve: Norrin–Fz4CRD 1:2 complex crystal structure (blue line). Middle curve: Norrin–Fz4CRD 2:2 complex crystal structure (red line). Top curve: modelled Norrin–Fz4CRD 2:2 complex crystal structure (missing regions for Norrin and Fz4CRD N- and C-termini are modeled into the crystal complex structure; green line). Structural models are shown in cartoon representation. The bottom left inset shows the experimental (black circles) and calculated (coloured lines) Guinier region. The bottom right inset shows the experimental (black circles) and calculated (coloured lines) pair distance distribution $P(r)$ curves.

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The following figure supplements are available for figure 4:

Figure supplement 1. Protein complex production and structural properties of Norrin–Fz4CRD complex.

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Figure supplement 2. Structural comparison of cystine-knot growth factor monomers and their ternary complexes.

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Figure supplement 3. No large conformational changes upon complex formation.

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Figure supplement 4. Structural comparison of Norrin–Fz4CRD complex with MBP-Norrin.

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the Fz4 binding site. Norrin mutants that lose binding to Fz4\textsubscript{CRD} also fail to induce the luciferase reporter activity, in agreement with the SPR results (Figure 6—figure supplement 1) and prior genetic data (Xu et al., 2004; Smallwood et al., 2007). Taken together, our structural and functional results suggest that Norrin uses \(\beta\) strands (\(\beta1-\beta2\) and \(\beta5-\beta6\)) for Fz4\textsubscript{CRD} binding rather than, as proposed by Bazan et al. (2012) using the loop between \(\beta1\) and \(\beta2\) (Bazan et al., 2012).

To determine the binding affinity of Norrin for different CRD of Fz receptors, we undertook a series of SPR experiments. The results (Figure 6—figure supplement 1F) show that Norrin has greatest affinity for Fz4\textsubscript{CRD} (Kd: 1 \(\mu\)M), low affinities for Fz5\textsubscript{CRD} (Kd: 42 \(\mu\)M) and Fz8\textsubscript{CRD} (Kd: 64 \(\mu\)M), and no binding to Fz7\textsubscript{CRD}. In combination, these results confirm that pairing Norrin with Fz4\textsubscript{CRD} provides
Figure 6. Biophysical and functional characterisation of Fz4 binding site. Surface representation of Norrin–Fz4_CRD complex in open book view. (A) Interface residues are coloured orange (Norrin) and blue (loop I), green (loop II), yellow (loop III), and cyan (Phe96) on Fz4_CRD. Norrin mutation sites used in functional assays are labelled (red, residues involved in Fz4_CRD binding; grey filled box, residues associated with diseases; black, residues located outside the Fz4 binding site). (B) Norrin and Fz4_CRD coloured by electrostatic potential from red (acidic; $-7 \, \text{k}_\text{B}T/e$) to blue (basic; $7 \, \text{k}_\text{B}T/e$). (C) Disease-associated mutations mapped onto the surface of Norrin and Fz4_CRD (purple, missense mutations; red, missense mutations of cysteine residues). (D) Surfaces colour-coded according to sequence conservation from white (not conserved) to black (conserved). (E) SPR results for Fz4_CRD binding to Norrin wild-type (WT) and Norrin V45E mutant. Inset SPR sensorgrams are of equilibrium-based binding assays with reference subtraction. (F) Luciferase Figure 6. continued on next page
selective and high affinity binding relative to interactions with other CRD of Fz receptors, in agreement with prior studies (Xu et al., 2004; Smallwood et al., 2007; Ke et al., 2013). However, it remains to be clarified whether the low affinity interactions of Norrin with other Fz receptors can play any functional role in vivo.

**Verification of GAG binding site**

To assess our putative binding site for GAGs (Figure 5E), we performed structure-guided mutagenesis and functional studies. Our heparin binding experiments confirmed that Norrin shows high affinity interaction with heparin (Figure 7—figure supplement 1A), consistent with previous studies (Perez-Vilar and Hill, 1997; Xu et al., 2004; Smallwood et al., 2007; Ohlmann et al., 2010), and further demonstrated Norrin–Fz4CRD complex binding to heparin (Figure 7A). The Norrin triple mutation R107E/R109E/R115L (R115L is a disease-associated mutation; Figure 2—figure supplement 2) impaired heparin binding (Figure 7B) and abolished signalling activity (Figure 7C). However, this mutant protein retained the ability to bind Fz4CRD (Figure 7D) with a 2:2 stoichiometry (Figure 7—figure supplement 1B). Ke et al. (2013) have reported MBP-Norrin binding to the Lrp6 ectodomain fragment comprising the first two tandem β-propeller-epidermal growth factor-like domain pairs (Lrp6_P1E1P2E2; Ke et al., 2013); we found both our wild-type and R107E/R109E/R115L mutant Norrin bind to Lrp6_P1E1P2E2 (Figure 7E,F). The Norrin K58N mutant (a disease-associated mutation; Figure 2—figure supplement 2) exhibited half of wild-type activity in our cell-based assay (Figure 7C), but did not affect Fz4CRD interaction (Figure 6—figure supplement 1C). These results are in agreement with previous functional studies (Smallwood et al., 2007), and suggest this area is a GAG binding site rather than that, as Ke et al. (2013) proposed, residues Arg107, Arg109, and Arg115 are involved in Lrp5/6 binding (Ke et al., 2013). HSPGs play important roles in the regulation of the Wnt signalling pathway (Malinauskas and Jones, 2014). Wnt signalling activity can be inhibited by treatment with exogenous heparin (Ai et al., 2003). Also, Jung et al. (2015) have reported that PG545, a heparan sulphate mimetic, can block Wnt binding to the cell surface, by competing with endogenous HSPGs, and inhibit Wnt signalling (Jung et al., 2015). For Norrin mediated Wnt/β-catenin signalling, we found that SOS could inhibit activity when pre-incubated with Norrin before stimulation of reporter cells (Figure 7—figure supplement 1D).

