

Structural Analysis of Wnt Signalling Pathways



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Abstract

Wnt signalling through its principal receptor Frizzled (Fzd) is one of the key determinants of metazoan development. Fzd is a member protein of the G-protein coupled receptor family, yet despite a wealth of functional information available on various aspects of its role within cells and during development, the molecular mechanism for Wnt-activated Fzd signalling has remained unknown. To shed light on this question, this project pursued a cryo-EM approach to obtain structures of different Fzd signalling complexes. To facilitate the assembly of complexes containing transmembrane regions of several proteins, as well as their subsequent examination by cryo-EM, I also explored the reconstitution of Fzd within two detergent-free systems, nanodiscs and SMALPs. Both systems posed unique challenges for obtaining well-behaved samples, ultimately proving to be unsuitable for sample examination by cryo-EM. I then returned to detergent systems, focusing on the assembly of Fzd4 in complex with an atypical ligand Norrin and bound Fab fragments, which, after an extensive sample optimization strategy, appeared to be too flexible for structure determination. This may support the theory that instead of functioning as a classic GPCR with defined intrahelical movements and a rigid structure upon activation, Fzd acts as a passive scaffold that brings different intra- and extra-cellular components together to result in signalling.

In parallel to this project, I successfully determined the structures of several small molecule inhibitors in complex with the Wnt deacetylase Notum via X-ray crystallography. This led to the identification of several key determinants controlling the affinity of molecules binding to the Notum catalytic site, thus aiding further drug development targeting this protein.

Declaration of work

All the work described herein is my own, except that detailed immediately below:

4. Structural characterization of Notum inhibitors:

- Treatment of purified hNot-cryst. with endoglycosidase H was performed by Dr Luca Vecchia.
- Crystallization and X-ray diffraction data collection of hNot-cryst. was done together with Dr Luca Vecchia.
- hNot-cryst. crystal soaking and mounting was completed by or with help of Dr Luca Vecchia and Dr Karl Harlos.
- Synthesis of Notum inhibitors and determination of their IC₅₀ values was performed by members of the Fish group (University College London).

Appendix I – Structure of SARS-CoV-2 Spike protein in complex with H11-D4 nanobodies:

- Isolation, affinity maturation, and purification of the H11-D4 nanobody was performed by Dr Jiandong Huo.
- Cryo-EM data collection of SARS-CoV-2 Spike in complex with H11-D4 was set up by Dr Loic Carrique and Dr Helen Duyvesteyn.
- Motion correction of movies in Relion v3.1 was performed by Dr Loic Carrique.

Contents

Abstract.....	2
Declaration of work	3
Table of figures	9
1. Introduction.....	15
1.1. General function of Wnt signalling in the human body	16
1.2. Wnt family of ligands.....	17
1.2.1. Wnt production, modification, and secretion	18
1.2.2. Transport of Wnt in the extracellular space	20
1.3. Notum is a negative regulator of Wnt activity	21
1.4. Thienopyrimidines as inhibitors of Notum	23
1.5. Frizzled (Fzd) family of receptors.....	24
1.5.1.Fzd structure and function	25
1.5.2. Fzd dimerization	28
1.6. Wnt-dependent signalling pathways	29
1.6.1. Canonical Wnt signalling	30
1.6.2. Non-canonical Wnt signalling	34
1.7. Specificity of Wnt and Fzd interactions and their functional outcomes	38
1.8. Norrin (Ndp).....	38
1.9. Wnt signalling in disease	41
1.9.1. Wnt signalling in cancer	41

1.9.2. Wnt signalling, aging, and neurodegenerative disease.....	42
1.10. Challenges in studying Wnt signalling	45
1.11. Cryo-EM of membrane proteins	47
1.12. Nanodisc reconstitution of membrane proteins.....	49
1.13. SMALPs – direct membrane protein solubilization from a native environment	50
1.14. Nanobodies as structural tools for membrane biology.....	50
1.15. Main goals of this study	53
2. Materials and methods	54
2.1. Constructs.....	54
2.1.1. Fzd	54
2.1.2. Dvl	55
2.1.3. Fzd co-receptors	56
2.1.4. Notum	56
2.1.5. Fzd ligands and afamin.....	57
2.2. Cloning and creation of expression constructs.....	57
2.3. Mammalian cell culturing	58
2.3.1. Freezing and thawing cells	58
2.3.2. Mammalian cell culture in flasks	59
2.3.3. Suspension cultures of mammalian cells.....	59
2.3.4. Culture of mammalian cells in roller bottles	60
2.4. Establishment of stable mammalian cell lines	60
2.5. Methods of protein expression	61

2.5.1. Transient transfection of suspension cultures.....	61
2.5.2. Transient transfection of cultures in roller bottles.....	62
2.5.3. Protein expression using stable cell lines in suspension	62
2.6. Protein production and purification	63
2.6.1. Frizzled	63
2.6.2. Dvl	65
2.6.3. Ndp	66
2.6.4. Notum	66
2.6.5. Nanobodies	67
2.6.6. F2.A Fab fragments	68
2.6.7. Nanodisc scaffold proteins	70
2.7. Nanodisc reconstitution.....	71
2.8. Protein extraction and purification using SMALPs	72
2.9. Protein complex formation in detergent.....	73
2.10. Protein expression tests	73
2.10.1. Using size-exclusion chromatography	73
2.10.2. Using fluorescence microscopy	74
2.11. Yeast Surface Display	74
2.11.1. Yeast library culturing and expansion	74
2.11.2. Magnetic-activated cell sorting (MACS)	75
2.11.3. Fluorescence-activated cell sorting (FACS).....	76
2.11.4. Enzyme-linked immunosorbent assay (ELISA)	78

2.12. Biolayer interferometry (BLI)	79
2.12.1. Protein biotinylation	79
2.12.2. Using the BLItz and Octet instruments	79
2.13. Electron Microscopy (EM)	80
2.13.1. Negative-stain EM.....	80
2.13.2. Cryo-EM Screening and Data collection.....	80
2.13.3. Cryo-EM data processing	81
2.14. Notum crystallization, data collection, and structure determination.....	83
3. Structural characterization of Fzd signalling complexes	86
3.1. Production of Fzd	87
3.1.1. Optimization of Fzd6 production	87
3.1.2. Exploring Fzd dimer formation	95
3.1.3. Stabilization of Fzd6 dimers.....	97
3.2. Expression and characterization of potential Fzd binding partners	101
3.2.1. Expression of co-receptors	101
3.2.2. Expression of Wnt	104
3.2.3. Dvl domain expression and interactions with Fzd6	106
3.2.4. Selection of nanobodies against Fzd6 using yeast surface display	112
3.3. Reconstitution of Fzd in detergentless systems	121
3.3.1. Fzd reconstitution in nanodiscs	121
3.3.2. Fzd reconstitution in SMALPs	134
3.3.3. Formation of Fzd4 and Fab fragment in SMALPs.....	143

3.4. Assembly and structural characterization of Fzd4/Ndp/Fab complex in detergent micelles	150
3.4.1. Optimization of Fzd4-1/Ndp3/F2.A-2 complex formation	150
3.4.2. Structural analysis of Fzd4-1/Ndp3/F2.A-2 complex	157
3.5. Discussion	165
4. Structural characterization of Notum inhibitors	171
4.1 Thienopyrimidine-derived Notum inhibitors	172
4.2. Heteroaryl inhibitors of Notum	177
4.3. Optimized heteroaryl inhibitors	182
4.4. Discussion	185
5. References.....	188
6. Appendix I – Structure of SARS-CoV-2 Spike protein in complex with H11-D4 nanobodies	214
6.1. Introduction	214
6.2. Expression and purification of SARS-CoV-2 Spike protein	215
6.3. SARS-CoV-2 Spike/H11-D4 cryo-EM data collection, processing, and modelling.....	216
6.4. Results	217
7. Appendix II – Supplementary data	222

Table of figures

Fig. 1. Production and secretion of mature Wnt.	19
Fig. 2. The active site of Notum	22
Fig. 3. Structure-activity relationships of a thienopyrimidine-based Notum inhibitor... 23	
Fig. 4. The Fzd family of receptors.	25
Fig. 5. Interaction between Fzd8 CRD and <i>X. laevis</i> XWnt8.	27
Fig. 6. hFzd5 CRD dimer in complex with palmitoleic acid	28
Fig. 7. Canonical Wnt signalling	31
Fig. 8. PCP pathway	35
Fig. 9. Ca ²⁺ signalling pathway.....	37
Fig. 10. Interaction between Ndp and Fzd4 CRD.....	39
Fig. 11. Reconstitution of transmembrane proteins within nanodiscs and SMALPs	49
Fig. 12. Yeast surface display of nanobody libraries.....	51
Fig. 13. Fzd Constructs	54
Fig. 14. Constructs of full-length Dvl1 and Dvl2	55
Fig. 15. Constructs of ROR2 and LRP6	56
Fig. 16. Notum crystallization construct.....	56
Fig. 17. Fzd ligand and afamin constructs	57
Fig. 18. Production of Fzd6-4 using transient transfection of HEK293S cells	88
Fig. 19. Production of Fzd6-4 using stable HEK293S GnTI ⁻ TetR cell lines.....	90
Fig. 20. Fzd6-4 expression test	91
Fig. 21. Production of cleaved and uncleaved Fzd6-5.....	94
Fig. 22. Purification of Fzd6-5/Fzd6-8 dimer	96
Fig. 23. Predicted Fzd6-5 dimer transmembrane domain interface.....	97
Fig. 24. Purification of Fzd6-5 dimer mutants.....	99

Fig. 25. Fluorescence of stable HEK293S GnTI ⁻ TetR cell lines expressing various Fzd co-receptor constructs.....	102
Fig. 26. FACS of HEK293S GnTI ⁻ TetR cells expressing ROR2-1 and LRP6-3	102
Fig. 27. Fluorescence of sorted HEK293S GnTI ⁻ TetR cell lines expressing LRP6-3.	103
Fig. 28. Expression test of stable HEK293S GnTI ⁻ TetR cells co-expressing Wnt3A or Wnt5A with afamin (AFM).....	105
Fig. 29. Dvl construct design.	106
Fig. 30. Purification of Dvl DEP domains.....	107
Fig. 31. Purification of Dvl PDZ domains.....	108
Fig. 32. Purification of Dvl PDZ-DEP domain constructs	109
Fig. 33. Interaction between Fzd6-6 and Dvl2 constructs	110
Fig. 34. SEC of Fzd6-5 and Dvl2-DEP mixture	111
Fig. 35. FACS of yeast cells expressing the nanobody library.....	113
Fig. 36. Titration of biotinylated Fzd6-7 onto selected yeast cells.....	114
Fig. 37. Sequences of nanobodies selected via ELISA.....	115
Fig. 38. Large-scale purification of selected nanobodies	117
Fig. 39. Interaction between Fzd6-7 and purified nanobodies.	118
Fig. 40. Interaction between Fzd6-7 and QE124.....	119
Fig. 41. ELISA measuring the interaction between Fzd6-7 and QE124.	120
Fig. 42. Purification of MSP2N2	122
Fig. 43. Reconstitution of Fzd6-5 within MSP2N2 nanodiscs.	124
Fig. 44. Optimisation of POPC content during Fzd6-5 reconstitution within MSP2N2 nanodiscs.	125
Fig. 45. Examination of Fzd6-5/MSP2N2/POPC (0.2:1:190) nanodiscs by negative-stain EM	126

Fig. 46. Reconstitution of Fzd6-5 within MSP1D1 nanodiscs.	127
Fig. 47. Examination of Fzd6-5/MSP1D1/POPC (0.07:1:65) nanodiscs by negative-stain EM	128
Fig. 48. Optimisation of POPC content within MSP1E3 nanodisc reconstitution mixtures	129
Fig. 49. Optimisation of Fzd6-5/MSP1E3/POPC nanodisc formation	130
Fig. 50. Examination of Fzd6-5/MSP1E3/POPC (0.01:1:130) nanodiscs by negative- stain EM.....	133
Fig. 51. Purification of Fzd6-5 SMALPs.....	135
Fig. 52. Cryo-EM of Fzd6-5 SMALPs	136
Fig. 53. Purification of Fzd6-5/Wnt5A-1 SMALPs	138
Fig. 54. Purification of Fzd4-1/Ndp1 SMALPs.....	140
Fig. 55. Cryo-EM of Fzd4-1/Ndp1 SMALPs.	142
Fig. 56. Fzd4-1 and F2.A complex	144
Fig. 57. Aggregation upon addition of F2.A to Fzd4/Ndp1 SMALPs.....	146
Fig. 58. Optimisation of Fzd4-1/Ndp1/F2.A-2 concentration on cryo-EM grids.....	148
Fig. 59. Purification of Ndp3	151
Fig. 60. Fzd4-1 and Ndp3 complex formation.....	152
Fig. 61. Fzd4-1 and Ndp3 complex formation at different Fzd4-1 : Ndp3 molar ratios	153
Fig. 62. Further optimisation of Fzd4-1 and Ndp3 complex formation	154
Fig. 63. Fzd4-1/Ndp3 complex formation in the absence of CHS	156
Fig. 64. Fzd4-1/Ndp3/F2.A-2 complex formation.....	157
Fig. 65. Large-scale purification of Fzd4-1/Ndp3-F2.A-2 complex.....	158
Fig. 66. Large-scale purification of cleaved Fzd4-1/Ndp3/F2.A-2 complex.....	160

Fig. 67. Cryo-EM of cleaved Fzd4-1/Ndp3/F2.A-2 complex.....	161
Fig. 68. Cryo-EM data processing of the Fzd4-1/Ndp3/F2.A-2 complex	162
Fig. 69. Cryo-EM data processing of the Fzd4-1/Ndp3/F2.A-2 complex (cont.).....	164
Fig. 70. Purification of hNot-Cryst.....	173
Fig. 71. Inhibitor '67' in complex with Notum.....	174
Fig. 72. Electron density of Notum inhibitors '1128' and '1147'	176
Fig. 73. Heteroaryl inhibitors of Notum	177
Fig. 74. Inhibitor '267' in complex with Notum.....	178
Fig. 75. Inhibitor '223' in complex with Notum.....	179
Fig. 76. Inhibitor '263' in complex with Notum.....	180
Fig. 77. Inhibitor '276' in complex with Notum.....	181
Fig. 78. Improved heteroaryl inhibitors of Notum.....	182
Fig. 79. Inhibitor '1233' in complex with Notum.....	183
Fig. 80. Inhibitors '1185' and '1241' in complex with Notum.....	184
Fig. 81. Notum inhibitors '223' and '1043'	185

A. fig. 1. Purification of SARS-CoV-2 Spike protein	216
A. fig. 2. Cryo-EM data processing of SARS-CoV-2 Spike protein in complex with H11-D4.....	218
A. fig. 3. Model of SARS-CoV-2 Spike protein in complex with H11-D4.....	219
A. fig. 4. Alignment of SARS-CoV-2 Spike/H11-D4 with a SARS-CoV-2 RBD/ACE2 complex	221

S. fig. 1. SEC of Fzd6-5(M237C/A348C) expressed at 30° C	222
S. fig. 2. Localization of different proteins expressed in HEK293S GnT ^I - TetR cells .	222
S. fig. 3. Reducing SDS-PAGE of Dvl1-PDZ without sample heating.....	223

S. fig. 4. Selection of yeast cells for FACS.	223
S. fig. 5. FACS detecting nanobody expression levels in a synthetic yeast surface display library.....	224
S. fig. 6. ELISA selection of nanobodies from a yeast surface display library	225
S. fig. 7. MSP2N2 expression test	225
S. fig. 8. Second and third purification of Fzd6-5 SMALPs	226
S. fig. 9. Examination of Fzd6-5/Wnt5A SMALPs by cryo-EM	227
S. fig. 10. Purification of 3C-cleaved and uncleaved Fzd4-1 in DDM.....	228
S. fig. 11. Cryo-EM processing of Fzd6-5 in SMALPs.....	230
S. fig. 12. Cryo-EM processing of Fzd4-1/Ndp1 in SMALPs.....	231
S. fig. 13. Cryo-EM processing of Fzd4-1/F2.A in DDM	232
S. fig. 14. Cryo-EM processing of Fzd4-1/F2.A in DDM.....	233
S. fig. 15. Final cryo-EM volume of Fzd4-1/F2.A in DDM.....	233
S. fig. 16. Cryo-EM processing of Fzd4-1/Ndp3/F2.A-2 in DDM.....	234
S. fig. 17. Cryo-EM processing of Fzd4-1/Ndp3/F2.A-2 in DDM.....	235
S. fig. 18. Final cryo-EM volume of Fzd4-1/Ndp3/F2.A-2 in DDM	235
S. fig. 19. Cryo-EM processing of Fzd4-1/Ndp3/F2.A-2 in DDM (cleaved).....	236
S. fig. 20. Cryo-EM 2D class averages of Fzd4-1/Ndp3/F2.A-2 in DDM (cleaved) ...	237
S. fig. 21. Heterogenous refinement of Fzd4-1/Ndp3/F2.A-2 complex in DDM.....	237
S. fig. 22. Validation of the structure of Notum in complex with compound 263	240
S. fig. 23. Cryo-EM processing of SARS-CoV-2 Spike in complex with H11-D4 nanobody	242
S. fig. 24. Cryo-EM 2D classification of SARS-CoV-2 Spike in complex with H11-D4 nanobody	242

S. Table 1. Cryo-EM data collection and processing statistics.....	229
S. Table 2. X-ray data collection and refinement statistics for Notum in complex with various compounds.....	238
S. Table 3. X-ray data collection and refinement statistics for Notum in complex with various compounds (cont.)	239
S. Table 4. Cryo-EM data collection and refinement statistics for SARS-CoV-2 Spike protein in complex with H11-D4 nanobody.....	241

1. Introduction

1.1. General function of Wnt signalling in the human body

The development of all multicellular organisms starts from a single cell, which then grows, divides, differentiates, migrates, and adheres to other cells in a very strict, genetically predetermined way to ensure the formation of a mature, healthy organism that is capable of surviving and interacting with its environment (Alberts et al., 2002). This is no simple task, requiring the coordination of many different cellular and intercellular processes in an extremely precise fashion, therefore it is initiated and carried out by a myriad of tightly regulated, interconnected biochemical mechanisms and signalling pathways that continue to exert their influence on the cells of an organism all throughout its lifetime to maintain both tissue homeostasis and renewal (Pires-daSilva and Sommer, 2003).

Signalling by the Wingless/Int (Wnt) family of secreted glycoproteins is one of the core regulatory pathways imparting shape and directionality on developing tissues in metazoan development. This ancient and evolutionarily conserved pathway has a pivotal role in coordinating the establishment of organized body plans within organisms ranging from sponges to humans (Logan and Nusse, 2004). Wnt signalling pathways can alter gene expression, cytoskeleton dynamics, and the activity of various other signalling pathways, thus regulating a vast range of cellular processes, including cell cycle kinetics, growth, polarity, differentiation, proliferation, cell fate, organelle dynamics, and migration. Together, these pathways act to control higher-order processes of tissue morphogenesis, homeostasis, and plasticity, organogenesis, neural patterning and synaptogenesis, as well as maintenance of stem cell populations.

Due to these roles in cell and tissue function, it is unsurprising that Wnt signalling pathways are implicated in a variety of pathophysiological disorders (Ng et al., 2019). These include functions for Wnt signalling in oncogenic transformation, tumour progression, and metastasis. Altered Wnt signalling has also been implicated in severe birth defects and neurological developmental disorders. Components of this system are thus viewed as important pharmacological targets for the development of novel medications.

Despite the importance of the Wnt pathway in both normal cell function and disease states, structural mechanistic details of the molecular processes underlying Wnt signalling have remained mysterious. This is due to the large number of Wnt family ligands, receptors, co-receptors, and modulators involved. This creates a high level of complexity that enables different signalling outcomes, but complicates meaningful structural analysis. Furthermore, the production of high quantities of purified Wnt for biochemical studies has proven challenging, thus limiting structural and mechanistic analysis of the signalling process (Mihara et al., 2016). In the remainder of this chapter, I will briefly summarize what is known about the properties and functions of the components of Wnt signalling pathways, starting with the family of Wnt ligands, then moving on to the receptor Frizzled and other components implicated in signalling.

1.2. Wnt family of ligands

Members of the Wnt family are ~40 kDa secreted glycolipoproteins (Miller, 2001). They are regarded as the primary activators of canonical Wnt signalling due to their ability to bind Fzd with high affinity and elicit a biological response. The mammalian genome encodes 19 different Wnt proteins, which all are predicted to share the same unique α -helical fold rich in disulphide bridges. Despite the structural similarity of the

Wnt family members, knockout mice deficient in individual Wnts exhibit as many different phenotypes as there are Wnts, suggesting that each Wnt protein serves a specific function within the developing organism (van Amerongen and Berns, 2006). It is not clear whether this is due to their propensity to activate different Fzd signalling pathways to varying extents, or whether these phenotypic differences exist only due to their specific expression patterns.

1.2.1. Wnt production, modification, and secretion

After translation of Wnt-encoding mRNA and translocation of the polypeptide chain into the lumen of the endoplasmic reticulum (ER), Wnts are glycosylated. Experimental data have shown that Wnt1/3A/5A/5B/6/7B enter the ER and are glycosylated on at least two sites - N87 and N298 (residue positions according to hWnt3A) (Kikuchi et al., 2011). By contrast, Wnt5A has been shown to be modified on at least N114, N120, N311 and N325. The glycans attached to Wnt3A are sensitive to glycosidase F and endoglycosydase H, indicating that Wnt3A is modified with high-mannose or hybrid-type N-linked glycans. However, the impact of differences in glycosylation on the activity of Wnts are not clear. Mutations removing Wnt glycosylation sites often result in impaired secretion, suggesting they are important for biogenesis of active Wnt (Komekado et al., 2007). However, Wnts still maintain their activity when deglycosylated *in vitro*, suggesting it is not crucial for their signalling function (Kurayoshi et al., 2007).

The majority of Wnts are also acylated by the ER membrane-bound O-acyltransferase Porcupine (PORCN) (Fig. 1) (Torres et al., 2019). This enzyme attaches 14-16 carbon-long, monounsaturated fatty acids to Wnt proteins utilising a dedicated pool of fatty acyl-CoA generated by the action of stearoyl CoA-desaturase SCD1. In mice, there are

4 different isoforms of PORCN (A-D), all of which are generated by a single gene by alternative splicing, but their individual roles in Wnt modification are not clear (Tanaka et al., 2000). The most common type of acylation found in mature Wnt is the palmitoylation of a conserved serine residue (S209 in Wnt3A). This modification is required for efficient secretion of Wnt, as well as playing a crucial role in the activation of signalling (Hosseini et al., 2018).

The release of glycosylated, acylated Wnt from the cell is mediated by the protein Wntless (Wls) (Fig. 1). This evolutionarily conserved eight-pass transmembrane carrier protein is localized to the endoplasmic reticulum (ER). Following acylation of Wnt by PORCN, it is directly loaded onto Wls, which shields its lipid modification in order to prevent aggregation (Nygaard et al., 2021). The Wnt/Wls complex is then transported to the plasma membrane via the Golgi apparatus and exocytic vesicles. The mechanism of Wnt release from Wls is unclear, but the prevailing theory is that is associated with acidification of the Wnt/Wls containing vesicles (Coombs et al., 2010).

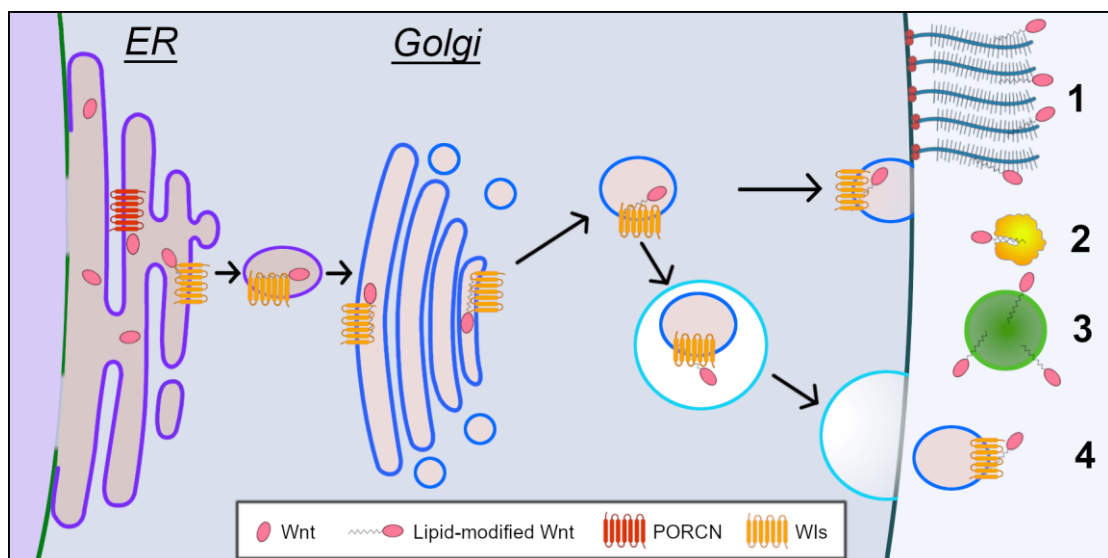


Fig. 1. Production and secretion of mature Wnt. Following synthesis, immature Wnt is glycosylated and lipidated by PORCN. It is then recognized and bound by Wls in the Golgi apparatus, which mediates its secretion to the extracellular space. Due to its hydrophobic nature, secreted Wnt in the extracellular space is found either (1) bound to proteoglycans; (2) bound to carrier proteins; (3) within lipoprotein particles; or (4) on the surface of exocytic vesicles. Adapted from Nusse and Clevers, 2017.

1.2.2. Transport of Wnt in the extracellular space

The presence of a lipid moiety confers extreme hydrophobicity to the Wnt molecule, which restricts its diffusion in the extracellular space. Several mechanisms have been implicated in mediating Wnt transport outside of cells, such as the binding to carrier proteins, interaction with proteoglycans, or incorporation within lipoprotein particles or exosomes.

One of the first proteins identified as a carrier of Wnt in the extracellular space is the secreted Wingless-interacting molecule (Swim). This lipocalin-family protein binds and sequesters the lipid moiety of Wg (a Wnt homologue in the fly), thus facilitating long-range signalling (Mulligan et al., 2012). In humans, lipocalins with similar activity have not been identified, although α -albumin (also known as afamin) has been shown to bind Wnt, thus making it soluble in solution (Mihara et al., 2016). Finally, secreted Frizzled-related proteins (sFRPs) are also known to recognize Wnt in a fashion similar to its receptor (Galli et al., 2006). Although generally associated with inhibition of their signalling activities, sFRPs might also play a role in Wnt transport.

In addition to carrier proteins, Wnt is also known to be concentrated on the surface of cells by interacting with heparan sulfate (HS)-containing proteoglycans (Fig. 1.1) (Reichsman et al., 1996). Of particular importance are the glypicans Dally and Dlp, which are known to sequester fly Wnt homologue Wg and modulate its signalling activities (Mii and Takada, 2020). Recent structural studies have shown that the lipid moiety of Wnt can be shielded from the aqueous environment by a hydrophobic pocket present in Dlp, thus preventing its aggregation and enabling restricted diffusion across the surface of the cell (McGough et al., 2020). A similar pocket is not present in Dally, therefore suggesting it might facilitate Wnt diffusion by some other mechanism, such as

interactions via its heparan sulfate chains (Dhoot et al., 2001). In humans, six types of glypican (GPC1-6) have been implicated in regulating Wnt signalling, which might function in a fashion similar to their fly homologues (Kikuchi et al., 2011).

Wnt can also spread in the extracellular environment via high-density lipoprotein (HDL) particles or exosomes (Fig. 1.3/4). Wnt3A and Wnt5A have been shown to co-purify with lipoprotein particles from cultured media, and their release from the cell surface directly corresponds to the concentration of HDL present, suggesting that they play a specific role in Wnt transport (Kaiser et al., 2019; Neumann et al., 2009). Both mammalian and *Drosophila* Wnts can also be present on the outer leaflet of exosomes, with one study indicating that exosome-bound Wg is responsible for 20-40% of total signalling activity within *Drosophila* cell cultures (Beckett et al., 2013; Gross et al., 2012). Since Wls can also be found in exosomes (Koles et al., 2012), it is possible that a fraction of Wnt/Wls-containing vesicles are transported to multivesicular bodies for exosome generation instead of direct secretion at the plasma membrane.

In conclusion, the transport of Wnt in the extracellular space relies on a variety of mechanisms. The specific biochemical properties of each Wnt protein could potentially bias it towards utilization of specific transport modes, thus modulating their long- and short-range activity. The role of co-operation between these mechanisms is still unclear, although it is likely to be important for coordinating mammalian development.

1.3. Notum is a negative regulator of Wnt activity

Notum is a highly conserved, secreted antagonist of Wnt signalling, which belongs to the α/β -hydrolase superfamily (Fig. 2.A) (Giráldez et al., 2002). Notum was initially thought to act as a phospholipase shedding glypicans, and thus bound Wnt, from the cell

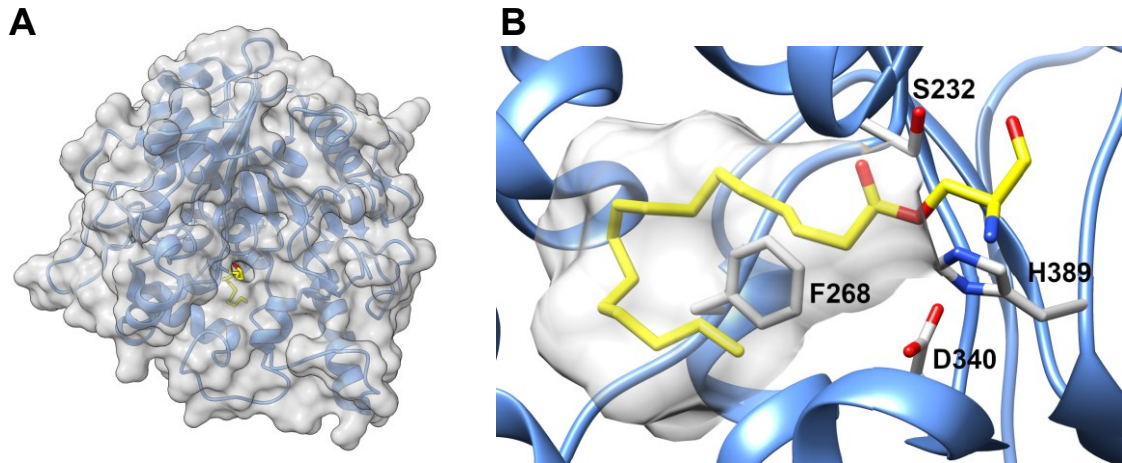


Fig. 2. The active site of Notum. **A** – The overall structure of Notum. **B** – O-palmitoleoylated serine bound within the hydrophobic cavity of Notum. The carbonyl group of palmitoleoyl moiety positions itself close to S232, which forms a catalytic triad with H389 and D340. The long aliphatic chain of the substrate is positioned within the adjacent hydrophobic cavity. Carbon atoms of the substrate depicted in yellow, while the surface of the protein shown in grey. Based on Kakugawa et al., 2015 (PDB ID: 4UZQ), which contains an S232A mutation (here shown as serine).

surface by removing their glycosylphosphatidylinositol anchors. It is now known that this small glycoprotein acts by cleaving off the palmitoleate moiety from Wnt, thus rendering it inactive (Kakugawa et al., 2015). To carry out its function, Notum interacts with glypicans, bringing it in close proximity of Wnt. It then binds cis-unsaturated fatty acyl modifications of Wnt via a large hydrophobic cavity present on its surface. The entrance of the cavity is located next to a catalytic DHS triad (D340, H389, S232 in hNotum) (Fig. 2.B). The catalytic serine is positioned in the vicinity of the fatty acid carboxylate group, enabling Notum to act as a carboxylesterase. This activity results in the removal of palmitoleate from Wnt, thus preventing it from initiating signalling.

Notum plays a key role in synaptogenesis. It is present in the extracellular space surrounding developing synaptic boutons, where it negatively regulates synapse formation (Kopke et al., 2017). Null mutants of Notum show increased synapse number and strength, similarly to overstimulation of canonical Wnt/Fzd signalling. Inhibition of Notum in mice has also been shown to restore the regenerative capacity of stem cells in aged tissue (Pentinmikko et al., 2019). Since one of the hallmarks of neurodegenerative

disease (NDD) is the loss of neurons (Taoufik et al., 2018), Notum could therefore be a potential target for the development of inhibitors for use in the treatment of NDDs.

1.4. Thienopyrimidines as inhibitors of Notum

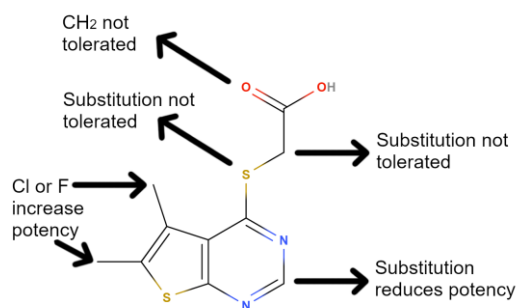


Fig. 3. Structure-activity relationships of a thienopyrimidine-based Notum inhibitor. Based on Tarver et al., 2016.

The first small-molecule inhibitors of Notum to be discovered were thienopyrimidine-based compounds, which exhibit nanomolar IC₅₀ values *in vitro*. They were identified using a high-throughput assay using purified Notum and a substrate generating fluorescent

reaction products (trisodium 8-octanoyloxypyrene-1,3,6-trisulfonate, OPTS) (Tarver et al., 2016). The structure of these Notum inhibitors consists of a thienopyrimidine ring system modified with a thioglycolate group on its 6-membered ring, as well as various aliphatic groups on its 5-membered ring (Fig. 3).

Chemical modification of thienopyrimidines has indicated that substitutions to the linker between the ring system and the carboxylate group are poorly tolerated, whereas addition of electron-rich aliphatic groups, such as chlorine or fluorine, lead to improvements of measured IC₅₀ values *in vitro* (Fig. 3) (Tarver et al., 2016).

These thienopyrimidine-based compounds were developed as treatments of osteoporosis, and they indeed showed bone density and strength improvements in mouse models by stimulating Wnt signalling (Tarver et al., 2016). Unfortunately, the presence of a carboxylate group results in poor blood-brain barrier penetration (Manallack et al., 2012), thus making this class of compounds unsuitable for treating NDDs.

1.5. Frizzled (Fzd) family of receptors

Members of the Frizzled family of proteins are the principal receptors responsible for recognising Wnt and initiating Wnt signalling (Huang and Klein, 2004). Frizzled proteins (Fzds) belong to the class F-type family of GPCRs (which only contain Fzd and Smoothened), but their homology to other GPCRs is mostly confined to the seven-transmembrane (7TM) α -helical bundle. Fzds are extremely widespread within metazoa, and most cell types are thought to express at least one Fzd family member. There are 10 different Fzd proteins, all of which are mostly localized to the plasma membrane, although certain Fzds are thought to prefer activation of specific downstream signalling cascades (Dijksterhuis et al., 2014), either canonical, non-canonical, or both, but the exact outcomes of signalling most likely depend on cellular context and the presence of signalling co-factors.

Specific Fzds have been associated with different physiological effects, for instance, it is known that Fzd5 plays a role in synaptogenesis and the development of Alzheimer's disease (Li et al., 2011; Sahores et al., 2010), Fzd4 is required for the proper development of microvasculature in the eye (Xu et al., 2004), Fzd10-mediated signalling is the most common culprit of colorectal cancer (Zhan et al., 2016), whereas Fzd6 seems to contribute to the development of various types of cancer and congenital defects (Corda and Sala, 2017; Naz et al., 2012). Similarly to Wnts, the plethora of different Fzd functions and roles in disease appears to stem from their differing expression patterns, as well as their preference for specific signalling pathway activation.

Since Fzds are GPCRs, and there are crystal structures available of their extracellular domains (Nile and Hannoush, 2018), the general architecture of the Fzd receptor

structure is known. Despite this, it is still unclear how exactly do these receptors mediate signalling. Do they function similarly to classical GPCRs, where defined intrahelical movements within the transmembrane bundle result in activation of specific downstream effectors (Schulte and Bryja, 2007), or do they act as a passive scaffold that only serves to bridge different intra- and extracellular components together upon ligand binding? What we do know for certain is that upon binding Wnt or other agonists, Fzd brings together several co-receptors and intracellular binding partners that result in specific signalling pathway activation (Schulte, 2015).

1.5.1.Fzd structure and function

Fzd proteins are 500-700 amino acids in length, forming an extracellular N-terminal cysteine-rich domain (CRD), a seven-transmembrane helical bundle (which shares its topology with other members of the GPCR superfamily), as well as a long and disordered intracellular C-terminal tail (Huang and Klein, 2004).

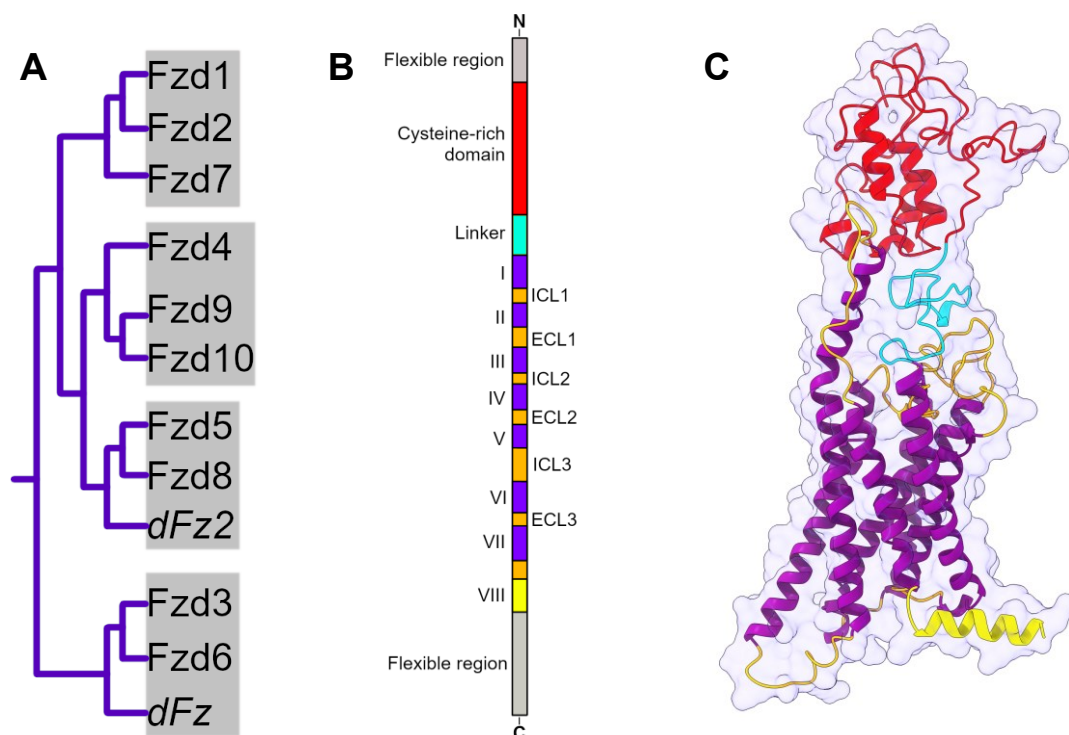


Fig. 4. The Fzd family of receptors. **A** – Phylogenetic tree of human and *D. melanogaster* Fzds. Based on Fredriksson et al., 2003. **B** – A general structure of Fzd. Helices labelled I – VIII. Intracellular and extracellular loops labelled as ICL and ECL respectively. **C** – homology model of Fzd6 based on the structure of *Smoothed* (PDB ID: 5L7D, Byrne et al., 2016).

Mammals contain 10 different Fzd proteins, which fall into 4 main clusters (Fig. 4.A) based on sequence identity: Fzd1/Fzd2/Fzd7 – approximately 75% shared identity between each Fzd pair, Fzd5/Fzd8 – 70%, Fzd3/Fzd9/Fzd10 – 65%, Fzd3/Fzd6 – 50% identity, whereas proteins between different clusters share only 20-40% identity (Fredriksson et al., 2003). Similarly to other GPCRs, most Fzd genes lack introns, except for hFzd5 and *D. melanogaster* dFzd2, which have one intron each, although the whole open reading frame is encoded as a single exon.

Highest levels of homology between different Fzds is found within the first 25 residues at the N-terminus of the intracellular tail, in the region close to the intracellular side of the plasma membrane that usually forms the intracellular helix VIII in classical GPCRs (Fig. 4.B/C) (McQuate et al., 2017). This region contains a conserved KTXXXW motif, which is essential for canonical Wnt signalling. Its primary role is believed to be with the binding of the intracellular protein Dishevelled (Dvl), which acts as the main mediator of Wnt signalling downstream of Fzd (Wong et al., 2003). Mutations in this motif, as well as ICLs 1 and 3 of Fzd, have been shown to abolish Wnt signalling, whereas the sequences flanking the conserved motif either directly or indirectly influence the ability of Fzd to interact with Dvl (Tauriello et al., 2012).

The transmembrane domain of Fzds is similar to other GPCRs (Fig. 4.B/C) - it contains conserved cysteines in extracellular loops (ECLs) 1 and 2, as well as a series of charged residues at the C- and N-terminus of intracellular loop 3 (ICL3). The major difference is the lack of the DRY motif at the C-terminus of transmembrane helix 3, which is important for heterotrimeric G-protein activation by class A GPCRs (Wright et al., 2019).

The N-terminal CRD is responsible for recognising Wnt ligands. It is formed by 120-125 residues with 10 conserved cysteines, all of which form cysteine bridges (Dann et

al., 2001). It is mostly α -helical, forming a unique fold with a compact, globular structure with a distinctive hydrophobic groove (Fig. 5.A). This groove is responsible for recognizing the palmitoleate modification present on Wnt (Fig. 5.B) (Janda et al., 2012). In addition, Wnt also makes interactions at a hydrophobic CRD site opposite to the lipid-binding groove, thus ‘grasping’ Fzd from both sides of its extracellular domain.

No structural information is available on the linker domain between the CRD and TMD of Fzd. It is hydrophilic, varies in length from 40 to 100 amino acids, and it is highly divergent between Fzd subtypes (Fredriksson et al., 2003). It has no known function, although it has been implicated in Dvl recruitment (Valnohova et al., 2018), while Fzd4 linker mutants exhibit strong familial exudative vitreoretinopathy phenotypes (DeBruine et al., 2017a).

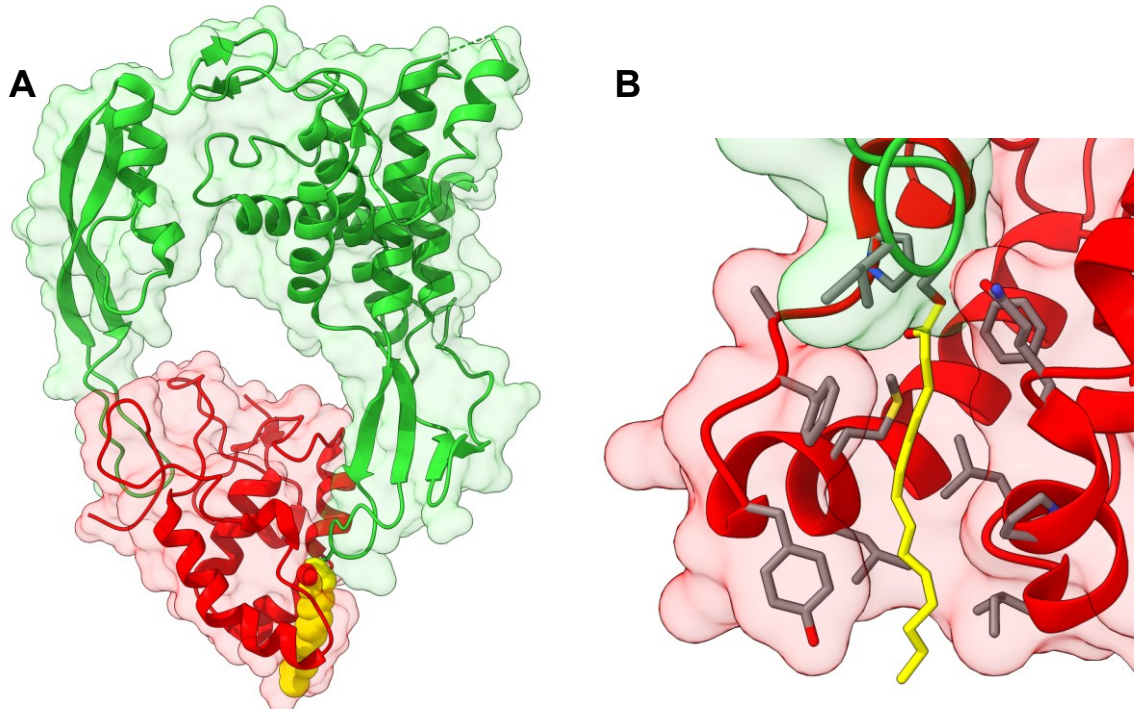


Fig. 5. Interaction between Fzd8 CRD and *X. laevis* XWnt8. **A** – Fzd8 CRD and XWnt8 complex. CRD – red; XWnt8 – green. The carbon atoms of O-palmitoleoylate modification shown in yellow. **B** – Recognition of O-palmitoleoylate by Fzd8 CRD. Residues within 5 Å distance of the O-palmitoleoylate shown as sticks. Based on Janda et al., 2012 (PDB ID: 4F0A).