**Mapping a potential Lrp5/6 binding site on Norrin**

Norrin interaction with co-receptor Lrp5/6ECD (Figure 7E) is essential for signal activation (Xu et al., 2004; Ke et al., 2013). To identify Norrin residues potentially involved in Lrp5/6ECD binding, we assessed solvent exposure, disease-association, and lack of involvement in Fz4CRD or GAG binding (Figure 2—figure supplement 2). Five residues (Lys54, Arg90, Arg97, Gly112, and Arg121) were highlighted by this analysis and form a continuous, positively charged, concave patch (Figure 8A). Notably, a negatively charged region of the Lrp6ECD surface has been implicated in ligand binding (Ahn et al., 2011; Bourhis et al., 2011; Chen et al., 2011; Cheng et al., 2011). We therefore focused on the positively charged concave surface of Norrin as a potential Lrp5/6 binding site (Figure 8A), interestingly, this putative binding site has a partially overlap, at Lys54, with the residue suggested to be involved in Lrp5/6 interaction by Ke et al. (2013). To test our proposed location for the Lrp5/6 binding site, we generated the disease-associated Norrin mutant R121W (Arg121 is a mutational hotspot; Figure 2—figure supplement 2). This mutation substantially impairs signalling activity (Figure 8—figure supplement 1A), but retains the ability to interact with Fz4CRD (Figure 8—figure supplement 1B) and heparin (Figure 8—figure supplement 1C). However, we found the R121W mutation reduced protein solubility and stability during protein
Figure 7. Verification of Norrin GAG binding site. Heparin affinity chromatography of (A) Norrin–Fz4CRD complex and (B) Norrin R107E/R109E/R115L–Fz4CRD complex. Protein elution profiles (left panel) were monitored by absorbance at 280 nm (blue curves) for a NaCl gradient (0.25–2 M; black dashed lines). Input sample, flow-through (green line) and peak fractions (red line) were analysed on SDS-PAGE (right panel). Norrin-Fz4CRD complex was eluted at 1.3 M NaCl concentration. (C) Luciferase reporter assays for Norrin mutations (coloured green) in the GAG binding site. Grey filled boxes highlight disease-associated residues (Figure 2—figure supplement 2). (D) SPR binding assay of Norrin R107E/R109E/R115L mutant and Fz4CRD interaction. Sensorgrams (top panel) and fitted plots of equilibrium binding response (bottom panels) for a series of concentrations of Fz4CRD are shown. Figure 7. continued on next page
production and in heparin binding assays. Analyses of additional Norrin mutants in biophysical and cellular assays will be required to verify the putative Lrp5/6 binding site. Taken together, the current data suggest three distinct and independent binding sites on Norrin for Fz4, Lrp5/6, and GAGs (Figure 8B). This arrangement of binding sites likely enables Norrin to form a ternary complex.

Structural comparison of Norrin–Fz4CRD with Wnt8–Fz8CRD

As Norrin and Wnt both trigger the canonical Wnt/β-catenin pathway, we compared their modes of action. *Xenopus* Wnt8 (Figure 9A) has been described as using ‘thumb’ and ‘index finger’ regions to grasp mouse Fz8 CRD at two distinct sites (Janda et al., 2012). In site 1, a palmitoleoyl group (PAM) covalently linked to the tip of the thumb inserts into a groove in Fz8 CRD, removal of this PAM moiety suppresses Wnt signalling activity (Kakugawa et al., 2015; Zhang et al., 2015). In site 2, the index finger contacts a hydrophobic pocket. We superposed Norrin–Fz4 CRD with Wnt8–Fz8 CRD. There are no major structural differences between the Fz4 CRD and Fz8 CRD (r.m.s. deviation of 1.3 Å over 110 equivalent Cα atoms; Figure 9A), and the structural elements that mediate site 1 PAM binding in Fz8 CRD are largely conserved in Fz4 CRD (Figure 9B). The Norrin binding site on Fz4 CRD (~800 Å² buried area) overlaps with site 2 on Fz8 CRD (~400 Å² buried area; Figure 8A), in agreement with previous mutational mapping studies (Smallwood et al., 2007). The position of the Wnt8 index finger overlaps with Norrin β1 and β2, and, unexpectedly, these β strands show some structural equivalence with Wnt8 (Figure 9C). Site 2 Wnt8 residues are strictly conserved in all Wnts, and the apolar residues in the corresponding positions on Norrin are associated with disease mutations (Figure 9C).

We also used our superposition of the Fz4 CRD and Fz8 CRD structures (Figure 9A) to identify the determinants of the Norrin binding specificity for Fz4 CRD (Figure 6-figure supplement 1F). In Fz4 CRD loop I (Figure 9D), Asn55 is replaced by Fz8 CRD Gly45, a change that would abolish interaction with Norrin Ser34 in the complex (Figure 5B). In Fz4 CRD loop II (Figure 9E), the substitution of Lys109 by Fz8 CRD Asp99 would introduce an unfavorable electrostatic interaction with Norrin Asp46 (Figure 5C). Thirdly, in Fz4 CRD loop III (Figure 9F), hydrogen bonds and salt...
bridges to Norrin would be lost on replacing Asn152 and Glu160 with Fz8 CRD Gly142 and Asp150 respectively (**Figure 5D**). Consistent with this analysis, these residue substitutions have been reported to affect Fz4 CRD binding to Norrin (**Smallwood et al., 2007**), and Fz4 CRD is unique in containing this particular combination of residues (**Figure 2—figure supplement 2**).

**Discussion**

Overall, our analyses provide several advances for our understanding of Norrin and Wnt signalling. Firstly, our results give fresh insight into the role of HSPGs. HSPGs have been proposed to regulate the local distribution of ligand and receptor at the cell surface, potentially acting as an introductory agency for ligand and receptor (**Lin and Perrimon, 1999; Baeg et al., 2001; Malinauskas et al., 2011; Malinauskas and Jones, 2014**). We have discovered a GAG binding site that may span Norrin and Fz4 CRD (**Figure 5E**). Interestingly, **Smallwood et al. (2007)** found the binding affinity of Norrin with Fz4 CRD is enhanced in the presence of heparin (**Smallwood et al., 2007**). We propose that the extended GAG binding site may allow co-receptor HSPGs to recruit secreted Norrin for interaction with Fz4 CRD and to co-localize Norrin and Fz4 receptor, similar to the role of HSPGs in Wnt signalling (**Reichsman et al., 1996; Baeg et al., 2001; Fuerer et al., 2010**). For example, HSPGs have been shown to regulate the Wnt morphogenetic gradient (**Lin and Perrimon, 1999; Baeg et al., 2004**). Also, **Capurro et al. (2014)** have reported that Fz4 CRD binds to the GAGs of the human HSPG Glypican-3 and that these interactions are involved in Wnt signal complex formation (**Capurro et al., 2014**). Secondly, we show the Norrin dimer binds separately to two molecules of Fz4 CRD (**Figure 4**), in contrast to the 1:1 complex of Wnt8–Fz8 CRD (**Janda et al., 2012**). Our discovery of the Fz4 and GAG...
binding sites, and analysis of a potential Lrp5/6 binding region, maps out distinct binding surfaces on Norrin (Figure 8B), which provide a framework in which to understand the effects of inherited mutations and probe the overall architecture of the ternary complex (Norrin–Fz4<sub>CRD</sub>–Lrp5/6<sub>ECD</sub>). Thirdly, we determine how Norrin structurally mimics Wnt for site 2 binding surfaces on the Fz ectodomain (Figure 9A). Interestingly, previous analyses using water-soluble ‘mini-Wnt’ proteins, which cannot contribute site 1 binding, have raised the possibility that site 2 binding to the CRD of Fz receptors can activate canonical Wnt/β-catenin signalling albeit weakly (Janda et al., 2012; von Maltzahn et al., 2013). Our findings indicate that the site 2 binding mode is central to signalosome formation for Norrin mediated signalling.

We used SPR experiments to establish the binding affinity for Norrin–Fz4<sub>CRD</sub> complex formation. The K<sub>d</sub> value of 1.1 μM for the interaction between Norrin and Fz4<sub>CRD</sub> we report here (Figure 6—figure supplement 1A) is weaker than previously published results (Xu et al., 2004; Ke et al., 2013). This discrepancy is likely due to our SPR binding assays being carried out with monomeric Fz4<sub>CRD</sub>. Xu et al. (2004) used an enzyme-linked immunosorbent assay to give an affinity of 3–4 nM for mouse Norrin fused with C-terminal alkaline phosphatase binding to mouse Fz4<sub>CRD</sub> dimerized by a C-terminal Fc fusion (Xu et al., 2004). Ke et al. (2013) reported K<sub>d</sub> values of 11 nM and 5 nM for the interaction between MBP-Norrin and Fc-tagged dimeric Fz4<sub>CRD</sub> using an AlphaScreen luminescence assay and biolayer interferometry, respectively (Ke et al., 2013). It is noteworthy that as Fc-dimerized Fz4<sub>CRD</sub> may mimic Fz4 receptor dimerization at the cellular surface, these tighter binding affinities may be more indicative of Norrin binding in the physiologically relevant environment. Similarly, our K<sub>d</sub> value of 2.87 μM for Norrin binding to Lrp5<sub>P1E1P2E2</sub> in an SPR based assay (Figure 7E) differs from the K<sub>d</sub> value of 0.45 μM reported by Ke et al. (2013) based on an homologous AlphaScreen competition assay using unlabeled MBP-Norrin against biotinylated MBP-Norrin for interaction with Lrp5<sub>P1E1P2E2</sub> (Ke et al., 2013).

Our studies reported here, in combination with previous findings for Norrin and Wnt signalling, are consistent with Norrin-induced receptor clustering and signalosome formation. Inactive pre-dimerized Fz4 may engage with homodimeric Tsps-12 to enhance receptor clustering (Kaykas et al., 2004; Ke et al., 2013). Norrin binding generates ternary complex formation by Fz4, Lrp5/6 and the GAGs of HSPGs to trigger signalling, which is enhanced in the presence of Tsps12. In the cytoplasm, Dishevelled binds to the C-terminal tail of Fz4 and self-assembles to oligomer (Schwarz-Romond et al., 2007), leading to Axin recruitment to the cytoplasmic domain of Lrp5/6 for phosphorylation and signalosome formation (Bilic et al., 2007).