1.5.2. Fzd dimerization

There is evidence that hints at the possibility that the oligomerization of Fzd is important for its signalling activities. Both Fzds and their CRDs have a propensity to homodimerize (Carron et al., 2003; Kaykas et al., 2003), and this can be modulated by Wnt stimulation (Nile et al., 2017; Petersen et al., 2017).

The crystal structures of Fzd CRDs show a dimer interface (Fig. 6) that is conserved between all Fzd CRDs except Fzd4 (Nile and Hannoush, 2018). This interface results in an extended U-shaped hydrophobic groove formed by the two CRDs. Individual grooves in the two CRDs converge at the dimer interface, which can completely bury a variety of free cis-fatty acids present in the crystallization mix. Interestingly, crystal structures of Fzd4 CRD lacking the conserved α -helical CRD dimer interface show the protein is still able to homodimerize in the presence of unsaturated fatty acids (DeBruine et al., 2017b). These observations suggest that the binding of Wnts to Fzd may stimulate their homodimerization in a lipid-dependent manner.

In addition to the conserved interfaces on Fzd CRDs, studies have suggested the presence of another homodimerization interface at the TMD, potentially formed by transmembrane helices IV and V (similarly to class B GPCRs). Evidence for this comes from studies of Fzd1 and Fzd2 lacking their CRD domains (Kaykas et al., 2003), as well

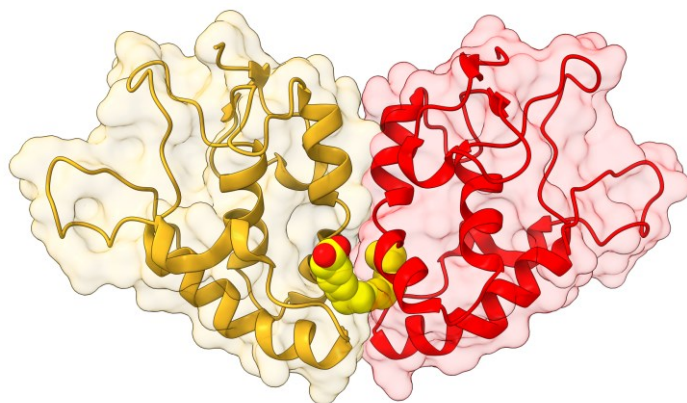


Fig. 6. hFzd5 CRD dimer in complex with palmitoleic acid. hFzd5 CRDs shown in red and gold. Palmitoleic acid shown as spheres. Based on Nile et al., 2017 (PDB ID: 5URY).

as mutants of full-length Fzd6 (Petersen et al., 2017), all of which have been reported to autodimerize. Interestingly, dissociation of Fzd6 homodimers was shown to initiate non-canonical signalling in the absence of ligand stimulation, thus suggesting it might play a role in signalling pathway selectivity.

Despite these observations, there is no conclusive evidence suggesting that Fzd homodimerization or homodimer dissociation is a general mechanism required for Wnt signalling *in vivo*. Most studies examining Fzd dimerization state within cells have relied on Fzd overexpression (Carron et al., 2003; DeBruine et al., 2017b; Petersen et al., 2017), therefore raising the possibility that Fzd might not be able to homodimerize at biologically relevant concentrations. Then again, forced homodimerization of *X. laevis* Xfz7 transmembrane domains leads to elevated levels of canonical signalling in comparison to wild-type protein (Carron et al., 2003), which suggests it must play a role in mediating signalling.

1.6. Wnt-dependent signalling pathways

The Wnt/Fzd system is capable of initiating downstream signalling through a variety of different pathways with specific cellular outcomes (Kikuchi et al., 2011). Despite their importance and decades of study, the exact mechanisms of various signalling pathway activation remain obscure.

It has been assumed that the binding of specific Wnts to a particular Fzd is responsible for determining the nature of the signalling output (Kikuchi et al., 2011). In this model, interactions between a particular Fzd family member and its intracellular binding partner Dishevelled (Dvl) and various co-receptors would result in a specific cellular response, usually associated with a particular signalling pathway, such as the canonical β -catenin pathway, or the non-canonical planar cell polarity (PCP) and Ca^{2+} pathways.

The different cellular responses associated with each pathway have led to the identification of various intracellular components required for mediation of specific signalling pathway activation, which have subsequently been classified as belonging to one of these aforementioned pathways.

Speaking against this simple model, several Wnt/Fzd combinations have been shown to activate multiple pathways simultaneously, or to activate only a specific pathway in certain cell types or conditions (Dijksterhuis et al., 2014). This poses the question - what exactly is it that serves as the determinant for specific signalling pathway activation? Studies have indicated that an interaction between Fzd and Wnt is not a strict requirement for the initiation of signalling (Hendrickx and Leyns, 2008; Povelones et al., 2005), whereas receptor, co-receptor and intracellular binding partner presence and phosphorylation status (Kikuchi et al., 2011; Verheyen and Gottardi, 2010), along with signalling complex endocytosis (Feng and Gao, 2015; Yamamoto et al., 2008), phospholipid and proteoglycan presence (McGough et al., 2020; Tanneberger et al., 2011), lipid raft microdomains (Sezgin et al., 2017), and intracellular pH (Cruciat et al., 2010) all seem to play a role in the determining when and which Wnt signalling pathway is activated. Such a wealth of different influencing factors makes it extremely difficult to pick out the contributions to the signalling pathway selection process, although this does provide ample opportunities for context and cell type-dependent signalling, which are key for the establishment of organized body plans.

1.6.1. Canonical Wnt signalling

After the discovery of genes responsible for the establishment of planar cell polarity in the fly (Gubb and García-Bellido, 1982), various studies started focusing on the role of Wnt signalling in animal development. This led to the identification of the Wnt β -

catenin signalling pathway. Since it was the first Wnt pathway characterized in detail, it is also referred to as the canonical pathway.

The basic framework of the β -catenin pathway (Fig. 7) was established by studies in fly, worm, frog, fish and mouse (Komiya and Habas, 2008), and it is characterized by the stabilization of cytosolic β -catenin in response to its activation.

Metazoan cells usually contain two distinct pools of β -catenin. The first one is found at adherens junctions, where it acts as a cadherin binding protein responsible for regulating cell-cell adhesion (Valenta et al., 2012). This pool is very stable and exhibits low levels of turnover. The other pool is free β -catenin in the cytosol, which, in the absence of canonical Wnt signalling, has high rates of turnover due to the activity of the β -catenin

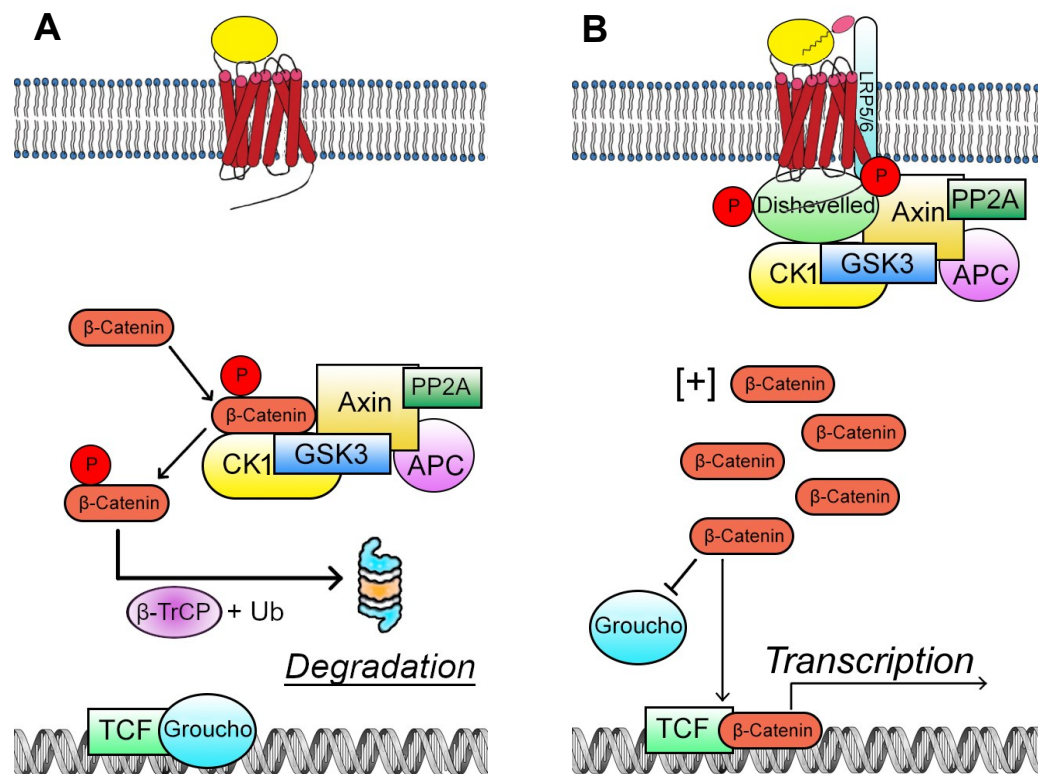


Fig. 7. Canonical Wnt signalling. In the absence of Wnt signalling (**A**), β -catenin is phosphorylated by the β -catenin destruction complex consisting of GSK3, Axin, CK1, APC, and PP2A. This results in β -catenin ubiquitinylation by β -TrCP and subsequent degradation. Wnt stimulation (**B**) results in the formation of a complex consisting of Fzd, Wnt, LRP5/6, and Dishevelled. It sequesters the β -catenin destruction complex from the cytosol, resulting in accumulation of β -catenin, which can then promote the activity of TCF/LEF transcription factors.

destruction complex consisting of glycogen synthase kinase 3 (GSK3), adenomatosis polyposis coli (APC), axin, casein kinase 1 (CK1), and protein phosphatase 2A (PP2A) (Kimelman and Xu, 2006). The complex targets β -catenin for destruction via the action of GSK3 and CK1, which phosphorylate degron motifs within this protein (CK1 – S45, GSK3 – T41/S37/S33) (Liu et al., 2002). These modifications enable β -catenin to be recognized and ubiquitinated by the β -Transducin repeat-containing E3 ubiquitin protein ligase (β -TrCP), thus directing it to proteolytic degradation by proteasomes.

Our current understanding of β -catenin signalling is as follows. First, a Wnt molecule interacts with the Fzd receptor and a co-receptor, the lipoprotein receptor-related protein (LRP5/6) (Komiya and Habas, 2008). LRP5/6 are single-pass transmembrane proteins containing an approximately 1400 amino acids-long extracellular domain, which consists of 4 beta-propeller domains which alternate with four EGF-like repeats, thus forming 4 distinct modules referred to as P1E1-P4E4 (Chen et al., 2011). These are then followed by 3 low-density lipoprotein receptor (LDLR) type A repeats, a transmembrane α -helix and a relatively short ($\sim$ 200 amino acids) intracellular loop domain. The P1E1-P4E4 domains contain binding sites for Wnt and modulators of Wnt signalling. The primary role of these interaction sites is thought to be bringing LRP in close proximity of Fzd via the interaction of Wnt, thus forming a ternary complex. LRP constructs lacking the entirety of the extracellular domain are capable of constitutively activating signalling in mammalian cells, thus suggesting that the extracellular domain of LRP has an autoinhibitory function, which might be relieved upon interaction with Wnt (Brennan et al., 2004). Finally, the 3 LDLR repeats in the LRP5/6 extracellular domain directly interact with some Fzds (such as Fzd5), whereas the P1E1-P4E4 domains are able to bind the extracellular loops of Fzd8, thus inhibiting Wnt signalling (Ren et al., 2015).

Wnt binding to the receptor/co-receptor complex is followed by the recruitment of scaffold protein dishevelled (Dvl) to the cell surface, where it interacts with the conserved motif of Fzd located on its helix VIII, and other binding sites in the intracellular loops of Fzd (Tauriello et al., 2012; Wong et al., 2003). Elevated local concentrations of Dvl in the vicinity of the membrane result in its polymerization via its DIX and DEP domains (Gammons et al., 2016; Schwarz-Romond et al., 2007). This results in increased avidity of Dvl for axin, which also contains a DIX domain, and is thus recruited to the cell membrane along with other components of the destruction complex (Fiedler et al., 2011; Parker and Neufeld, 2020). CK1 and GSK3 can then phosphorylate conserved PPPSP motifs found within the intracellular tails of LRP5/6, which further inhibit GSK3 activity and increase affinity of the destruction complex to the cell surface (Niehrs and Shen, 2010; Stamos et al., 2014). Dvl also binds and activates phosphatidylinositol-4-kinase-type II α , and phosphatidyl-4-phosphate-5-kinase-type I, thus stimulating phospholipid production in the vicinity of Fzd signalling complexes (Pan et al., 2008). This includes PIP2, which is bound by APC membrane recruitment 1 protein (AMER1). This membrane-associated protein interacts with CK1, axin, and GSK3 (Tanneberger et al., 2011), thus further stimulating β -catenin destruction complex sequestration from the cytosol. Finally, canonical Wnt signalling is also associated with increased rates of axin dephosphorylation (Willert et al., 1999). This reduces its affinity for other components of the destruction complex (Kim et al., 2013), while also promoting the recruitment of RING E3 ubiquitin ligase SIAH ('seven in absentia homologue'), thus resulting in axin degradation (Ji et al., 2017).

All of the aforementioned mechanisms act to sequester and inhibit the action of the β -catenin destruction complex. This results in elevated β -catenin concentrations in the cytosol and subsequent transport to the cell nucleus, where it interacts with members of

the TCF/LEF family of transcription factors (T-cell specific factor and lymphoid enhancer-binding factor) (Hurlstone and Clevers, 2002). In the absence of β -catenin, they form a repression complex with transducin-like enhancer protein (TLE) and Groucho, which recruit histone deacetylases to mediate transcriptional repression. Interaction with β -catenin displaces Groucho, allowing TCF/LEF to recruit basal transcriptional machinery, such as the TATA box-binding protein (TBP), as well as transcriptional co-activators such as CBP/p300 and Brg1, which stimulate the transcription of a variety of genes required for stimulating cell proliferation and differentiation, including c-Myc, cyclin D1, Axin2, COX-2 and iNOS (Gammons and Bienz, 2018; Hurlstone and Clevers, 2002).

1.6.2. Non-canonical Wnt signalling

It would be an oversimplification to classify all non-canonical signalling pathways under the same descriptor, since they are extremely diverse, have different functions, and exhibit a level of complexity that we have only recently started to appreciate. Non-canonical signalling has been less studied in comparison to the β -catenin-involving counterpart, so even less information is known about the mechanisms underlying their action. Normally, Wnt/Fzd signalling pathways are categorised depending either on their physiological outcomes or the downstream signalling partners they activate.

1.6.2.1. Planar cell polarity pathway

As previously mentioned, the planar cell polarity (PCP) pathway was the first one discovered that led to the identification of the Wnt and Fzd genes (Gubb and García-Bellido, 1982). Within flies, it is responsible for establishing planar cell polarity, which controls the orientation of epithelial structures on the fly surface, such as cuticle hairs, sensory bristles, and ommatidia in the eye. The same process in vertebrates also controls

primary axis formation, cell morphology and migration during gastrulation (particularly dorsal mesodermal cells), hair follicle organization, and formation of stereocili in the sensory epithelium of the inner ear (Seifert and Mlodzik, 2007).

Similarly to the canonical pathway, PCP signalling is initiated via the formation of a Wnt/Fzd complex, which subsequently recruits Dvl (Fig. 8) (Wang, 2009). Instead of Lrp5/6, the resulting signalling complex potentially recruits tyrosine kinase co-receptors, such as ROR1/2 or Ryk, which are capable of binding Wnt via Fzd-like CRDs or WIF domains (Roy et al., 2018). Their intracellular kinase domains have not been shown to have kinase activity, and the role of these proteins within PCP signal transduction is not clear, although their knockouts result in PCP phenotypes (Kikuchi et al., 2011).

Following recruitment, Dvl stimulates the activity of two separate but parallel signalling cascades through its PDZ and DEP domains (Komiya and Habas, 2008). The PDZ

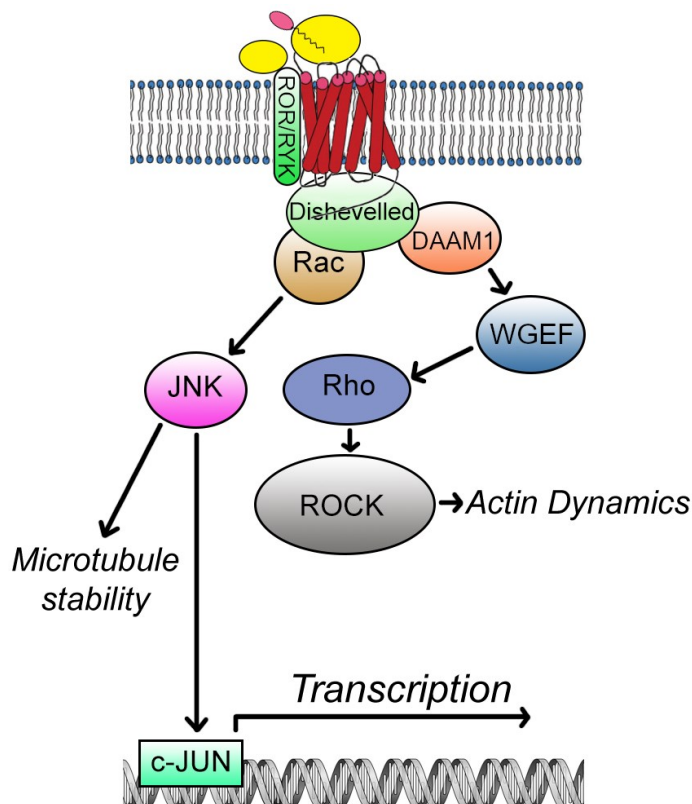


Fig. 8. PCP pathway. Wnt and Fzd interaction results in the recruitment of Dishevelled (and possibly co-receptors ROR1/2 and RYK). Dvl then activates Rac and Rho Ser/Thr kinases, which alter actin dynamics and microtubule stability.

domain recruits and activates DAAM1 (dishevelled associated activator of morphogenesis 1), which in turn stimulates the guanine exchange factor WGEF. It subsequently activates the small GTPase RhoA, thus stimulating the activity of downstream effectors Rho-associated kinase ROCK and myosin, both of which act to modify the kinetics and organization of actin. In parallel to the signalling mediated by PDZ, the DEP domain of Dvl activates the small GTPase Rac, which stimulates the transcription factor activity of JNK, which promotes actin filament turnover (Habas et al., 2003; Li et al., 1999). The combined actions of Rho and Rac thus regulate the actin cytoskeleton architecture and directionality, giving rise to polarity and enabling the migration of cells.

1.6.2.2. Calcium Pathway

The Wnt/Fzd calcium signalling pathway was first discovered during studies of *Xenopus* embryos, where the injection of Wnt-encoding RNA resulted in elevated concentrations of intracellular calcium (Slusarski et al., 1997a). Since then, studies have shown that several Fzd/Wnt complexes can result in release of Ca^{2+} from the ER lumen (Komiya and Habas, 2008; McQuate et al., 2017; Schulte and Wright, 2018). This process is pertussis toxin (PTX)-sensitive, indicating that it relies on the activity of heterotrimeric G proteins belonging to the $\text{G}_{i/o}$ family (Halleskog and Schulte, 2013; Slusarski et al., 1997b). Although the exact mechanisms that lead to Ca^{2+} increase are unclear, the $\text{G}_{\beta\gamma}$ subunits of activated $\text{G}_{i/o}$ -family proteins have been shown to activate phospholipase C (Oude Weernink et al., 2007). This enzyme releases inositol triphosphate from the cell membrane, which can then activate calcium channels present in ER membranes, thus leading to calcium release. In opposition to this, overexpression of Dvl constructs lacking the DIX domain in *Xenopus* is able to initiate calcium

signalling in a PTX-insensitive manner, so the involvement of heterotrimeric G proteins in Wnt signalling remains controversial (Sheldahl et al., 2003).

Increase of intracellular calcium levels results in the activation of various effectors in the cell (Fig. 9). These include protein kinase C and calmodulin-dependent protein kinase II (CaMKII), which regulate cell adhesion and migration by affecting focal adhesion formation and actin dynamics (Fogh et al., 2014; Okamoto et al., 2007).

CaMKII, as well as another Ca^{2+} activated protein calcineurin, have also been shown to inhibit canonical Wnt signalling via activation of TAK1/NLK or NFAT respectively, thus regulating cell fate decisions and playing an important role in embryogenesis (De, 2011; Ishitani et al., 2003; Saneyoshi et al., 2002).

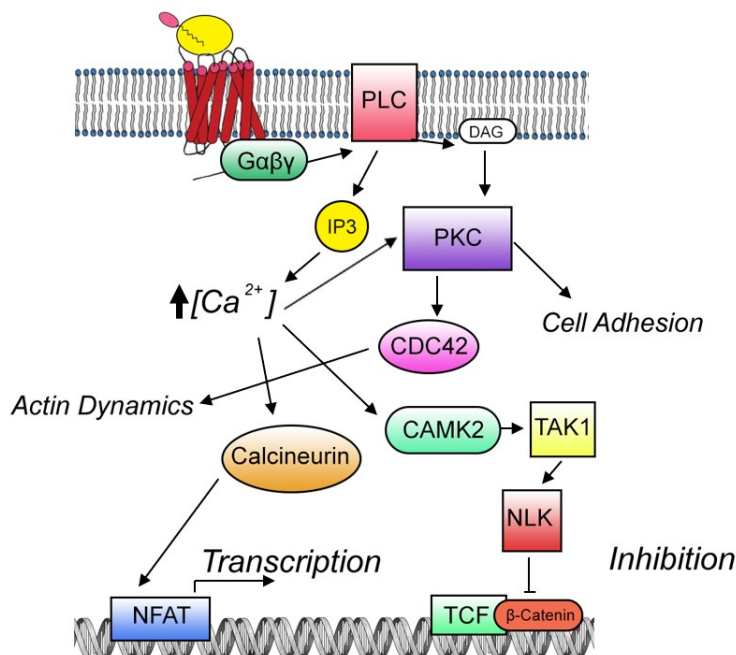


Fig. 9. Ca^{2+} signalling pathway. The Wnt/Fzd complex stimulates the activity of heterotrimeric G-proteins, thus leading to increased Ca^{2+} levels within the cell. This promotes cell adhesion and affects actin dynamics, while also inhibiting the canonical Wnt signalling pathway.

1.7. Specificity of Wnt and Fzd interactions and their functional outcomes

Mammalian cells express a large number of different Wnts and Fzds, which in some cases can exhibit preferences for particular Wnt/Fzd complex formation and specific signalling pathway activation. Wnt8 stimulation has only been reported to result in intracellular β -catenin accumulation, Wnt4 – Rho and Rac activation, while Wnt11 can activate both canonical and non-canonical PCP and Ca^{2+} pathways (Chien et al., 2009). Although the exact molecular determinants of different pathway selectivity are not known, it appears that the type of Wnt present usually plays a more important role in this process, since the majority of Fzds can mediate both canonical and non-canonical signalling pathway activation upon exposure to different Wnt types (Dijksterhuis et al., 2014). Due to the large number of different factors involved in modulating this system, the exact cellular response to Wnt stimulation probably depends on the cellular context.

1.8. Norrin (Ndp)

Norrin (Ndp) is an atypical Fzd agonist. The gene it is encoded by is also referred to as the Norrie disease gene, which is an X-linked congenital retinal dysfunction which usually exhibits itself as blindness at birth (Chen et al., 1992). Mutations in this gene (or related Wnt signalling components, such as Fzd4) also often lead to familial exudative vitreoretinopathy (FEVR) (Chen et al., 1993). These associated diseases exemplify the function of Ndp, which is responsible for regulating the development of retinal vasculature within the developing embryo.

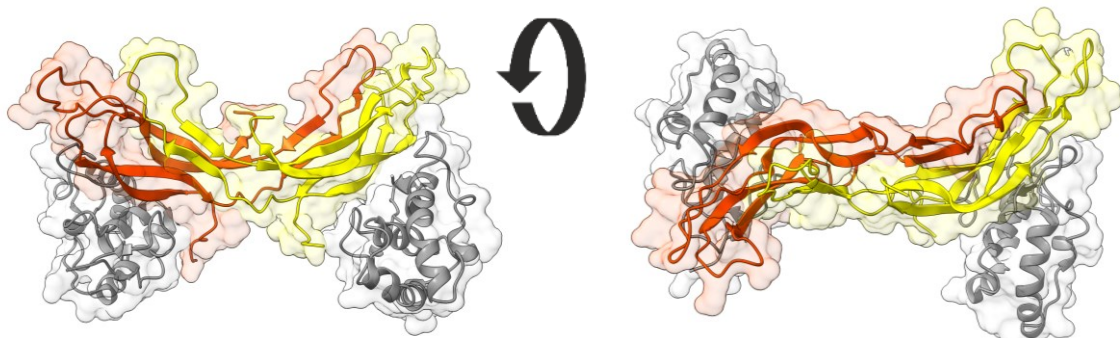


Fig. 10. Interaction between Ndp and Fzd4 CRD.

Ndp dimer - yellow and orange. Fzd4 CRDs - grey. Based on Shen et al., 2015 (PDB ID: 5CL1).

Ndp contains a cysteine knot-like domain, which is only active as a covalent dimer via a cysteine bridge (Fig. 10) (Ke et al., 2013). It has no structural similarity to Wnt and it is not lipid-modified, despite being able to activate Fzd-mediated β -catenin signalling, so it might be an example of convergent evolution, although in contrast to most Wnts it is only able to bind and activate Fzd4. Interestingly, Fzd9 and Fzd10 are close homologues of Fzd4, and they also contain the majority of residues required for binding Ndp, although they cannot initiate signalling. One of the potential reasons for this might be the involvement of TSPAN12 in Ndp/Fzd signalling activation, which might act as a co-receptor for the Ndp/Fzd4 complex along with LRP5 to initiate β -catenin signalling (Lai et al., 2017).

As previously mentioned, Ndp is not lipid modified, making it more suitable for purification and further biochemical and structural studies in comparison to Wnt. Crystal structures of NDP and Fzd4 CRD show that they form a 2:2 complex, where the binding surface of the Ndp dimer overlaps with that of the Wnt finger, leaving the hydrophobic lipid binding groove on the Fzd CRD unoccupied (Shen et al., 2015). The necessity for an Ndp homodimer, where each constituent monomer can interact with a single Fzd CRD also hints at the role of Fzd dimerization and oligomerization in initiating signalling. Studies using bioluminescence resonance energy transfer have shown that the addition of NDP to Fzd4 CRDs results in Fzd dimer formation in

solution as well as on cell membranes, also leading to the discovery that Fzd4 can also homodimerize in the absence of ligands via an additional dimerization surface formed by its transmembrane helices IV and V (Bang et al., 2018). Interestingly, Wnt5A has also been shown to induce Fzd4 dimerization, although the monomer arrangement within this dimer is most likely different from Ndp due to the physical restraints associated with Wnt binding requirements (DeBruine et al., 2017b). This hints at specific differences in the initiation of canonical Fzd signalling mediated by Ndp.

The ability to obtain larger quantities of purified Ndp have also enabled more detailed study in regards to the structural changes associated with Fzd receptor activation.

Hydrogen:deuterium exchange (HDX) has been used to identify regions within full-length Fzd4 that are associated with Ndp binding (Bang et al., 2018). The results show that upon Ndp binding Fzd4 undergoes conformational changes in its extracellular loop 1, intracellular loop 3, extracellular side of transmembrane helix VI, as well as the linker between the CRD and the TMD. Mutations in the Fzd4 linker region diminish β -catenin accumulation upon Ndp stimulation, as well as reduce the K_d values for the interaction between Ndp and Fzd4 CRD, thus suggesting that the linker region of Fzd4 has an important role for initiating signalling. Furthermore, HDX data indicated that Fzd4 helix VIII containing the conserved KTXXXW motif required for the activation of canonical signalling undergoes partial unfolding upon Ndp stimulation (Bang et al., 2018). The significance of this is not clear, although it could hint at a potential mechanism for how Fzds are able to transduce signals across the cell membrane.

1.9. Wnt signalling in disease

Due to its involvement in almost every aspect of cellular function, Wnt signalling is often the causative factor for pleiotropic pathologies of the human, such as cancers, neurodegenerative disease, skeletal malformations, and birth defects (Ng et al., 2019). Mutations and/or deregulation in any step of Wnt signalling can result in a variety of conditions, while presenting a wealth of potential therapeutic targets.

1.9.1. Wnt signalling in cancer

Due to its role in promoting cell growth and proliferation, Wnt/Fzd signalling in the context of disease has been most often studied in association with cancer. Abnormal Wnt signalling is associated with many cancers, such as colorectal, breast, cervical, and hematopoietic cancers, all of which exhibit altered levels of Wnt signalling (Koni et al., 2020).

Solid tumours are not just a random overgrowth of cells – they exhibit complex interactions between a variety of different cell types, thus sharing many similarities with developing organs (Egeblad et al., 2010). Because of this, both canonical and non-canonical Wnt signalling have been implicated in tumour formation, survival, and progression, although the role of β -catenin signalling has been studied most extensively (Kato, 2017).

Increased β -catenin signalling is important for tumourigenesis, cancer stem cell proliferation and maintenance (Patel et al., 2019), although it does not appear to arise primarily from elevated signalling at the cell membrane. Instead, it is most commonly caused by mutations in the β -catenin destruction complex components, with APC being mutated in approximately 70% of all colorectal cancers (Rowan et al., 2000; Zhan et al.,

2016). Despite this, mutations in proteins regulating Fzd levels present on the cell surface, such as LGR5 and RNF43, are also thought to play a role in aberrant β -catenin signalling in cancer (Giannakis et al., 2014; Lau et al., 2014; Xu et al., 2019). Wnt/ Ca^{2+} further contributes to cancer stem cell maintenance and differentiation by increased levels of NFAT target gene transcription, which also leads to increased therapeutic resistance (Katoh, 2017). Finally, all Wnt signalling pathways are implicated in cancer metastasis due to their requirement for epithelial-mesenchymal transition (Zhan et al., 2016). Due to its primary role being the regulation of cytoskeleton dynamics, the PCP pathway is particularly important for this process, with elevated PCP signalling leading to higher rates of surrounding tissue invasion (Katoh, 2005).

Wnt signalling is also responsible for shaping the tumour microenvironment. For instance, tumour-infiltrating macrophages express high levels of Wnt2 and Wnt5A, which have been implicated in the adenoma-carcinoma transition in colorectal cancer (Smith et al., 1999). These cells also secrete Wnt7B, which activates vascular endothelial cell proliferation in the vicinity of the tumour, thus stimulating angiogenesis (Rao et al., 2007). Similarly, cancer-associated fibroblasts also secrete high levels of Wnt2, which, in addition to promoting cancer cell growth, also plays a role in extracellular matrix remodelling and cancer cell invasion (Kramer et al., 2017). Wnts in the tumour microenvironment also lead to immunoevasion by promoting a regulatory state of dendritic cells, thus inhibiting effector T cell activation (Spranger et al., 2015; Suryawanshi and Manicassamy, 2015).

1.9.2. Wnt signalling, aging, and neurodegenerative disease

One of the principal outcomes of normal aging in the human brain is reduced synaptic strength, function, and plasticity, which results in diminished cognitive function (Torres

and Cardenas, 2020). This is caused by alterations to various pathways crucial for synapse integrity, including Wnt signalling (Palomer et al., 2019). Aging brain shows lower overall expression levels of both Wnt and Fzd proteins, whereas the production of Wnt signalling inhibitors (such as Sfrp1 and Dkk1) is increased (Palomer et al., 2019). This is also accompanied by a general reduction in the proliferation and differentiation capacities of hippocampal precursor cells, which tend to become quiescent, thus resulting in worsening memory. Boosting Wnt signalling in the brains of aging mice models has been shown to result in improved cognitive function, thus further supporting the connection between diminished Wnt signalling and age-related brain dysfunction (Vargas et al., 2014).

Age is also the primary risk factor for neurodegenerative disease (NDD). This extensive group of neurological disorders is most commonly associated with the accumulation of intra- and extracellular protein aggregates due to protein misfolding and disrupted proteostasis as a consequence of the aging brain (Dugger and Dickson, 2017). The presence of these aggregates results in major disruptions to the cellular biochemistry of the brain, such as increased oxidative stress, altered mitochondrial dynamics, elevated intracellular calcium levels, diminished autophagic integrity, and activation of inflammatory signalling (Chung et al., 2018; Sweeney et al., 2017). The combination of all of these effects results in cell death, thus leading to neuronal loss and progressive decline of brain cognitive and motor functions.

Wnt signalling in relation to NDD has been most studied in the context of Alzheimer's Disease (AD). It is characterized by the accumulation of extracellular A β amyloid plaques and intracellular neurofibrillary tangles formed by hyperphosphorylated tau protein. Both types of aggregates exhibit cytotoxic effects as described above, thus

leading to the usual symptoms associated with AD – memory loss, reduced motor functions, behavioural issues, and dementia (Breijyeh and Karaman, 2020). A growing amount of evidence suggests that disruption of Wnt signalling is a major factor leading to synapse loss during AD progression, and restoration of normal Wnt signalling could be useful in combatting this disease.

The brains of AD patients exhibit reduced levels of Wnt signalling (García-Velázquez and Arias, 2017). BACE1 - β -amyloid precursor protein cleaving enzyme responsible for generating A β , is transcriptionally repressed by active TCF4, therefore diminished canonical signalling by Wnt is responsible for increased A β amyloid plaque formation (Parr et al., 2015). It also leads to elevated levels of GSK3, which is involved in tau hyperphosphorylation and thus neurofibrillary tangle assembly (Salcedo-Tello et al., 2014). Wnt signalling is also responsible for the establishment and maintenance of the blood-brain barrier, the integrity of which is reduced in AD, thus further contributing to brain dysfunction (Liebner et al., 2018).

Taken together, these observations indicate that reduced Wnt signalling plays an important role in AD pathogenesis and progression. Stimulation of the β -catenin pathway results in decreased generation of intra- and extracellular aggregates, limits their toxicity, and increases cognitive function in disease models, therefore suggesting that Wnt pathways could be useful targets for therapeutic intervention (Jia et al., 2019).

1.10. Challenges in studying Wnt signalling

Wnt signalling and its importance within metazoa has been obvious for several decades, so there has been a large interest to decypher the role of its individual components not only for the goal of developing new drugs, but also purely for gaining a deeper understanding of mammalian development.

Unfortunately, the Wnt/Fzd system is not very conducive for scientific research due to the involvement of various signalling pathways and a plethora of ligands, receptors, co-receptors, and modulators, often with no obvious differences in function or role in specific signalling outcomes. It seems likely that minor differences in Wnt signalling pathway component activity (together with their expression levels, post-translational modifications, and cellular context) all play a role in specific Wnt signalling pathway activation and the resulting cellular response, therefore, to fully understand the mechanisms governing the initiation and transduction of Wnt signalling, studies should take into account these factors to form a unified picture of what is happening upon their activation. Unfortunately, that is very difficult to do, since the role, function, and regulation of many proteins involved in Wnt signalling has not been elucidated.

As previously described, the properties of Wnts make them very difficult to be produced in large quantities. Even if that was not the case, the signalling activity of purified Wnt might differ from Wnt bound to carriers or extracellular structures present *in vivo*. Because of this, biochemical studies examining the function of individual Wnt signalling pathway components have been rare. Instead, most of the information on this topic comes from cellular studies, where the expression levels of individual proteins involved in signalling have been altered in a specific cell type, and the outcomes measured only as a particular cellular response. Usually this is the activation of

TCF/LEF transcription factors, due to a relatively simple TCF promoter based assay being available for measuring it (Molenaar et al., 1996), whereas the measurement of PCP and Ca^{2+} pathway activation is much more difficult. Thus, the role of various components involved in Wnt signalling is usually condensed to a single readout, making their individual contributions and roles impossible to discern.

Another obstacle lies in the fact that the initiation and cellular outcomes of Wnt signalling seems to depend on events that take place in the cellular plasma membrane following stimulation. Contrary to the relatively simple receptor/ligand/co-receptor model, Wnt signalling also depends on lipid microdomains and lipid composition of the membrane, as well as the presence of additional transmembrane proteins and proteoglycans (Azbazdar et al., 2021; Bengoa-Vergniory and Kypta, 2015).

Furthermore, activation of the β -catenin pathway results in the formation of large complexes of transmembrane proteins and intracellular, membrane-associated signalling mediators (DeBruine et al., 2017a). Therefore, to identify a specific sequence of events leading to downstream signalling pathway activation, research should be conducted within the context of the membrane. Unfortunately, work with isolated membrane proteins or their complexes is often challenging, and to elucidate the role of various weakly-interacting co-factors or lipids would require experiments to be performed in the absence of detergent. The number of studies focusing on transient transmembrane complexes in isolation is small, thus underlining the challenges present in this field.

1.11. Cryo-EM of membrane proteins

One of the principal methods for studying the structure of relatively large protein assemblies is cryo-electron microscopy (cryo-EM). A detailed description of this technique is beyond the scope of this work, but, in brief, purified protein samples are flash-frozen on carbon-supported grids, forming a nanometer-thick layer of vitreous ice with embedded protein particles in various orientations (Passmore and Russo, 2016). The grid is then exposed to a high-energy electron beam that interacts with the sample, thus leading to radiation damage and generation of contrast. The resulting image, consisting of sample projections convoluted with a point-spread function of the microscope, is then recorded as a movie with direct electron detector cameras (Bhella, 2019). The contrast between the particles and the surrounding ice is very small, and the raw movies are blurred due to the movement of sample under the influence of the electron beam. Therefore, before further data processing, movies need to be motion- and aberration-corrected (Sigworth, 2016). The particles present within the movies are subsequently classified by 2D averaging, allowing the selection of particles with the highest quality that are then used to produce 3D maps representing the distribution of Coulomb potential within the sample (Marques et al., 2019). These maps can then be used for modelling of the protein or protein complex of interest. The recent decade has shown a tremendous increase of high resolution models obtained using this method due to technological advancements and a broader accessibility of the technique (Renaud et al., 2018).

Cryo-EM has also been successfully applied to various membrane proteins to determine their structure, which are particularly notorious for their resistance to crystallization. This is because the detergent micelles that surround the transmembrane domains of

detergent-solubilized protein severely limit the amount of protein surface residues that can participate in the formation of crystal contacts when traditional vapour diffusion methods are used (Parker and Newstead, 2016). To avoid this issue, lipid cubic phase crystallography can be used, which requires even larger quantities of purified sample and adds additional variables to the condition screening process, while still exhibiting low success rates. Cryo-EM overcomes these problems, but others still remain. Protein flexibility is the main factor preventing high resolutions of final models, therefore the sample needs to be rigid. The presence of detergent also causes several issues (Hauer et al., 2015). First, free detergent monomers interact with the electron beam, thus reducing the overall contrast of the sample. Second, the detergent alters the surface tension of the liquid sample during the blotting and vitrification procedure, resulting in gradients of ice thickness across the grids. Since particle distribution is affected by this property, the presence of detergent thus leads to higher variations in particle distribution across the grid, making it more difficult to find areas suitable for imaging. Finally, the detergent micelle surrounding the transmembrane domains of the protein reduces their contrast significantly. This can lead to difficulties of aligning individual particles during 2D and 3D processing if the protein does not contain large intracellular or extracellular domains.

1.12. Nanodisc reconstitution of membrane proteins

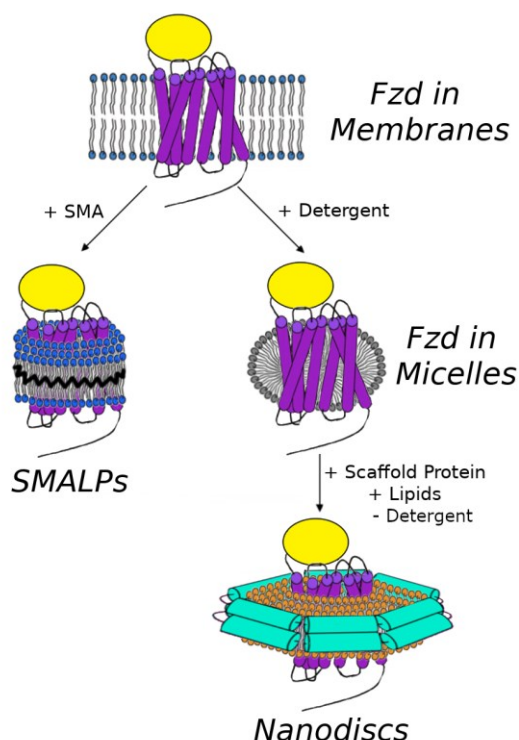


Fig. 11. Reconstitution of transmembrane proteins within nanodiscs and SMALPs. Fzd used here as an example, but the principles are the same for other transmembrane proteins as well. To obtain Fzd-containing nanodiscs, cell membranes from cells expressing Fzd are first solubilised using a suitable detergent. This leads to the extraction of Fzd embedded in detergent micelles. The resulting sample is then incubated in the presence of nanodisc scaffold proteins and lipids at a specific ratio. Subsequent removal of detergent results in the formation of nanodiscs containing Fzd. To obtain SMALPs containing Fzd, cell membranes are incubated with styrene : maleic acid copolymer (SMA), which leads to the extraction of Fzd from the cell membrane surrounded by a belt of native lipids and SMA.

The use of nanodiscs for transmembrane protein study was developed by Sligar et al., who showed that engineered apolipoprotein scaffolds can be combined with phospholipids to form disc-like particles with a defined diameter containing a lipid bilayer (Denisov et al., 2004). If the disc reconstitution procedure is performed in the presence of detergent-solubilized protein, the protein can be reconstituted within these discs, thus resulting in transmembrane protein samples without the presence of free detergent (Fig. 11) (Denisov and Sligar, 2016). A successful reconstitution relies on using the right scaffold, sample, and lipid ratios in the reaction mixture, as well as the properties of scaffold and lipid types used (Ritchie et al., 2009). The lipid bilayer resembles the native environment of transmembrane proteins much more closely than detergent micelles, thus providing greater stability to the sample. A variety of proteins have been reconstituted within nanodiscs, for use in both biophysical and structural studies which might benefit from the absence of detergent.

1.13. SMALPs – direct membrane protein solubilization from a native environment

Another technology for membrane protein solubilization in the absence of detergent is styrene:maleic acid lipid particles (SMALPs). These polymers consist of alternating hydrophobic styrene and polar maleic acid monomers, thus giving them both a hydrophobic and a charged character (Lee et al., 2016). By combining these polymers with isolated cell membranes, the polymer is capable of entering and solubilising them, forming disc-like particles containing constituent cell membrane components, that is, native lipids and transmembrane proteins (Xue et al., 2018). In comparison to nanodiscs, the protein does not first need to be purified or detergent solubilized (Fig. 11), thus enabling direct extraction of transmembrane proteins (or transmembrane protein complexes) from their native environment.