Previously reported mice genetic studies have demonstrated that expression of ectopic Norrin can rescue pathological retinal vascularization (Ohlmann et al., 2005, 2010). In addition, the pathological progresses of Norrie disease and familial exudative vitreoretinopathy are highly related to age-related macular degeneration and diabetic retinopathy (Ye et al., 2010; Ohlmann and Tamm, 2012). Further investigation of the therapeutic possibilities for retinal diseases has been hampered by the difficulty of producing recombinant Norrin proteins. In this study, we provide a method to produce fully active untagged Norrin in mammalian cells (Figure 1). Our recombinant Norrin opens up new avenues to explore for the treatment of genetic retinal diseases and other ophthalmic disorders.

More generally, Norrin as a Wnt mimic, may have potential as a reagent in regenerative medicine (Clevers et al., 2014). Wnt signalling is important for tissue homeostasis throughout life (Clevers and Nusse, 2012). Multiple Wnt extracellular antagonists function to modulate Wnt signalling (Malinauskas and Jones, 2014), these include Dickkopf and Sclerostin, which bind to Lrp5/6, as well as Wnt inhibitory 1 and sFRPs, which sequester Wnt. Aberrant Wnt signalling (insufficient or excessive) is implicated in diseases such as neurodegeneration and tumorigenesis, respectively (Clevers and Nusse, 2012; Anastasis and Moon, 2013). Interestingly, our Norrin mutants (used to verify the GAG and putative Lrp5/6 binding sites) retain Fz4<sub>CRD</sub> binding but lose the ability to activate signalling. These properties are similar to those of the monoclonal antibody OMP-18R5 which can bind to the CRDs of Fz1, 2, 5, 7 and 8. OMP-18R5 inhibits tumour growth (Gurney et al., 2012) and has just completed phase I clinical trials (Kahn, 2014). Engineered Norrin mutants could similarly serve as blocking agents, but with specificities tailored to target Fz4 or other individual Fz receptors.
Materials and methods

Construct design, cloning, and mutagenesis

Synthetic complementary DNA (cDNA) clones (codon-optimized for expression in mammalian cells) of human Norrin (UniprotKB/Swiss-prot Q00604) were obtained from GeneArt (Life Technologies, UK). The cDNA templates of human receptors Fz4 (IMAGE ID: 40082087), Fz7 (IMAGE ID: 4549389), Lrp6 (IMAGE ID: 40125687), and Tspan-12 (IMAGE ID: 5275953) and mouse receptors Fz5 (IMAGE ID: 40088671) and Fz8 (IMAGE ID: 8861081) were purchased from SourceBioScience (UK). All expression constructs reported here are derived from the pHlsec vector backbone (Aricescu et al., 2006). The human Norrin wild-type (residues 25–133) construct of SUMO-Norrin (Figure 1A) and Norrin mutant constructs for heparin affinity binding assays were tagged N-terminally with the murine Igκ-chain secretion signal, followed by a Strep-II tag, 8xHis tag, a mammalian expression codon-optimized Saccharomyces cerevisiae SUMO (UniprotKB/Swiss-prot Q12306; residues 2–96) (Peroutka et al., 2008), and a Human Rhinovirus (HRV)-3C protease cleavage site. They were tagged C-terminally with a TETSQVAPA sequence derived from bovine rhodopsin (Rho-1D4) that is recognized by the Rho-1D4 monoclonal antibody (Molday and MacKenzie, 1983). The construct of Norrin (residues 25–133) was cloned into the pHlsec vector (Aricescu et al., 2006) in frame with a C-terminal Rho-1D4 (Figure 1A). For large-scale protein expression, the CRD constructs for human Fz4 (residues 42–179) and Fz7 (residues 42–179) as well as mouse Fz5 (residues 31–176) and Fz8 (residues 30–170) were cloned into a modified pHlsec vector (pHLsec-mVenus-12H), containing a C-terminal HRV-3C protease cleavage site followed by a linker, monoVenus (Nagai et al., 2002), and a tandem 6×His tag. Human Lrp6 P1E1P2E2 (residues 1–631) construct was cloned into a modified vector for stable cell line generation, pNeoSec (Zhao et al., 2014), in frame with a C-terminal 10×His tag. For luciferase reporter assays, the full-length constructs of the human receptors Fz4 (residues 1–537), Lrp6 (residues 1–1613), and Tspan-12 (residues 1–305) were cloned into the pLEXm-1D4 vector carrying a C-terminal Rho-1D4 tag. Norrin wild-type and mutants for biophysical and cellular assays were obtained from GeneArt (Life Technologies, UK) and cloned into the pHL-Avitag 3 vector encoding a C-terminal BirA recognition sequence (Aricescu et al., 2006). Mutant proteins were secreted at similar levels to the wild-type proteins. Constructs were verified by DNA sequencing (Source Bioscience, UK).

Western blot assays

For western blot, HEK293T (ATCC CRL-11268) cells were transfected with the DNA using Lipofectamine 2000 (Life Technologies, UK) according to the manufacturer’s instructions. The conditioned media were collected 2 days post transfection and were analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels transferred onto a nitrocellulose membrane (GE Healthcare Life Sciences) with Rho-1D4 monoclonal antibodies (Flintbox, University of British Columbia, Canada) as primary antibody and goat anti-mouse IgG-horseradish peroxidise conjugate (Sigma). The signal was visualized by Enhanced Chemiluminescence western blotting detection kit (ECL, GE Healthcare Life Sciences).

Protein production and purification

Norrin wild-type and mutants were expressed in HEK293T cells (Aricescu et al., 2006) in the presence of 4 mM valproic acid (Backliwal et al., 2008). For crystallization experiments, Fz4_CRD was produced in HEK293T cells in the presence of 5 µM of the class I α-mannosidase inhibitor, kifunensine (Chang et al., 2007). Norrin in complex with Fz4_CRD was co-expressed in HEK293T cells in the presence of kifunensine and valproic acid. For all other experiments, recombinant proteins were expressed in HEK293T cells. The Norrin conditioned media were passed through 1D4-affinity beads covalently coupling purified Rho-1D4 antibody to CnBr-activated Sepharose 4 Fast Flow (CnBr-1D4; GE Healthcare Life Sciences) and eluted in 25 mM Tris, pH 7.5, 0.5 M NaCl, 10% (wt/vol) Glycerol, 0.5% (wt/vol) CHAPS, 250 µM TETSQVAPA peptide (GenScript). The eluted sample was incubated with Glutathione S-Transferase (GST)-tagged HRV-3C protease to remove the SUMO-tagged fusion protein. The cleaved Norrin was purified by CnBr-1D4 followed by SEC (Superdex 200 10/300 GL High Performance, GE Healthcare Life Sciences) in either 10 mM HEPES, pH 7.5, 0.7 M NaCl, 0.5% (wt/vol) CHAPS or acetate buffer, pH 4.0, 0.5 M NaCl, 0.5% (wt/vol) CHAPS. For purification of Fz4_CRD, the
conditioned media were dialyzed and recombinant proteins were purified by IMAC (TALON beads, Clontech, Mountain View, CA). The purified sample was dialyzed against 25 mM Tris, pH 7.5, 0.5 M NaCl, 10% (wt/vol) Glycerol and treated with GST-tagged Flavobacterium meningosepticum endoglycosidase-F1 (Endo-F1) (Chang et al., 2007) and His-tagged HRV 3C protease. The deglycosylated and cleaved sample was further purified by IMAC and further polished by SEC (Superdex 75 16/600 column, GE Healthcare Life Sciences) in 10 mM HEPES, pH 7.5, 0.15 M NaCl. Purification of Fz5CRD, Fz7CRD, and Fz8CRD followed the same procedure to that described above, except protein was expressed in HEK293T cells and the treatment by Endo-F1 was omitted. Norrin–Fz4CRD complex was isolated from dialyzed conditioned media by IMAC. The eluted sample was dialyzed and treated with GST-tagged HRV-3C protease and Endo-F1. The deglycosylated and cleaved complex was further purified by IMAC and GST-affinity beads and subsequently isolated by SEC (Superdex 200 16/600 column, GE Healthcare Life Sciences) in 10 mM HEPES, pH 7.5, 0.7 M NaCl. For preparation of methylated proteins, the purified sample was subject to subject to surface lysine methylation (Walter et al., 2006) and further purified by SEC (Superdex 200 16/600 column, GE Healthcare Life Sciences). The selenomethionine (Se-Met) labelled protein was prepared as described previously (Aricescu et al., 2006). A stable HEK293 GnT1(−) cell line (Reeves et al., 2002) for Lrp6P1E1P2E2 protein production was generated as reported previously (Zhao et al., 2014) and protein was purified following our established procedure (Chen et al., 2011).

Crystallization and data collection
Concentrated proteins (Norrin, 5 mg/ml; Fz4CRD, 60 mg/ml; Norrin in complex with Fz4CRD including native and methylated proteins, 10–12 mg/ml) were subjected to sitting drop vapor diffusion crystallization trials in 96-well Greiner plates consisting of 100 nl protein solution and 100 nl reservoir using a Cartesian Technologies dispensing instrument (Walter et al., 2005). Crystallization plates were placed in at The Automation Partnership storage vault maintained at 294 K and imaged via a Veeco visualization system. Methylated Norrin–Fz4CRD complex crystallized in 0.1 M Bicine, pH 9.0, 10% (wt/vol) PEG6000, Norrin crystal form I in 0.1 M sodium acetate, pH 5.0, 5% (wt/vol) PGA-LM, 30% (wt/vol) PEG550MME, Norrin crystal form II in 0.1 sodium acetate, pH 5.0, 5% (wt/vol) PGA-LM, 4% (wt/vol) PEG2000MME, 24% (wt/vol) PEG550MME, Norrin crystal form III in 0.1M citrate, pH 5.0, 30% (wt/vol) PEG6000, Fz4CRD crystal form I in 1.6 M tri-sodium citrate, pH 6.5, and Fz4CRD crystal form II in 0.1 M HEPES, pH 7.5, 0.1 M NaCl, 1.6 M ammonium sulfate. For the Norrin–Fz4CRD–SOS complex, protein complex was mixed with 10 mM SOS (Toronto Research Chemicals Inc.) prior to crystallization and crystals were obtained in 0.1 M Tris, pH 8.0, 0.15 M NaCl, 8% (wt/vol) PEG8000. For cryoprotection, crystals were soaked in mother liquor supplemented with 30% (vol/vol) glycerol for methylated Norrin–Fz4CRD, with 20% (vol/vol) PEG2000 and 10 mM SOS for Norrin–Fz4CRD–SOS, with 30% (vol/vol) PEG550MME for Norrin crystal form II, with 30% (vol/vol) glycerol for Norrin crystal form III, with 1.8 M tri-sodium citrate, pH 6.5 for Fz4CRD crystal form I, and with 23% (vol/vol) sucrose for Fz4CRD crystal form II and subsequently flash-cooled by dipping into liquid nitrogen. The crystals of Norrin crystal form I were frozen directly. Data were collected at 100 K at Diamond Light Source (Oxfordshire, UK) at beamlines 103 (Norrin Se-Met), 104 (methylated Norrin–Fz4CRD and Norrin crystal form II and III), 104-1 (Norrin–Fz4CRD–SOS), and 104 (Norrin crystal form I and Fz4CRD crystal form I and II). Diffraction data were indexed and integrated using XIA2 (Winter, 2010) coupled with XDS or IMOSFLM, and scaled and merged using Aimless (Evans and Murshudov, 2013). A subset of 5% of randomly selected diffraction data were used for calculating Rfree (Brunger, 1993).