1.14. Nanobodies as structural tools for membrane biology

Although nanodiscs and SMALPs alleviate the effects of free detergent on the quality of cryo-EM micrographs, as well as resulting in better transmembrane protein stability, they still obscure the transmembrane parts of solubilized proteins, and, as previously mentioned, sufficient mass of the protein particles needs to extend from the transmembrane region to facilitate downstream data processing.

One widely used approach to overcome some of these limitations is to exploit nanobodies – variable domains of heavy-chain-only antibodies found in camelids and sharks (Könning et al., 2017). In contrast to regular antibodies around 120 kDa in size,

they are only 15 kDa in size, and have proven useful in membrane protein crystallization due to the relatively simple production process and their ability to recognize small or hidden epitopes on the surface of transmembrane proteins (Pardon et al., 2014). Upon binding to their target protein, they are able to stabilize the protein, increase its mass, provide additional crystallization contacts, as well as alter protein solubility, thus making nanobodies a useful addition to the structural biologist's toolkit. Their small size has been a limiting factor for their use in cryo-EM studies, but it has recently been shown that nanobodies can be conjugated to larger scaffold proteins, thus significantly boosting the size of cryo-EM particles (Brunner and Schenck, 2020).

The biggest limitation to a wider use of nanobodies has been the requirement of camelids for immunization with target protein. Few research institutions have access to their own animals for such purposes. Therefore, to boost access to this technology, several synthetic nanobody libraries have been developed. They are usually based either

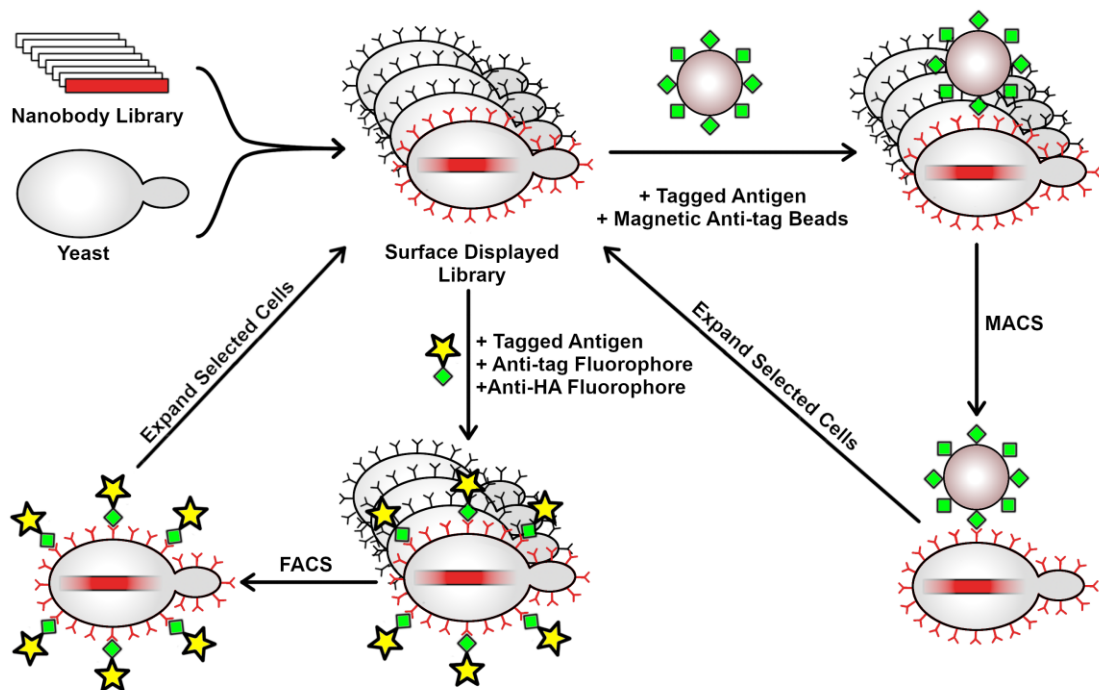


Fig. 12. Yeast surface display of nanobody libraries. Plasmids encoding individual nanobodies linked to a membrane anchor are used to transform yeast cells. Expression results in the display of nanobodies on the yeast surface. This allows the selection of yeast cells expressing nanobodies binding the antigen of interest via magnetic- and fluorescence-activated cell sorting (MACS and FACS, respectively).

on phage or yeast surface display technology, thus allowing them to be used in a regular lab setting (Fig. 12) (McMahon et al., 2018; Moutel et al., 2016; Zimmermann et al., 2018).

1.15. Main goals of this study

The main aims of this thesis were to provide additional structural mechanistic understanding of the Wnt signalling through its principal receptor Fzd. For any signalling pathway, its initiation is usually the key step that determines all subsequent physiological outcomes. Therefore, the key question that has interested me has been the structure of Fzd signalling complexes, which have not been reported previously. The determination of these structures would allow us to answer several important questions about how Wnt signalling functions, including the mechanisms of signal transduction across the cell membrane and different signalling pathway selection.

The main goals of this study were to:

1. Obtain high quantities of purified, detergent-solubilized Fzd.
2. Reconstitute Fzd in detergent-free systems.
3. Explore the expression and properties of Fzd ligands, co-receptors, and Dvl.
4. Form different Fzd signalling complexes and determine their structure.

In the second part of this work, I have studied Notum and candidate small molecule inhibitors by X-ray crystallography in collaboration with the Fish group at UCL. The aim was to rationalize the effect of different modifications present on the molecule in respect to their inhibition efficiency. This information would then be used to develop better inhibitors of Notum that could potentially be used for the treatment of NDDs.

2. Materials and methods

2.1. Constructs

2.1.1. Fzd

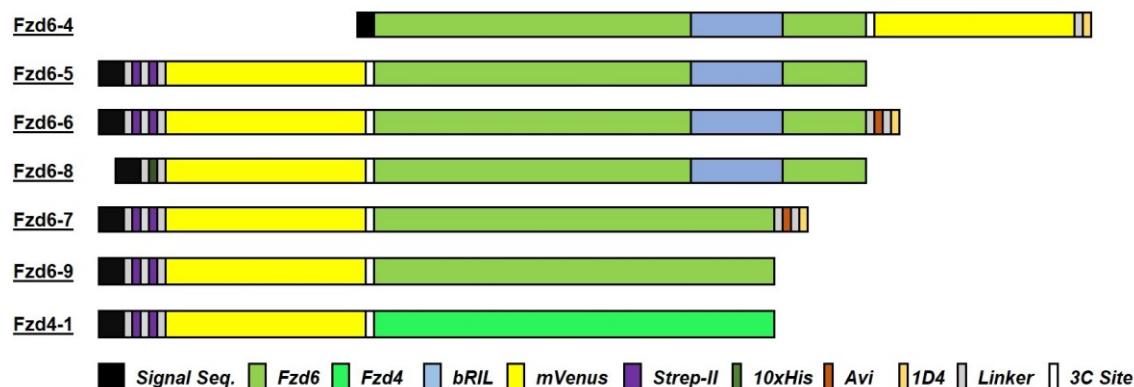


Fig. 13. Fzd Constructs. N-terminus on the left. Black - chicken RPTP σ signal sequence (except for Fzd6-4, where it designates the native hFzd6 signal sequence). Green – hFzd6₍₁₉₋₅₀₇₎-C500S, in case of b⁵⁶²RIL (bRIL) insert it consisted of residues 1-401 and 407-507. Lime – hFzd4₍₁₋₄₀₋₅₁₅₎-C507S. Blue - b⁵⁶²RIL₍₂₃₋₁₂₇₎-M29W-H124I (bRIL) insert, often used to improve GPCR stability. Yellow – mVenus fluorescent protein tag (Kremers et al., 2006). Purple – Strep-II affinity tag (two sequential Strep-II tags are also known as a Twin-Strep tag). Dark green – 10xHis affinity tag. Orange – Avi-tag, a 15 amino acid biotin ligase (BirA) binding site used for enzymatic *in vitro* biotinylation. Gold – 1D4 epitope tag. Grey – linker sequences (2-5 amino acids consisting of G, S, T). Exception is the linker between two sequential Strep-II tags (15 amino acids). White – HRV 3C protease cleavage site.

The starting point for the design of human Fzd6 (Uniprot ID O60353.2) constructs was Fzd6-4. It contained Fzd6 residues 1-507 (including the native signal sequence) with a C500S mutation and a b⁵⁶²RIL insert (Uniprot ID P0ABE7.1, residues 23-127, mutations M29W, H124I), which replaced Fzd6 intracellular loop 3 (residues 401-407). Human Rhinovirus 3C protease cleavage site (LEVLFQGP), mVenus fluorescent protein tag (Kremers et al., 2006) and a 1D4 epitope tag (TETSQVAPA) were also found at the C-terminus of the construct.

Most constructs of Fzd6 were based on Fzd6-5, which contained a chicken RPTP σ signal sequence at its N-terminus, followed by Twin-Strep-tag, mVenus, 3C cleavage site, and Fzd6 (residues 19-507, C500S) with a b⁵⁶²RIL insert. Additional modifications included Avi- and 1D4-tags at the C-terminus (Fzd6-6, Fzd6-7), reconstitution of the

original Fzd6 ICL3 via the removal of b⁵⁶²RIL (Fzd6-7, Fzd6-9), as well as a replacement of the Twin-Strep-tag with a 10xHis-tag (Fzd6-8).

Fzd4-1 was generated by replacing the Fzd6 sequence in the Fzd6-9 construct with human Fzd4 (Uniprot ID Q9ULV1.2, residues 40-515, C507S).

2.1.2. Dvl

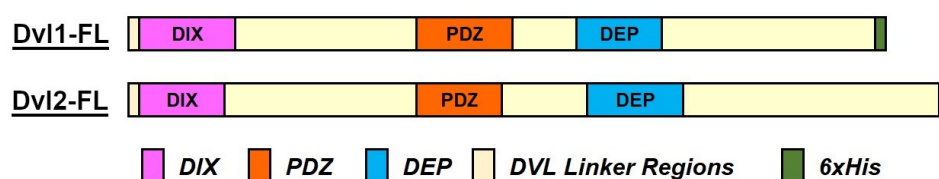


Fig. 14. Constructs of full-length DVL1 and DVL2. N-terminus on the left. Magenta – DIX domains (residues 1-85 for Dvl1, 12-91 for Dvl2). Orange – PDZ domains (residues 251-323 for Dvl1, 268-351 for Dvl2). Blue – DEP domains (residues 425-499 for Dvl1, 426-507 for Dvl2). Beige – Dvl domain linker regions. Green – 6xHis-tag.

Various constructs of human Dvl1 (Uniprot ID O14640.2) and Dvl2 (Uniprot ID O14641.1) were designed using full-length (FL) constructs (Fig. 14). Dvl1-FL was missing a part of the linker region just before the DEP domain (residues 378-403). Dvl1 domain boundaries were defined as follows: DIX (1-85), PDZ (251-323), DEP (425-499) (Choi et al., 2010). The Dvl2 domains were defined as DIX (13-91), PDZ (268-351), DEP (426-507) using sequence alignment to Dvl1 and previously published structures (PDB ID: 3CBY, 5SUY). All constructs contained a C-terminal 6x-His tag. Residues C338 and C493 in Dvl1 and C354, C412, C501 in Dvl2 were mutated to serines.

2.1.3. Fzd co-receptors

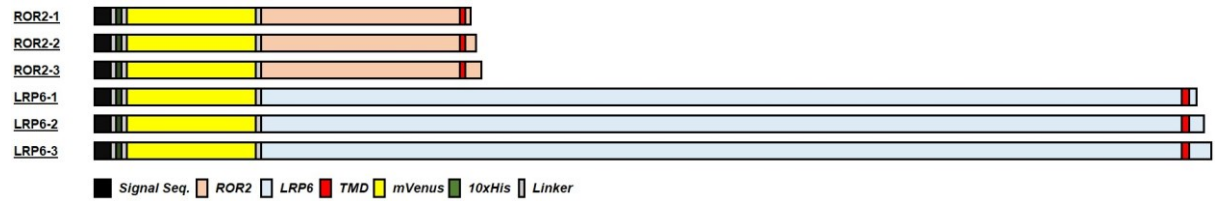


Fig. 15. Constructs of ROR2 and LRP6. N-terminus on the left. Black - chicken RPTP σ signal sequence. Yellow – mVenus fluorescent protein tag. Dark green – 10xHis affinity tag. Grey – linker sequences (2-5 amino acids consisting of G, S, T). Salmon – ROR2. Residues 34-427 (ROR2-1), 34-430 (ROR2-2), or 34-434 (ROR2-3), with C426S and C428S mutations if applicable. Blue – LRP6. Residues 20-1396 (LRP6-1), 20-1399 (LRP6-2), or 20-1406 (LRP6-3), with mutations C1394S and C1399S present if applicable. Red – predicted transmembrane regions of ROR2 and LRP6.

Constructs of human ROR2 (Uniprot ID Q01974.2) contained its extracellular domain and transmembrane region, while the intracellular regions were omitted, except for the inclusion of either 3, 6, or 10 residues following the predicted transmembrane region. The three constructs contained ROR2 residues 34 – 427 (ROR2-1), 34-430 (ROR2-2), or 34-434 (ROR2-3), with mutations C428S and C426S if applicable (Fig. 15).

A similar strategy was also employed for the generation of human LRP6 constructs (Uniprot ID O75581.2), residues 20-1396 (LRP6-1), 20-1399 (LRP6-2), 20-1406 (LRP6-3), also including mutations C1399S and C1394S.

2.1.4. Notum

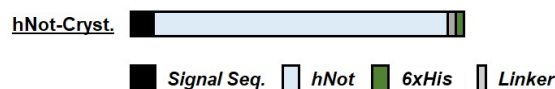


Fig. 16. Notum crystallization construct. N-terminus on the left. Black – baculovirus ecdysteroid UDP-glucosyltransferase signal sequence. Blue – hNotum₍₈₁₋₄₅₁₎-C330S. Green – 6xHis-tag. Grey – a 3 amino acid linker sequence (GTK).

The construct hNot-Cryst. used for crystallization of human Notum (Uniprot ID Q6P988.2) contained only the globular core of the protein (residues 81-451), with a C330S mutation.

2.1.5. Fzd ligands and afamin

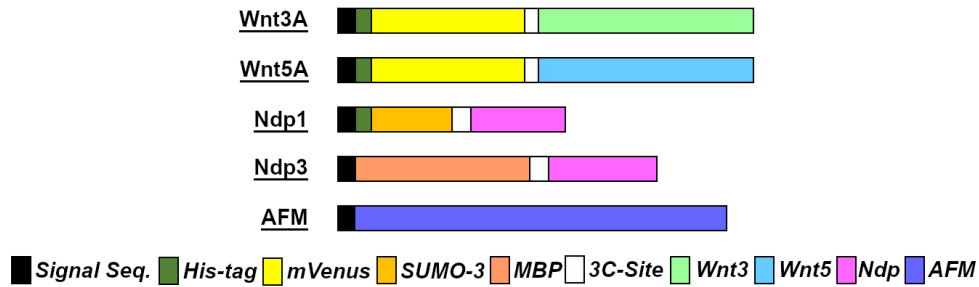


Fig. 17. Fzd ligand and afamin constructs. N-terminus on the left. Black - chicken RPTP σ signal sequence. Green – 10xHis-tag. Yellow – mVenus fluorescent protein tag. Lime – hWnt3A₍₅₇₋₂₅₂₎. Light blue – hWnt5A₍₆₂₋₃₈₀₎. Orange – SUMO-3 tag (used to improve protein solubility). Magenta – hNdp₍₂₅₋₁₃₃₎. Salmon – maltose binding protein tag (used for affinity purification and to improve protein solubility). Dark blue – human afamin (AFM₍₂₂₋₅₉₉₎). White – HRV 3C protease cleavage site.

Constructs of human Wnt3A (Uniprot ID: P56704, residues 57-252) and Wnt5A

(Uniprot ID: P41221, residues 62-380) contained a chicken RPTP σ signal sequence at its N-terminus, followed by a 10xHis-tag, mVenus, and a 3C protease cleavage site.

Human Ndp (Uniprot ID: Q00604, residues 25-133) was tagged either with 8xHis-SUMO-3 (Luo et al., 2018) or Maltose Binding Protein (MBP) tags (Ndp1 and Ndp3, respectively), also containing an Igk signal sequence and a 3C cleavage site. Construct of human afamin (AFM, Uniprot ID: P43652, residues 22-599) contained only a chicken RPTP σ signal sequence at its N-terminus.

2.2. Cloning and creation of expression constructs

The starting point for cloning the target constructs were either plasmids containing the genes of interest already present in the lab, or the required genes ordered from AddGene. Their sequences were confirmed via sequencing (Eurofins) before commencing any further manipulations.

To obtain higher quantities of plasmid DNA, it was used to transform subcloning efficiency DH5 α competent *E. coli* cells (Invitrogen) following manufacturer's instructions, and plasmid DNA extracted from the resulting cultures using a QIAprep

SPIN Miniprep Kit (QIAGEN). To assemble the construct of interest, genes were subcloned into vectors containing the required tags via restriction enzyme double digest (enzymes obtained from NEB) and ligation (DNA Ligation Kit LONG, TaKaRa).

If no suitable restriction sites required for construct assembly were available, they were introduced via PCR (Pyrobest DNA Polymerase, TaKaRa), using primers containing the restriction site sequence at their 5'-end. Overlap extension PCR was used for the introduction of mutations. Mutagenic primers consisted of sequences complementary to the gene of interest at both 5'- and 3'- ends of the mutagenesis site, with the length and T_m of each region being 25-30 bp and $\sim 60^\circ\text{C}$ respectively. These primers were then used as either forward or reverse primers in two separate PCRs (Pyrobest DNA Polymerase, TaKaRa), followed by gel extraction (QIAquick Gel Extraction Kit, QIAGEN) and a single PCR reaction combining the two reaction products.

All primers were designed with a C or G nucleotide at their 3'- end. If not used for overlap extension PCR, the sequences complementary to the gene of interest had a T_m of $\sim 60^\circ\text{C}$ and a length of 25-30 bp. All primers were analyzed for self-annealing and secondary structure formation using IDT OligoAnalyzer. Primers were ordered from Sigma-Aldrich, and PCR performed using a T100 thermal cycler (Bio-Rad).

2.3. Mammalian cell culturing

2.3.1. Freezing and thawing cells

To freeze mammalian cells, a T75 flask (Greiner) containing cell cultures of interest was washed with PBS (Gibco), subsequently adding 5 ml of 0.25% trypsin-EDTA (Gibco) and incubating the flask for 3 min at 37°C . The cells were resuspended in 15 ml DMEM/F-12 (Gibco) supplemented with 10% FBS (Gibco) and 10% DMSO added,

and transferred to a 50 ml tube for centrifugation at 100 x g for 3 min. The media was then carefully removed and the cell pellet gently resuspended in 6 ml DMEM/F-12/10% FBS/10% DMSO. The resulting cell suspension was then aliquoted into 2 ml cryogenic tubes (Nunc), which were placed in a CoolCell (Corning) and stored at -80° C overnight. The next day the cryovials were transferred to vapour-phase liquid nitrogen for long-term storage.

To thaw cells, a cryogenic tube containing cells of interest was retrieved from vapour-phase liquid nitrogen storage and placed in a 37° C waterbath, swirling the vial until the media was almost thawed. The cells were then transferred to a 50 ml tube, and 15 ml DMEM/F-12/10% FBS slowly added. The cells were centrifuged for 3 min at 100 x g, the media removed, and the cells resuspended in 12 ml DMEM/F-12/10% FBS supplemented with 1x NEAA (Gibco). The cell suspension was then added to a T75 flask, which was placed in a humidified incubator set at 37° C and 5% CO₂.

2.3.2. Mammalian cell culture in flasks

Cells growing in flasks were split 1 : 2 upon reaching 90% confluency, which took approximately 2-3 days for HEK293S cells. During this process, the media was removed from the flask, and the cells washed with PBS and trypsinized as described above. The required volume of DMEM/F-12/10% FBS/1x NEAA was then added, and the cell suspension redistributed between the required number of flasks.

2.3.3. Suspension cultures of mammalian cells

Suspension cultures of mammalian cells were started by taking two ~90% confluent T175 flasks containing the cell cultures of interest. The cells were washed with PBS, trypsinized, and transferred to a 50 ml tube containing 30 ml FreeStyle 293 Expression Medium (Gibco) supplemented with 10% FBS. The cells were centrifuged at 100 x g

for 3 min, the media removed, and the cells resuspended in either 100 ml FreeStyle/1% FBS or PEM/1% FBS (Gibco). The suspension was then transferred to a conical glass flask, which was placed in a shaking incubator operating at 37° C and 8% CO₂.

The cell density of suspension cultures was measured every 2-3 days, and the cells were split 1:2 upon reaching a density of ~3 m/ml by adding more media to the growing cultures.

2.3.4. Culture of mammalian cells in roller bottles

T175 flasks containing ~90% confluent cell cultures were washed with PBS and trypsinized, followed by the addition of 20 ml DMEM (Gibco) supplemented with 10% FBS. 12.5 ml of cell suspension was then added to a 2.3 L ribbed cell culture roller bottle (Greiner) containing 180 ml DMEM/10% FBS. Cell cultures were then incubated in a roller rig operating at 37° C, 5% CO₂.

2.4. Establishment of stable mammalian cell lines

Establishment of stable cell lines expressing the protein constructs of interest (COI) were performed according to Elegheert, et al., 2018.

DNA encoding COI was subcloned into a pHR-CMV-TetO₂ transfer plasmid, between its AgeI and KpnI restriction sites. 10 µg of the transfer plasmid containing COI were then mixed with 10 µg of both psPAX2 and pMD2.G packaging and envelope plasmids, as well as 75 µl polyethylenimine (1 mg/ml, 25 kDa branched, Merck) in a sterile 1.5 ml tube. The total volume of the mixture was increased to 500 µl by the addition of DMEM/F-12, and the mixture then incubated for 15 min.

A T75 flask containing Lenti-X 293T cell culture (TaKaRa) was washed with PBS, subsequently adding 11.5 ml DMEM/F-12/2% FBS and 0.5 ml of the prepared DNA mixture. The flask was then placed in a humidified incubator operated at 37° C, 5% CO₂. After 72 h, the media containing the lentiviral particles was collected, supplemented with 6 ml DMEM/F-12/10% FBS, and filtered using a 0.45 µm syringe filter (Millipore). 18 µl of 10 mg/ml polybrene solution (Merck) was added to the lentiviral particles and the resulting mixture gently agitated.

A T75 flask containing HEK293S GnTI⁻ TetR cell culture was washed with PBS, and 18 ml of lentivirus-containing media was added to the cells. They were then placed in a humidified incubator operated at 37 C° and 5% CO₂ for 5 days. The cells were then used for subsequent culturing or frozen.

2.5. Methods of protein expression

2.5.1. Transient transfection of suspension cultures

400 µg of pHL-sec vector encoding COI was added to a 1.5 ml tube containing 80 µl of chloroform and centrifuged for 10 min at 26,000 x g, while two 50 ml tubes containing 20 ml FreeStyle/1% NEAA were prepared. The upper aqueous phase of the vector/chloroform mixture was transferred to one of the prepared 50 ml tubes, while 1.2 ml PEI MAX 40K (Polysciences) was added to the other. The contents of both tubes were then mixed and incubated for 15 min at room temperature (RT).

800 ml of suspension cell culture at ~2 m/ml density was centrifuged at 400 x g for 7 min to remove media. The cell pellet was then resuspended in the transfection mixture and added to a 2 L conical glass flask containing 80 ml FreeStyle/1% NEAA, which was then placed in a shaking incubator operating at 37° C and 8% CO₂ for 5 h. The

volume of cell suspension was then increased to 800 ml by the addition of either FreeStyle or PEM supplemented with 1% FBS and 1 mM valproic acid (VPA), and the culture then placed in a shaking incubator for protein expression (3 days for membrane proteins, 7 days for soluble proteins). After the expression period, cells were harvested by centrifugation at 1,000 x g, 7 min, flash-frozen in liquid nitrogen and stored at -80° C.

2.5.2. Transient transfection of cultures in roller bottles

Transfection mixtures containing 500 µg of pHL-sec vector and PEI MAX 40K were prepared as described above, using DMEM instead of FreeStyle. The media in roller bottles containing ~95% confluent cell cultures was replaced with 200 ml DMEM/2% FBS, subsequently adding 50 ml of the transfection mixture. If required, kifunensine (Sigma-Aldrich) was added to the cell cultures to a final concentration of 1 mg/ml. The cultures were then placed in a roller rig operating at 37° C, 5% CO₂ for 5 days before harvesting conditioned media by centrifugation at 1,000 x g for 7 min.

2.5.3. Protein expression using stable cell lines in suspension

To induce protein expression in HEK293S GnTI⁻ TetR cell cultures in suspension, 5-10 µg/ml doxycycline and 1 mM VPA was added to the media. The cells or conditioned media were subsequently harvested after 2-3 days (for membrane proteins) or 7 days (for secreted proteins) following induction.

2.6. Protein production and purification

2.6.1. Frizzled

2.6.1.1. *Twin-Strep-tagged constructs*

Frozen pellets of cells containing the protein of interest were thawed on ice and resuspended in resuspension buffer (25 mM HEPES, pH 8.0, 0.3 M NaCl, 1 mM EDTA, supplemented with protease inhibitors from Roche), using 3 ml of buffer per 1 g of cell pellet. 10% DDM 5:1 CHS (w/v) was then added to a final concentration of 1%, and the mixture incubated at 4° C for 90 min with agitation.

The resulting cell lysate was then centrifuged at 60,000 x g for 30 min at 4° C. The supernatant was filtered using a 0.22 µm syringe filter, diluted two-fold using resuspension buffer, and loaded onto a 5 ml Strep-Tactin XT column (IBA Lifesciences) using a flow rate of 1 ml/min. Before use, the column was equilibrated with wash buffer (25 mM HEPES, pH 8.0, 0.3 M NaCl, 1 mM EDTA, 0.03% DDM 5:1 CHS), and the procedure performed at 4° C. After loading of sample, the column was washed with 50 ml wash buffer.

If cleavage of affinity tags was to be performed, a solution of 5 µg/ml 3C protease (produced in-house, containing a C-terminal 6xHis-tag) in wash buffer was injected onto the column, which was then stored at 4° C overnight. The following morning protein was eluted using 30 ml wash buffer and a 1 ml/min flowrate. Alternatively, if cleavage was not required, the protein was eluted from the column with 20 ml buffer BXT (IBA Lifesciences) using a 0.2 ml/min flowrate.

The eluate was concentrated to ~250 µl using a 100 kDa MWCO Vivaspinn column (Sartorius) operated at 4,000 x g and 4° C. The concentrated sample was transferred to a

1.5 ml tube, centrifuged at 26,000 x g for 15 min at 4° C, and injected onto a Superose 6 Increase 10/300 GL column (GE) equilibrated with SEC buffer (10 mM HEPES, pH 7.5, 150 mM NaCl, 0.03% DDM 5:1 CHS) using an Äkta pure (GE) operating at 8° C.

Size-exclusion chromatography (SEC) fractions were subjected to non-reducing SDS-PAGE, and the peaks containing the protein of interest were pooled, concentrated using a 100 kDa MWCO concentrator, and either used immediately or frozen in liquid nitrogen for storage at -80° C.

2.6.1.2. 1D4-tagged constructs

To purify 1D4-tagged constructs, a procedure similar to Twin-Strep-tagged construct purification was utilized, except the suspension (25 mM HEPES, pH 7.5, 300 mM NaCl, protease inhibitors from Roche) and wash buffers (25 mM HEPES, pH 7.5, 300 mM NaCl, 0.03% DDM 5:1 CHS) were different. For affinity purification, 30 µl of CnBr-1D4 resin were added per 1 g of solubilized cell pellet. The resin was then incubated for 2 h at 4° C, washed with 40 RV (resin volume) wash buffer, and finally incubated overnight at 4° C with 2 RV of 500 µM solution of 1D4 peptide (Cube Biotech) in wash buffer. The eluate was then collected and used for SEC as described previously.

2.6.1.3. Decahistidine-tagged constructs

Decahistidine-tagged constructs were purified similarly to 1D4-tagged constructs, except a 5 ml HisTrap FF column (GE) was used for affinity chromatography step. The column was pre-equilibrated with wash buffer, and clarified cell lysates loaded using a flow rate of 1 ml/min. The column was then washed three times with 20 column volumes (CV) of wash buffer supplemented with either 20, 50, or 100 mM imidazole.

Finally, the protein was eluted with wash buffer containing 500 mM imidazole. All steps were performed at 4° C.

2.6.2. Dvl

Chemically competent *E. coli* Rosetta 2(DE3) cells (Novagen) were transformed with pET-22b vectors encoding Dvl constructs while lacking the pelB secretion signal. A single colony was then used to inoculate a 30 ml preculture of LB medium supplemented with 100 µg/ml ampicillin and 34 µg/ml chloramphenicol. The preculture was placed in a shaking incubator operated at 37° C, 250 rpm. The following day 20 ml of preculture were used to start the main culture (1 L) using the same media composition and incubation conditions. Once OD₆₀₀ had reached 0.8, protein expression was induced by the addition of 1 mM IPTG. The culture was then placed overnight in a shaking incubator operating at 28° C, 250 rpm. Cells were harvested by centrifugation at 8,000 x g for 30 min at 4° C, flash-frozen in liquid nitrogen, and stored at -80° C.

Cell pellets were resuspended in 30 ml buffer A (50 mM TRIS-HCl, pH 8.0, 500 mM NaCl, protease inhibitors from Roche) and lysed using a Sonics Vibra-Cell VCX500 sonicator (microtip, 35% amplitude, 5/1s pulse, 3 x 1 min). Lysates were clarified by centrifugation at 50,000 x g for 30 min at 4° C, and the supernatant loaded onto a 5 ml HisTrap FF column pre-equilibrated with buffer A. The column was then washed three times with 20 CV buffer A supplemented with either 20, 50, or 100 mM imidazole. Finally, the protein was eluted with buffer A containing 500 mM imidazole.

The eluate was concentrated using a Vivaspin column with an appropriate molecular weight cut-off, centrifuged at 26,000 x g for 15 min, and injected onto a Superdex 75 HiLoad 16/600 GL column equilibrated with 50 mM TRIS-HCl, pH 8.0, 300 mM NaCl using an Äkta pure (GE) operating at 8° C.

SEC fractions were subjected to reducing SDS-PAGE, and the peaks containing the protein of interest were pooled, concentrated, and either used immediately or frozen in liquid nitrogen for storage at -80° C.

2.6.3. Ndp

Conditioned media from HEK293S GnTI⁻ TetR cell cultures stably expressing Ndp constructs was filtered using a 0.22 µm Steritop filter (Merck) and diafiltrated using Ndp buffer (20 mM HEPES, pH 7.5, 500 mM NaCl) and a QuixStand benchtop system equipped with a 50 kDa MWCO cartridge (GE). The diafiltrate was then recirculated over 5 ml of pre-equilibrated amylose resin (NEB) for 3 h using a flow rate of 10 ml/min. The resin was then washed with 100 ml Ndp buffer and subsequently incubated with 30 ml of the same buffer supplemented with 20 mM maltose. The eluate was then collected, concentrated using a 100 kDa MWCO Vivaspinn column, centrifuged at 26,000 x g for 15 min, and injected onto a Superdex 200 10/300 GL column equilibrated with Ndp buffer using an Äkta pure (GE) operating at 8° C.

SEC fractions were subjected to SDS-PAGE, and the peaks containing the protein of interest were pooled, concentrated, and used immediately.

2.6.4. Notum

Production of Notum was performed similarly to Ndp, with slight differences.

Conditioned media obtained from transiently transfected HEK293T cell cultures in roller bottles was diafiltrated against buffer A (10 mM HEPES, pH 7.5, 500 mM NaCl, 25 mM imidazole), and a 10 kDa MWCO QuixStand cartridge was used. The diafiltrate was loaded onto a 5 ml HisTrap FF column (pre-equilibrated with buffer A) using a flow rate of 3 ml/min. The column was then washed two times with 15 CV buffer A,

which was supplemented with 50 mM imidazole for the second wash. The protein was eluted with buffer A supplemented with 500 mM imidazole.

The eluate was then concentrated using a 30 kDa MWCO Vivaspin column, centrifuged at 26,000 x g for 15 min, and injected onto a HiLoad 16/60 Superdex 75 pg column equilibrated with 10 mM HEPES, pH 7.5, 150 mM NaCl using an Äkta pure (GE) operating at 8° C.

SEC fractions were subjected to SDS-PAGE, and the peaks containing the protein of interest were pooled, concentrated, flash-frozen in liquid nitrogen, and stored at -80° C.

2.6.5. Nanobodies

All nanobodies (Nbs) were produced according to Steyaert et al. (Pardon et al., 2014) Competent *E. coli* WK6 cells were transformed with pMESy4 vectors encoding the Nb of interest. The cells were then cultured overnight at 37° C on LB agar plates containing 100 µg/ml ampicillin and 2% glucose. A single colony was used to inoculate 20 ml LB supplemented with 100 µg/ml ampicillin, 2% glucose and 1 mM MgCl₂, which was then placed in a shaking incubator operating at 37° C, 190 rpm. Next day 3 ml of preculture was used to start a main culture with 330 ml TB media supplemented with 100 µg/ml ampicillin, 0.1% glucose, and 1 mM MgCl₂. Nb expression was induced by adding IPTG to a final concentration of 1 mM IPTG once culture OD₆₀₀ had reached 0.7. The cells were then cultured overnight at 25° C, 190 rpm.

Cells were harvested by centrifugation at 7,000 x g for 10 min. Supernatant was discarded, the cell pellet resuspended in 5 ml ice-cold TES buffer (200 mM TRIS, pH 8.0, 0.5 mM EDTA, 500 mM sucrose), and the suspension then incubated at 4° C with agitation overnight. 30 mL 0.25x TES buffer were then added, followed by another

incubation step for 45 min at 4° C with agitation. Supernatant was then collected by centrifugation at 8,000 x g for 30 min and filtered with a 0.22 µm syringe filter.

The supernatant was loaded onto a 5 ml HisTrap column equilibrated with PBI buffer (50 mM phosphate, pH 7.0, 1 M NaCl), using a flow rate of 1 ml/min. The column was then washed with 6 CV PBI buffer and 6 CV PBI buffer supplemented with 10% PBII buffer (50 mM phosphate, pH 7.0, 150 mM NaCl, 500 mM imidazole). Nb was eluted with 6 CV PBII buffer.

The eluate was then concentrated using a 3 kDa MWCO Vivaspin column, centrifuged at 26,000 x g for 15 min, and injected onto a Superdex 75 10/300 GL column equilibrated with 10 mM HEPES, pH 7.5, 150 mM NaCl using an Äkta pure (GE) operating at 8° C.

SEC fractions were subjected to reducing SDS-PAGE, and the peaks containing the protein of interest were pooled, concentrated, flash-frozen in liquid nitrogen, and stored at -80° C.

2.6.6. F2.A Fab fragments

Competent *E. coli* Rosetta 2(DE3) cells were transformed with an RH2.2 vector encoding the heavy and light chains of F2.A Fab (obtained as a kind gift from the Sidhu lab, University of Toronto). The heavy and light chains of F2.A contained C-terminal 6xHis- and FLAG-tags respectively, with the former tag being removed in the F2.A-2 construct. The cells were plated out on agar supplemented with 100 µg/ml ampicillin and 34 µg/ml chloramphenicol. After an overnight incubation at 37° C, a single colony was used to inoculate 30 ml 2xYT media supplemented with 100 µg/ml ampicillin and 34 µg/ml chloramphenicol. The culture was placed in a shaking incubator operating at

37° C, 250 rpm and grown overnight. 10 ml of this starter culture was then used to inoculate 300 ml 2xYT media supplemented with 100 µg/ml ampicillin and 34 µg/ml chloramphenicol. The cells were cultured at 37° C, 250 rpm, and protein expression was induced by the addition of 1 mM IPTG once OD₆₀₀ reached 0.8. The temperature was then reduced to 28° C, and the cells grown overnight.

Cells were harvested by centrifugation 12,000 x g for 30 min. Supernatant was discarded, and the cell pellet resuspended in 20 ml PBS supplemented with 2 mM MgCl₂ and protease inhibitor cocktail from Roche. The suspension was then sonicated (3 x 1 min, 5 s : 2 s pulse, microtip, 35% amplitude, 66,666 energy, Sonics Vibra-Cell VCX500). The lysate was clarified by centrifugation at 50,000 x g for 30 min, and the resulting supernatant filtered using a 0.22 µm filter. Protein A resin (100 µl per 100 ml culture, GE) was then added, and the suspension incubated overnight at 4° C with agitation.

Protein A resin was washed with 30 ml PBS supplemented with 2 mM MgCl₂, and Fab eluted using 6 ml of elution buffer (50 mM NaH₂PO₄, 100 mM H₃PO₄, 140 mM NaCl, pH 2.8) into a tube containing 1 ml of neutralization buffer (750 mM Na₂HPO₄, 140 mM NaCl, pH 9.0). The sample was concentrated to ~500 µl using a 30 kDa MWCO Vivaspın column and subsequently heat-treated for 60 min at 60° C.

The sample was centrifuged at 26,000 x g for 15 min, and injected onto a Superdex 200 10/300 GL column equilibrated with 10 mM HEPES, pH 7.5, 150 mM NaCl using an Äkta pure (GE) operating at 8° C.

SEC fractions were subjected to non-reducing SDS-PAGE, and the peaks containing the protein of interest were pooled, concentrated, flash-frozen in liquid nitrogen, and stored at -80° C.

2.6.7. Nanodisc scaffold proteins

30 ml LB media (supplemented with 30 µg/ml kanamycin and 34 µg/ml chloramphenicol) were inoculated with a single colony from freshly streaked *E. coli* Rosetta 2 (DE3) cells transformed with a pET-28a vector encoding the MSP scaffold protein. The culture was incubated overnight at 37° C, 250 rpm, and then used to inoculate 1 L TB supplemented with 10 µg/ml kanamycin and 34 µg/ml chloramphenicol. The cells were grown at 37° C, 250 rpm. Once culture OD₆₀₀ had reached 0.8, protein expression was induced with 1 mM IPTG. The incubator temperature was then reduced to 28° C, and the culture grown overnight.

Cells were harvested by centrifugation at 8,000 x g for 15 min and resuspended in 50 ml of 20 mM sodium phosphate buffer, pH 7.4, supplemented with protease inhibitors from Roche. 1% Triton X-100 was added, and the cells lysed by sonication (microtip, 3 x 1 min, 5 s : 2 s pulse, 66,666 energy, Sonics Vibra-Cell VCX500). Lysate was clarified by centrifugation at 50,000 x g for 30 min, filtered using a 0.22 µm filter, and loaded onto a 5 ml HisTrap FF column pre-equilibrated with 40 mM phosphate buffer, pH 7.4, using a flow rate of 1 ml/min. The column was then washed with 10 CV of buffer 1 (40 mM TRIS-HCl, pH 8.0, 300 mM NaCl, 1% Triton X-100), buffer 2 (40 mM TRIS-HCl, pH 8.0, 300 mM NaCl, 50 mM Na-Cholate, 20 mM imidazole), and buffer 3 (40 mM Tris/Hcl, pH 8.0, 300 mM NaCl, 50 mM imidazole). The protein was eluted with elution buffer (40 mM Tris-HCl, pH 8.0, 300 mM NaCl, 400 mM imidazole).

The eluate was concentrated using a 3 kDa MWCO Vivaspin column, centrifuged at 26,000 x g for 15 min, and injected onto a Superdex 75 10/300 GL column equilibrated with 20 mM Tris-HCl, 100 mM NaCl, 0.5 mM EDTA, pH 7.4, using an Äkta pure system operating at 8° C.

SEC fractions were subjected to reducing SDS-PAGE, and the peaks containing the protein of interest were pooled, concentrated, flash-frozen in liquid nitrogen, and stored at -80° C.

2.7. Nanodisc reconstitution

POPC chloroform solution (Avanti Polar Lipids) was transferred to a glass test tube, wrapped in foil, and placed in a dessicator overnight. A buffer consisting of 10 mM HEPES, pH 7.5, 150 mM NaCl, 50 mM Na-cholate was then added to the dried POPC film to yield a final POPC concentration of 20 mg/ml. The POPC film was then solubilized by placing it in a water bath sonicator set to 37° for 2 h.

Purified MSP scaffold and POPC solution were then mixed to yield the desired MSP : POPC molar ratio, supplemented with 0.03% DDM 5:1 CHS, and incubated for 1 h at 4° C. Purified Fzd protein was then added to the mixture to yield the desired Fzd : MSP : POPC molar ratio, followed by incubation for 2 h at 4° C. Pre-wetted Bio-Beads SM-2 resin (Bio-Rad) was then added (normally 500 mg/ml), and the reconstitution mixture incubated overnight at 4° C with agitation.

To obtain nanodiscs containing Fzd, the affinity chromatography and SEC steps previously used to purify the Fzd sample were repeated.

2.8. Protein extraction and purification using SMALPs

Cell pellets obtained from HEK293S GnTI⁻ TetR suspension cultures stably expressing the proteins of interest were resuspended in 30 ml of lysis buffer (10 mM HEPES, pH 7.5, 1 mM EDTA, protease inhibitors from Roche), incubated for 2 h at 4° C with agitation, and sonicated (1 min, 5 s : 2 s pulse, microtip, 35% amplitude, 66,666 energy, Sonics Vibra-Cell VCX500). The membranes were collected by centrifugation at 120,000 x g for 45 min at 4° C, and washed twice by resuspension in 30 ml wash buffer (10 mM HEPES, pH 7.5, 1 M NaCl) and removal of supernatant by centrifugation at 120,000 x g for 45 min at 4° C.

Washed membranes were then resuspended in SMA solubilization buffer (50 mM TRIS-HCl, pH 8.0, 500 mM NaCl, 10% glycerol) to a final membrane concentration of 80 mg/ml. 5% SMA Xiran SL 30010 solution (Polyscope) in SMA solubilization buffer was then added to resuspended membranes at a 1 : 1 v/v ratio, and the resulting mixture incubated at room temperature for 3 h.

The resulting SMALP solution was then clarified by centrifugation at 120,000 x g at 4° C for 45 min, and the supernatant collected and diluted 10-fold using 10 mM TRIS, pH 8.0, 100 mM NaCl. To isolate SMALP particles containing the protein of interest, the supernatant was then used to perform affinity chromatography suitable for the target protein, omitting detergent in all buffers used and adjusting the pH to 8.0. SEC was usually not used for analysis of the final sample.

2.9. Protein complex formation in detergent

Fresh samples of 3C-treated Fzd4-1, Ndp-3, and F2.A were used for complex formation. Before use, Ndp-3 and F2.A samples were supplemented with 0.03% DDM 5:1 CHS. The desired amount of Fzd4-1 was dispensed into a 1.5 ml tube, followed by the addition of Ndp3, usually to yield a 1 : 1 molar ratio of Fzd4-1 and Ndp-3. The sample was incubated for 30 min, followed by the addition of F2.A at a 1:2 Fzd4-1 : F2.A molar ratio. Optionally, 3C protease (produced in-house, containing a C-terminal 6xHis-tag) was added to the sample at a final concentration of 5 µg/ml. The final mixture was then either incubated overnight at 4° C. or used immediately.

To assess complex formation, 3 ul of the sample were injected onto a Superose 6 Increase 3.2/300 GL column equilibrated with 10 mM HEPES, pH 7.5, 150 mM NaCl, 0.03% DDM 5:1 CHS, using a Shimadzu Prominence system operating at 8° C. For large-scale purification of Fzd4-1/Ndp-3/F2.A complex, the sample was concentrated using a 100 kDa MWCO Vivaspın column and injected onto a Superose 6 Increase 10/300 GL column equilibrated with 10 mM HEPES, pH 7.5, 150 mM NaCl, 0.03% DDM, using an Äkta pure system operating at 8° C.

2.10. Protein expression tests

2.10.1. Using size-exclusion chromatography

Small-scale expression tests of fluorescent Fzd constructs were performed by using 6-well plates containing confluent HEK293S GnTI⁻ TetR cell cultures expressing the protein of interest. The cells were removed by aggressive washing with PBS, which was subsequently removed by centrifugation at 100 x g for 5 min. The resulting cell pellet

was resuspended in 0.2 ml of 10 mM HEPES, pH 7.5, 150 mM NaCl, 0.03% DDM 5:1 CHS, and incubated for 90 min at 4° C with agitation.