Structure determination and refinement
The structure of Norrin crystal form I was solved using highly redundant single-wavelength anomalous dispersion data merged from four data sets and collected at the Se K absorption edge. HKL2MAP (Sheldrick, 2010) was used to identify the Se sites, which were then fed into PHENIX AUTOSOL (Adams et al., 2002), resulting in an interpretable density modified electron map generated by RESOLVE (Terwilliger, 2003). An initial model generated by BUCCANEER (Cowtan, 2006) was used to solve the high-resolution native structures. The structure of Fz4CRD was determined by molecular replacement (MR) in PHASER (McCoy, 2007) using mouse Fz8CRD (PDB ID: 1IJY) as the search model, which was modified by CHAINSAW. For the determination of methylated Norrin–Fz4CRD, Norrin was
used as search model for MR in Phaser (McCoy, 2007) to obtain the initial phases. The additional electron density corresponding to Fz4CRD was clearly discernible after density modification with PARROT (Cowtan, 2010). Subsequently, the complex structure was solved by searching for Fz4CRD with MR in Phaser (McCoy, 2007). All other structures were solved by MR in Phaser (McCoy, 2007) using the refined Norrin and Fz4CRD structures as search models. The models were completed by manual rebuilding in COOT (Emsley and Cowtan, 2004) and refinement in REFMAC5 (Murshudov et al., 1997) and PHENIX (Adams et al., 2010). The crystallographic statistics are listed in Table 1. All models were validated with MOLPROBITY (Chen et al., 2010).

Structure analysis
Amino acid sequence alignments were constructed using ClustalW (Thompson et al., 1994). Structure superposition was performed within the CCP4 program suite using the SSM algorithm (Krissinel and Henrick, 2004). Electrostatic potential calculations were generated using APBS tools (Baker et al., 2001), surface sequence conservation was calculated using CONSURF (Ashkenazy et al., 2010) and interface areas of proteins were analyzed with the PISA web server (Krissinel and Henrick, 2007). High-quality images of the molecular structures were created with the PyMOL Molecular Graphics System (Version 1.5, Schrödinger, LLC). Schematic figures and other illustrations were prepared using Corel Draw (Corel Corporation).

Surface plasmon resonance equilibrium binding studies
SPR experiments were performed using a Biacore T200 machine (GE Healthcare Life Sciences) at 25°C in 10 mM HEPES, pH 7.5, 0.15 M NaCl, 0.005% (wt/vol) Tween20. For in vivo biotinylation (Penalva and Keene, 2004) Norrin wild-type or mutants in the pH-L-Avtag3 vector (Aricescu et al., 2006) were co-transfected with a pHLsec construct of BirA-ER (the synthetic BirA gene with a C-terminal KDEL sequence for retention in the endoplasmic reticulum) in HEK293T cells. Mutant proteins were secreted at similar levels to the wild-type proteins. The mammalian cell secretory pathway uses stringent quality control mechanisms to ensure that secreted proteins are correctly folded (Trombetta and Parodi, 2003). The biotinylated Norrin variants were immobilized onto the surface of a CMS sensor chip (GE Healthcare Life Sciences) on which approximately 8500 resonance units of streptavidin were coupled via primary amines. Fz4CRD proteins used as analytes were expressed in HEK293T cells to ensure full glycosylation and prepared as described above. The signal from SPR flow cells was corrected by subtraction of a blank and reference signal from a mock-coupled flow cell. In all analyses, the experimental trace returned to baseline line after a regeneration step with 100 mM phosphate pH 3.7, 2 M NaCl, 1% (wt/vol) CHAPS. The deglycosylated Norrin–Fz4CRD complex was measured at 1.02 and 2.14 mg/ml in 10 mM NaCl. Norrin was measured at 0.75 and 1.26 mg/ml in 10 mM HEPES, pH 7.5, 0.7 M NaCl, 0.5% (wt/vol) Tween20. The data were fitted to a 1:1 Langmuir adsorption model ($B = B_{\text{max}}C/(K_d + C)$, where B is the amount of bound analyte and C is the concentration of analyte in the sample) for the calculation of dissociation constant ($K_d$) values using Biacore Evaluation software (GE Healthcare Life Sciences). Data points correspond to the average from two independent dilution series.

Small-angle x-ray scattering experiment
Solution scattering data were collected at beamline BM29 of the European Synchrotron Radiation Facility (ESRF; Grenoble, France) at 293 K within a momentum transfer range of 0.01 Å$^{-1} < q < 0.45$ Å$^{-1}$, where $q = 4\pi\sin(\theta)/\lambda$ and 2θ is the scattering angle (Pernot et al., 2013). X-ray wavelength was 0.995 Å and data were collected on a Pilatus 1M detector. Fz4CRD was measured at 1.47 and 3.10 mg/ml (deglycosylated form) and 0.97 and 1.45 mg/ml (glycosylated form) in 10 mM HEPES pH 7.5, 0.15 M NaCl. Norrin was measured at 0.75 and 1.26 mg/ml in 10 mM HEPES, pH 7.5, 0.7 M NaCl, 0.5% (wt/vol) CHAPS. The deglycosylated Norrin–Fz4CRD complex was measured at 1.02 and 2.14 mg/ml in 10 mM HEPES, 0.5 M NaCl. Data reduction and calculation of invariants was carried out using standard protocols implemented in the ATSAS software suite (Petoukhov et al., 2012). A merged dataset was obtained by merging the low-angle part of the low-concentration dataset with the high-angle part of the high-concentration dataset. The Radius of gyration ($R_g$) was obtained from Guinier plot using AutoRg (Petoukhov et al., 2012). The maximum dimension of the particle ($D_{\text{max}}$) and Volume Porod ($V_p$ [nm$^3$]) were calculated by GNOM (Svergun, 1992). Molecular weights were obtained by (a) comparison with the reference bovine serum albumin (BSA) and (b) dividing the Porod Volume by 1.66 (Rambo and Tainer, 2011). Theoretical X-ray scattering patterns of structural models were calculated and fitted to experimental X-ray scattering curves using the program FoXS (Schneidman-Duhovny et al., 2010).
The Norrin, Fz4CRD and the Norrin–Fz4CRD complex solution structures were modeled starting from their respective crystal structures. Complex glycan structures and missing regions of N- and C-termini were added using the program Modeller (Eswar et al., 2003). All-atom simulations, and calculation and fitting of scattering patterns of Norrin, Fz4CRD and the Norrin–Fz4CRD complex were performed using the automated AllosMod-FoXS procedure (Guttman et al., 2013).

Size-exclusion chromatography coupled to multi-angle light scattering analysis
SEC-MALS experiments were performed by using SEC on an analytical Superdex S200 10/300 GL column (GE Healthcare Life Sciences) connected to online static light-scattering (DAWN HELOS II, Wyatt Technology, Santa Barbara, CA), differential refractive index (Optilab rEX, Wyatt Technology, Santa Barbara, CA) and Agilent 1200 UV (Agilent Technologies, Santa Clara, CA) detectors. Purified sample (F2CRD proteins at 50 μM or Norrin–Fz4CRD complex at 25 μM) was injected into a column equilibrated in 10 mM HEPES, pH 7.5, 0.15 mM NaCl. Molecular mass determination was performed using an adapted RI increment value (dn/dc standard value; 0.186 ml/g) to account for the glycosylation state. The theoretical molecular weight was predicated from amino acid sequence plus 2.35 kDa per N-linked glycosylation site for full glycosylated protein produced from HEK293T cells or 203 Da per site for deglycosylated protein produced from HEK293T cells in the presence of kifunensine (Chang et al., 2007) with limited glycosylation and treated with Endo-F. Data were analyzed using the ASTRA software package (Wyatt Technology, Santa Barbara, CA).

Luciferase reporter assay
The stable HEK293STF cell lines (Xu et al., 2004) carrying the Super Top Flash firefly luciferase reporter were split into 96-well plates and transfected 24 hr later with 200 ng DNA per well using Lipofectamine 2000 (Life Technologies, UK) according to the manufacturer’s instructions. For assessment of interface mutants used in SPR experiments, the DNA mix contained 80 ng Norrin plasmid, 40 ng each of Fz4 and Lrp6 plasmids, 20 ng each of Tspan-12 and constitutive Renilla luciferase plasmids (pRL-TK, Promega, Madison, WI). The firefly and Renilla luciferase activities were measured 48 hr later with Dual-Glo luciferase reporter assay system (Promega, Madison, WI) using an Ascent Lunimoskan luminometer (Labsystems). For evaluation of recombinant Norrin and SOS inhibition, the DNA mix (80 ng pLEXm plasmid, 40 ng each of Fz4 and Lrp6 plasmids, 20 ng each of Tspan-12 and pRL-TK plasmids) was used for transfection. Cells were stimulated 6 hr post transfection with 9 μg/ml Norrin, 9 μg/ml Norrin preincubated with 2 mM SOS for 15 min, or control 9 μg/ml Fetal Calf Serum (FCS). The Dual-Glo luciferase reporter assays were performed 48 hr later. The firefly luciferase activity was normalized to Renilla luciferase activity (relative light unit, RLU). Luciferase reporter assays were performed 3 times in triplicate.

Heparin affinity chromatography
Protein samples produced in HEK293T cells were freshly purified by SEC and then adjusted in 50 mM Tris, pH 7.5, 0.25 M NaCl. Purified protein (0.5 mg) was loaded onto a 1 ml HiTrap heparin HP column (GE Healthcare Life Sciences) equilibrated in 20 mM Tris, pH 7.5, 0.25 M NaCl and eluted with a linear NaCl gradient to 20 mM Tris, pH 7.5, 2 M NaCl, 5% (wt/vol) glycerol over 10 column volumes. Notably, we found that Norrin–Fz4CRD complex tends to partially disassemble (Fz4CRD detected in flow-through; Figure 7A) during sample preparation for the heparin binding assay (NaCl concentration was reduced from 0.5M to 0.25M).