Lysate was clarified by centrifugation at 26,000 x g for 30 min at 4° C. 10 ul of the final sample was then injected onto a Superose 6 Increase 10/300 GL column equilibrated with 10 mM HEPES, pH 7.5, 150 mM NaCl, 0.03% DDM 5:1 CHS using a Shimadzu Prominence system operating at 8° C. Presence of mVenus-tagged protein was detected by emission at 528 nm using an excitation wavelength of 515 nm.

2.10.2. Using fluorescence microscopy

Stable HEK293S GnTI⁻ TetR cell lines expressing mVenus-tagged protein of interest were cultured in a 6-well plate. Upon reaching ~90% confluency, protein expression was induced using 10 µg/ml doxycycline. After 72 h, the media was replaced with phenol-free DMEM (Gibco), and the cells imaged using a Nikon Eclipse TE2000U fluorescence inverted microscope equipped with a Hamamatsu ORCA-285 CCD camera. Images were taken using a YFP fluocube with a 600 ms exposure time.

2.11. Yeast Surface Display

2.11.1. Yeast library culturing and expansion

An aliquot of *S. cerevisiae* cells (protease-deficient strain BJ5465) containing a synthetic yeast display nanobody library (McMahon et al., 2018) was obtained as a gift from the Kruse lab. The aliquot was thawed at 30° C and resuspended in 1 L of Yglc4.5-Trp media (containing 3.8 g yeast synthetic drop-out medium supplements without tryptophan (Sigma-Aldrich), 6.7g yeast nitrogen base (Sigma-Aldrich), 10.4 g Na-citrate, 7.4 g citric acid monohydrate, 100 units Pen-Strep (Gibco), 20 g glucose, pH 4.5). The cells were incubated in a shaking incubator overnight at 30° C, 230 rpm. The

culture was then expanded to 3 L using the same media and incubated at 30° C, 230 rpm for 48 h. Cells were collected by centrifugation at 3,500 x g for 5 min and resuspended in Yglc4.5-Trp supplemented with 10% DMSO to a final cell density of 10¹⁰ cells/ml. 2 ml aliquots of resuspended cells were then distributed between cryogenic tubes and frozen using a CoolCell freezing chamber placed at -80° C.

2.11.2. Magnetic-activated cell sorting (MACS)

Yeast cells containing the synthetic yeast display nanobody library were centrifuged at 3,500 x g for 3 min, and the cell pellet resuspended in 50 ml -Trp+Gal media (containing 3.8 g/L yeast synthetic drop-out medium supplements without tryptophan 6.7 g/L yeast nitrogen base, 100 units Pen-Strep, 20 g galactose, pH 6.0). The cells were centrifuged again at 3,500 x g for 3 min, the supernatant removed, and the cell pellet resuspended in 500 ml -Trp+Gal media. Cells were then incubated at 25° C, 220 rpm for 72 h.

The cell density of yeast culture was then measured, and 5 x 10¹⁰ cells (10 x the estimated diversity of the naïve library) were dispensed in a 15 ml tube. The cells were centrifuged at 3,500 x g for 3 min at 4° C, supernatant removed, and the pellet resuspended in 10 ml of chilled selection buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 0.1% (w/v) BSA, 5 mM maltose, 0.03% DDM 5 : 1 CHS). The cells were then centrifuged, the supernatant removed, and the pellet resuspended in 4.9 ml selection buffer (SB).

100 µl Streptavidin microbeads (Miltenyi Biotec) were added to the resuspended cells, followed by incubation for 40 min at 4° C with agitation. The sample was then centrifuged at 3,500 x g for 3 min at 4° C, supernatant aspirated, and the pellet resuspended in 5 ml SB. The sample was then added to an LD column pre-equilibrated

with SB (Miltenyi Biotec) while placed in a MidiMACS separator (Miltenyi Biotec), which was washed with additional 2 ml SB. Flowthrough was collected, centrifuged at 3,500 x g for 3 min at 4° C, supernatant aspirated, and the pellet resuspended in 5 ml SB containing 1.5 µM of 3C-cleaved Fzd6-7, which was previously biotinylated using BirA500 kit (Avidity) and subjected to an additional SEC step. The sample was incubated at 4° C for 60 min with agitation, centrifuged at 3,500 x g for 3 min at 4° C, the supernatant removed, and the cell pellet resuspended in 4.9 ml SB. 100 µl Streptavidin microbeads were added, and, following incubation at 4° C for 20 min with agitation, the cells were washed twice by centrifugation at 3,500 x g for 3 min at 4° C and resuspension in 3 ml SB buffer. The sample was then added to an LS column (Miltenyi Biotec) pre-equilibrated with SB while placed in a MidiMACS separator. The column was washed with 8 ml SB and then removed from the MidiMACS separator. 5 ml SB were added to the column and the sample immediately eluted. The collected cells were then centrifuged at 3,500 x g for 3 min at 4° C, supernatant removed, and the pellet resuspended in 3 ml -Trp+Glc media (containing 3.8 g/L yeast synthetic drop-out medium supplements without tryptophan 6.7 g/L yeast nitrogen base, 100 units Pen-Strep, 20 g glucose, pH 6.0). The cells were then placed in a shaking incubator operating at 30° C, 220 rpm for 24 h.

2.11.3. Fluorescence-activated cell sorting (FACS)

Selected yeast cells were centrifuged at 3,500 x g for 3 min, and the cell pellet resuspended in 50 ml -Trp+Gal media. The cells were centrifuged again at 3,500 x g for 3 min, the supernatant removed, and the cell pellet resuspended in 500 ml -Trp+Gal media. Cells were then incubated at 25° C, 220 rpm for 72 h.

The cell density of yeast culture was then measured, and $\sim 10^6$ cells dispensed into each of the 4 pre-prepared 1.5 ml tubes containing 500 μ l SB. The cells were centrifuged at 3,500 x g for 3 min at 4° C, supernatant removed, and the pellets resuspended in 100 μ l SB. 0.5 μ g anti-HA-tag mouse antibody labelled with Alexa Fluor 488 (AF488, ThermoFisher) was added to the first tube (nanobody expression control), 0.5 μ g Streptavidin-Alexa Fluor 647 (AF647, ThermoFisher) and 1.5 μ M biotinylated, 3C-cleaved Fzd6-7 added to the second (Fzd6-7 binding control), all three aforementioned reagents added to the third tube (sample for sorting), and none added to the last tube (unlabelled control). The tubes were then incubated at 4° C for 15 min, and the cells washed by centrifugation at 3,500 x g for 3 min at 4° C, supernatant removal, resuspension in 700 μ l SB, followed by another round of centrifugation, supernatant removal, and resuspension in 100 μ l SB.

The cells were sorted using a SH800 cell sorter (Sony Biotechnology) equipped with a 100 μ m microfluidic sorting chip, using 488 nm and 638 nm lasers for detecting AF488 and AF647 respectively. Following calibration using the AutoSetup Beads (Sony Biotechnology), AF488- and AF647-labelled samples were used for fluorescence compensation. The control samples were then sorted, maintaining the event rate at 1,000 events per second, and recording 100,000 events in total. The main population of cells on the FSC-A/BSC-A plot ($\sim 80\%$ of all cells), were used to generate an FSC-A/FSC-H plot, from which the main population of cells was again selected ($\sim 64\%$ of all cells, S. fig. 4). The AF488 signal of these cells was plotted against the AF647 signal, enabling the control sample plots to be used to set sorting gates that would be used to collect cells exhibiting both nanobody expression and Fzd6-7 binding. The AF647- and AF488-labelled yeast cells were then sorted using the the previously set gates. The experiment

was performed using an event rate of 300 events per second, recording 1,000,000 events in total, and cells exhibiting significant levels of AF647 and AF488 signal were sorted in a tube containing 2 ml -Trp+Glc media using the sorting mode 'Normal'. The cells were then placed in a shaking incubator operating at 30° C, 220 rpm for 24 h.

2.11.4. Enzyme-linked immunosorbent assay (ELISA)

100 µl of 0.01 mg/ml Streptavidin solution in PBS was added to every well of a 96-well Nunc MaxiSorp plate. The plate was then covered with an adhesive seal and incubated at 4° C overnight. The wells were washed five times with PBS, blocked with 2% BSA (w/v) in PBS for 2 h at room temperature, and washed again five times with buffer E (10 mM HEPES, 150 mM NaCl, 0.03% DDM 5:1 CHS). 100 µl of 5 µg/ml biotinylated Fzd6-7 in buffer E was then added to every other row of the plate, followed by incubation for 1 h at room temperature with agitation and five subsequent washes with buffer E. Nanobody-containing periplasmic extracts were obtained as previously described (see 'Protein production and purification – nanobodies'). The extracts were 5-fold diluted in buffer E supplemented with 0.2% BSA, 100 µl of the diluted extract added to Fzd6-7-coated and uncoated wells, and the plate incubated for 1 h at room temperature with agitation. The plate was then washed five times with buffer E. A solution containing Anti-polyHistidine mouse (HIS-1, 4,000x dilution of ascites fluid, Sigma-Aldrich) and anti-mouse IgG (Fc specific)–peroxidase antibodies (1,000x dilution, Sigma-Aldrich) was prepared in buffer E and incubated for 30 min at room temperature. 100 µl of the antibody solution was then added to each well of the plate, which was incubated for 1 h at room temperature with agitation. After five washes with buffer E, TMB and peroxide solution was added to the wells, using reagents from the TMB peroxidase EIA substrate kit (Bio-Rad). The peroxidase reaction was stopped

after 10 min by adding 100 μ l 2M sulfuric acid to each well. Finally, 430 nm absorbance values of each well were measured using a Tecan Infinite 200 plate reader.

2.12. Biolayer interferometry (BLI)

2.12.1. Protein biotinylation

Purified samples of avi-tagged proteins were biotinylated using the BirA biotin-protein ligase kit (Avidity). A reaction mixture was prepared, containing 40 μ M of the avi-tagged sample, 50 mM bicine, pH 8.3, 10 mM ATP, 10 mM MgOAc, and 50 μ M d-biotin. 2.5 μ g of BirA ligase was then added for every 10 nmol of substrate. The sample was then incubated overnight at 4° C with agitation, subsequently repeating the SEC procedure suitable for the biotinylated sample.

2.12.2. Using the BLItz and Octet instruments

The operation of both BLItz and Octet instruments (FortéBio) was similar, except the volume of buffer or sample used was different for each system (4 μ l and 220 μ l respectively). The sample buffer used was 10 mM HEPES, pH 7.5, 150 mM NaCl, 0.1% BSA, 0.03% 5:1 CHS. All experiments used Octet Streptavidin biosensors (FortéBio), which were hydrated in the sample buffer for 10 min before use. The biosensor was placed in the instrument and equilibrated with sample buffer. Biotinylated, cleaved Fzd6 constructs were then loaded onto the surface of the biosensor using sample buffer supplemented with 50 μ g/ml protein. The biosensor was equilibrated again with sample buffer, subsequently exposing it to the analyte in sample buffer at a specific concentration. The biosensor was finally equilibrated again with the sample buffer.

2.13. Electron Microscopy (EM)

2.13.1. Negative-stain EM

Purified samples at a concentration ranging from 5 to 50 µg/ml were dispensed on a glow-discharged Formvar/Carbon 300 Cu mesh grids (Agar Scientific) and incubated for 40 s. The samples were then blotted away via filter paper, immediately washing the grids two times with ddH₂O and once with 0.75% uranyl formate. The grids were then incubated in 0.75% uranyl formate for 40 s, after which the stain solution was blotted away. The grids were then left to dry for 40 min, after which they were stored in a cool, dark place until imaging. The samples were then examined using a Tecnai T12 transmission electron microscope (FEI) operated at 120 kV. Images were taken at a nominal magnification of 42,000 x with a calibrated pixel size of 2.63 Å, using a 2.5 µm defocus.

2.13.2. Cryo-EM Screening and Data collection

3.5 µl of sample were applied to glow-discharged grids using the plasma cleaner PDC-002-CE (Harrick Plasma). The ranges of sample concentrations used for screening were 0.1-1 mg/ml for samples without detergent and 0.5-10 mg/ml for samples containing detergent. Different copper grids covered with holey carbon film were used for sample screening depending on the sample type. For detergent-containing samples Quantifoil R1.2/1.3 200 Cu mesh grids (EMS) were used, for SMALPs - Quantifoil R2/2 200 Cu mesh grids (EMS), for SARS-CoV-2 Spike - C-Flat R2/1 200 Cu mesh grids (EMS). The grids were blotted using Vitrobot Mark IV (Thermo Fisher Scientific) operated at 100% humidity, 4° C, 2 – 5 s blotting time and -15 force. For SARS-CoV-2 Spike protein a blotting time and force of 6 s and -1 were used respectively. Grids were then plunged in liquid ethane, clipped, and stored in liquid nitrogen until screening.

Sample screening was performed using a Glacios 200 kV microscope equipped with a Falcon 3 detector (Thermo Fisher Scientific) operated in linear mode. Micrographs for estimating sample quality were taken using a nominal defocus value of 3.5 μm , 92,000 x magnification, and $\sim 60 \text{ e}^-/\text{\AA}^2$ dose after manually setting the Z-height. If particle quality and distribution appeared to be suitable, grid squares and holes with suitable particle density and ice thickness for data collection were manually selected using EPU version 2.5 (Thermo Fisher Scientific). Following beam alignment, automated astigmatism and coma correction was performed on carbon-containing regions using the built-in auto-functions of EPU. A carbon- and copper-free region was then used to record the camera gain reference. Preliminary data collection on the Glacios microscope was performed using a 92,000 x magnification with a calibrated pixel size of 1.54 $\text{\AA}$, $\sim 60 \text{ e}^-/\text{\AA}^2$ dose, and 40 frames per movie in linear mode, using a defocus range of 3 – 1.2 μm . Data collection for the Fzd6-5 in SMALPs, Fzd4-1/F2.A in DDM, and Fzd4-1/Ndp-3/F2.A-2 complex was performed using a Titan Krios G2 electron microscope (Thermo Fisher Scientific) operating at 300 kV, equipped with a K2 camera (Gatan) and a GIF Quantum energy filter (Gatan) with a 20 eV slit. Before data collection, beam alignments, coma and astigmatism correction, as well as the recording of the gain reference was performed similarly as on the Glacios microscope, with the inclusion of a Gatan Imaging Filter (GIF) tuning step. Movies were collected in counting mode, using EPU version 2.5 (Thermo Fisher Scientific). The imaging parameters were different for each dataset collected on the Krios, for details see S. Table 1.

2.13.3. Cryo-EM data processing

Data processing was performed using cryoSPARC v3.2 (Punjani et al., 2017). The exact workflow and parameters used in each step of the data processing procedure varied between each dataset being processed, so the following is a generalized overview of a

typical workflow. The processing details for each sample can be found at S. fig. 11-13, 16, 19.

Global motion correction and alignment of movies was performed using a 5 x 5 patch-based alignment. The resulting full-frame micrographs were then used for estimating contrast transfer function (CTF) using GCTF (v.1.06) (Zhang, 2016). Micrographs exhibiting high levels of motion, low CTF resolution estimates, or ice contamination were discarded. Non-template-driven (blob-based) particle picking was performed, specifying a range of the expected particle diameter, followed by 2D classification. The classes resembling the expected shape of the particles were then used for template-based particle picking. The picked particles were then used for several rounds of 2D classification. Each round usually consisted of 20-25 iterations, using a batchsize of 500 – 1500 particles per class and a circular mask suitable to the size of the particles. During the initial rounds of 2D classification a 10 Å resolution limit was imposed. The worst classes were discarded before each new round. If the 2D classes obtained had much higher quality than the ones used for template-driven picking, it was repeated before proceeding with additional rounds of 2D classification. The final set of particles obtained from 2D classes was then subjected to per-particle motion correction and used for *ab initio* 3D map generation, followed by a mixture of homogenous and heterogenous refinements. Visual representations of the final models were generated using Chimera 1.14 (Pettersen et al., 2004).

2.14. Notum crystallization, data collection, and structure determination

200 nl purified Notum at 5 mg/ml and 100 nl of reservoir solution (0.1 M Na-citrate, 4.1 pH, 1.4 M $(\text{NH}_4)_2\text{SO}_4$) were dispensed in 96-well Swissci/MRC plates for crystallization using the sitting-drop vapour diffusion method. The plates were sealed and kept at room temperature for 2 weeks, after which orthorhombic crystal formation was evident.

Compounds to be used for soaking Notum crystals were provided in powder form by Dr Paul Fish (UCL, London, UK). Crystal soaking was performed by unsealing the wells containing crystals, and 300 nl of reservoir solution supplemented with ~5 mg/ml of the compound of interest and 40% ethylene glycol were then added to the sitting drop. The plate was then resealed and incubated for 24 h at room temperature. Soaked crystals were subsequently mounted using MicroLoops (MiTeGen) with an aperture size of 100 μm and immediately flash-frozen in liquid nitrogen for storage in UniPucks (MiTeGen) until data collection.

X-ray diffraction data sets were collected at 100 K using the I04-1 beamline at the Diamond Light Source (Didcot, UK) and an X-ray wavelength of ~0.92 Å. All crystals of Notum were orthorhombic, exhibiting $\text{P2}_1\text{2}_1\text{2}_1$ symmetry with one protein molecule per asymmetric unit, and approximate cell dimensions of 60, 72, and 78 Å (a, b, c). All unit cell angles were 90°. Data were scaled and integrated using Xia2 (Winter, 2009) with default settings. The phases for each dataset were determined by molecular replacement using Phaser (McCoy et al., 2007) with default settings and a previously published Notum structure (Kakugawa et al., 2015, PDB ID: 4UZ1) with all ligands and water molecules removed. The same model was the rigid-body fitted within the resulting electron density map using phenix.refine (Adams et al., 2011) with all other

refinement options ignored. This was followed by further refinement using phenix.refine (3 cycles, coordinate refinement in real and reciprocal space, as well as individual B-factor refinement, using a maximum likelihood target function, default turn-libration-screw (TLS) parameters, and a 0.5 scale factor for X-ray/stereochemistry weight). Non-crystallographic symmetry was not imposed at any step of data processing. The resulting model was then compared against the calculated 2Fo-Fc and Fo-Fc electron density maps in COOT (Emsley et al., 2010). Regions of the model that did not fit the calculated electron density map were manually rebuilt in COOT. The new models were re-refined using phenix.refine using the same parameters as before. This process of refining, manual map/model inspection, and model rebuilding was repeated several times, until no further improvements in the $R_{\text{work}}/R_{\text{free}}$ values were observed.

Another round of phenix.refine was then performed, using the ‘Update waters’ option, as well as imposing anisotropic individual B-factor refinement in addition to the previously used parameters. The iterative process of phenix.refine and manual model building in COOT was repeated (disabling the ‘Update waters’ option). The positions of each water molecule within the model were adjusted based on the electron density present at their location. Large blobs of unexplained 2Fo-Fc and Fo-Fc electron density close to the surface of the protein model were examined, subsequently placing SO_4^{2-} , citrate³⁻, or ethylene glycol molecules within them if deemed appropriate. As the refinement progressed, these molecules, as well as water, were removed if their addition resulted in strong negative density in the Fo-Fc electron density maps.

Once no further improvements in $R_{\text{work}}/R_{\text{free}}$ values were observed, the density within the catalytic site of Notum (in the vicinity of serine 232) was examined. A structure of the ligand that was used to soak the crystal was then fitted, if deemed appropriate. The

model and restraints of the ligand were generated using eLBOW (Moriarty et al., 2009) with default settings, providing a SMILE code as the input. The previously described refinement procedure was repeated several times, adjusting the position of the ligand and the surrounding residue sidechains to achieve optimal fit against the calculated 2Fo-Fc and Fo-Fc electron density maps. This was done until no further improvements in the $R_{\text{work}}/R_{\text{free}}$ values were observed. If the final model was to be submitted to the PDB, its geometry was validated using the wwPDB validation server (Berman et al., 2003). Visual representations of the final models were generated using Chimera 1.14 (Pettersen et al., 2004).

3. Structural characterization of Fzd signalling complexes

3.1. Production of Fzd

Previous work in the group indicated that human Fzd6 could be expressed and might be amenable to further structural studies. Furthermore, Fzd6 has been shown to form dimers within cell membranes, and to bind both Dvl and heterotrimeric G-proteins (Kilander et al., 2014; Petersen et al., 2017). These features would potentially be valuable to increase the size of the particles for cryo-EM. Thus, hFzd6 seemed to be a good starting point for my project.

3.1.1. Optimization of Fzd6 production

The procedure for purifying membrane proteins in the lab at the time relied on transient expression in HEK293S GnT1⁻ cells, as well as the use of 1D4-tagged protein constructs, since affinity purification using anti-1D4 beads had been shown to yield highly pure membrane protein samples by other members of our lab in the past. The first construct of Fzd6 that I analyzed was Fzd6-4 (Fig. 13), with most of its C-terminal tail truncated, a bRIL insert that is commonly used to thermostabilize GPCRs, a C-terminal mVenus, which reportedly helped with the solubility of the protein, and a C500S mutation in the remaining intracellular region to prevent protein aggregation in the absence of reducing agents.

For the initial purification of Fzd6-4, a 12 L suspension culture of HEK293S GnT1⁻ cells was used for transient transfection with a Fzd6-4 construct within a pHL-sec vector. After three days the culture was harvested to give 40 g of cells, which were immediately used for subsequent DDM solubilization and 1D4 purification without 3C cleavage. After overnight elution from 1D4 resin with 1D4 peptide, the resin remained fluorescent, indicating that elution of 1D4-tagged Fzd by the 1D4 peptide was not a very efficient process. Because both the 1D4 tag and 1D4 peptide have similar affinities

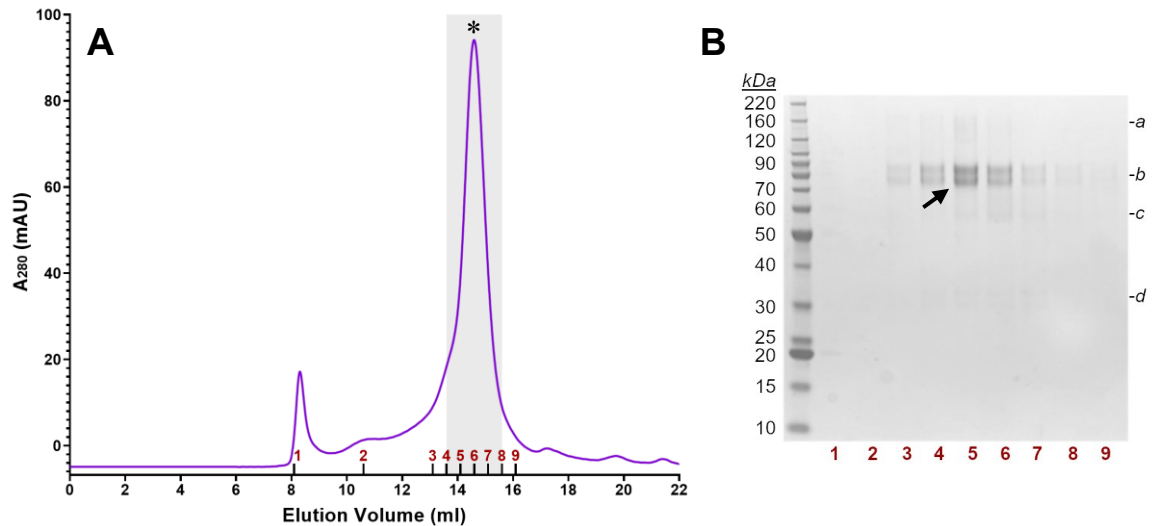


Fig. 18. Production of Fzd6-4 using transient transfection of HEK293S GnT1⁻ cells. **A** – SEC profile. Main Fzd6-4 peak shaded grey. Asterisk denotes peak containing the protein of interest. SEC performed using a Superose 6 Increase 10/300 GL column. **B** – SDS-PAGE of SEC fractions. *a* – higher molecular weight species; *b* – Fzd6-4; *c/d* – contaminants. Arrow denotes bands corresponding to the protein of interest. Experiment performed under non-reducing conditions.

to the 1D4 antibody, this suggests that much higher concentrations of 1D4 peptide would be required to efficiently elute the column.

The resulting SEC peak for the 1D4-eluted material was symmetrical (Fig. 18.A), with a height of 95 mAU. When the final sample was examined using non-reducing SDS-PAGE, the protein, which had an expected size of approximately 100 kDa, and migrated as two bands at around 85 and 75 kDa (Fig. 18.B.b).

In addition to these main bands, there were other minor species present at ~160 kDa (Fig. 18.B.a), 60 and 30 kDa (Fig. 18.B.c and d). The lower molecular weight bands were excised from the gel and sent off for N-terminal sequencing, showing that they are fragments of Fzd6-4 generated by cleavage between the CRD and the TMD. Consistent with this information, the cell culture media following centrifugation was fluorescent, which could be explained by Fzd6-4 N-terminal cleavage and subsequent release of mVenus into the media.

In conclusion, this initial trial showed that protein expression levels, sample purity, and purification efficiency needed to be improved for the production of Fzd for structural studies. A 95 mAU SEC peak from 12 L of mammalian suspension culture is a low yield, which could be due to the combined effects of using a transient expression system, protein cleavage, as well as inability to elute protein efficiently from the 1D4-antibody resin, which remained highly fluorescent following elution. To overcome these issues, I decided to take a multi-pronged approach.

First, I changed expression systems. The use of transient transfection is not efficient for large scale production of proteins, because only ~60% of the cells will express the tagged protein of interest (Elegheert et al., 2018). Furthermore, generation of DNA and large scale transfection are quite costly and time-consuming processes, so a stable cell line should not only give higher yields, but also be easier to work with. I thus decided to use a lentiviral stable cell-line generation system, which has been shown to result in transfection rates close to 100%, while also the use of inducible expression allowed me to grow cells and then express my target protein only when required.

Second, to increase sample purity and purification efficiency, I decided to create a new construct containing an N-terminal Twin-Strep tag. If the reason for these additional bands on the SDS-PAGE was Fzd6 cleavage, this would mean that the current C-terminal 1D4 tag also co-purifies cleaved Fzd6 transmembrane domains still found within cells after cleavage. Placing the tag at the N-terminus would mean that any cleaved CRDs containing the tag would be washed away during cell pelleting, thus allowing the purification of only intact Fzd still found within cell membranes. Finally, the Twin-Strep system has been shown to be highly efficient at binding proteins, while

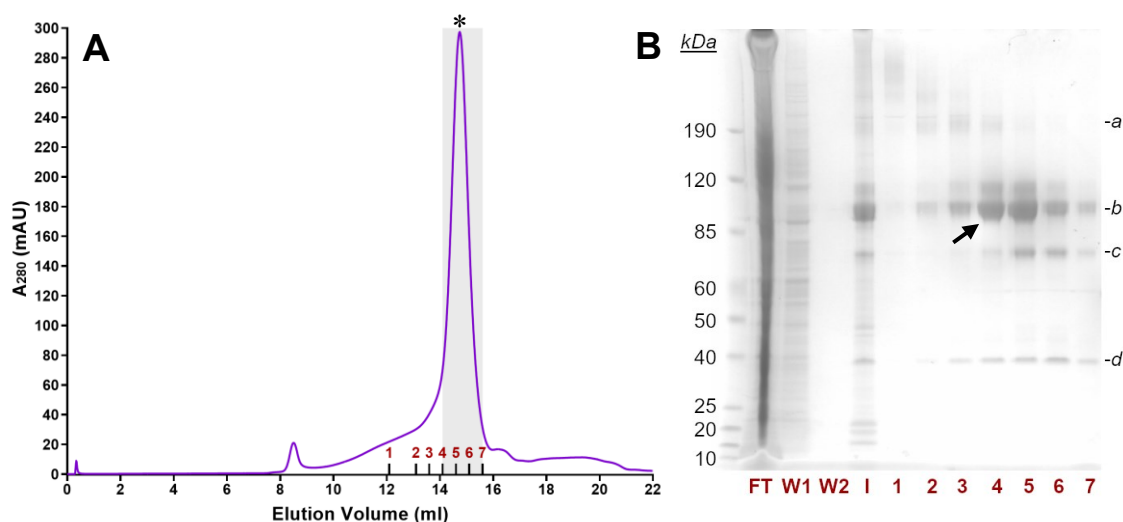


Fig. 19. Production of Fzd6-4 using stable HEK293S GnTII- TetR cell lines. **A** – SEC profile. Main Fzd6-4 peak shaded grey. Asterisk denotes peak containing the protein of interest. SEC performed using a Superose 6 Increase 10/300 GL column. **B** – SDS-PAGE of SEC fractions. FT – flowthrough from the affinity chromatography (AC) column; W1/W2 – AC column washes. I – sample used for performing SEC. *a* – higher molecular weight species; *b* – Fzd6-4; *c/d* – contaminants. Arrow denotes band corresponding to the protein of interest. Experiment performed under non-reducing conditions.

biotin used for elution has a much higher affinity for streptactin than the Twin-Strep peptide, therefore enabling higher protein recovery rates from the resin.

To test whether the lentiviral system gave increased yields, I first subcloned the same Fzd6-4 construct within the lentiviral constructs, and then used it to generate stable HEK293S GnTII- TetR cell lines. This approach also placed the expression of this gene under the control of a tetracycline repressor, therefore allowing me to induce protein expression by the addition of doxycycline. For my first purification I used 9 g of cell pellet from 1 L of cell culture and proceeded with the same 1D4 purification protocol as before. This time, using almost 4 times less material, I obtained 3 times more protein (Fig. 19.A), thus showing that stable cell lines were better suited for the production of Fzd than transient expression systems. Due to the increased protein concentrations, protein degradation bands (Fig. 19.B.c/d) were also more obvious, thus underlining the need to alter Fzd6 construct design.

I then used the same stable cell line to perform expression tests, to determine the best conditions for Fzd expression. To do this, I set up small-scale expression tests, by splitting cells containing Fzd6-4 into 6-well plates after induction, exposing them to different conditions, finally measuring Fzd6-4 levels present in cell lysates using fluorescence size-exclusion chromatography (FSEC). The conditions tested were expression time, the addition of valproic acid (VPA) to boost expression, induction at different cell densities and different media, as well as using different amounts of doxycyclin for induction (Fig. 20).

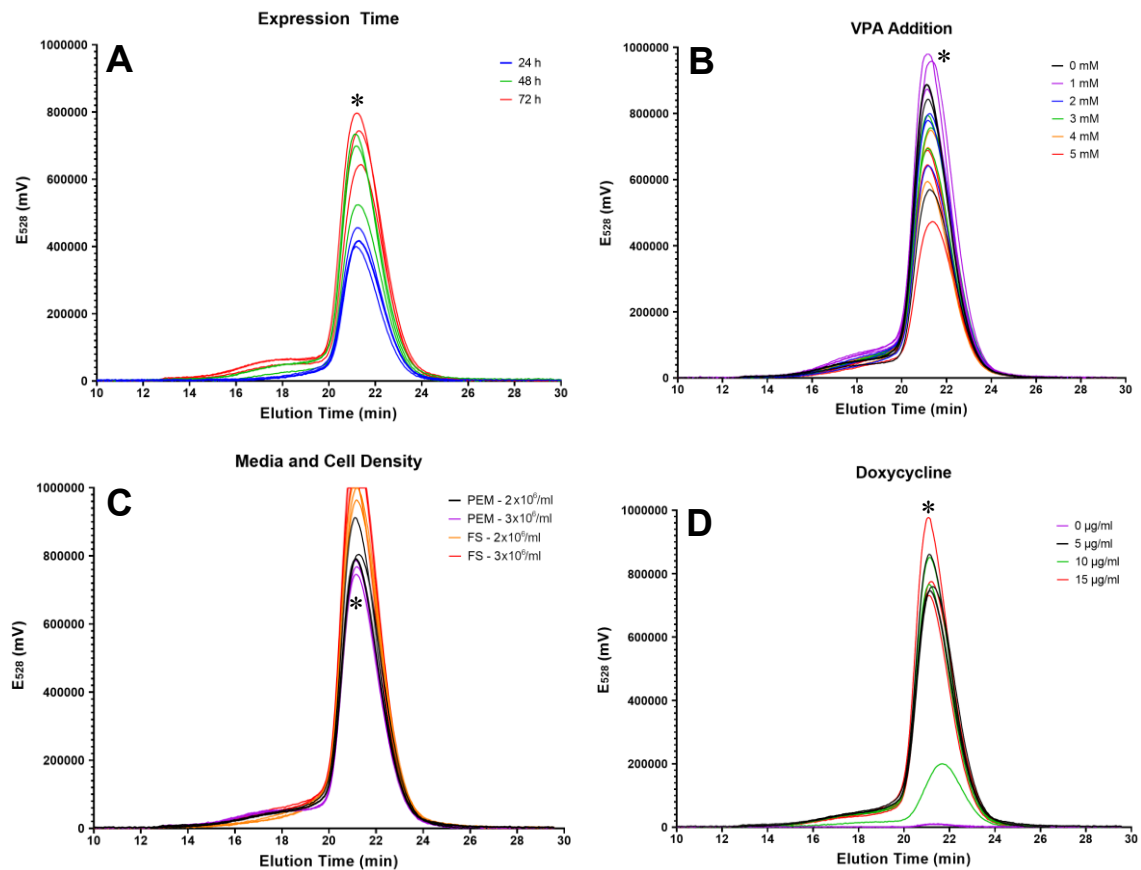


Fig. 20. Fzd6-4 expression test. **A** – *expression time*. Protein expression was allowed to proceed for 24-72 h following induction. **B** – *VPA concentration*. Cell media was supplemented with 0-5 mM valproic acid (VPA). **C** – *Media type and cell density at induction*. Protein expression was performed in either Protein Expression Media (PEM) or Freestyle 293 Expression Media (FS), inducing protein expression at either 2×10^6 /ml or 4×10^6 /ml cell density. **D** – *doxycycline concentration*. Different doxycycline concentrations used for induction of protein expression were tested, ranging from 0-15 μ g/ml. Graphs show FSEC curves of stable HEK293S GnTI⁻ TetR cell lysates obtained using a Superose 6 3.2/300 GL column and a flow rate of 0.08 ml/min. Asterisks denote peaks containing the protein of interest. Three samples were prepared and examined for each condition tested. Before lysis, confluent cells within a single well of a 6-well plate were induced with 10 μ g/ml doxycycline and cultured for 72 h (unless otherwise noted).

Harvesting cells after 72 h resulted in the highest amounts of protein produced from the three time points tested (Fig. 20.A), although the difference between 48 and 72 h was not large. The aggregate peak (eluting at 18 min) increased with longer expression times, so I decided to use a 48 h expression time to obtain the good expression level with reduced aggregation compared to 72 h.

Addition of 1 mM valproic acid (VPA) slightly boosted Fzd expression (Fig. 20.B), but higher concentrations of this histone deacetylase inhibitor resulted in reduced yield, so any subsequent Fzd expression was performed in the presence of 1 mM VPA. The reason for this effect is unclear, but suggests VPA has detrimental effects on normal cell function at concentrations above 1 mM.

In comparison to PEM media used for protein expression in the past, Freestyle 293 Expression Media was able to support higher cell densities during expression (12×10^6 /ml versus 7×10^6 /ml for PEM media after 48 h of protein expression). This led to higher protein yields (Fig. 20.C), particularly when protein expression was induced at a cell density of 3×10^6 /ml. In contrast, the Fzd yields of cells growing in PEM media appeared to be even smaller when induced at higher densities, suggesting that in this medium cells probably start dying sooner if kept at peak density for prolonged periods of time. Because of these observations, all future protein expression in suspension cultures of mammalian cells were performed using Freestyle media.

Finally, the use of different doxycycline concentrations for inducing Fzd6-4 expression did not result in any significant difference in protein expression levels (Fig. 20.D). In the absence of doxycycline no Fzd6-4 was expressed, whereas addition of 5 μ g/ml already resulted in levels of expression that did not significantly increase as the

doxycycline concentrations were increased. Therefore, protein expression in subsequent experiments was performed using 5 µg/ml doxycycline.

To increase sample purity and boost yields further, I created a new construct called Fzd6-5 (Fig. 13), which contained an N-terminal Twin-Strep tag followed by mVenus, Fzd6 with a bRIL insert, C500S mutation and a truncated C-terminus. It was stably inserted into HEK293S cells using the lentiviral system, which were then used for protein production using the parameters determined by the expression test.

For the first purification of this construct, 2.5 grams of cell pellet obtained from 250 ml of mammalian cell culture were used. Despite the smaller amount of starting material compared to the previous expression trials, the final SEC peak had a height of 450 mAU (Fig. 21.A). Thus, the combined use of the lentiviral system, modified protein construct, and optimized expression conditions allowed me to obtain much higher yields of Fzd6. The SEC profile was slightly altered, with an additional peak eluting slightly sooner than the the main peak (at 14 ml using Superose 6 Increase 10/300 GL). Furthermore, the final protein was also much cleaner than before as indicated by SDS-PAGE, with Fzd6 cleavage bands at 30 and 60 kDa absent (Fig. 21.B), although the higher molecular weight band observed previously (Fig. 19.B.a) was still present, corresponding to the additional peak observed in the SEC profile.

The Fzd6-5 construct also contained a 3C protease site, which enabled the removal of the Twin-Strep and mVenus tags from the protein. When this was done, the main protein peak present in the SEC profile eluted slightly later, as expected (Fig. 21.A), while SDS-PAGE showed that the main bands thought to correspond to Fzd did indeed show reduced molecular weight (Fig. 21.C.b.2), indicating that the cleavage was

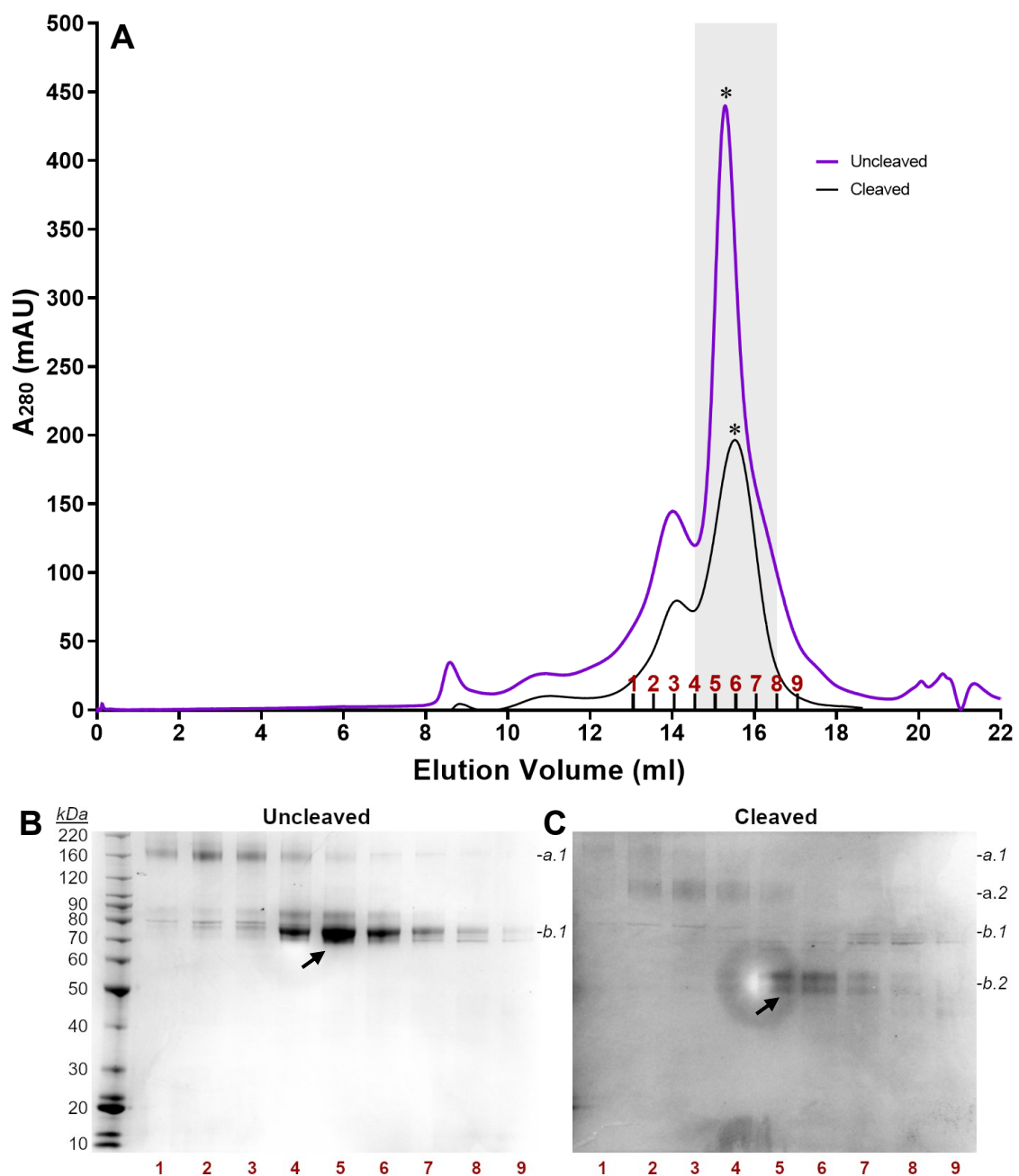


Fig. 21. Production of cleaved and uncleaved Fzd6-5. **A** – SEC curves. The main Fzd6-5 peak shaded grey. Asterisks denote peaks containing the proteins of interest. SEC performed using a Superose 6 Increase 10/300 GL column. **B and C** – SDS-PAGE of uncleaved and cleaved Fzd6-5. a.1 and a.2 – higher molecular weight bands; b.1 – uncleaved Fzd6-5; b.2 – cleaved Fzd6-5. Arrows denote bands corresponding to the proteins of interest. Experiments performed under non-reducing conditions.

successful. The increased migration distance of Fzd6-5 on SDS-PAGE also revealed

additional bands present at a position that was previously occupied by bands formed by

the uncleaved Fzd6-5. Mass spectrometry identified these proteins as bovine serum

albumin present in the cell culture, as well as major heat-shock proteins BiP, HSP70-1,

and HSP70-8. These could potentially bind immature Fzd that might be co-purified with the correctly folded protein. Interestingly, the higher molecular weight bands (Fig. 21.C.a.1/2) double the molecular weight of Fzd6-5 also migrated further on the gel following 3C treatment, therefore supporting the idea that they might belong to Fzd6 dimers.

3.1.2. Exploring Fzd dimer formation

The attempts to purify Fzd6-5 showed additional bands on non-reducing SDS-PAGE gels that were double the size of the predicted Fzd6-5 molecular weight (Fig. 21.B/C, bands a.1 and a.2). These bands seemed to migrate further following 3C treatment, thus hinting that they might belong to Fzd6-5 dimers. Several publications have suggested that Fzds form dimers in cell membranes (DeBruine et al., 2017b; Petersen et al., 2017), therefore it could be possible that the purification procedure could result in the presence of these dimers in the final sample.

To test whether this was indeed the case, I modified the Twin-Strep-tagged Fzd construct to contain a 10xHis-tag (Fzd6-8, Fig. 13). HEK293S GnTI⁻ TetR cells were then co-transduced with Fzd6-5 and Fzd6-8 to create stable cell lines. After inducing protein expression, I first purified the sample using a StreptactinXT column, followed by a nickel affinity chromatography. If Fzd dimers were present in the preparations, there should be a population of protein containing both Twin-Strep- and 10xHis-tagged Fzd6.

The resulting SEC resulted in a single peak (Fig. 22.A), corresponding to the previously observed faster-eluting peak produced by Fzd6-5. SDS-PAGE confirmed that this pooled peak contained a major band at a molecular weight corresponding to Fzd6-5 and Fzd6-8 (Fig. 22.B.b). Western blots confirmed that the peak contains both Fzd6-5 and

Fzd6-8 (Fig. 22.C/D), thus confirming that Fzd6 is capable of forming dimers. Some minor higher molecular weight species were observed by SDS-PAGE (Fig. 22.B.a), and these were also detected by Western blotting for Fzd6-5 and Fzd6-8 (Fig. 22.C/D). Due to the single SEC peak these may have arisen in sample preparation for SDS-PAGE or reflect stable dimers of the protein that have not dissociated in the presence of SDS.

In conclusion, these results support the idea that Fzd6 is capable of forming dimers, and that it is possible to purify them using detergent-based solubilization of cells. It should be noted that the additional dimer peak observed during SEC of Fzd6-5 was not present when purifying Fzd6-4. This could be due to the fact that the expression levels of Fzd6-5 were much higher than Fzd6-4, thus indicating that Fzd6 concentration either within cell membranes or during purification is important to maintain Fzd6 in a dimeric state.

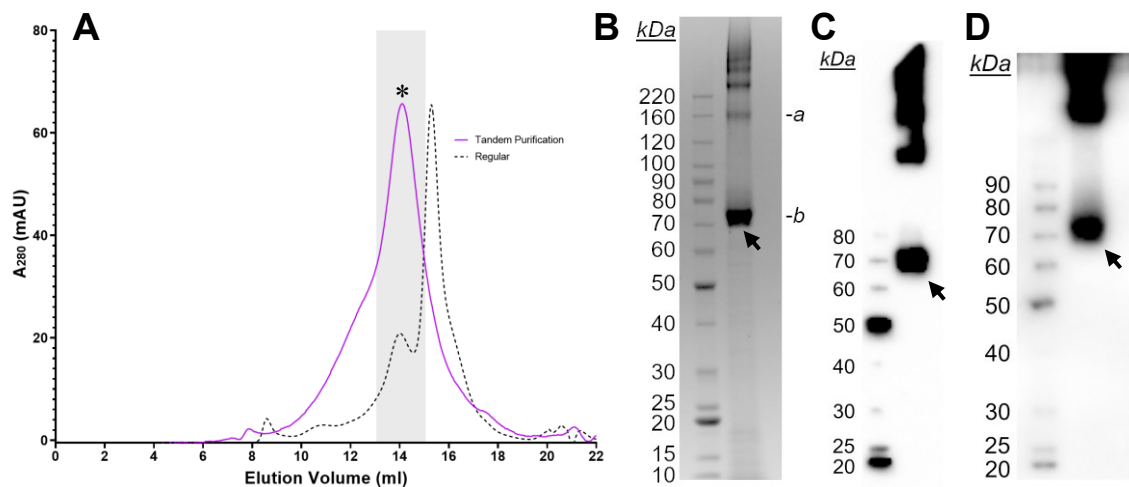


Fig. 22. Purification of Fzd6-5/Fzd6-8 dimer. **A** - SEC curves. Tandem purification used to purify the Fzd6-5/Fzd6-8 dimer, whereas regular purification used a single affinity chromatography step used to obtain the Fzd6-5 monomer (curve scaled for comparison). The main dimer peak shaded grey. Asterisk denotes peak containing the sample of interest. SEC performed using a Superose 6 Increase 10/300 GL column. **B** - SDS-PAGE of the pooled peak. a - higher molecular weight species; b - Fzd6-5. **C** - anti-Twin-Strep western blot of the final sample detecting Fzd6-5. **D** - anti-6xHis western blot detecting Fzd6-8. Arrows denote bands corresponding to the proteins of interest. Experiments performed under non-reducing conditions.

3.1.3. Stabilization of Fzd6 dimers

Fzd6 dimers would be appealing for further study – the increased particle size would make the sample more suitable for cryo-EM analysis, while also enabling characterization of the Fzd6 dimer interface. However, for this approach the interface between the Fzd6 monomers would need to be stabilized. Current papers indicate that this interface is located between TMD helices 4 and 5 (Petersen et al., 2017), which is also supported by the currently available homodimer structures of the closely related F-type GPCR smoothened (PDB ID: 4JKV).

To model the potential dimer interface of Fzd6, I created a full-length homology model of Fzd6 based on the published structure of Smoothened using SwissModel (Fig. 23). I then used the resulting homology model to identify potential residue pairs that would be amenable to the introduction of disulphide bridges in order to stabilize Fzd6 dimers.

The residues would have to be located in the vicinity of the putative dimer interface, as

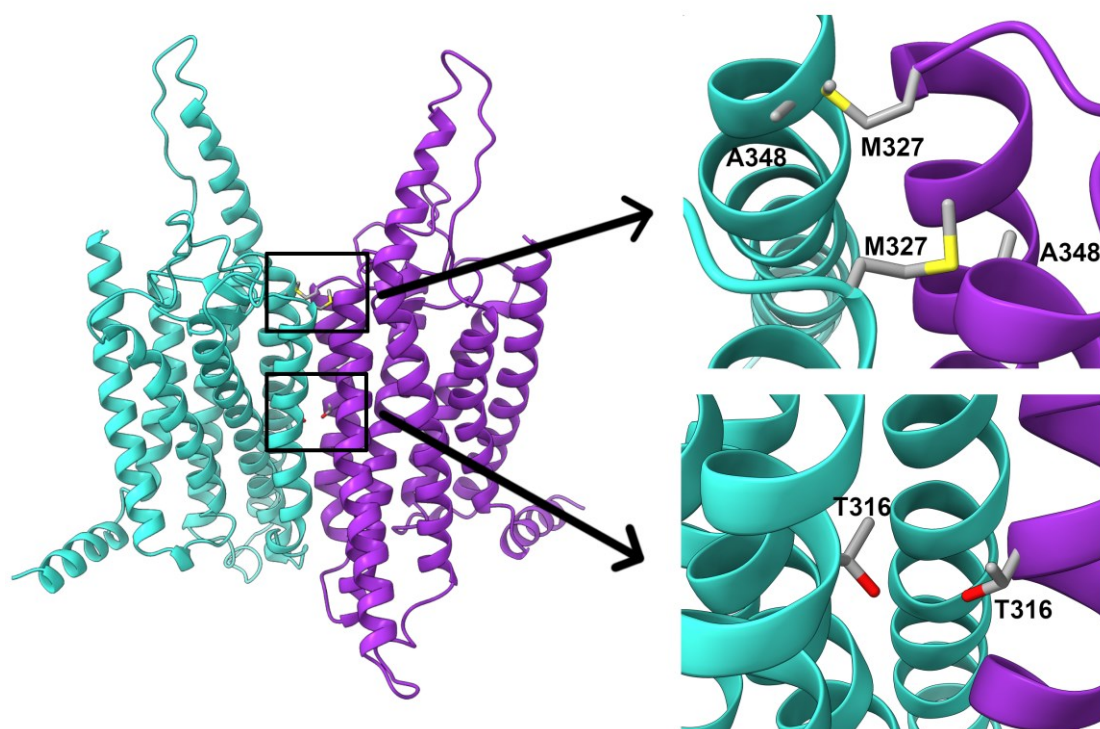
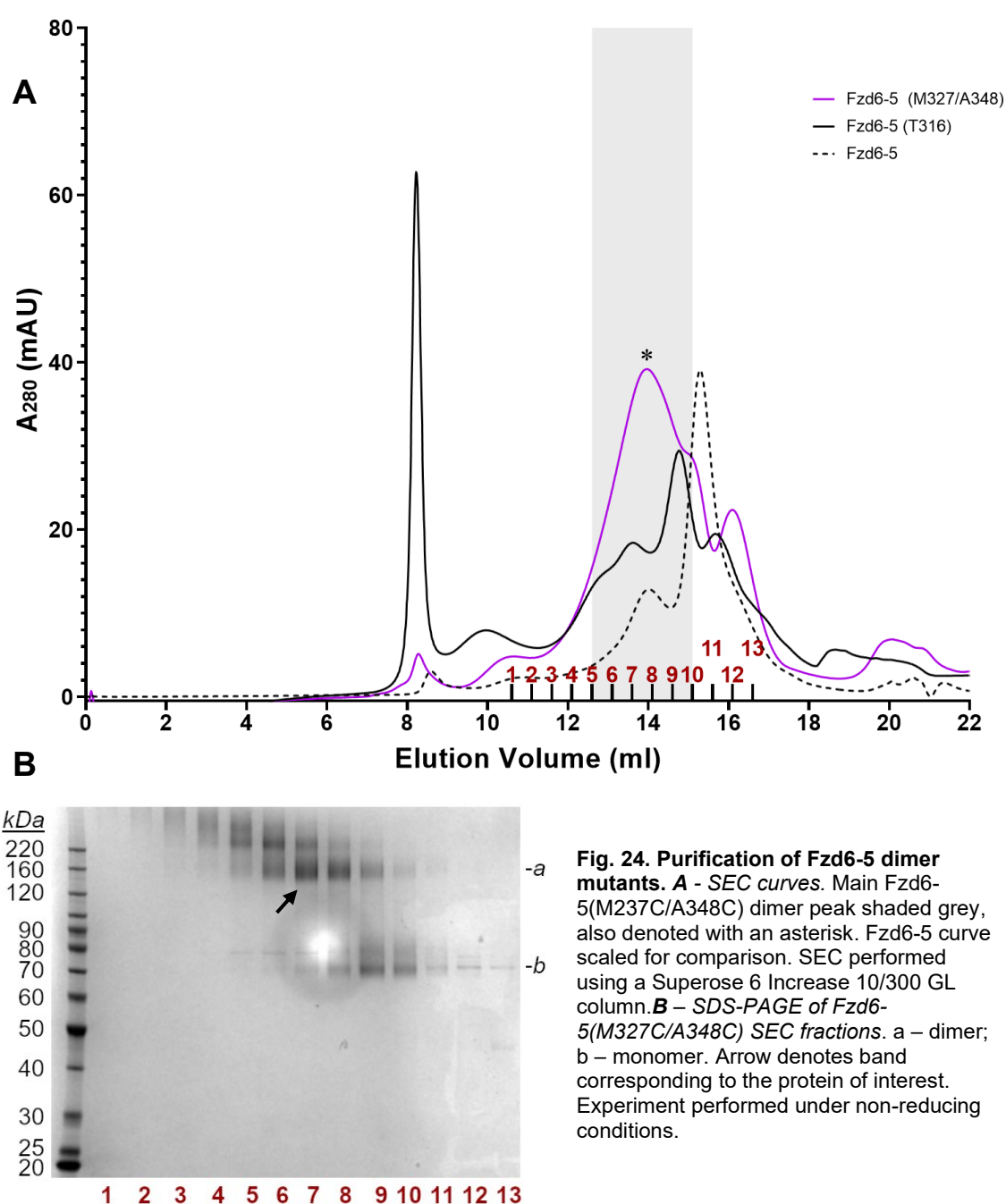


Fig. 23. Predicted Fzd6-5 dimer transmembrane domain interface. Model based on the structure of human Smoothened dimer (Wang et al., 2013, PDB ID: 4JKV) and a homology model of Fzd6 based on Smoothened (Byrne et al., 2016, PDB ID: 5L7D). CRDs omitted for clarity.

well as in close proximity of one another. Based on these requirements, I identified M327 and A348 pair near the top of Fzd6 TMD, as well as T316 in the middle of the TMD helical bundle (Fig. 23). The latter forms a pair with another T316 on the other monomer, meaning that a single mutation to cysteine should be sufficient for the introduction of the disulfide bridge. I then proceeded by generating stable HEK293S GntI⁻ TetR cell lines expressing Fzd6-5(M327C/A348C) and Fzd6-5(T316C) mutants. The purification of the M327/A348 mutant yielded much lower amounts of total protein in the final sample than normal (40 mAU versus 450 mAU from a 2.5 g cell pellet) (Fig. 24.A). Despite this, it seemed that the stabilization of the Fzd6 dimer might have been partially successful, as indicated by the highest peak in the SEC profile eluting at the same volume as the Fzd6-5 dimer peak. Unfortunately, the monomer peak was still present, partially overlapping with the dimer peak, thus making it impossible to isolate fractions with only the dimer present. In addition, the dimer peak itself was quite broad, with SDS-PAGE indicating that higher molecular weight species of Fzd6 might also be present (Fig. 24.B). In an attempt to optimize the production of this Fzd6-5(M327C/A348C), I tested protein expression at a lower temperature of 30° C, while also increasing expression time to 72 h, but this did not lead to any differences in the final quality of the sample (S. fig. 1).

The SEC profile of the Fzd6-5(T316C) mutant was even more complex than the one produced by the M327C/A348C mutant, consisting of several overlapping peaks. In addition, the highest peak eluted at a volume between the previously observed Fzd6 monomer and dimer peaks. For these reasons, this mutant was not characterized further.

In conclusion, the Fzd6-5 M327C/A348C mutant did indeed show increased propensity to dimerize, although the process was not very efficient, still leading to a major proportion of the overall sample being found in a monomeric state, whereas the T316A mutant was unable to convincingly stabilize Fzd6 dimers. There could be several explanations for this. First of all, the dimer interface model based on homology models might not be accurate enough to permit the selection of residues optimal for the introduction of disulphide bridges. Second, the residues selected might not have been



positioned within a distance and with an interresidue geometry suitable for disulphide bridge formation. Third, the position of the cysteine pairs within the Fzd6 protein might not have exposed them to a sufficiently oxidative environment to result in cysteine oxidation (particularly for T316). Finally, the introduction of a disulphide bridge within the positions used could result in significant alterations of the protein structure, thus leading to their degradation, or that Fzd6 dimers are actively removed from cells due to some unknown mechanism as suggested by the overall lower levels of expression for both of the mutants tested.

3.2. Expression and characterization of potential Fzd binding partners

3.2.1. Expression of co-receptors

One of the goals in elucidating the mechanism of Wnt signalling is to study complexes between Fzd and its co-receptors. To pursue this goal I asked whether it was possible to express two co-receptors LRP6 and ROR2. The constructs encoding these two co-receptors were tagged with mVenus and truncated a few residues after the predicted transmembrane helices (Fig. 15), because the signalling capabilities of the intracellular regions might result in abnormal cell growth and co-receptor degradation, while the presence of long, flexible linkers might interfere with efficient expression.

To assess co-receptor expression levels by stably transfected mammalian cells, they were examined under a fluorescence microscope (Fig. 26). The ROR2 constructs seemed to express well, with the protein localising to the cell membranes indicating proper folding (S. fig. 2), with ROR2-1 showing the highest levels of protein present.

In contrast, the cells expressing LRP6 constructs exhibited very low levels of fluorescence under the same imaging conditions, with the longer LRP6-3 construct giving a slightly stronger signal than LRP6-1. Since the constructs encoding LRP6 co-receptors were quite large (~1380 bp), it was possible that the low levels of LRP6 expression could be due to reduced lentiviral transfection efficiency. To obtain stable cell lines expressing higher levels of LRP6, I performed fluorescence-assisted cell sorting to isolate the populations of cells exhibiting the highest levels of mVenus fluorescence. In contrast to cells expressing ROR2-1, which showed a large, distinctive population of highly fluorescent cells (Fig. 25.A), less than 45% of cells expressing

LRP6-3 (Fig. 25.B) exhibited significant levels of fluorescence, and these were ~10 times less fluorescent than the ROR2-1 cells. Furthermore, the FACS peaks containing fluorescent and non-fluorescent cells had significant overlap. Both of the

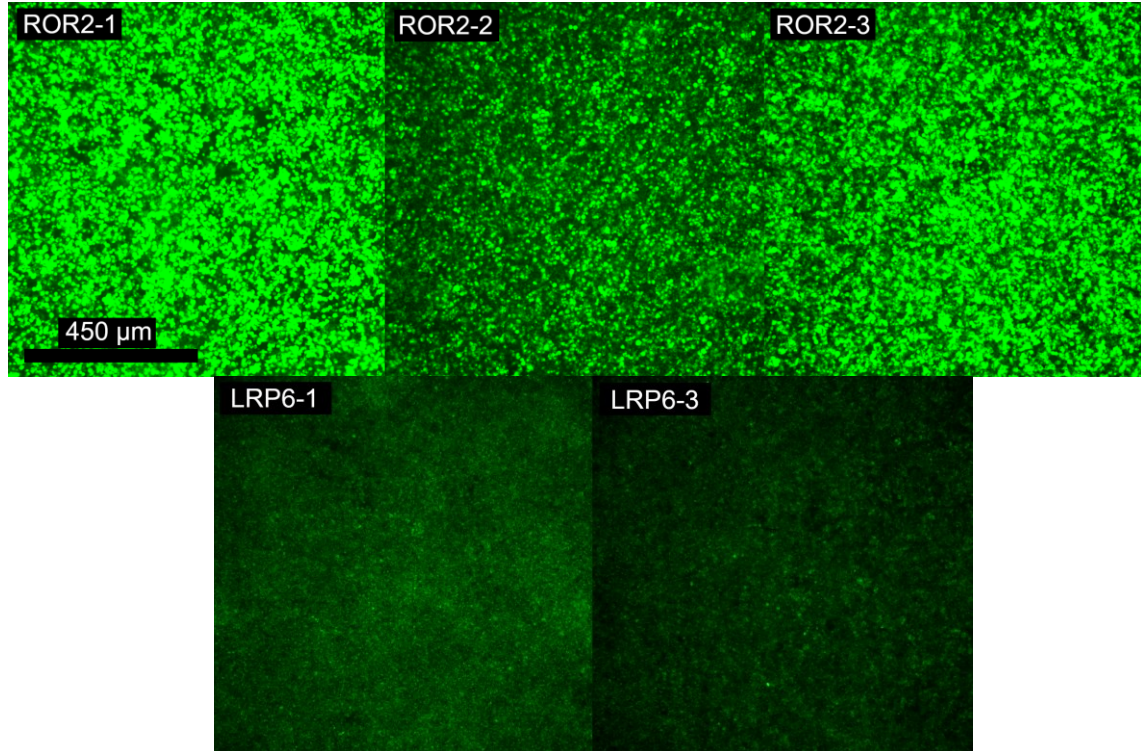


Fig. 26. Fluorescence of HEK293S GnTI⁻ TetR cell lines expressing various Fzd co-receptor constructs. All cells were imaged following induction of protein expression for 72 h with 10 μ g/ml doxycycline. 600 ms exposure.

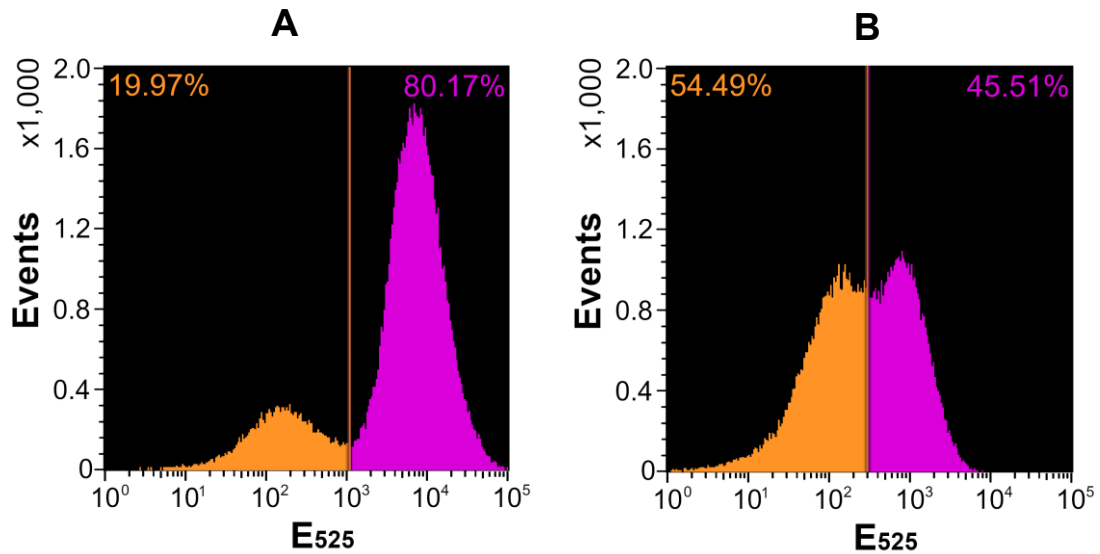


Fig. 25. FACS of HEK293S GnTI⁻ TetR cells expressing ROR2-1 and LRP6-3. **A** – ROR2-1. **B** – LRP6-3. Cells were sorted using the fluorescence signal of mVenus present on the constructs and using an excitation wavelength of 488 nm following induction of protein expression for 72 h with 10 μ g/ml doxycycline. mVenus signal was detected using emission at 525 nm. 100000 cells were sorted for each sample. Collected cells shown in magenta.

aforementioned observations meant that it was not possible to isolate cells expressing higher levels of LRP6-3 using FACS.

Due to the large size of the LRP6 constructs, longer protein expression induction times might be required to result in the production of mature protein. I therefore decided to examine the fluorescence of LRP6-3 expressing cells over the course of 8 days (Fig. 27), although the results were inconclusive. At day 6 some cells exhibited higher levels of fluorescence, although this result was not consistent across all the cells present.

In conclusion, the preliminary results presented here suggest that LRP6 constructs tested do not appear to be suitable for high levels of protein production, whereas ROR2 could be a suitable candidate for further biochemical experiments.

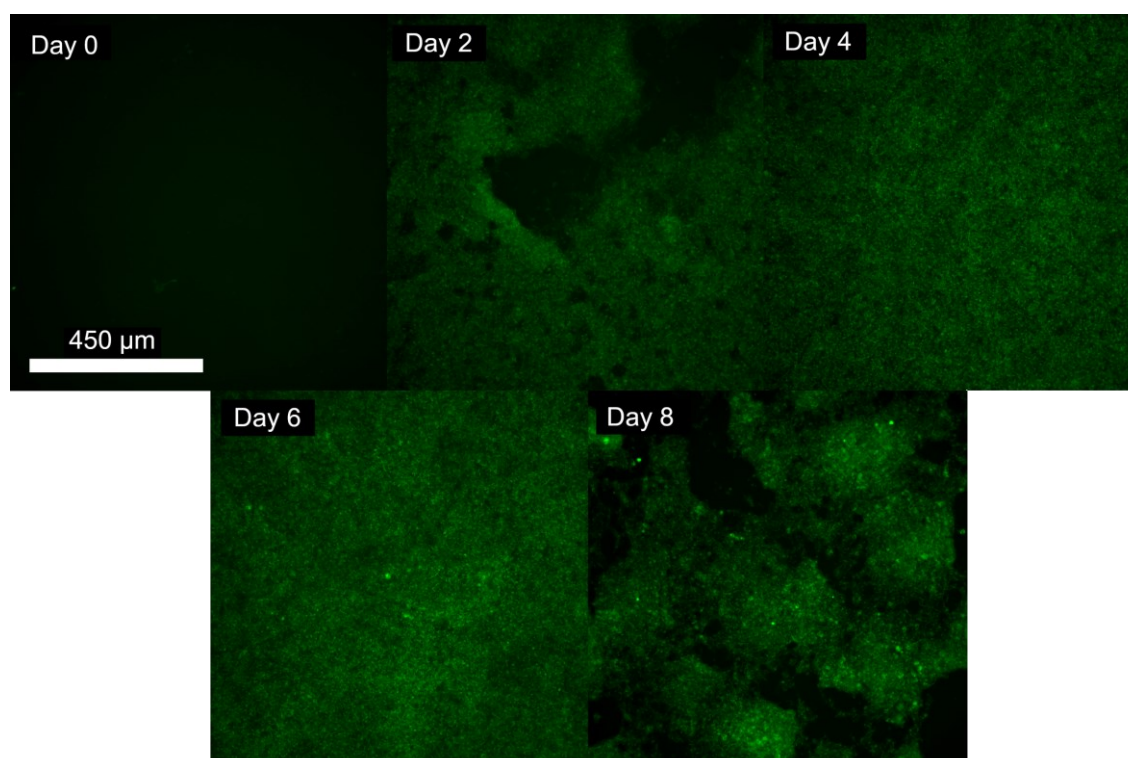


Fig. 27. Fluorescence of sorted HEK293S GnT1⁻ TetR cell lines expressing LRP6-3. All cells were imaged following induction of protein expression with 10 μ g/ml doxycycline for the time period specified.

3.2.2. Expression of Wnt

The production of large quantities of purified, active Wnt proteins is necessary for structural studies of its mechanism of action. However, these studies are hampered by the presence of a lipid modification on the Wnt molecule, which interferes with protein expression and solubilisation. Some studies have reported that co-expression of Wnt in the presence of a carrier protein capable of sequestering its lipid tail can significantly increase yield (Mihara et al., 2016). Afamin, a human plasma vitamin E-binding glycoprotein, is one such carrier protein, therefore I decided to explore the possibility of using it to produce soluble Wnt.

To assess the ability of cells to produce Wnt, I generated stable HEK293S GnTI⁻ TetR cell lines co-expressing afamin and either mVenus-tagged Wnt3A or Wnt5A. I choose these Wnts due to them being the two standard Wnts usually used in studies to activate either canonical or non-canonical Wnt signalling pathways respectively. All cell lines exhibited very high levels of fluorescence, which were equivalent to the fluorescence found within cell lines expressing the optimized Fzd6-5 constructs (Fig. 28.A). Since Wnts are known to associate with cell surfaces following secretion, it is very likely that these high levels of fluorescence could arise from secreted Wnt remaining bound to cell surface proteoglycans (S. fig. 2). This would imply little Wnt is released from the cell into the media.

I therefore analysed media from cells co-expressing mVenus-tagged Wnt5A and afamin by FSEC (Fig. 28.B). It gave a high, sharp peak eluting at the column void volume, followed by a broad peak eluting across the volume of the column. This broad peak was centered at an elution volume corresponding to ~250 kDa based on the behaviour of the 203 kDa Fzd6-5 dimer in a DDM micelle.

Since the predicted molecular weight of a mVenus-tagged Wnt5A (64 kDa) and afamin (87 kDa) complex would be 151 kDa, it appears that this peak consists of a mixture of Wnt5A and afamin subunits, complexes, or aggregates. Although clearly not suitable for further structural characterization, the Wnt5A produced by these stable cell lines could still exhibit functional activity, which merits further study.

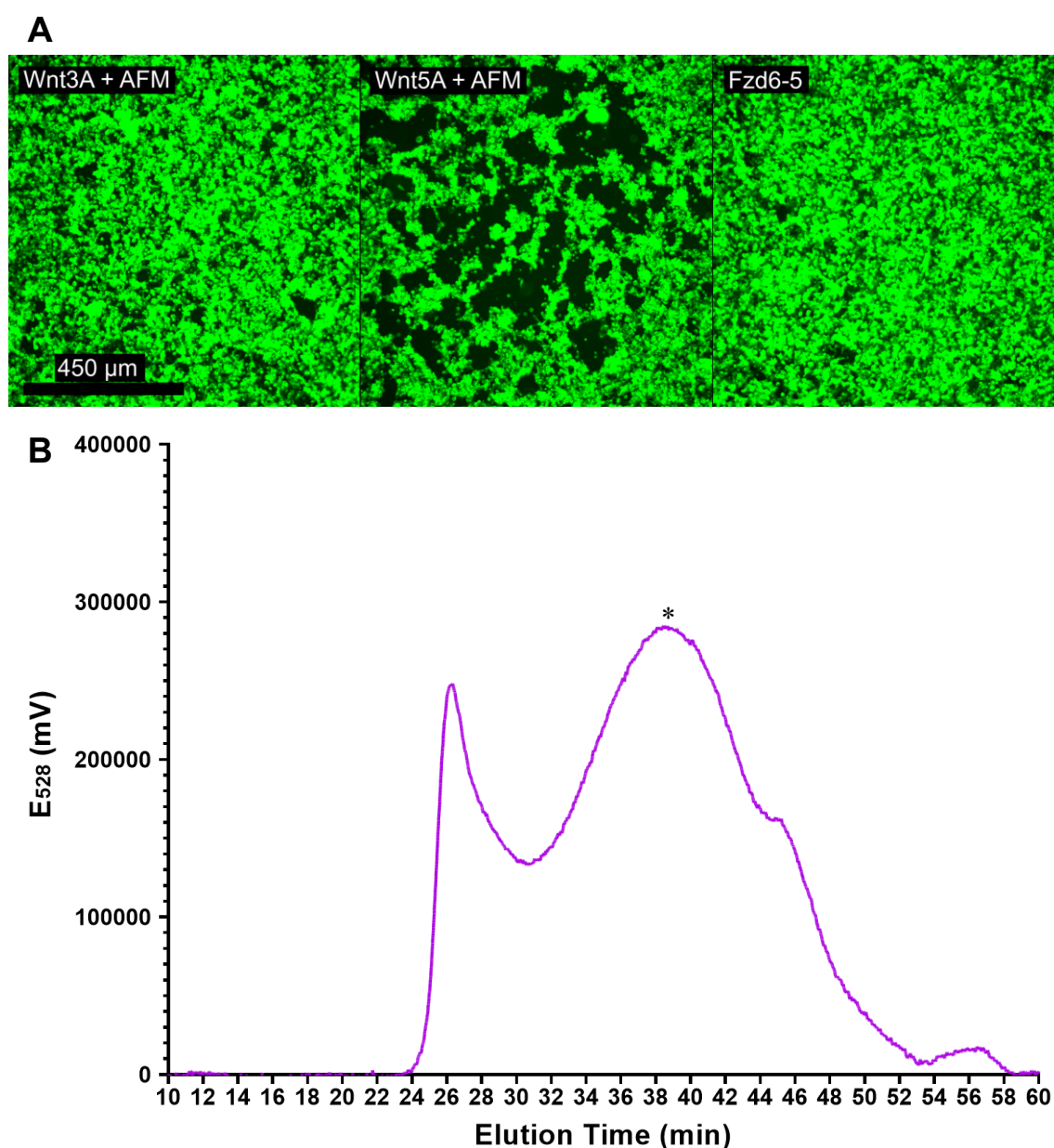


Fig. 28. Expression test of HEK293S GnTI⁻ TetR cells co-expressing Wnt3A or Wnt5A with afamin (AFM). **A** – fluorescence of cells co-expressing Wnt and afamin. Fzd6-5 used as a control. **B** – FSEC of media of cells co-expressing Wnt5A and afamin. Asterisk denotes the main peak present. FSEC performed using a Superose 6 Increase 3.2/300 GL column and a flow rate of 0.04 ml/min. All cells were imaged and FSEC performed following induction of protein expression for 72 h with 10 μ g/ml doxycycline.

3.2.3. Dvl domain expression and interactions with Fzd6

Dvl is considered to be crucial for mediating signal transduction following Fzd activation. Its production and incorporation within isolated signalling complexes could thus give new insights into the mechanisms of Wnt signalling.

There are three members of the Dvl family of proteins (Dvl1, Dvl2, Dvl3) found within mammals, although Dvl1 and Dvl2 are the most commonly expressed forms within cells. Since the DIX, PDZ, and DEP domains are connected via long linker regions, while the presence of DIX domain results in multimer formation, I attempted to express different combinations of PDZ and DEP domains of both Dvl1 and Dvl2 either with or without the linker regions present. Furthermore, Dvl proteins contain several cysteine residues, which would result in protein aggregation when attempting to use them for complex formation with Fzd in the absence of reducing agents. To overcome this, I mutated the cysteine residues present within the constructs to serines.

The various 6xHis-tagged constructs of Dvl1/2 DEP and PDZ domains were subcloned into a pET22b vector with the pelB periplasmic secretion signal absent (Fig. 29). They were then expressed in *E. coli* and subsequently subjected to nickel affinity chromatography and SEC.

The results showed that all constructs of Dvl2 could be expressed reasonably well (Fig. 30.C, Fig. 31.C, Fig. 32.C), showing well-defined peaks on SEC profiles, with the Dvl2

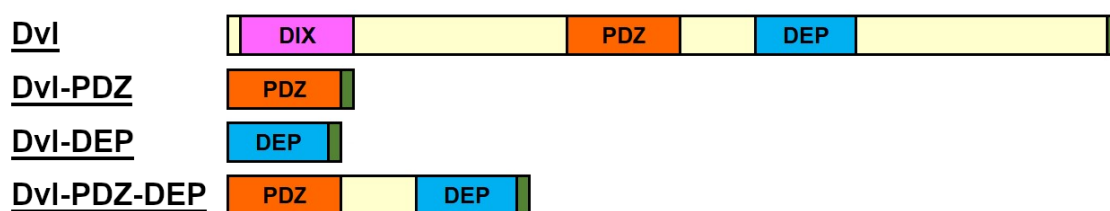


Fig. 29. Dvl construct design. N-terminus on the left. Magenta – DIX domains (residues 1-85 for Dvl1, 12-91 for Dvl2). Orange – PDZ domains (residues 251-323 for Dvl1, 268-351 for Dvl2). Blue – DEP domains (residues 425-499 for Dvl1, 426-507 for Dvl2). Beige – Dvl domain linker regions. All constructs tagged with a 6xHis-tag at the C-terminus (dark green).

DEP domain expressing the best (Fig. 30.C). All Dvl2 constructs also resulted in the co-purification of several contaminants, as indicated by the presence of extra peaks during SEC, as well as on SDS-PAGE (Fig. 30.D, Fig. 31.D, Fig. 32.D). They were well-separated from the peaks containing clean target protein, except for the Dvl2-PDZ-DEP construct, where extra bands were present also in the fractions belonging to the main peak (Fig. 32.D). Interestingly, the peaks belonging to Dvl2 PDZ construct seemed to aggregate upon heating in SDS loading buffer, resulting in smears present on reducing

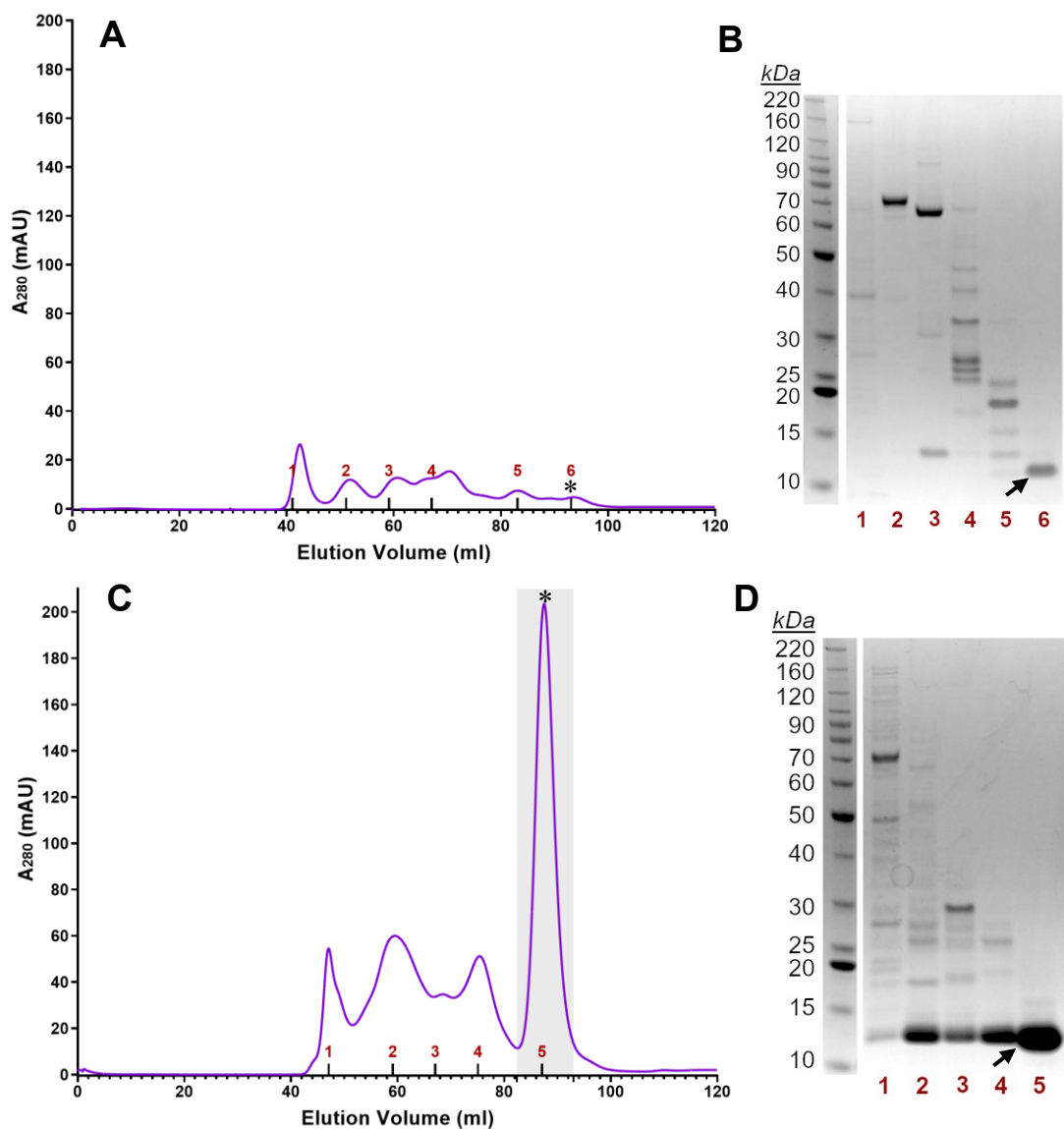


Fig. 30. Purification of Dvl DEP domains. **A** – DVL1-DEP SEC. **B** – Reducing SDS-PAGE of DVL1-DEP SEC fractions. **C** – DVL2-DEP SEC. Main peak shaded grey. **D** – Reducing SDS-PAGE of DVL2-DEP SEC fractions. Predicted molecular weights of Dvl1 and Dvl2 DEP domains are 11.1 and 11.7 kDa respectively. Asterisks denote SEC peaks containing the protein of interest. SEC performed using a Superdex 75 HiLoad 16/600 GL column. Arrows denote bands corresponding to the proteins of interest. Experiments performed under non-reducing conditions.

SDS-PAGE, although these could be overcome if the sample was not heated before loading (Fig. 34.B).

In contrast to Dvl2, the only Dvl1 construct that expressed well was Dvl1-PDZ (Fig. 31.A), which, similarly to Dvl2-PDZ, also produced smears on SDS-PAGE when heated (Fig. 31.B, S. fig. 3). The purification of other Dvl1 constructs resulted in SEC profiles that were either too chaotic due to the presence of contaminants (Dvl1-PDZ-

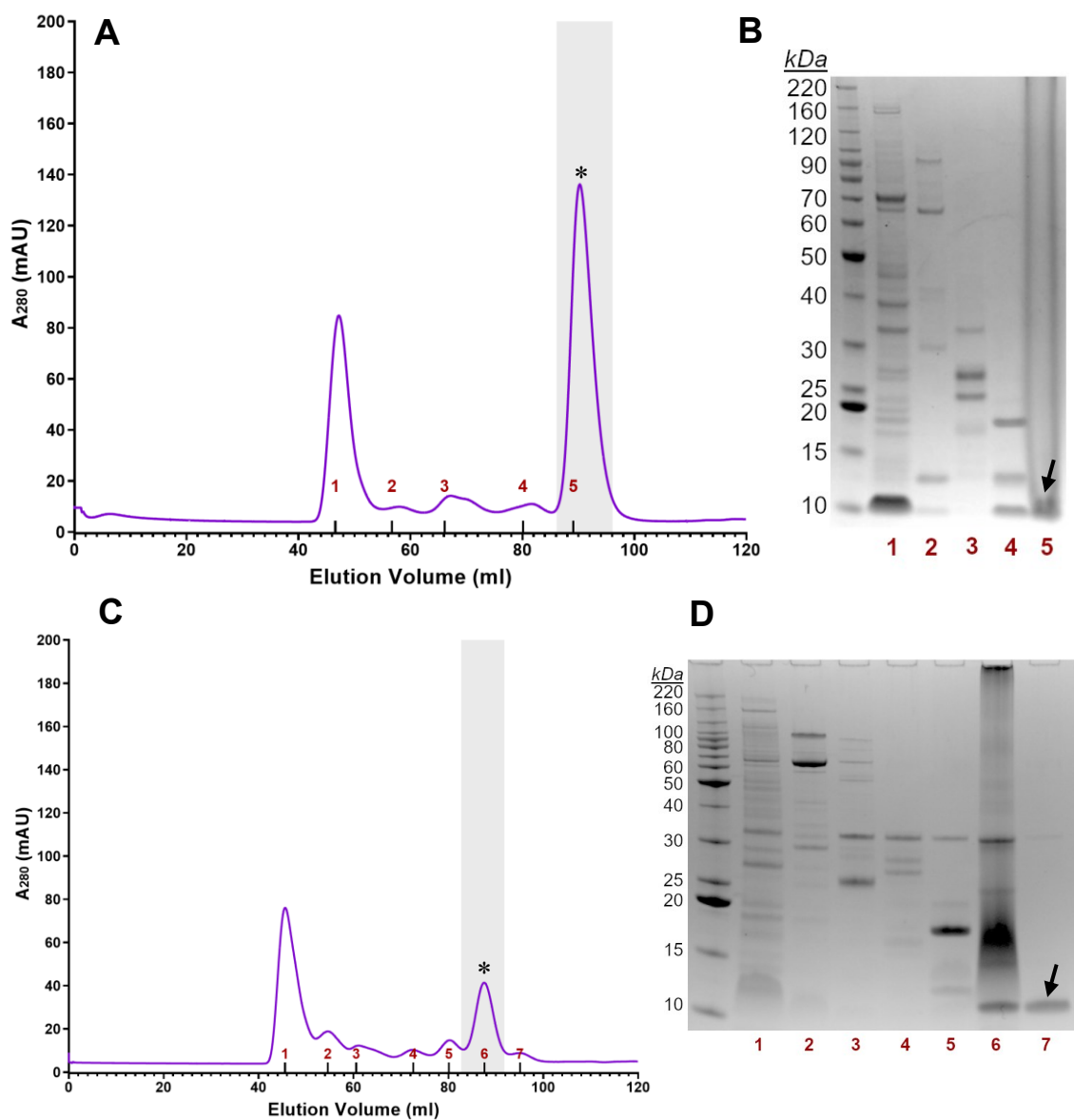


Fig. 31. Purification of Dvl PDZ domains. **A** – DVL1-PDZ SEC. **B** – Reducing SDS-PAGE of DVL1-PDZ SEC fractions. **C** – DVL2-PDZ SEC. **D** – Reducing SDS-PAGE of DVL2-PDZ SEC fractions. Main peaks of SEC curves shaded grey. Predicted molecular weights of Dvl1 and Dvl2 PDZ domains are 10.7 and 11.1 kDa respectively. SEC performed using a Superdex 75 HiLoad 16/600 GL column. Arrows denote bands corresponding to the proteins of interest. Experiments performed under non-reducing conditions.

DEP, Fig. 32.A/B), or with peaks that were too low to obtain sufficient amounts of protein (Dvl1-DEP, Fig. 30.A/B).

Since all constructs of Dvl2 could be produced, I wanted to test whether they could potentially interact with the Dvl recognition motif KTXXXW found within helix VIII of inactive Fzd6-5. To do so, I generated a new construct of Fzd6-5 containing a C-terminal Avi-tag (Fzd6-6, Fig. 13), which could be biotinylated for binding studies, and used it to perform preliminary biolayer interferometry (BLI) experiments (Fig. 33).