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**Author contributions**

T-HC, Development of a new mammalian expression system, Conception and design, Acquisition of data from protein production to structural and functional experiments, Analysis and interpretation of data, Drafting or revising the article; F-LH, Acquisition of data (tissue cultures, protein production, and functional assays), Analysis and interpretation of data; MZ, Acquisition of data (crystallography), Revising the article; KH, Acquisition of data (crystallography); JE, Acquisition of data (SEC-MALS and SAXS), Drafting SAXS section; EYJ, Conception and design, Drafting or revising the article

**Additional files**

**Major datasets**

The following datasets were generated:

| Author(s)       | Year | Dataset title                                             | Dataset ID and/or URL                                                                 | Database, license, and accessibility information                                                                 |
|-----------------|------|-----------------------------------------------------------|--------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------|
| Chang TH, Hsieh FL, Zebisch M, Harlos K, Jones EY | 2015 | Crystal structure of Norrin in complex with the cysteine-rich domain of Frizzled 4 and sucrose octasulfate | http://www.rcsb.org/pdb/explore/explore.do?structureId=5BQC | Publicly available at RCSB Protein Data Bank (Accession No. 5BQC).                                             |
| Chang TH, Hsieh FL, Harlos K, Jones EY          | 2015 | Crystal structure of Norrin in complex with the cysteine-rich domain of Frizzled 4 -Methylated form | http://www.rcsb.org/pdb/explore/explore.do?structureId=5BQE | Publicly available at RCSB Protein Data Bank (Accession No. 5BQE).                                             |
| Chang TH, Hsieh FL, Harlos K, Jones EY          | 2015 | Crystal structure of Norrin, a Wnt signalling activator, Crystal Form I | http://www.rcsb.org/pdb/explore/explore.do?structureId=5BPU | Publicly available at RCSB Protein Data Bank (Accession No. 5BPU).                                             |
| Chang TH, Hsieh FL, Harlos K, Jones EY          | 2015 | Crystal structure of Norrin, a Wnt signalling activator, Crystal Form II | http://www.rcsb.org/pdb/explore/explore.do?structureId=5BQ8 | Publicly available at RCSB Protein Data Bank (Accession No. 5BQ8).                                             |
| Chang TH, Hsieh FL, Harlos K, Jones EY          | 2015 | Crystal structure of Norrin, a Wnt signalling activator, Crystal Form III | http://www.rcsb.org/pdb/explore/explore.do?structureId=5BQ8 | Publicly available at RCSB Protein Data Bank (Accession No. 5BQ8).                                             |
| Chang TH, Hsieh FL, Harlos K, Jones EY          | 2015 | Crystal structure of the cysteine-rich domain of human Frizzled 4 - Crystal Form I | http://www.rcsb.org/pdb/explore/explore.do?structureId=5BPB | Publicly available at RCSB Protein Data Bank (Accession No. 5BPB).                                             |
### The following previously published datasets were used:

| Author(s) Year | Dataset title                                                                 | Dataset ID and/or URL                                                                 | Database, license, and accessibility information                                                                 |
|----------------|--------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------|
| Chang TH, Hsieh FL, Harlos K, Jones EY 2015 | Crystal structure of the cysteine-rich domain of human Frizzled 4 - Crystal Form II | http://www.rcsb.org/pdb/explore/explore.do?structureId=5BQP                             | Publicly available at RCSB Protein Data Bank (Accession No. 5BQP).                                               |
| Dann III CE, Hsieh JC, Rattner A, Sharma D, Nathans J, Leahy DJ 2001 | Crystal structure of the cysteine-rich domain of mouse Frizzled 8 (Mfz8)           | http://www.rcsb.org/pdb/explore/explore.do?structureId=1IY                               | Publicly available at RCSB Protein Data Bank (Accession No. 1IY).                                               |
| Ke J, Harikumar KG, Erice C, Chen C, Gu X, Wang L, Parker N, Cheng Z, Xu W, Williams BO, Melcher K, Miller LJ, Xu HE 2013 | Crystal Structure of Norrin in fusion with Maltose Binding Protein                  | http://www.rcsb.org/pdb/explore/explore.do?structureId=4MYF2                             | Publicly available at RCSB Protein Data Bank (Accession No. 4MYF2).                                              |
| Janda CY, Waghray D, Levin AM, Thomas C, Garcia KC 2012 | Crystal structure of XWnt8 in complex with the cysteine-rich domain of Frizzled 8 | http://www.rcsb.org/pdb/explore/explore.do?structureId=1IY                               | Publicly available at RCSB Protein Data Bank (Accession No. 1IY).                                               |
| Dann III CE, Hsieh JC, Rattner A, Sharma D, Nathans J, Leahy DJ 2001 | Crystal structure of the Cysteine-rich domain of secreted Frizzled-related protein 3 (Sfrp-3,Fzb) | http://www.rcsb.org/pdb/explore/explore.do?structureId=1IY                               | Publicly available at RCSB Protein Data Bank (Accession No. 1IY).                                               |
| Stiegler AL, Burden SJ, Hubbard SR 2009 | Crystal Structure of the Frizzled-like Cysteine-rich Domain of MuSK                | http://www.rcsb.org/pdb/explore/explore.do?structureId=3HKL                              | Publicly available at RCSB Protein Data Bank (Accession No. 3HKL).                                               |
| Nachtergaele S, Whalen DM, Mydock LK, Zhao Z, Malinauskas T, Krishnan K, Ingham PW, Covey DF, Siebold C, Rohatgi R 2013 | Crystal structure of the Smoothened CRD, native                                    | http://www.rcsb.org/pdb/explore/explore.do?structureId=4C79                             | Publicly available at RCSB Protein Data Bank (Accession No. 4C79).                                               |
| Rana R, Carroll CE, Lee HJ, Bao J, Marada S, Grace CR, Guibao CD, Ogden SK, Zheng JJ 2013 | Solution structure of Smoothened                                                 | http://www.rcsb.org/pdb/explore/explore.do?structureId=2MAH                             | Publicly available at RCSB Protein Data Bank (Accession No. 2MAH).                                               |

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