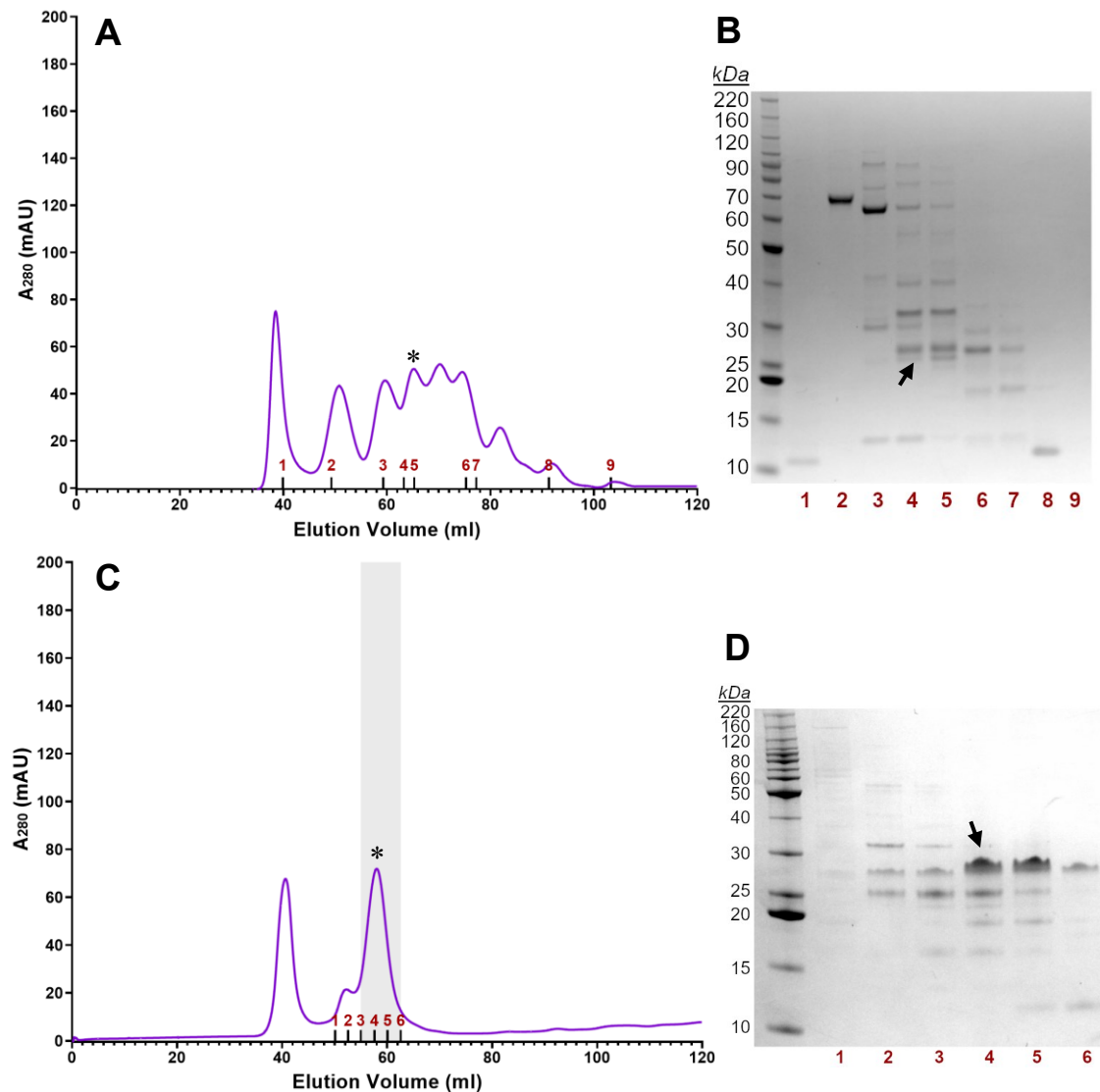


Fig. 32. Purification of Dvl PDZ-DEP domain constructs. **A** – DVL1-PDZ-SEP SEC. **B** – Reducing SDS-PAGE of DVL1-PDZ-DEP SEC fractions. **C** – DVL2-PDZ-DEP SEC. Main peak shaded grey. **D** – Reducing SDS-PAGE of DVL2-PDZ-DEP SEC fractions. Predicted molecular weights of Dvl1 and Dvl2 PDZ-DEP constructs are 26.4 and 28.2 kDa respectively. SEC performed using a Superdex 75 HiLoad 16/600 GL column. Arrows denote bands corresponding to the proteins of interest. Experiments performed under non-reducing conditions

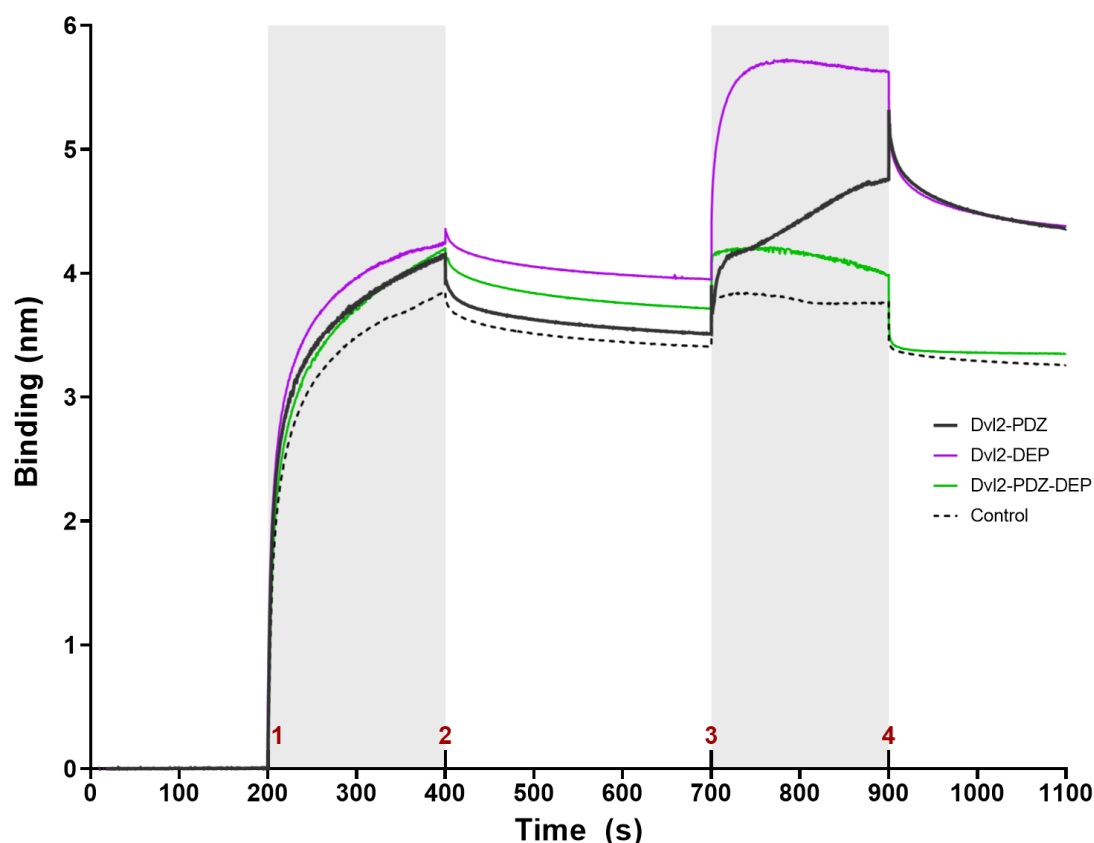


Fig. 33. Interaction between Fzd6-6 and Dvl2 constructs. Interactions measured using the Blitz BLI system and streptavidin biosensors. 1 – Loading of biotinylated Fzd6-6 (50 µg/ml). 2 – Wash. 3 – Addition of Dvl2 samples (100 µM), 4 – wash. Control performed in the absence of analyte. Experiment repeated 4 times, representative results shown.

Fzd6-6 was loaded onto streptavidin-coated tips, and the binding of different Dvl2 constructs evaluated by applying them at 100 µM. Only the Dvl2-DEP construct showed a curve indicative of specific binding, whereas the application of Dvl2-PDZ resulted in a steady rise in signal, corresponding to non-specific interactions. Interestingly, the experiment using the Dvl2-PDZ-DEP domain did not result in any signal compared to a control, despite containing the Dvl2-DEP region.

These results indicated the DVL2-DEP domain as being the most likely candidate for recognising the conserved motif on Fzd6, whereas the absence of signal for the Dvl2-PDZ-DEP domain suggests that the presence of the PDZ domain might somehow interfere with the recognition of the Fzd6 motif by the DEP domain. Due to the low sensitivity of the BLItz system used, no data on the binding constants for these

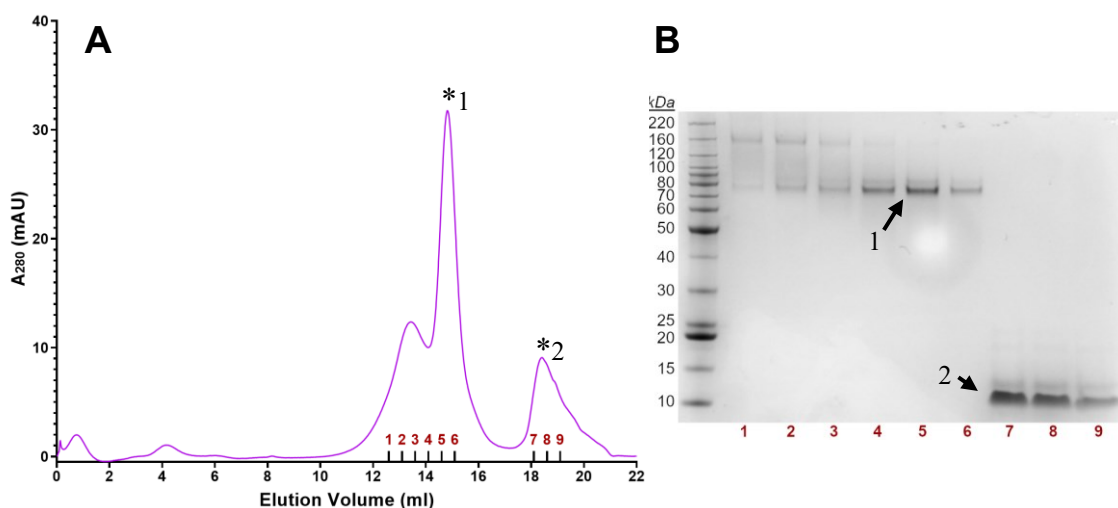


Fig. 34. SEC of Fzd6-5 and Dvl2-DEP mixture. Purified samples of Fzd6-5 and Dvl2-DEP were mixed at a 1:1.5 molar ratio. **A** – SEC curve. Asterisks denote peaks containing the proteins of interest (1 – Fzd6-5, 2 – Dvl2-DEP). SEC performed using a Superose 6 Increase 10/300 GL column. **B** – SDS-PAGE. Arrows denote bands corresponding to the proteins of interest (1 – Fzd6-5, 2 – Dvl2-DEP). Experiment performed under non-reducing conditions.

constructs could be obtained. It should be noted that the other constructs of Dvl2 could still potentially interact with the intracellular parts of Fzd6, such as the ICL3, although in Fzd6-6 this is replaced by a bRIL insert, therefore such interactions would go undetected. Furthermore, the position of the Avi tag at the C-terminus of Fzd6-6 could sterically hinder the interactions between the PDZ domain of Dvl2, although this did not appear to be the case for Dvl2-DEP.

To explore the possibility of forming Fzd6/Dvl2-DEP complexes for further study, I co-incubated Fzd6-5 and Dvl2-DEP at a 1 : 1.5 molar ratio, subsequently performing SEC. Unfortunately, both components eluted as separate peaks (Fig. 34), thus indicating the inactive-state Fzd6 does not bind Dvl2-DEP with an affinity that would be sufficiently high for complex isolation. It has been reported that Fzd helix VIII containing the KTXXXW Dvl-binding motif undergoes conformational changes in the presence of detergents, therefore similar experiments would be worth repeating in the absence of detergent.

3.2.4. Selection of nanobodies against Fzd6 using yeast surface display

A key goal of this project was to obtain the structures of Fzd signalling complexes by cryo-EM. Assuming that the final complex would be imaged either in detergent micelles or in any detergentless system aimed at maintaining the solubility of Fzd, the particles would need to have considerable amount of mass extending away from the transmembrane regions of the complex, so that the cryo-EM data processing software would be able to properly align the particles. One of the potential ways to achieve this would be via nanobodies. Although quite small by themselves, they could be fused to additional scaffolds that would increase their size considerably. Furthermore, they might also stabilize the complex itself, thus resulting in higher final resolutions.

Currently the most prevalent method for obtaining nanobodies against a protein of interest is via llama immunization. This takes several months, since gaining access to llama farms for immunization, as well as the immunization process itself, requires time. During the last couple of years several artificial nanobody libraries have been developed to mimic the complexity of the recombination capabilities of the llama immune system, thus enabling nanobody selection via either phage or yeast display methods. The potential advantages are the reduced times needed to go from purified protein to selected nanobodies, as well as the lack of animal immunization. Then again, the risk is that since the possible nanobody combinations within these libraries are limited, they might not yield high-affinity nanobodies for a particular protein of interest.

To obtain nanobodies (Nbs) against Fzd6, I used the system created by McMahon et al., 2018. It has been created by mining the PDB for published Nb structures and sequences, thus biasing the library towards nanobody scaffolds that might be useful for structure

determination, also introducing extra variability within specific regions of the VHH variable loop 3 via PCR mutagenesis.

The idea behind the selection process is shown in Fig. 12. I started with a biotinylated construct of Fzd6 lacking the bRIL insert called Fzd6-7 (Fig. 13) to prevent the selection of Nbs against bRIL. I then performed several rounds of magnetic-activated cell sorting (MACS) on yeast cells expressing the Nb library on their surface, which were incubated using biotinylated Fzd6-7 and paramagnetic streptavidin beads (negative selection was also performed to eliminate any yeast cells that would recognize the streptavidin beads directly). Finally, I performed FACS on yeast cells incubated with Fzd6-7, fluorescently labelled streptavidin, and a fluorescently labelled antibody recognising the HA epitope tag present on the stalk tethering the Nbs to the surface of the yeast cell. This allowed me to estimate the Nb expression and Fzd6-6 binding levels of each yeast cell sorted.

The results (Fig. 35) showed that there was a subpopulation of yeast cells that both expressed Nbs and contained bound Fzd6-7. Although this only made up 0.31% of all

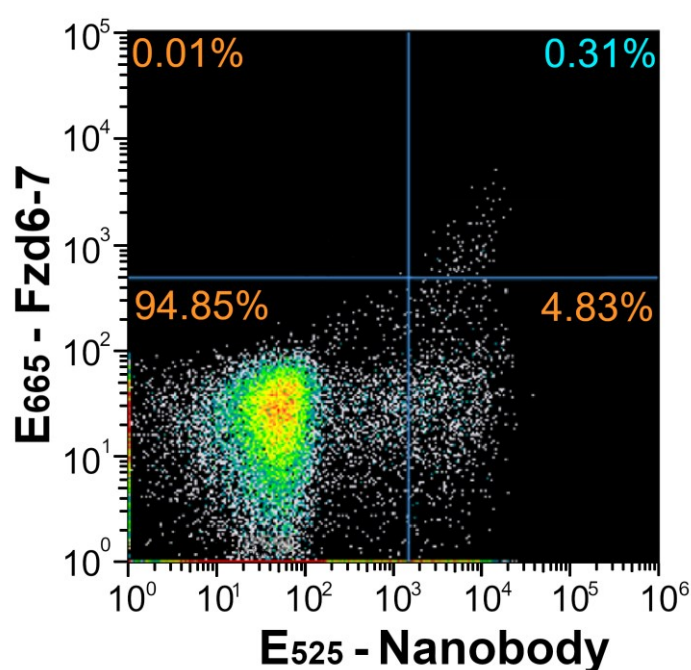


Fig. 35. FACS of yeast cells expressing the nanobody library. Before the experiment, the yeast cells were incubated with anti-HA antibodies labelled with AF488, biotinylated and cleaved Fzd6-7, and streptavidin-AF647. Nanobody expression levels on the yeast surface and Fzd6-7 binding were measured using emission at 525 and 665 nm wavelengths respectively. The quadrants were defined so that all unlabelled cells would be found in the lower left quadrant. Values in orange and cyan indicate the percentage of cells (out of 100000 total) found in each quadrant. Cells in the top-right quadrant were selected for further experiments.

the cells sorted, this was due to the fact that the yeast cells tended to express Nbs in a somewhat stochastic fashion, with only about 15-20% of all yeast cells expressing Nbs at any time (S. fig. 5), which was similar to the findings of the authors of this nanobody library. Furthermore, the gates for sorting were set in a very conservative fashion, using

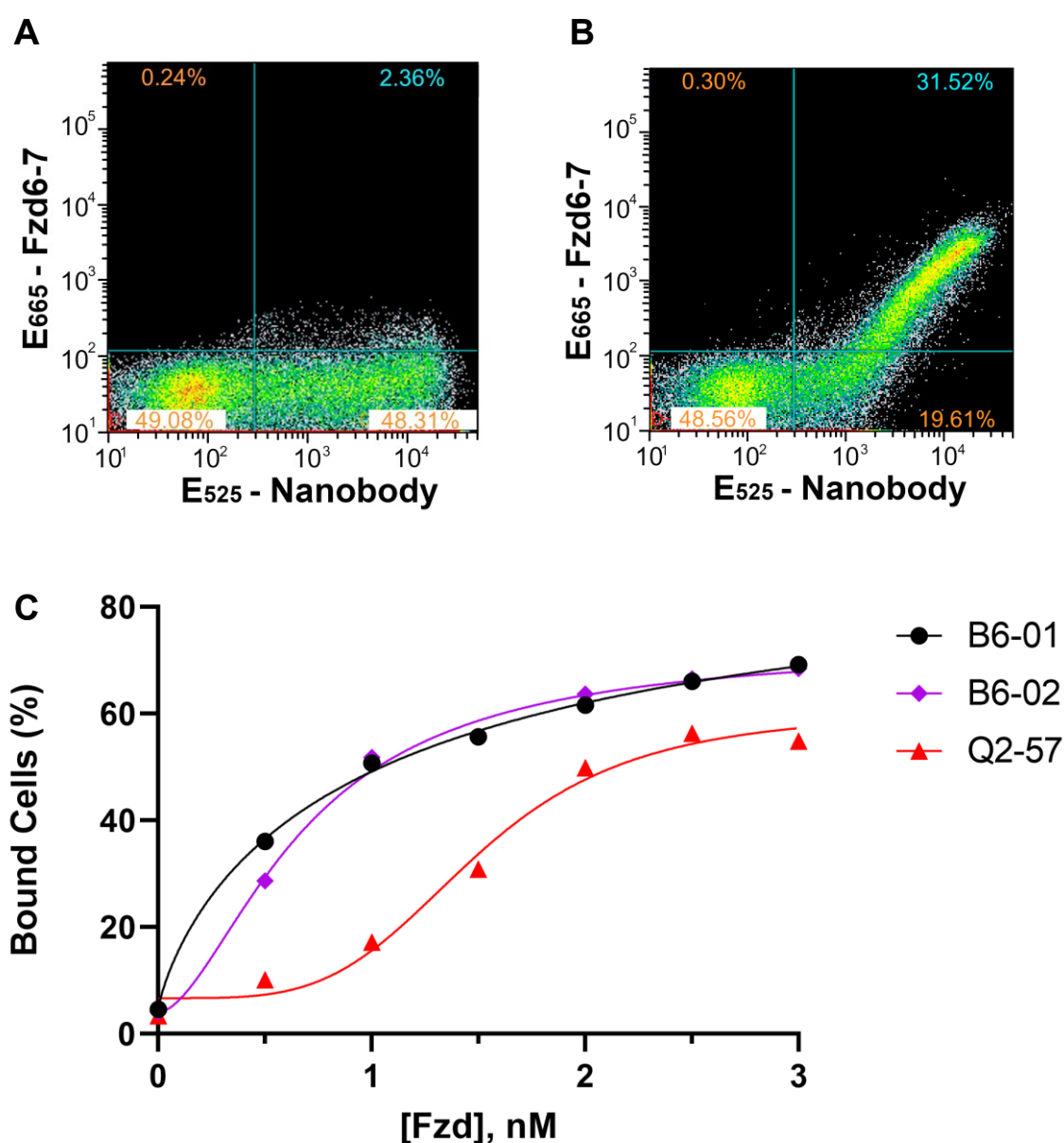


Fig. 36. Titration of biotinylated Fzd6-7 onto selected yeast cells. **A** – FACS of selected, nanobody expressing yeast cell B6-01 incubated with 0 nM Fzd6-7. **B** – FACS of selected, nanobody expressing yeast cell B6-01 incubated with 3 nM Fzd6-7. **C** – percentage of nanobody-expressing yeast cells binding Fzd6-7 at various concentrations. Three yeast cells selected from previous experiments (B6-01, B6-02, Q257) were used. Before the experiment, the yeast cells were incubated with anti-HA antibodies labelled with AF488, biotinylated and cleaved Fzd6-7, and streptavidin-AF647. Nanobody expression levels on the yeast surface and Fzd6-7 binding were measured using emission at 525 and 665 nm wavelengths respectively. The quadrants were defined so that all unlabelled cells would be found in the lower left quadrant. Values in orange and cyan indicate the percentage of cells (out of 100000 total) found in each quadrant.

Fzd6-7-unlabelled yeast cells expressing Nbs, so that all cells found in the upper right quadrant would only contain yeast cells binding Fzd6-7 with a high level of certainty.

To test whether the selected yeast cells bound Fzd6-7 in a specific fashion, I plated them out and selected single colonies for further culturing. I then repeated the FACS experiment, incubating the yeast cells with varying concentrations of Fzd6-7 present. As predicted, increasing concentrations of Fzd6-7 resulted in higher amounts of cells with Fzd6-7 labelling present (Fig. 36.A/B), and the levels of Nb on the surface of the cells directly correlated with the level of Fzd6-7 binding, thus indicating that the selected yeast cells did indeed specifically bind Fzd6-7. If the percentage of cells binding Fzd6-7 (from the total amount of cells expressing Nbs) was plotted against Fzd6-7 concentration, I could see that the EC₅₀ values for saturation of yeast cells expressing Nb were in the low nanomolar range, thus further confirming that the anti-Fzd6-7 Nb selection process was successful (Fig. 36.C).

The nanobody-encoding plasmids were then extracted from the selected yeast cells and subcloned into bacterial expression vectors. To introduce an additional selection step that would also take into account Nb expression levels, I performed a small-scale

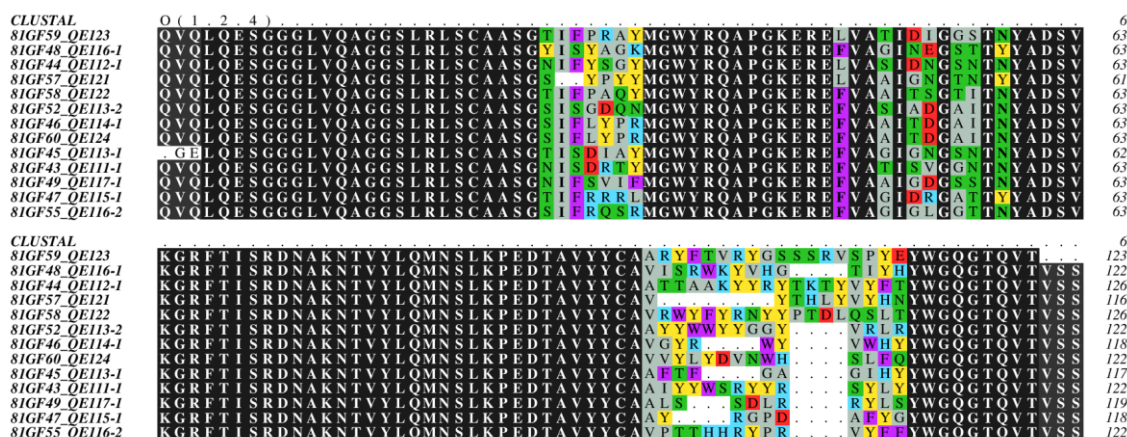


Fig. 37. Sequences of nanobodies selected via ELISA. Identical sequence regions labelled black. Negatively charged residues – red; positively charged residues – blue, aliphatic residues – grey, phenylalanine – purple, tyrosine – yellow.

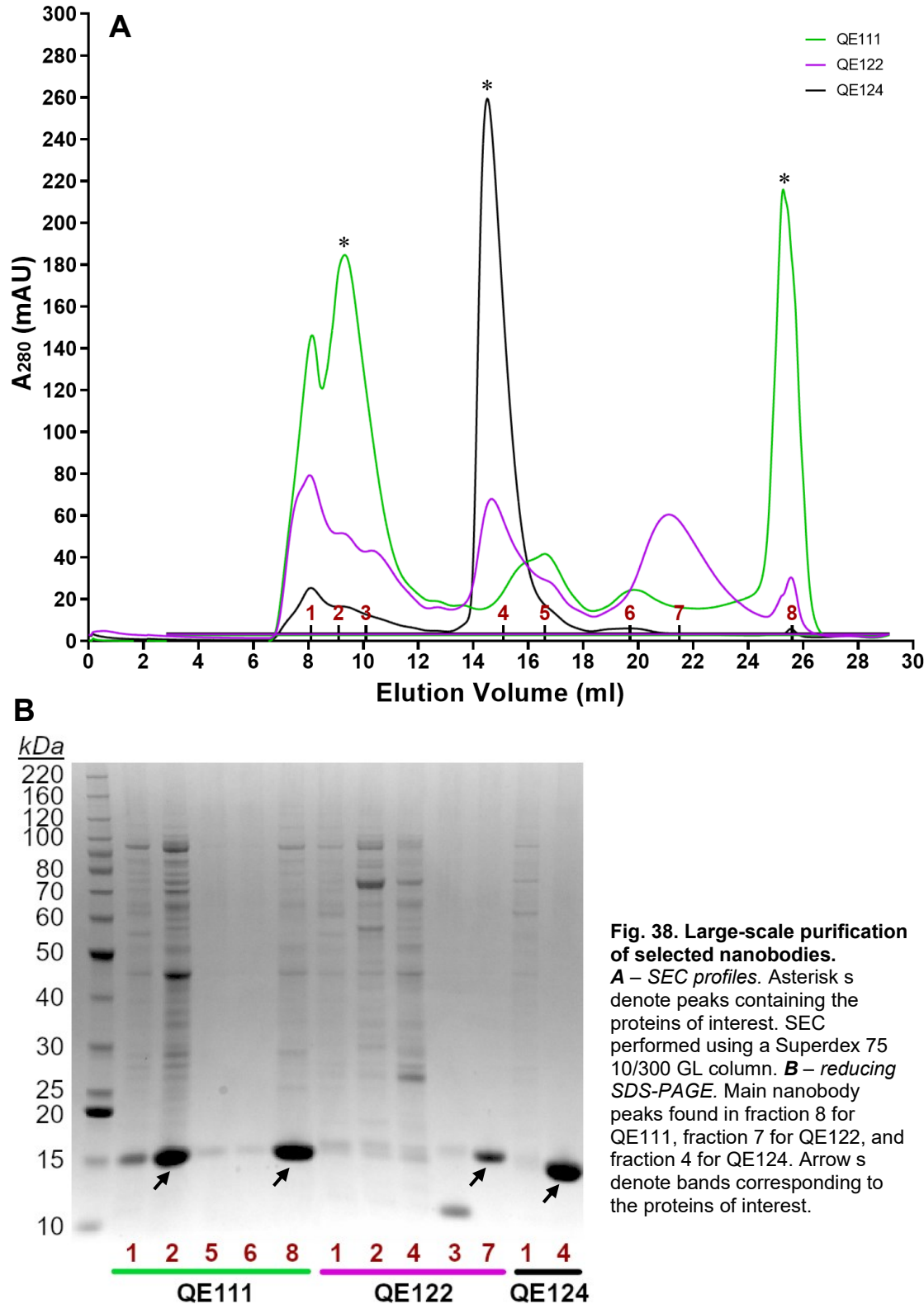
expression test, using the resulting cell lysates to perform an ELISA using plates coated with Fzd6-7 (S. fig. 6). Nbs yielding signal were then sequenced (Fig. 37).

The alignment of Nb sequences clearly showed the presence of the three variable loops, whereas other regions were identical, thus matching the design of this Nb library.

Usually selection of Nbs obtained via llama immunization show sequence convergence in the variable loop 3, which allows classification of selected Nbs within families. This presence of families is due to the action of the llama immune system, which positively selects only for the strongest binders of the antigen. In this case, the lack of such a mechanism resulted in Nb sequences that were almost unique for each Nb sequenced. The potential disadvantage of this could be that some of the Nbs might have reduced affinity for the target protein, while the advantage could be that the Nbs present could potentially sample a larger variety of epitopes on the target protein.

The nanobodies showing the strongest signal on ELISA were then subjected to large scale purification using nickel affinity chromatography and SEC (Fig. 38.A). The Nbs behaved in an unusual fashion – although some Nbs produced single isocratic peaks, different Nbs tended to elute at different volumes, which was usually delayed in comparison to the expected elution volume of ~15 ml (using a Superdex 75 10/300 GL column). Furthermore, SEC and subsequent SDS-PAGE (Fig. 38.B) indicated the presence of various higher molecular weight contaminants. This would indicate that the selected Nbs have a high propensity to interact with various other proteins, as well as with the SEC column itself, although this is unlikely for such small, supposedly well-ordered proteins.

The purified Nbs were then used to perform preliminary binding experiments using the Blitz system (Fig. 39). QE122 did not exhibit signal that would correspond to specific interactions with Fzd6-7 – the sudden increase in signal following addition of QE122



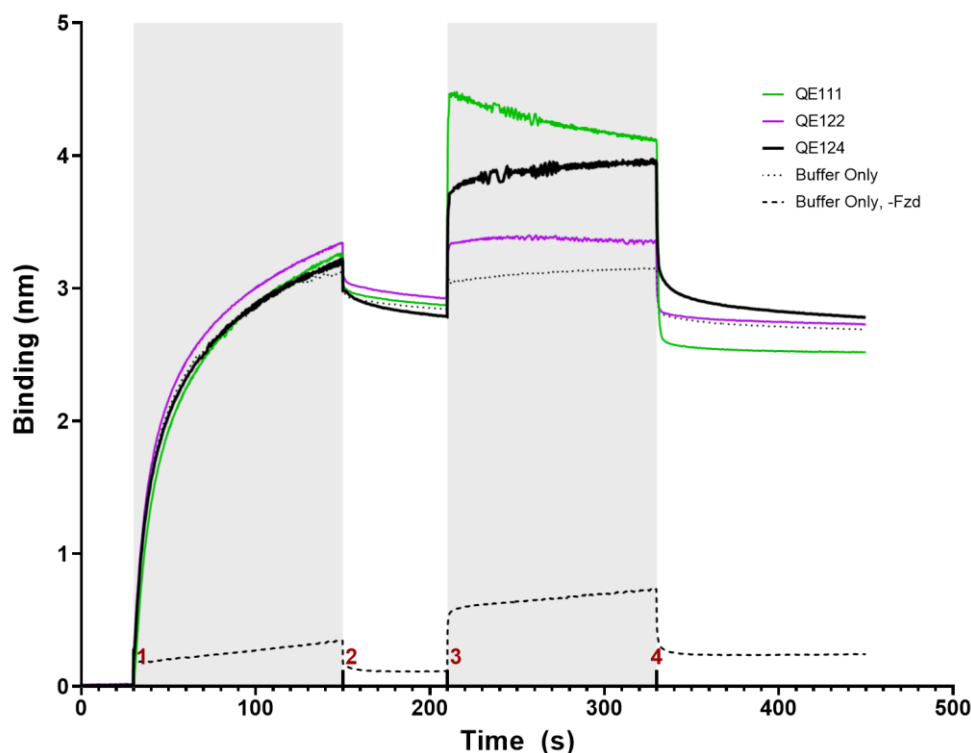


Fig. 39. Interaction between Fzd6-7 and purified nanobodies. Interactions measured using the Blitz BLI system and streptavidin tips. 1 – Loading of biotinylated Fzd6-6 (50 $\mu\text{g/ml}$). 2 – Wash. 3 – Addition of nanobody samples (100 μM), 4 – wash. “Buffer Only” indicates that no nanobody was present during step 3. “-Fzd” indicates that no biotinylated Fzd6-6 was loaded onto streptavidin tips during step 1. Experiment repeated twice, representative results shown.

was approximately the same height as for the control run conducted in the absence of Fzd (bold, dashed line, Fig. 39), while the subsequent curve remained relatively flat.

The addition of QE111 resulted in a significant increase of signal in comparison to the control, although the shape of the curve was abnormal, presenting a sharp increase of signal followed by a slow dropoff. Out of the Nbs tested, QE124 showed the best binding profile with a sharp increase followed by a more slow ascent.

Since QE124 produced a promising binding curve during the Blitz experiment, while also behaving well during SEC (eluting at the expected volumes), it was selected for further characterization using the Octet BLI system. Unfortunately, application of the Nb sample at concentrations as high as 1 μM did not produce any signal (Fig. 40). After repeated attempts to optimize the Octet experiment by varying Fzd6-7 and QE124 concentrations, as well as buffer conditions, while not seeing any improvements of

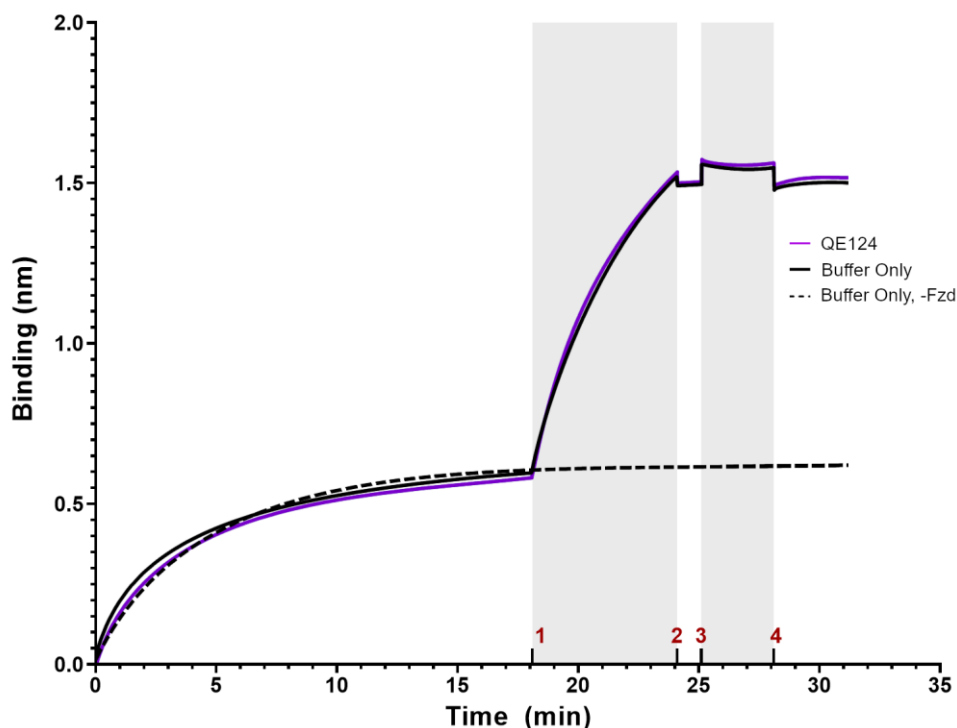


Fig. 40. Interaction between Fzd6-7 and QE124. Interactions measured using the Octet BLI system and streptavidin tips. 1 – Loading of biotinylated Fzd6-7 (50 $\mu\text{g/ml}$). 2 – Wash. 3 – Addition of nanobody samples (1 μM), 4 – wash. “Buffer Only” indicates that no nanobody was present during step 3. “-Fzd” indicates that no biotinylated Fzd6-6 was loaded onto streptavidin tips during step 1. Experiment repeated six times, representative results shown.

signal, I decided to evaluate the binding of purified QE124 to biotinylated Fzd6-7 by ELISA. The results indicated that QE124 is only able to bind Fzd6-7 at concentrations higher than 1 μM (Fig. 41).

In conclusion, despite all previous experiments over the course of the Nb selection process indicating that high-affinity Fzd6-7 binders should be present in the final samples, this was not the case. The Nbs selected using the yeast surface display library were capable of binding Fzd6-7 while expressed on the surface of the yeast, but, when expressed in isolation, they were actually quite low-affinity binders. This suggests that the high levels of signal obtained during FACS using nanomolar concentrations of Fzd6-7 (Fig. 36.C) probably resulted from a high copy number of individual Nbs found on the surface of the yeast cells, thus leading to strong avidity effects, which created the appearance of a successful selection process. Furthermore, the high levels of signal

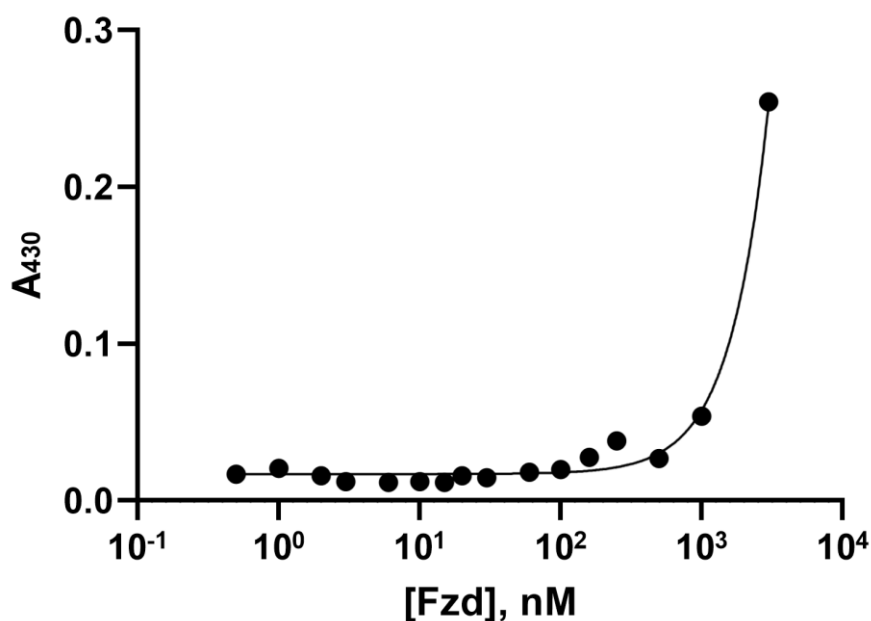


Fig. 41. ELISA measuring the interaction between Fzd6-7 and QE124. ELISA plates were coated with biotinylated Fzd6-7. Experiment was performed only once.

observed in the initial ELISA experiments must have been present due to high concentrations of Nb in the cell lysates used. Finally, the abnormal behaviour of purified Nbs on SEC column (as well as the presence of a large number of contaminants) suggests that the random mutations introduced within the Nbs as a part of the library generation process might actually result in reduced nanobody solubility, or the absence of a more-thorough negative selection process against a large variety of proteins usually found within camelids make it more likely for the selected nanobodies to non-specifically bind a large number of proteins. It is possible that the nanobodies identified by this selection process could be useful for further mutagenesis to create Nb moieties exhibiting higher affinity for Fzd6, although the low starting affinity and strange behaviour of selected Nbs suggests that it would require a considerable amount of effort, which eliminates the time-saving aspect of using this library.

3.3. Reconstitution of Fzd in detergentless systems

3.3.1. Fzd reconstitution in nanodiscs

Since the ultimate aim of this work was to obtain cryo-EM structures of Fzd signalling complexes, the presence of detergent in the final samples would be detrimental. As previously mentioned, empty detergent micelles and monomers present in vitrified cryo-EM grids reduce sample contrast and alter the properties of the thin aqueous layer during the grid preparation process. In addition, since it has been suggested that Fzds might function as dimeric or multimeric species, these interactions could be disrupted by detergent molecules, whereas the structure of intracellular regions of Fzd is altered in the absence of a lipid bilayer. Finally, in regards to future perspectives, the interactions between Fzd and co-receptors could also be disrupted by detergent. Therefore, the use of alternatives for transmembrane protein solubilization could lead to results that more accurately represent the native state of Fzd and enable further characterization of various Fzd signalling complexes.

I thus decided to explore the possibility of reconstituting purified Fzd6 protein within nanodiscs. Since the eventual goal of this would be to form large signalling complexes within the nanodisc, not reconstituting only the Fzd alone but together with potential ligands and co-receptors, it was decided to start by using relatively large nanodisc scaffolds, namely MSP2N2, which are capable of forming discs with a diameter of 16 nm (Ritchie et al., 2009).

Expression and purification of the MSP2N2 scaffold followed by SEC unexpectedly gave multiple peaks (Fig. 42.A). SDS-PAGE revealed a major band at the expected size of MSP2N2, and some lower molecular weight species (Fig. 42.B). Induction of protein expression at a lower temperature or different OD₆₀₀ values made no difference to the final quality of the protein (S. fig. 7). There are several possible explanations for the presence of different species within the SEC profile. MSP2N2 might interact with other proteins present in the cytosol, which would then be co-purified during the affinity purification procedure. Since almost all contaminants present on SDS-PAGE have reduced molecular weight in comparison to MSP2N2, they could also be MSP2N2 degradation products. Alternatively, MSP2N2 might either aggregate, or be found in various conformations within the sample. Nanodisc scaffolds have a shape that resembles a long, flexible belt, therefore it could fold back on itself to result in species with varying hydrodynamic radius. Previously published papers using MSP2N2 for nanodisc reconstitution had not reported SDS-PAGE gels or SEC profiles of its purification, so I decided to just collect one of the peaks from the SEC profile and use it for future nanodisc reconstitution experiments, keeping the production protocol

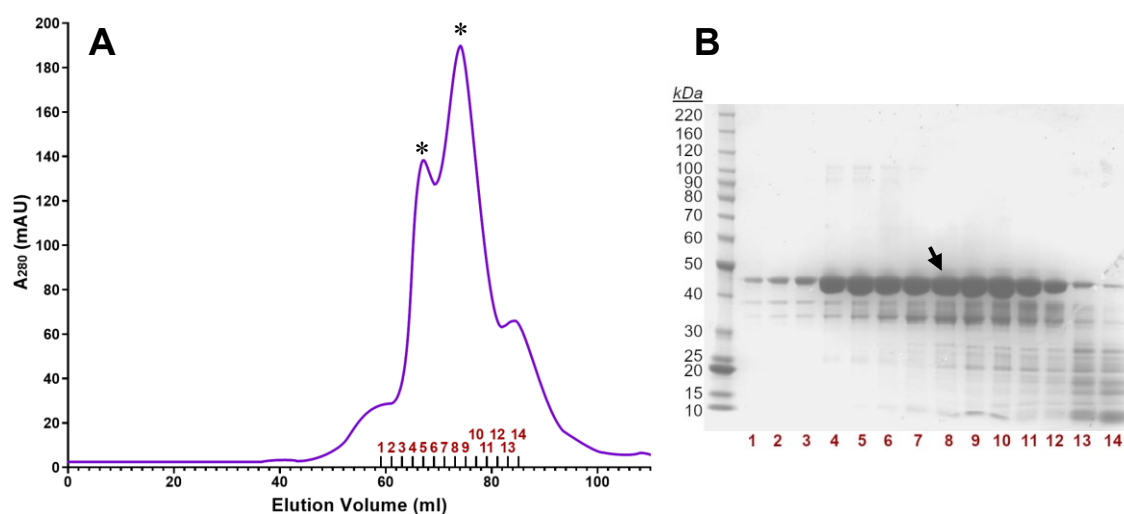


Fig. 42. Purification of MSP2N2. **A** – SEC. Asterisks denote peaks containing the protein of interest. SEC performed using a Superdex 75 HiLoad 16/600 GL column. **B** – reducing SDS-PAGE. Arrow denotes bands corresponding to the proteins of interest.

consistent. The presence of contaminants was a concern, but it should still allow me to optimize the right ratios for efficiently forming nanodisc particles.

I then attempted to reconstitute purified Fzd6-5 into nanodiscs using MSP2N2 and the lipid POPC, since this is the standard lipid most commonly used for nanodisc reconstitution, with a previously determined amount of POPC molecules that are found within the mature discs for a variety of scaffolds. As a starting point, I decided to use the scaffold and lipid ratio that was previously reported to be optimal for empty MSP2N2 nanodisc formation (1 : 240), also introducing Fzd6-5 into the reconstitution mixtures. To increase the success rates of Fzd incorporation within the discs without screening a large number of potential ratios, I prepared mixtures where both the scaffold and lipid were added in excess, using a ratio of scaffold and lipid that has been reported to be optimal for disk formation, so that the presence of Fzd6-5 would result only in minor deviations of final required POPC amounts present in the reconstitution mixtures.

After mixing all of the components together at specific protein : scaffold : lipid ratios, the detergent was removed by the addition of biobeads, and the resulting samples used to repeat Twin-Strep affinity purification to remove empty nanodiscs, subsequently using the resulting samples to perform SEC in order to assess the quality of the final samples (Fig. 43). The SEC profiles indicated that there was a predominant species present in the final Fzd-nanodisc samples, and it seemed to elute earlier than both Fzd and empty nanodiscs alone. The height of the peaks resulting from different mixtures indicated that the incorporation process is indeed more efficient if the scaffold and lipid were present in the mixtures in excess (e.g. 1.0:1:240 (red curve) versus 0.2:1:240

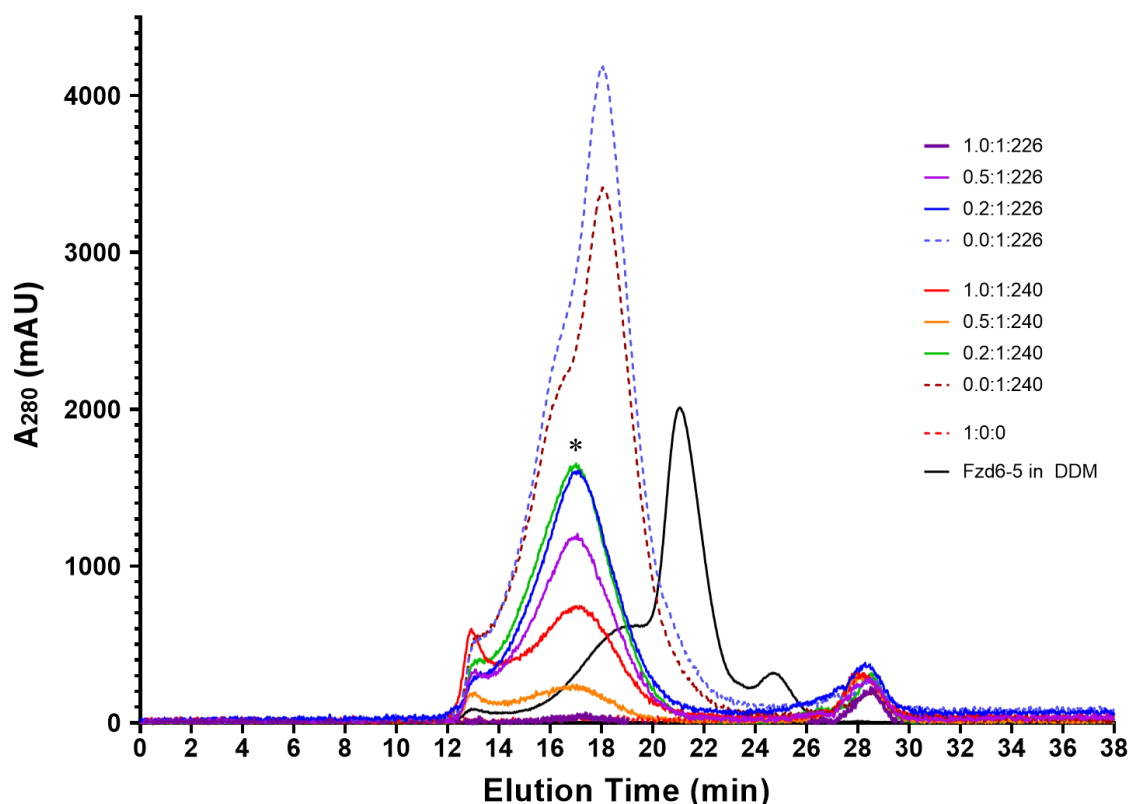


Fig. 43. Reconstitution of Fzd6-5 within MSP2N2 nanodiscs. Values indicate Fzd6-5: MSP2N2 : POPC molar ratios. Asterisk denotes SEC peaks suspected to contain the sample of interest. SEC performed using a Superose 6 Increase 3.2/300 GL column at a flow rate of 0.08 ml/min.

(green curve) Fzd6-5:MSP2N2:POPC ratio in Fig. 43). Despite this, the final Fzd-nanodisc peaks appeared quite broad, suggesting heterogeneity in nanodisc size.

This heterogeneity might be due to suboptimal MSP2N2 : POPC ratios, so I therefore decided to screen several ratios for empty nanodisc formation (Fig. 44). As the MSP2N2:POPC ratio was increased from 1:140 to 1:190 (purple and blue curves, Fig. 44), the SEC profile changed from two overlapping, low-intensity peaks to a single, sharp peak eluting slightly earlier than detergent-solubilized Fzd6-5 (dashed curve, Fig. 44). The left side of the peak produced by the 1:190 sample appeared to overlap with a small, earlier-eluting peak already present when using a 1:140 ratio. As the MSP2N2 : POPC ratio was increased further to 1:240 (green curve, Fig. 44), the profile collapsed back to the profile already observed with 1:140, but the highest MSP2N2:POPC ratio tested (1:300, red curve, Fig. 44) resulted in a sharp peak that eluted even quicker than

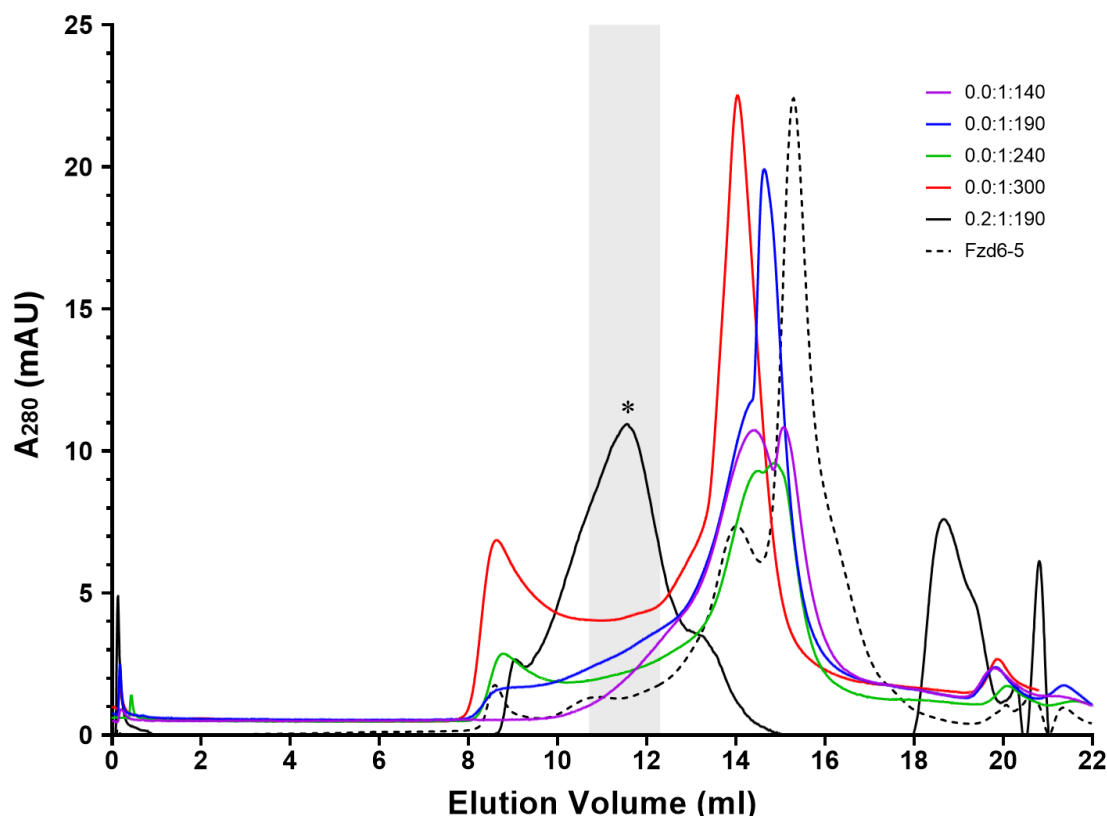


Fig. 44. Optimisation of POPC content during Fzd6-5 reconstitution within MSP2N2 nanodiscs. Values indicate Fzd6-5: MSP2N2 : POPC molar ratios. Peak selected for further examination (0.2:1:190) shaded grey and denoted with an asterisk. SEC performed using a Superose 6 Increase 10/300 GL column.

highest peak observed with the 1:190 sample. The predicted size of Fzd6-5 in a DDM detergent micelle is approximately 173 kDa, and the theoretical molecular weight of an MSP2N2 nanodisc containing 240 POPC molecules is 228 kDa. Taking into account previously observed protein separation characteristics of the Superose 6 Increase 10/300 GL column used in these experiments, it was assumed that the peak eluting closer to the peak produced by Fzd6-5 (1:190) would be the most likely candidate for containing properly formed MSP2N2 nanodiscs, whereas the peak produced by the 1:300 sample could be some other misformed nanodisc species with a higher molecular weight.

Unfortunately, SEC is not a suitable method for determining which peak contains properly formed nanodiscs with certainty. Commercially available protein standards for SEC column calibration are globular proteins, whereas nanodiscs are planar entities, therefore their molecular weight and elution volume relationships would not be

comparable due to differing relationships between the molecular weight and the Stokes radius of the sample.

I then performed a further round of MSP2N2 nanodisc reconstitution, using the scaffold and lipid in five-fold excess of Fzd6-5 (0.2:1:190). The resulting SEC peak (black curve, Fig. 44) exhibited profiles similar to my initial results (Fig. 43), with a broad peak eluting much earlier than both Fzd6-5 and empty MSP2N2 nanodiscs. This indicated that particles within the final sample had a much larger hydrodynamic radius in comparison to MSP2N2 nanodiscs without Fzd6-5 present, and that the sample was heterogenous. To confirm this, I assessed the homogeneity of the resulting nanodisc sample by negative-stain EM (Fig. 45), which showed that the particles found within the sample exhibited an extremely wide range of diameters (9 – 33 nm). Since the reported size of MSP2N2 nanodiscs is ~16 nm, it was obvious that the introduction of Fzd6-5 within the nanodisc reconstitution mixtures led to a disruption in nanodisc formation, which presented itself as high levels of heterogeneity.

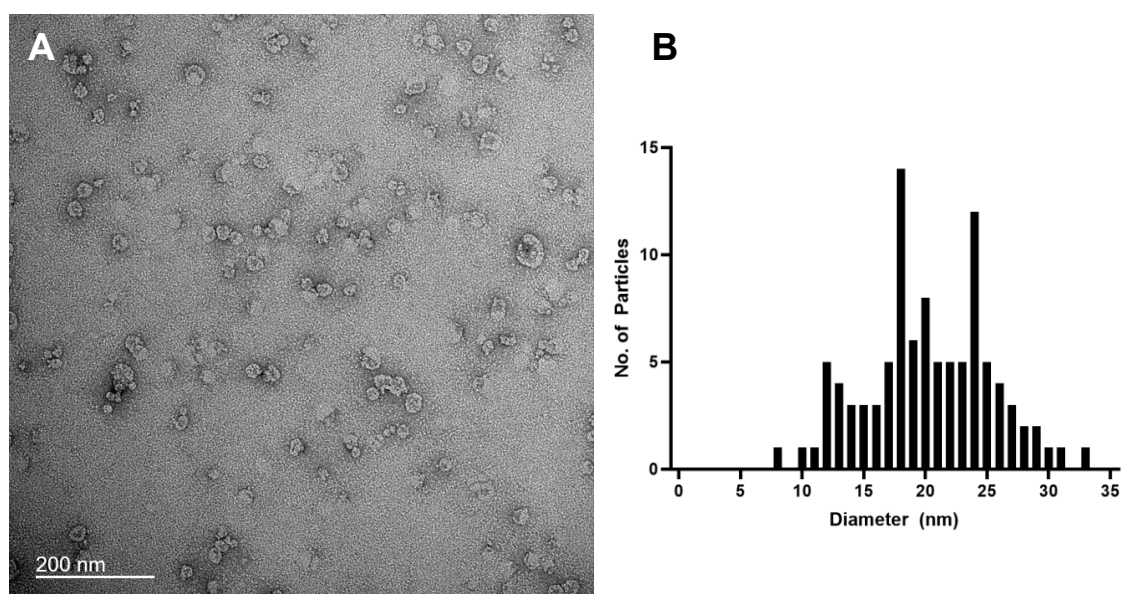


Fig. 45. Examination of Fzd6-5/MSP2N2/POPC (0.2:1:190) nanodiscs by negative-stain EM.

A – representative negative-stain EM image. Grids prepared using sample at a 5 µg/ml concentration. Micrograph was taken using a Tecnai T12 transmission electron microscope (FEI) operated at 120 kV, using a 42,000 x magnification. **B** – particle diameter distribution (100 particles total). To assess particle diameter, the longest possible dimension for each particle was measured, which would correspond to the diameter of the nanodisc.

The buffer conditions were the same when forming both empty and Fzd6-5 containing nanodiscs, and the diameter of the Fzd6-5 TMD is small in comparison to the reported diameter of MSP2N2 nanodiscs (4 nm versus 16 nm). Despite this, SEC and negative-stain EM data (Fig. 44, Fig. 45) suggest that the presence of Fzd6-5 within the reconstitution mixtures leads to a large disruption in reconstituted nanodisc structure. The reasons for this are unclear, although it may be due to a change in the nanodisc properties reducing their stability, or a technical issue during affinity chromatography of Fzd6-5-containing MSP2N2 nanodiscs.

The reconstitution experiments were then repeated using MSP1D1 scaffold, which has been reported to form nanodiscs with a diameter of ~10 nm, closer in size to the expected Fzd6 diameter. For the scaffold and POPC ratios, I decided to use the predetermined ratio of 1:65, using Fzd6-5 at a 15-fold excess.

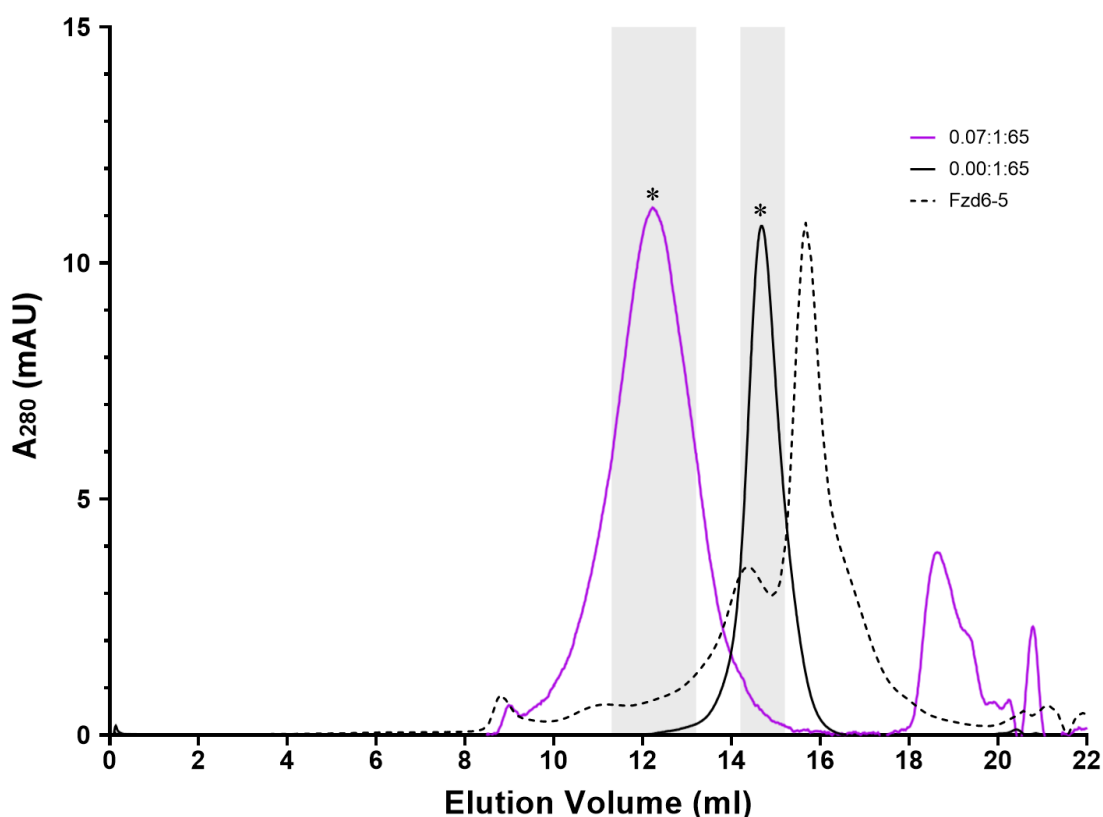
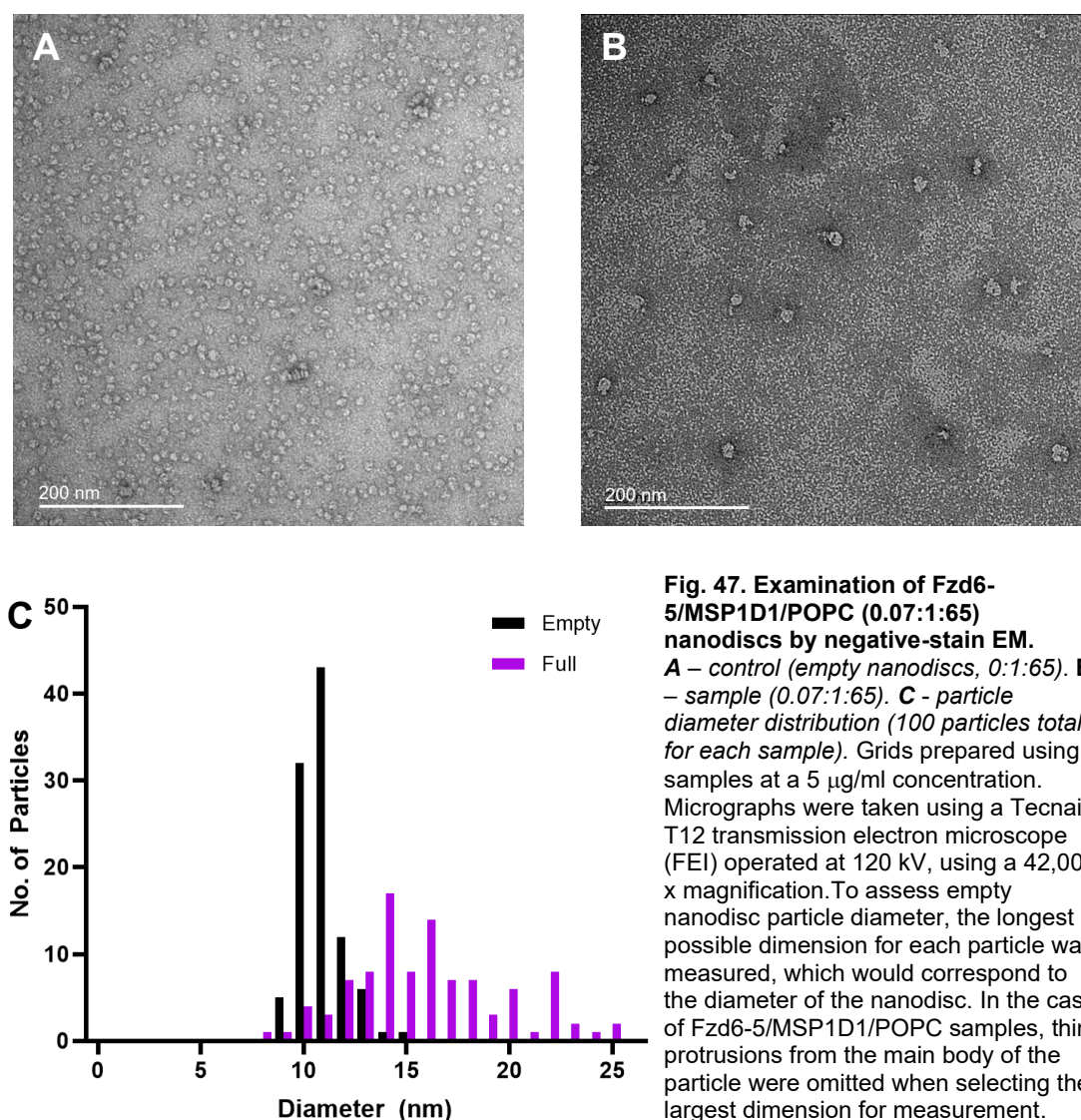


Fig. 46. Reconstitution of Fzd6-5 within MSP1D1 nanodiscs. Values indicate Fzd6-5: MSP1D1 : POPC molar ratios. Peaks selected for further examination shaded grey and denoted with an asterisk. SEC performed using a Superose 6 Increase 10/300 GL column.

The control reactions aimed at forming the MSP1D1 nanodisc in the absence of Fzd6-5 yielded a monodisperse peak on its SEC profile (Fig. 46), and upon examination of the sample by negative-stain EM, the empty nanodisc samples were homogenous, with the diameter of the disk ranging between 10 and 12 nm (Fig. 47.A/C). The inclusion of Fzd6-5 within the MSP1D1 reconstitution mixtures resulted in a single peak eluting faster than empty nanodiscs, and it was slightly more broad in comparison (Fig. 46). Despite this promising SEC result, the particles present within the final sample exhibited a wide range of diameters (9-25 nm), very different to the homogeneity of the empty MSP1D1 nanodiscs (Fig. 47.B/C).



The similar behaviour of MSP2N2 and MSP1D1 nanodiscs suggests that in both cases the heterogeneity of Fzd6-5-containing nanodiscs most likely arises due to the presence of Fzd6-5 itself. One possibility was that MSP2N2 nanodiscs were too large, whereas MSP1D1 nanodiscs too small, since Fzd6-5 is capable of forming dimers (Fig. 22) with a predicted diameter of ~ 8 nm. I therefore decided to test whether the slightly larger MSP1E3 nanodiscs with a reported diameter of ~ 12 nm might produce better results.

I started by evaluating the best MSP1E3 : POPC ratios for the formation of empty nanodiscs. In contrast to MSP2N2 (Fig. 44), all empty MSP1E3 nanodisc samples produced with a scaffold to POPC molar ratio higher than 1:50 produced a single peak on SEC profiles, eluting at an identical volume with varying POPC concentrations (Fig. 48). The main differences between the samples was the height of the main peak, as well as the presence of aggregate peaks eluting just before the main peak, which became

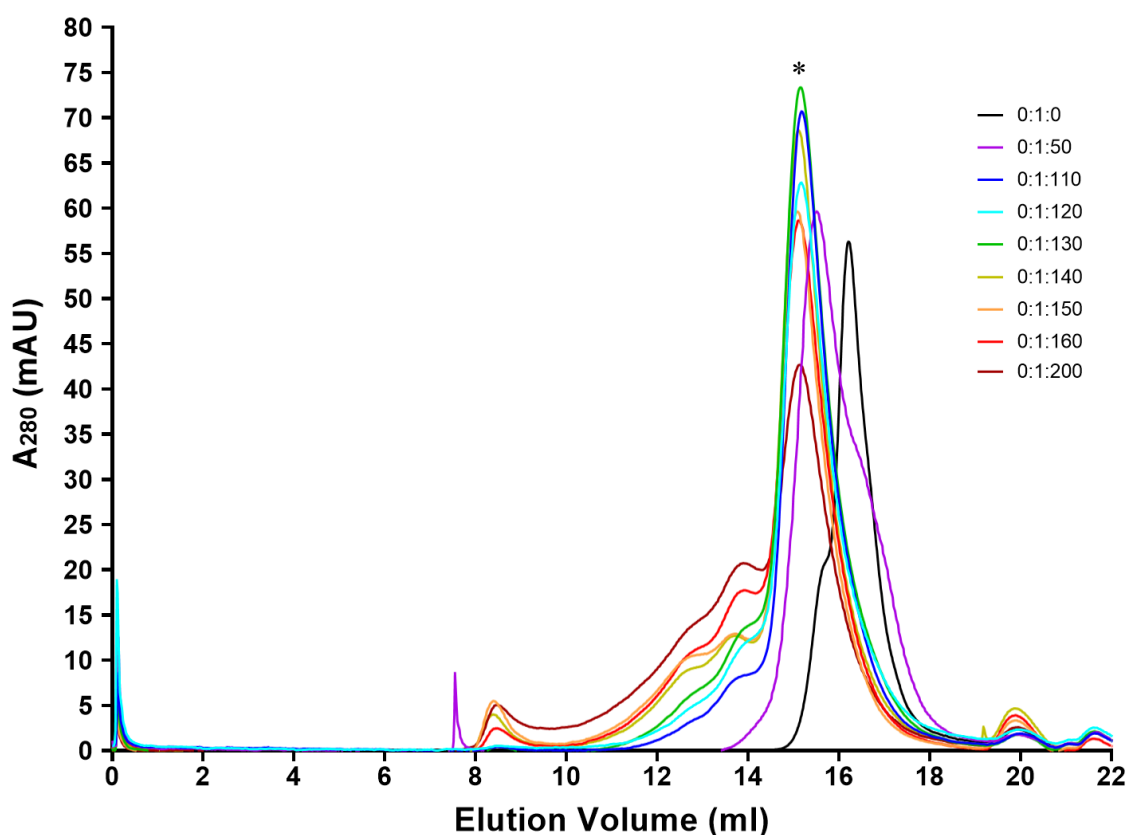


Fig. 48. Optimisation of POPC content within MSP1E3 nanodisc reconstitution mixtures. Values indicate Fzd6-5: MSP1E3 : POPC molar ratios. Asterisk denotes peak suspected to contain the sample of interest. SEC performed using a Superose 6 Increase 10/300 GL column.

more prominent with increasing amounts of POPC in the nanodisc reconstitution mixtures. Based on these results, I selected a MSP1E3 : POPC ratio of 1:130 for subsequent experiments, using a similar strategy as before, where empty nanodiscs were formed in excess in the presence of Fzd6-5.

The inclusion of Fzd6-5 into the MSP1E3 : POPC reconstitution mixture, resulted in a SEC profile which produced a distinctive peak, which eluted approximately 1.6 ml earlier than empty MSP1E3 nanodiscs and Fzd6-5 (Fig. 49). It was the smallest peak shift between full and empty nanodiscs observed so far (MSP1D1 – 2.4 ml, MSP2N2 – 3.2 ml, using Superose 6 Increase 10/300 GL), indicating that this peak might contain Fzd6-5 containing nanodiscs with a size that does not exceed that of the empty nanodiscs by a large amount. Unfortunately, the peak was not isocratic, being flanked by curves with an elevated amount of signal. This suggested that although there might

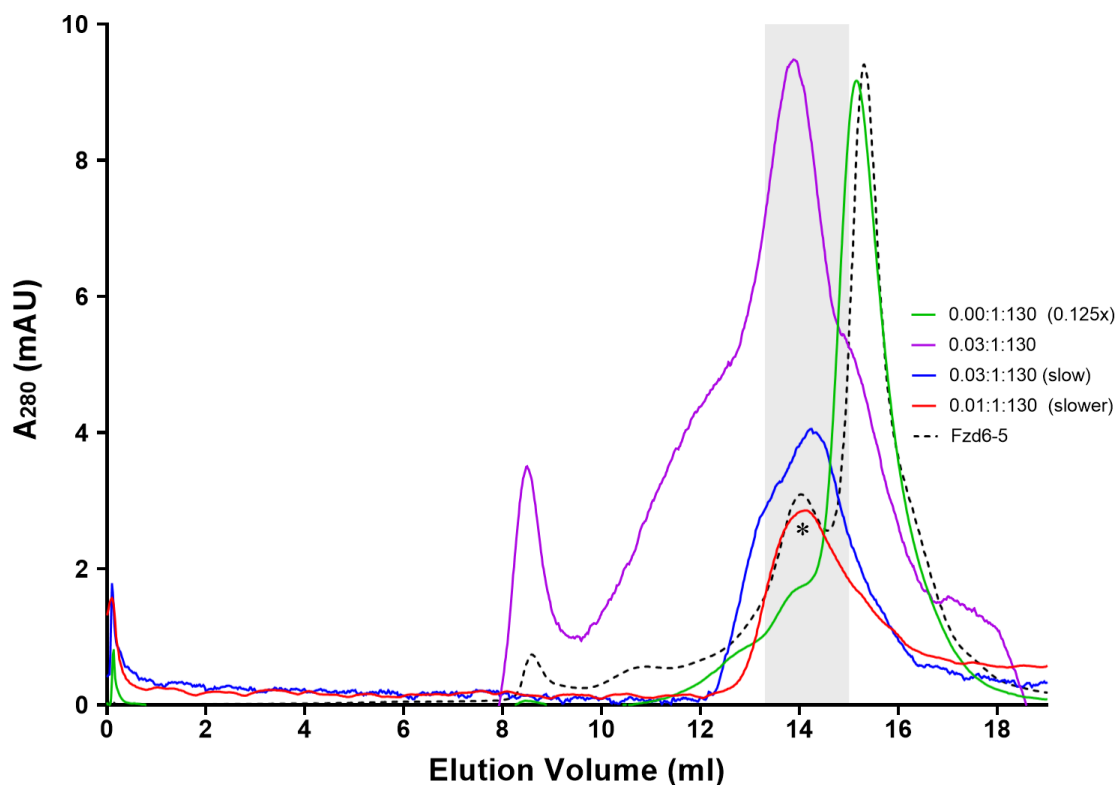


Fig. 49. Optimisation of Fzd6-5/MSP1E3/POPC nanodisc formation. For 0.03:1:130 (slow) and 0.01:1:130 (slower) samples, the total amount of biobeads required for removal of all detergent present was calculated and used, adding biobeads in either 2 or 6 batches with 30 min intervals between for the former and latter sample respectively. The peak of 0.01:1:130 (slower) selected for further examination shaded grey and denoted with an asterisk. SEC performed using a Superose 6 Increase 10/300 GL column.

be a predominant species present in the final sample, the nanodisc reconstitution process did not proceed efficiently enough to yield monodisperse sample. Since the previous optimization runs evaluating empty MSP1E3 nanodisc formation showed that minor variations the MSP1E3:POPC molar ratios should not make a major difference in the quality of the final disc samples (Fig. 48), something else must be at fault. One possibility would be the properties of Fzd6-5 itself, whereas the other was the kinetics of the reconstitution process itself.

I therefore decided to evaluate the effect of adding varying amounts of biobeads to the reconstitution mixture at different speeds. This might increase the quality of the final sample, since slower rates of detergent removal might result in a more orderly assembly of the nanodiscs, while biobeads themselves have been reported to bind not only detergent, but also lipid and transmembrane proteins, so their excess within the reconstitution mixtures might be detrimental to obtaining high quality samples. Previously reported protocols have suggested using 0.5 ml of biobeads per ml of sample added as a single batch, and I had followed this for all my previous reconstitutions. Instead, I decided to calculate the optimal amount of biobeads required for the removal of all detergent molecules within my sample based on previously reported biobead binding capacities of different lipids. The calculations indicated that I had been using ~6.5 x more biobeads than theoretically required.

I then repeated the Fzd6-5 and MSP1E3 nanodisc reconstitution experiments, using an amount of biobeads that was 1.1-fold in excess of the calculated amount, adding the total amount in either 2 or 6 batches with a 30 min incubation period between each addition (Fig. 49). By adjusting these two parameters, the final nanodisc samples yielded a much cleaner SEC profile, with a single, albeit slightly broad peak, being

present, although the shape of the peak was slightly better when the biobeads were added over a longer period of time. When examined by negative-stain EM, the final Fzd6-5-containing nanodisc sample appeared monodisperse and homogenous (Fig. 50.A). The average particle diameter was only slightly increased for full nanodiscs in comparison to the empty control (17 versus 16 nm), which was still higher than the reported MSP1E3 nanodisc diameter value of 12 nm (Fig. 50.B). Preliminary 2D class averaging showed that the majority of the nanodiscs present on the grids appeared to be ‘laying flat’, with a particle diameter varying a lot between individual classes, but this could be because the nanodisc top-views would all be placed within a single 2D class average. The preference for top-views also made it impossible to see any features within the nanodiscs that might belong to Fzd6-5, although the final samples used for grid preparation were fluorescent, indicating that the mVenus-tagged Fzd6-5 was present within the reconstituted nanodiscs.

In conclusion, the choice of nanodisc scaffold and the speed of detergent removal are important parameters for efficient reconstitution of transmembrane proteins within nanodiscs. The importance of biobead addition in particular appears to be overlooked in the literature. In regards to the use of MSP1E3 nanodiscs for future experiments, the amount of nanodisc sample obtained from the final experiments using 0.1 mg of purified Fzd6-5 yielded only 0.6 µg of nanodisc, indicating that the efficiency of the nanodisc reconstitution process was under 1%, making them unsuitable for signalling complex formation or further characterization by cryo-EM. It could be possible that the use of different lipids for nanodisc formation could result in higher yields, but due to the time already spent on optimizing nanodisc reconstitution and the emergence of SMALPs as a viable alternative for solubilising Fzd in the absence of detergent, this possibility was not explored further.

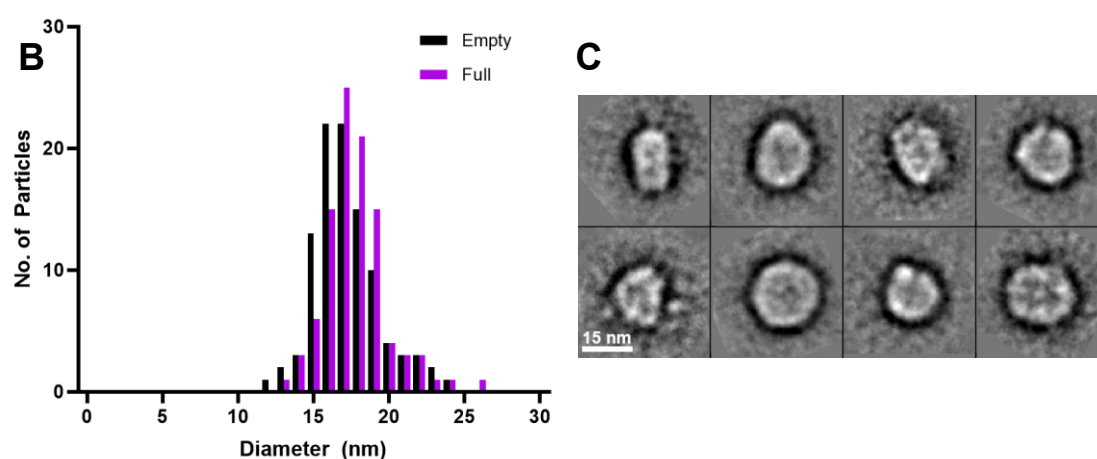
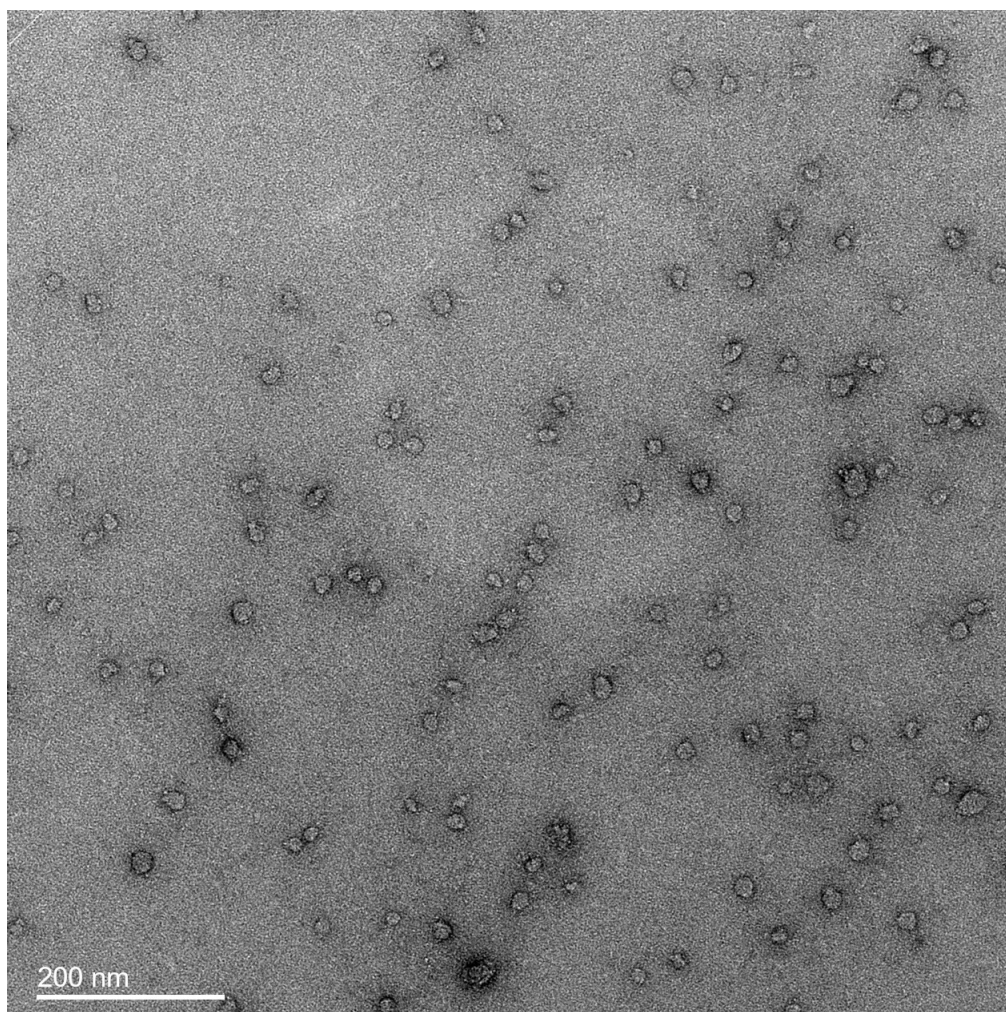


Fig. 50. Examination of Fzd6-5/MSP1E3/POPC (0.01:1:130) nanodiscs by negative-stain EM.
A – representative negative-stain EM micrograph. Grids prepared using samples at a 5 $\mu\text{g/ml}$ concentration. Micrographs were taken using a Tecnai T12 transmission electron microscope (FEI) operated at 120 kV, using a 42,000 x magnification. **B** – particle diameter distribution (100 particles total for each sample). The longest possible dimension for each particle was measured, which would correspond to the diameter of the nanodisc. **C** – 2D class averages. 30 micrographs were manually collected using a Tecnai T12 transmission electron microscope (FEI) operated at 120 kV, using a 42,000 x magnification and a defocus value of 1 μm . 1816 particles were manually picked, extracted using a box size of 38 nm, and subjected to several rounds of 2D classification to yield the final classes shown, consisting of 768 particles.

3.3.2. Fzd reconstitution in SMALPs

The low efficiency of Fzd6-5 reconstitution within nanodiscs made it an unsuitable method for studying higher-order complexes of Fzd by cryo-EM. A potential alternative would be to use SMALPs – styrene : maleic acid lipid particles. Similarly to nanodiscs, SMALPs have been shown to form disc-like particles containing lipids, but the major difference is that the SMA polymer is added directly to isolated cell membranes, extracting and solubilising transmembrane proteins from within them along with patches of native lipids. This would eliminate the need to use detergent altogether, while also resulting in a more native-like environment for the purified protein.

The first attempt at producing Fzd6-5-containing SMALPs yielded a clean SEC profile which closely resembled the profiles obtained from detergent-solubilized Fzd6-5, except that the peak eluted earlier from the column (Fig. 51.A). The SDS-PAGE also showed that the protein was very clean (Fig. 51.B), thus indicating that the procedure yielded a high-quality sample. Despite this initial attempt, subsequent SEC was not reproducible, and the chromatographic behaviour was unusual (S. fig. 8). This has been reported in previous publications using SMALPs, suggesting an interaction between the SMALP polymer and the SEC column resin (Qiu et al., 2018). Because of this, future SMALP preparations relied on SDS-PAGE for confirming the presence of the target protein, whereas the quality of final sample was assessed by EM.

To further assess the quality of SMALP particles, negative-stain EM could not be used – the uranyl salt stains used for negatively-staining grids have pH values in the range between 4 and 5, whereas the integrity of the SMALP particles depends on stable pH values above 7, under which the SMALP particles fall apart, resulting in sample aggregation (Fig. 51.C).

The Fzd6-5 SMALP samples were thus embedded in a thin, vitreous layer of ice on a holey carbon grid and examined by cryo-EM. Monodisperse particles were clearly

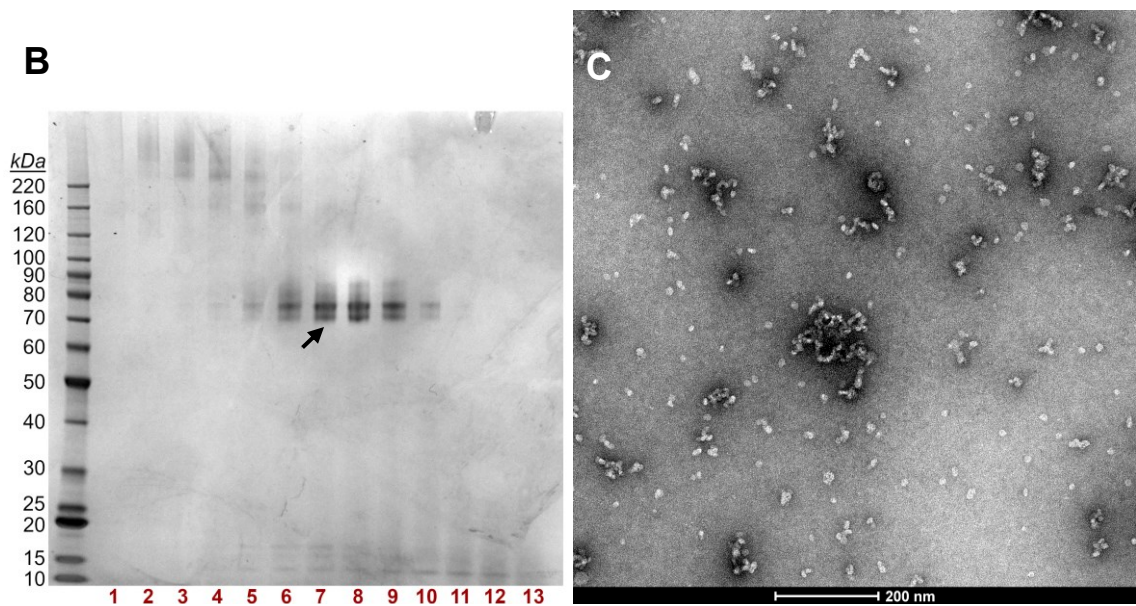
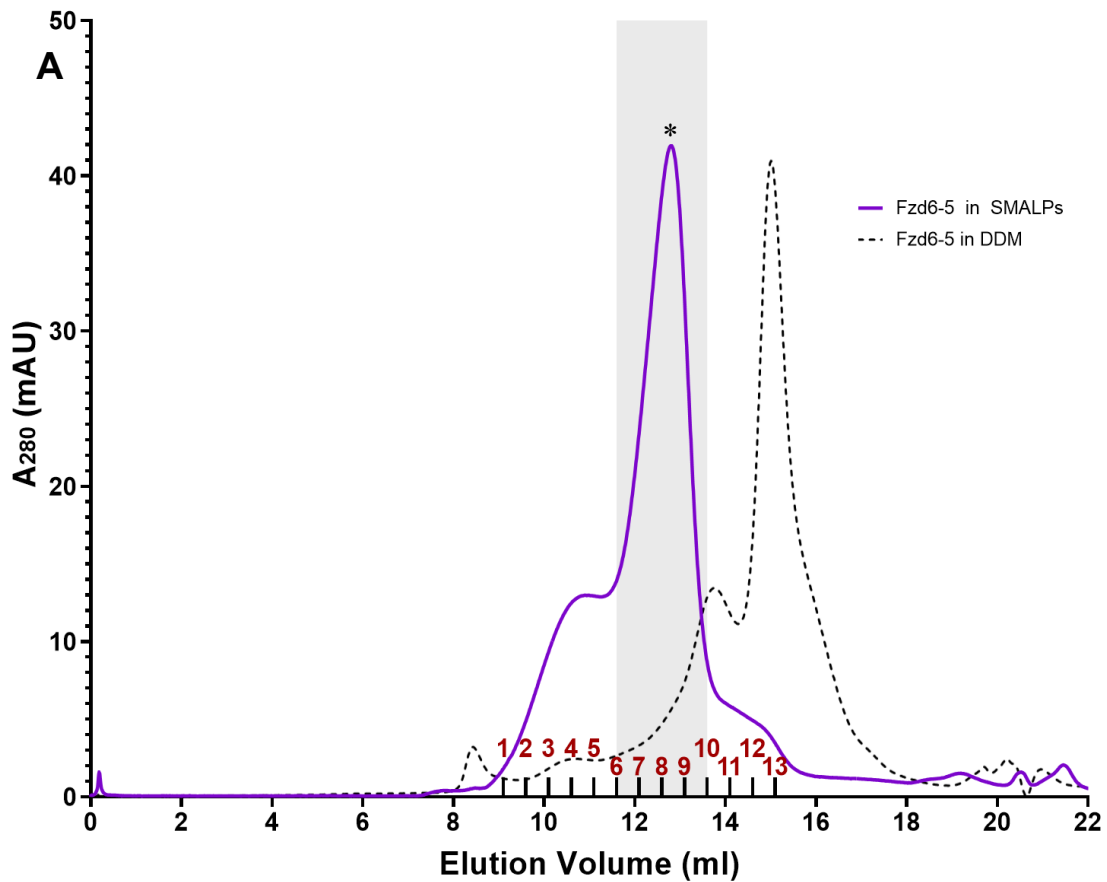


Fig. 51. Purification of Fzd6-5 SMALPs. **A** – SEC. Asterisk denotes peak containing the sample of interest. SEC performed using a Superose 6 Increase 10/300 GL column. **B** – SDS-PAGE. Arrow denotes bands corresponding to the protein of interest. Experiment performed under non-reducing conditions. **C** – examination by negative-stain EM. Grid prepared using sample at a 5 μ g/ml concentration. Micrograph taken using a Tecnai T12 transmission electron microscope (FEI) operated at 120 kV, using a 42,000 x magnification.

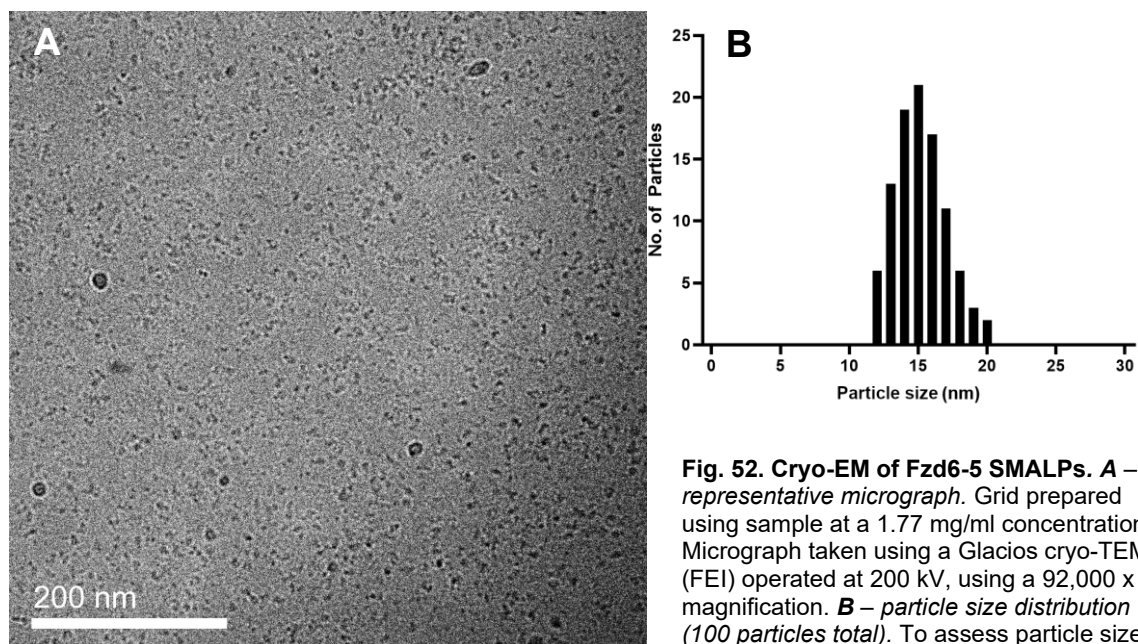
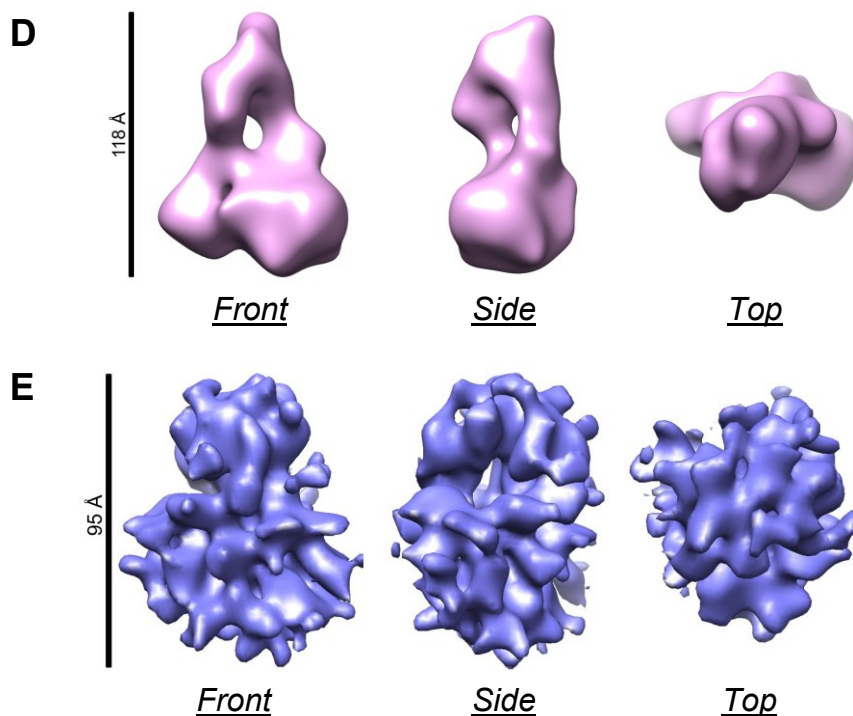
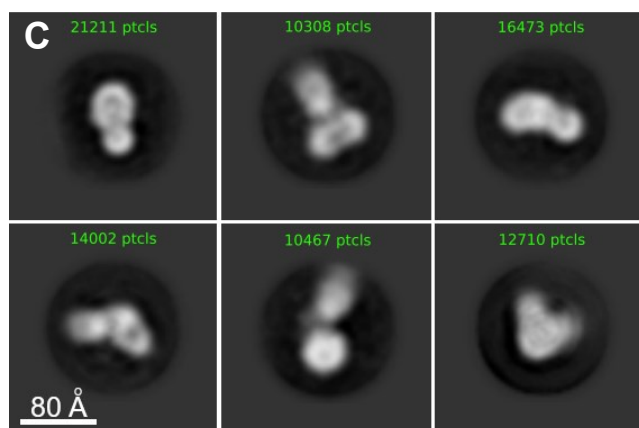


Fig. 52. Cryo-EM of Fzd6-5 SMALPs. A – representative micrograph. Grid prepared using sample at a 1.77 mg/ml concentration. Micrograph taken using a Glacios cryo-TEM (FEI) operated at 200 kV, using a 92,000 x magnification. **B – particle size distribution (100 particles total).** To assess particle size, the longest possible dimension for each particle was measured. **C – Representative 2D class averages.** 923 movies were collected using a Titan Krios cryo-TEM (FEI) operated at 300 kV, using a K2 camera in counting mode. Data processed in Cryosparc (v2.5.0). Number of particles (ptcls) within each class shown above each class. **D – Ab initio model.** The model was reconstructed using 76k particles. **E – refined model.** The model was refined using 76k particles to an estimated resolution of 12.6 Å. For details, see S. Table 1 and S. fig. 11.



present on grids (Fig. 52.A), and the sample appeared to be homogenous, with an average particle size of 15 nm (Fig. 52.B). 2D classification of the particles resulted in classes that indeed appeared to be formed by a disc-like object, with one or two extensions protruding from its surface (Fig. 52.C). 3D processing of the data further supported the presence of a protein (or its dimer) within the discs (Fig. 52.D), although further refinements resulted in noisy volumes with low resolution ($\sim 11\text{\AA}$).

In conclusion, the data from these initial Fzd6-5 SMALP purification trials indicated that the use of SMALPs is a viable strategy for the purification of Fzd in the absence of detergent. The process of purifying protein-containing SMALP particles also appeared to be much easier than using nanodiscs – after many failed attempts at obtaining homogenous Fzd6-5 nanodiscs, the use of SMALPs gave promising results on the first try, while also resulting in higher yields. Furthermore, the hints of Fzd dimer being present in the sample were very encouraging, indicating that the absence of detergent within the purification buffers might maintain native Fzd6 complexes formed on the cell surface in a dimeric state. The extensions extending from the SMALP discs as observed by cryo-EM appeared to lack enough mass or rigidity to enable efficient alignment of particles, therefore larger, more stable complexes of Fzd would have to be formed in order to obtain higher resolution cryo-EM structures. The downside of using SMALPs was that the samples behaved erratically on SEC columns, limiting the options of troubleshooting the purification procedure, which might be especially important when trying to isolate SMALP particles with Fzd complexes in the future.

To further increase the size of the Fzd6-5 SMALP particles while also potentially stabilising its extracellular domains, I attempted to isolate SMALPs from cells co-expressing both Fzd6-5 and 10xHis-tagged Wnt5A. Secreted Wnt5A appeared to

associate with cell membranes following secretion (Fig. 28.A), Wnts have been known to associate with carrier proteins, and secreted Wnt5A is thought to activate signalling via Fzd6. Therefore, co-expression of Fzd6-5 and Wnt5A should potentially result in the formation of Fzd6-5/Wnt5A complexes on the surface of the cell, which could subsequently be solubilized and purified by the use of SMALPs.

The final SMALP sample obtained following StreptactinXT and nickel affinity chromatography was examined by SDS-PAGE (Fig. 53.A) and a western blot against the His-tag present on Wnt5A (Fig. 53.B). The SDS-PAGE showed bands corresponding to Fzd6-5 (Fig. 53.A.b), a large streak of aggregated protein at the top of the wells (Fig. 53.A.a), as well as two faint bands migrating at a distance corresponding to a molecular weight of ~61 and 71 kDa (the predicted molecular weight of the used Wnt5A construct was 67 kDa). The western blot confirmed that these bands, along with the high molecular weight streak, contained Wnt5A.

It was later noted that the addition of SDS-PAGE loading buffer to the Fzd6-5/Wnt5A SMALP samples immediately resulted in visible aggregation of the sample, therefore the quality of the sample was evaluated by cryo-EM (S. fig. 9). The sample appeared

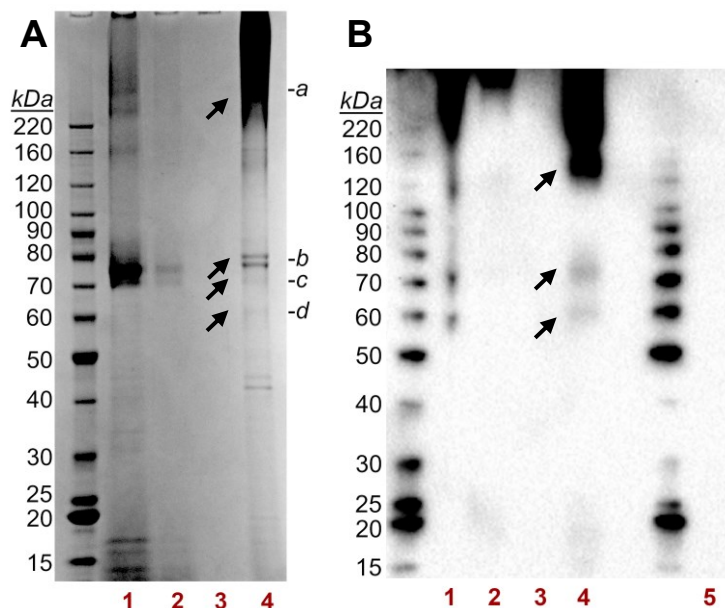


Fig. 53. Purification of Fzd6-5/Wnt5A-1 SMALPs.
A – SDS- PAGE. 1, 2 – Nickel affinity chromatography flowthrough and wash. 3 – flowthrough during concentration of final sample. 4 – Final sample. **B** – anti-6xHis western blot (detecting Wnt5A-1). 1 – cell membranes. 2 – solubilised SMALP solution. 3 – nickel affinity chromatography flowthrough. 4 – final sample. 5 – control (Fzd6-5 SMALPs). Arrows denote bands corresponding to the proteins of interest. a – aggregates; b – Fzd6-5; c/d – suspected Wnt5A-1 bands. Experiments performed under non-reducing conditions.

monodisperse and homogenous, although no major differences in particle size of morphology were observed. Based on this, the co-expression of Fzds and Wnts followed by subsequent SMA solubilization appears to be worth exploring further in the future for the formation of Wnt-containing SMALP signalling complexes, but the lack of differences in particle morphology and size meant that other routes for increasing particle size should be examined first.

I therefore decided to turn my attention to Fzd4, which has been reported to dimerize in the presence of the atypical ligand Ndp (Bang et al., 2018). It is less hydrophobic and thus easier to produce than Wnt, while its interaction with Fzd4 has been shown to result in conformational changes within the receptor (Bang et al., 2018). Since it binds two CRDs within the Fzd4 dimer, it should also reduce any potential flexibility within the extracellular regions of Fzd4, making it more amenable to structure determination by cryo-EM.

I generated a construct of Fzd4 based on the tagging strategy that was utilized for Fzd6-5, omitting the bRIL insert in the intracellular loop 3, in order to permit any future addition of other intracellular binding partners to the complex, as well as potentially minimize any steric hindrance between two copies of bRIL within the putative Fzd4 dimer. The expression of this construct, Fzd4-1 (Fig. 13), within HEK293S GnTI⁻ TetR cell lines was confirmed using a traditional, detergent-based purification (S. fig. 10).

A stable HEK293S GnTI⁻ TetR cell line co-expressing Fzd4-1 and a 6xHis- and SUMO-tagged Ndp (construct referred to as Ndp1) was then generated, and isolated cell membranes were subsequently solubilized using SMA polymer. Following affinity chromatography using streptactin and nickel resins, the sample was subjected to SEC, which resulted in a profile typical of SMALPs (Fig. 54.A). As previously mentioned,

many SMALP samples tend to interact with SEC column resins, although this does not apply to all membrane proteins solubilized using SMA polymer (Qiu et al., 2018). Although the exact reason for this is not known, it is most likely related to the

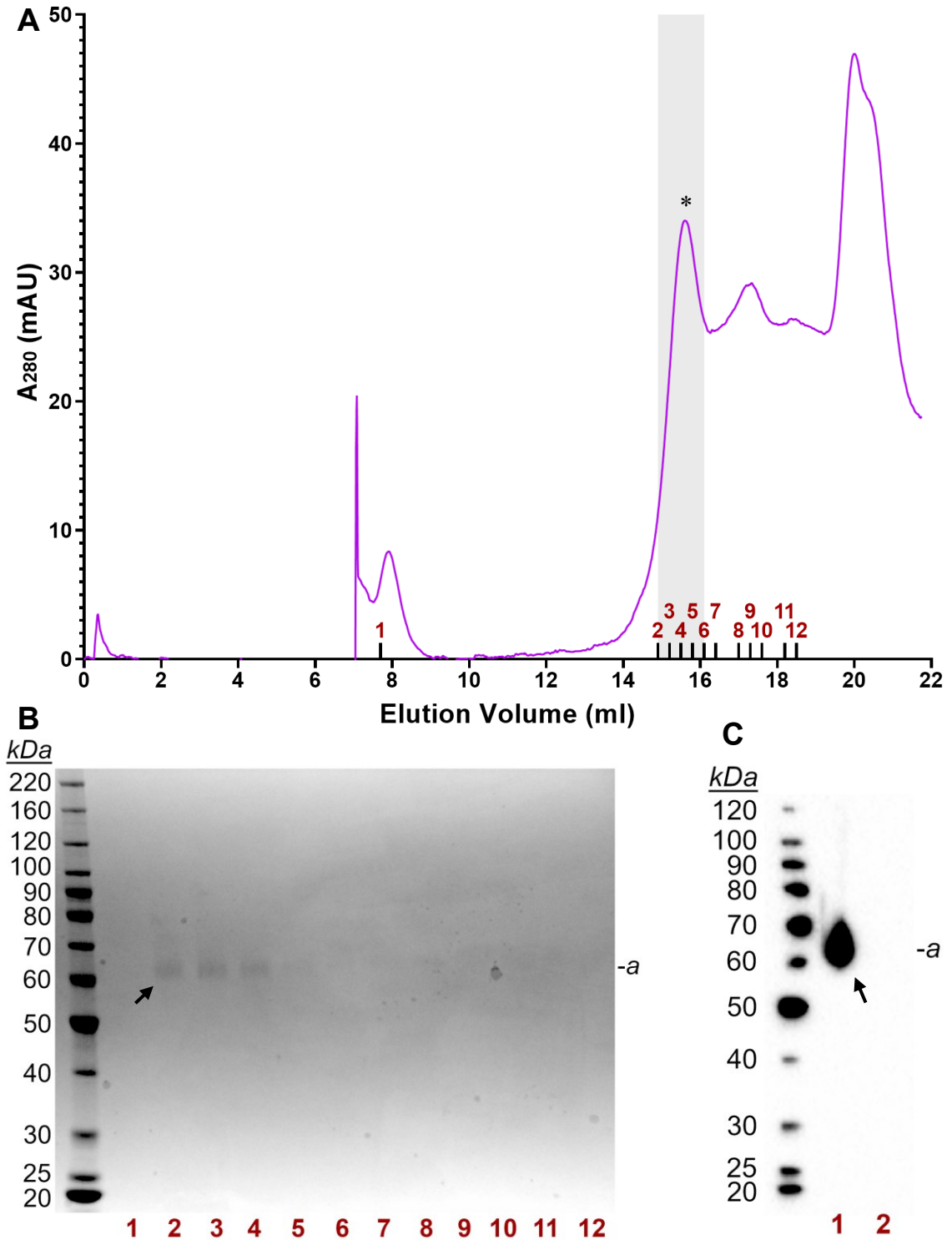


Fig. 54. Purification of Fzd4-1/Ndp1 SMALPs. **A** – SEC. Main protein-containing peak shaded grey and denoted with an asterisk. SEC performed using a Superose 6 Increase 10/300 GL column. **B** – SDS-PAGE. **C** – anti-6xHis western blot (detecting Ndp1). 1 – final sample. 2 – Fzd6-5 SMALP control. Arrows denote bands corresponding to the proteins of interest. Experiments performed under non-reducing conditions.

biochemical properties of the membrane protein present within the SMALP particle. It was hoped that the presence of the SUMO tag within Ndp1, known for its ability to improve protein solubility in solution, would sufficiently alter the biochemical properties of the Fzd4-1/Ndp1 complex within SMALPs to minimise the suspected interaction with the SEC resin. Unfortunately this was not the case, as indicated by the aberrant SEC profile (Fig. 54.A). SDS-PAGE of the resulting fractions produced a single band (Fig. 54.B), which corresponded both to previously observed, detergent solubilized bands of Fzd4-1 (S. fig. 10), while also being quite close to the 58 kDa predicted molecular weight of an Ndp1 dimer. I therefore performed an anti-6xHis western blot, which confirmed that the band indeed contains Ndp1 (Fig. 54.C.a).

The Fzd4-1/Ndp1 SMALP sample was examined by cryo-EM. In contrast to the Fzd6-5 SMALPs, the particles appeared to be distributed more unevenly across the grid, suggesting that the properties of Fzd4-1/Ndp1 complex make it more susceptible to aggregation at the air-water interface (Fig. 55.A). A preliminary data collection and processing showed 2D class averages that were significantly different from the Fzd6-5 SMALPs (Fig. 52.A), clearly having two different lobes of density present that emerge from a single base at one end of the particle (Fig. 55.B). This strongly suggested that Fzd4-1 within SMALP particles was found in dimeric form, although the presence of Ndp1 was not conclusive. Also, the class averages appeared to exhibit more features and less blur than the data obtained using Fzd6-5 SMALPs (Fig. 52.C), while still leading to 3D reconstruction resolutions that were too low for structure determination (S. fig. 12).

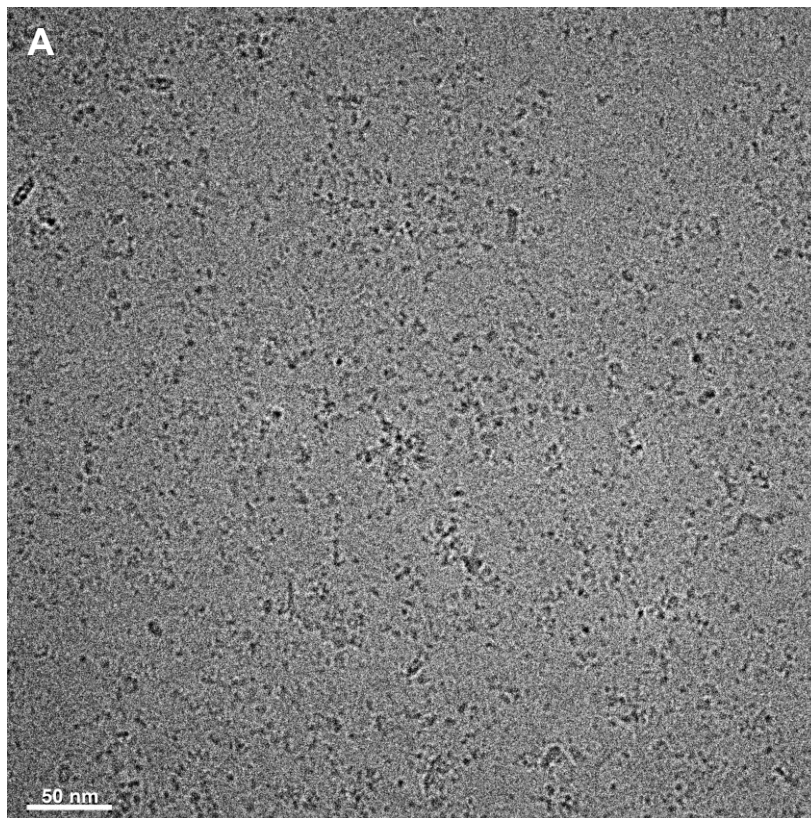
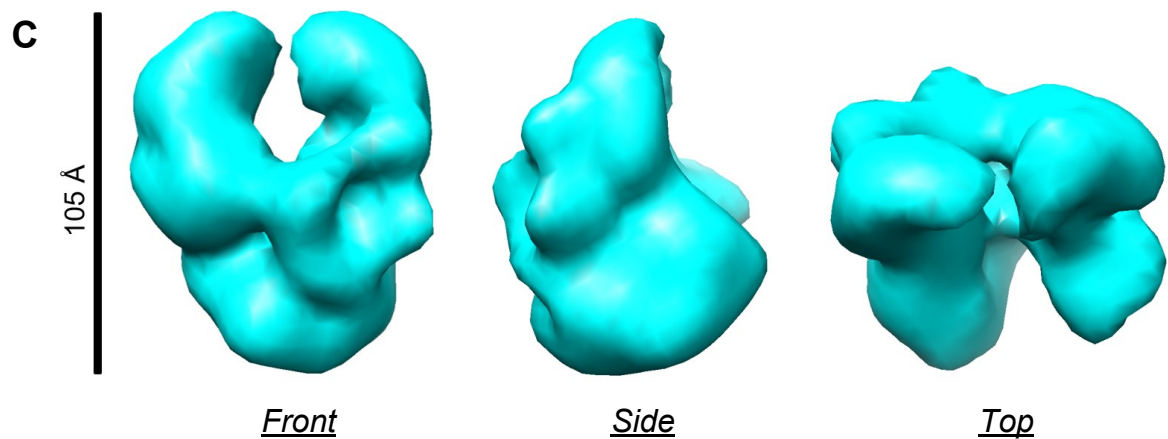
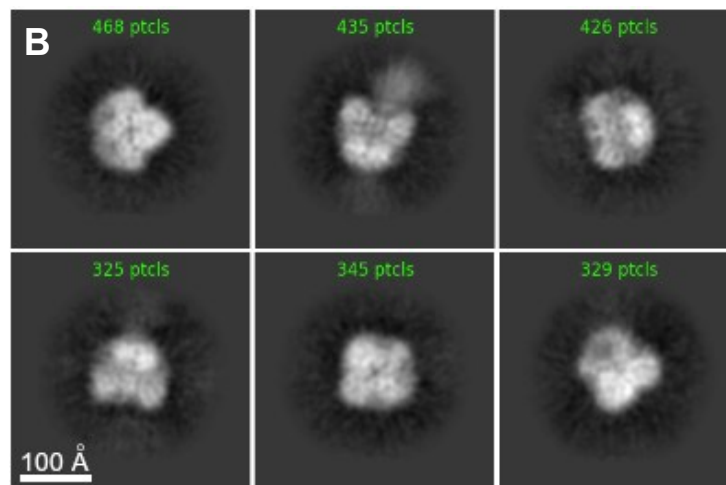


Fig. 55. Cryo-EM of Fzd4-1/Ndp1 SMALPs.

A – representative micrograph.

B – representative 2D class averages. Number of particles (ptcls) within each class shown above each class. **C** – final volume.

Volume refined using 23k particles. Grid prepared using sample at a 0.86 mg/ml concentration. Movies recorded using a Glacios cryo-TEM (FEI) operated at 200 kV, with a Falcon 3 camera at a 92,000 x magnification in linear mode. For details, see S. Table 1 and S. fig. 12.



3.3.3. Formation of Fzd4 and Fab fragment in SMALPs

Although Fzd4-1/Ndp1 SMALP particles showed significant improvements in cryo-EM data quality when compared to Fzd6-5 SMALPs, the lack of high resolution features during data processing indicated that the sample was still not suitable for structure determination. One of the reasons for this could be that the mass of the particles located outside of the SMALP disc was still not sufficiently large to allow efficient particle alignment during data processing. Because of this, I obtained an RH2.2 plasmid encoding a 6xHis-tagged heavy chain and a FLAG-tagged light chain of an anti-Fzd4 antigen-binding fragment (Fab) called F2.A, which had been reported to bind the extracellular domain of Fzd4 with nanomolar affinity, while maintaining its ability to bind Ndp (Pavlovic et al., 2018). This fragment would allow me to increase the mass of the Fzd4-1/Ndp1 SMALP particles by ~100 kDa, with each Fab binding to one monomer Fzd4-1. The molecular weight of the resulting Fzd4-1/Ndp1/F2.A complex would be ~230 kDa (with the tags cleaved), which should be sufficient for the determination of high resolution structures by cryo-EM.

F2.A was expressed in *E. coli* and purified using protein A resin. The SEC profile of F2.A samples consisted of a single, isocratic peak, while co-incubation of purified, detergent-solubilized Fzd4-1 and F2.A at a 1:1.5 molar ratio resulted in a faster-eluting peak, indicative of complex formation (Fig. 56.A). Examination of the resulting sample by cryo-EM confirmed that the formation of the Fzd4-1/F2.A complex was successful, with 2D class averages showing a large protrusion extending away from the detergent micelle (Fig. 56.B).

After confirming that F2.A is capable of binding Fzd4-1 extracellular domain, I tried adding it to purified Fzd4-1/Ndp1 SMALPs, which resulted in immediate precipitation of the sample (Fig. 57.A). I hypothesized that this could be due to interactions between the 6xHis-tag present on the heavy chain of F2.A – since SMALP polymer is negatively charged at pH 8, it could potentially interact with the 6xHis-tag. Histidine sidechains

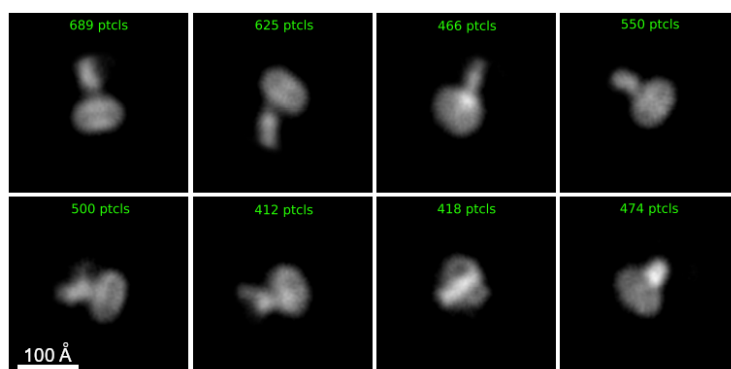
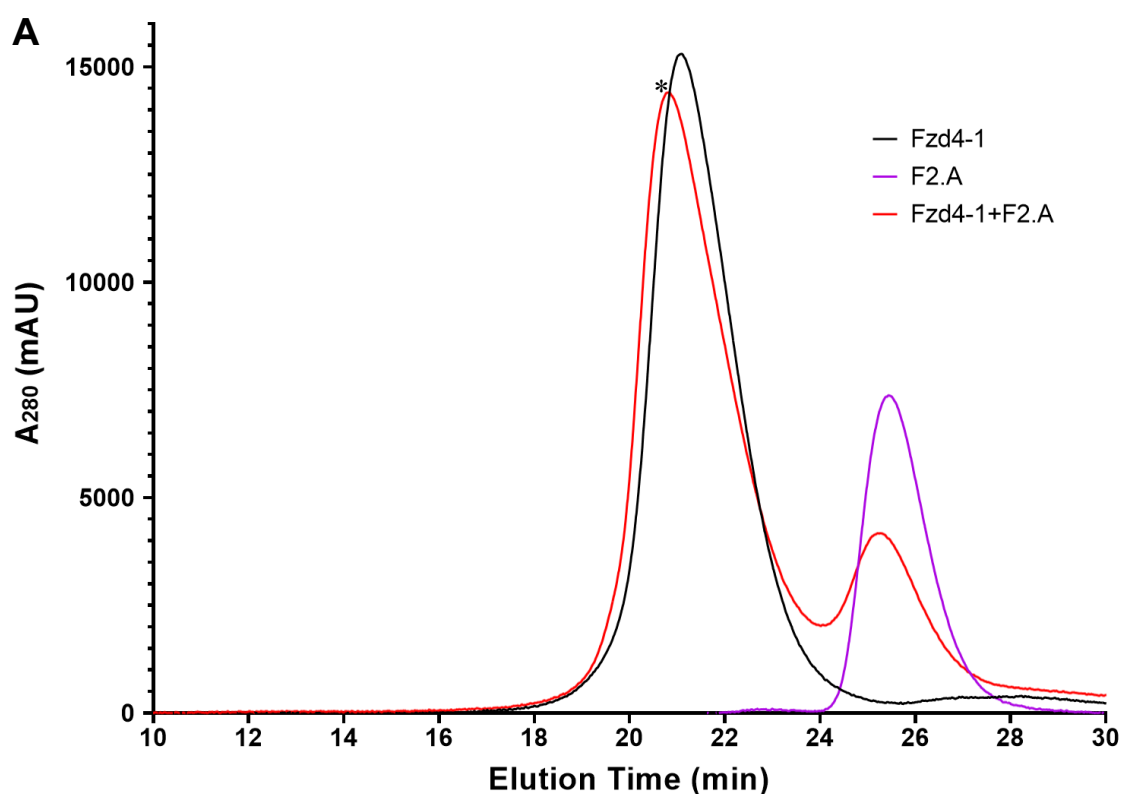


Fig. 56. Fzd4-1 and F2.A complex. **A** – SEC curves. Asterisk denotes peak containing the complex of interest. SEC performed using a Superose 6 Increase 3.2/300 GL column with a flow rate of 0.08 ml/min. **B** – Cryo-EM 2D class averages. Movies collected using a Titan Krios cryo-TEM (FEI) operated at 300 kV, using a K2 camera in linear mode. 2D classification performed using Cryosparc. Number of particles (ptcls) within each class shown above each class. For details, see S. Table 1, S. fig. 13., 14., 15.

have a pKa value of ~6, therefore they would be positively charged when present in the SMALP buffer.

Since two molecules of F2.A are expected to bind Fzd4-1/Ndp1 SMALPs, the interaction between the 6xHis-tags and the SMALP polymer would effectively result in the crosslinking of SMALP particles. To test this, I briefly incubated F2.A with carboxypeptidase, which should cleave off the C-terminal 6xHis-tag, and then added the cleaved Fab fragment sample to Fzd4-1/Ndp1 SMALPs. As predicted, the treatment prevented the formation of any observable aggregation (Fig. 57.B). It should be noted this effect could have also been caused by the carboxypeptidase treatment removing an excessive number of residues from the C-terminus of the F2.A heavy chain, thus disrupting its ability to interact with Fzd4-1. Despite this, later experiments using a construct of F2.A where the 6xHis-tag was absent did not lead to any observable precipitation, thus further supporting the idea that the 6xHis-tag was responsible for this effect.

The Fzd4-1 and Ndp1 present within the previously obtained SMALP samples were uncleaved, still containing the TwinStrep-mVenus and 10xHis-Sumo tags respectively. Since Ndp1 is a dimer, a single SMALP particle would also contain two copies of 10xHis-tags, which, similarly to F2.A, could also lead to sample aggregation and loss of yield. Fzd4-1/Ndp1 SMALP samples appeared to be soluble, and western blots had confirmed the presence of Ndp1 (Fig. 52.C). Despite this, examination of the Ndp1-containing SMALP samples by cryo-EM (Fig. 55.A) indicated that the particles present on the grids had not been dispersed as well as when Fzd6-5 SMALPs without Ndp1 were imaged (Fig. 52.A).

Therefore, to improve Fzd4-1/Ndp1 SMALP sample quality, I attempted to perform an overnight 3C protease cleavage of Fzd4-1 and Ndp1 following the first SMALP affinity purification step using streptactin resin. 3C protease was added to the Fzd4-1/Ndp1 SMALP sample (0.49 mg/ml) at a 1:100 w/w ratio, and the resulting mixture incubated overnight at 4° C. After applying the sample to the nickel affinity resin for the second affinity purification step, no protein was detected in the flowthrough, whereas elution produced fluorescent samples, indicating that the cleavage was not successful. I subsequently found out that the 3C protease used was also 6xHis-tagged, so it might have also be sequestered by the SMALP scaffolds, preventing it from carrying out cleavage efficiently. I repeated the experiment using the same concentrations of Fzd4-1/Ndp1 SMALPs and GST-tagged 3C protease, but this still did not lead to successful cleavage. Since it was still possible to pick out individual particles of Fzd4-1/Ndp1 SMALPs for cryo-EM data processing (Fig. 55), the future experiments were performed leaving the tags present on Fzd4-1 and Ndp1 intact.

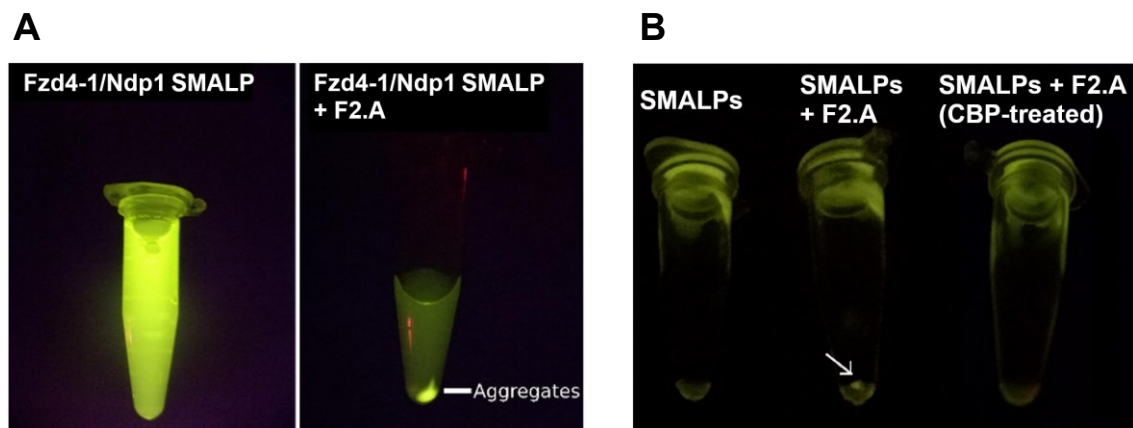


Fig. 57. Aggregation upon addition of F2.A to Fzd4/Ndp1 SMALPs. **A** – Addition of F2.A to Fzd4-1/Ndp1 SMALPs. The sample is fluorescent due to the presence of mVenus tag fused to Fzd4-1. In the absence of F2.A the solution remains fluorescent after centrifugation (10 min, 10,000 x g). Following addition of F2.A to the SMALP sample, precipitation is observed, as indicated by a fluorescent pellet following centrifugation. **B** – Addition of CBP-treated F2.A to Fzd4-1/Ndp1 SMALPs. F2.A addition was repeated, using small amounts of sample (50 μ l). SMALP samples in the absence or presence of F2.A (labelled as “SMALPs” and “SMALPs + F2.A” respectively) were centrifuged (10 min, 10,000 x g), and the tubes gently tilted sideways to detect the presence or absence of a fluorescent aggregate pellet (indicated by an arrow). F2.A sample (1 mg/ml) was then incubated with 0.1 mg/ml porcine pancreas carboxypeptidase B (CBP, Sigma-Aldrich) for 30 min at 4° C. The procedure for F2.A addition to SMALPs was repeated using the CBP-treated F2.A, which did not lead to the formation of an aggregate pellet following centrifugation. In all experiments F2.A (1 mg/ml) was added at a 1.5x molar excess to Fzd4-1/Ndp1 SMALPs (0.5 mg/ml).

After obtaining a new plasmid encoding F2.A without the 6xHis-tag present on its heavy chain (F2.A-2, Sidhu group, University of Toronto), I prepared a fresh sample of Fzd4-1/Ndp1 SMALPs, subsequently incubating it with F2.A-2 at a 1:1.5 molar ratio, which did not lead to any precipitation. The resulting sample was used to prepare cryo-EM grids. The particle distribution on the grids was very poor – although the presence of individual particles was apparent, the particles tended to clump together, overlapping with their neighbours (Fig. 58.A), which would make it impossible to pick out particles suitable for further processing. Screening of different sample concentrations, ice thickness, or grid types also did not lead to any improvements, with particles being clustered even at very low concentrations of sample (Fig. 58.B).

Fzd4-1/Ndp1 SMALP particles did not exhibit such severe levels of aggregation (Fig. 55) as Fzd4-1/Ndp1/F2.A-2 SMALPs (Fig. 58) on cryo-EM grids. Since no visible precipitation upon the addition of F2.A-2 to Fzd4-1/Ndp1 SMALPs was observed, the presence of F2.A-2 probably alters the properties of the particles in a way that leads to their aggregation when applied to cryo-EM grids. Unfortunately, this could not be overcome by varying the tested parameters of the grid preparation procedure.

In conclusion, the use of SMA for the extraction of transmembrane proteins and their complexes is a quick and simple way to obtain detergentless samples containing the protein of interest. Despite this, the inability to reliably perform SEC on the final sample, as well as the unpredictable behaviour of SMALPs in the presence of various tags used for protein purification, severely limit the possibilities of sample optimization for cryo-EM, especially when the sample in question is a multi-protein complex, where the effects of each individual component on sample behaviour would need to be assessed. Previous results indicated that the Fzd4/Ndp/F2.A complex is a viable target

for structure determination by cryo-EM, but the presence and behaviour of the sample

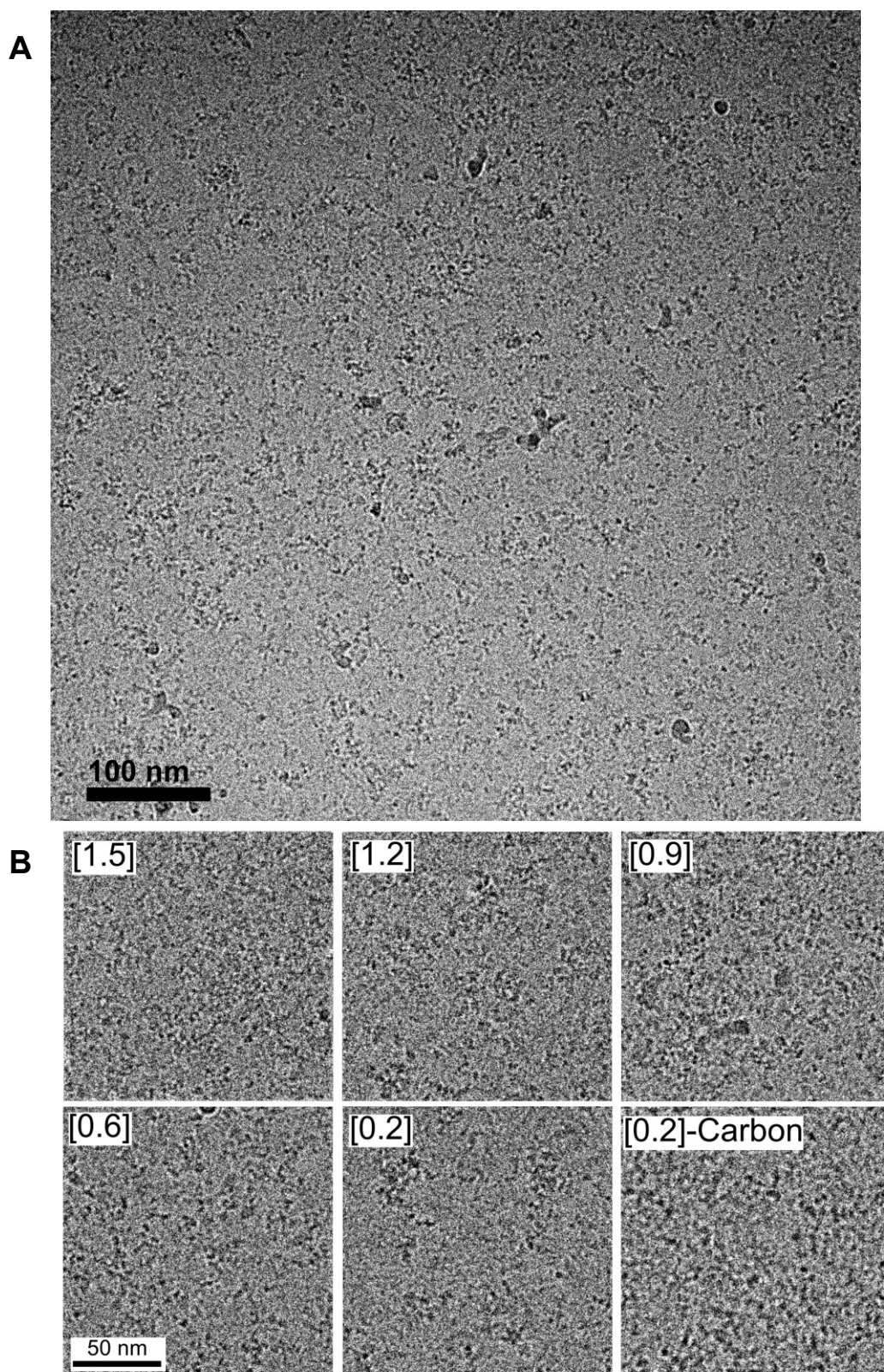


Fig. 58. Optimisation of Fzd4-1/Ndp1/F2.A-2 concentration on cryo-EM grids.

A – a representative micrograph (0.6 mg/ml). **B** – particle distribution at different sample concentrations (0.2 – 1.5 mg/ml). Micrographs recorded using a Glacios cryo-TEM (FEI) operated at 200 kV, with a Falcon 3 camera at a 96,000 x magnification in linear mode. Quantifoil R2/2 grids were used in all cases, except for one sample labelled “Carbon”, where a Lacey grid with a carbon support film was used. For details, see S. Table 1.

upon addition of each component would need to be more thoroughly evaluated to obtain good samples. Due to the aforementioned reasons, this was not possible with SMALPs, therefore I decided to return to the use of detergent-solubilized Fzd4.

3.4. Assembly and structural characterization of Fzd4/Ndp/Fab complex in detergent micelles

3.4.1. Optimization of Fzd4-1/Ndp3/F2.A-2 complex formation

Upon starting this project, I had hoped to prepare samples of Fzd signalling complexes in the absence of detergent. This would result in a more native-like environment for the final samples, while also improving the quality of cryo-EM data, thus leading to better structures. Since the use of nanodiscs or SMALPs did not result in samples that were optimal for cryo-EM, I decided to pursue a more classical detergent-based approach.

Previous results suggested that the Fzd4/Ndp/Fab complex was the most promising target for structure determination. The major obstacle for this was the fact that detergent-purified Fzd4-1 appears to be monomeric, although it has been reported that monomeric, detergent-solubilized Fzd4 can still undergo dimerization in the presence of purified Ndp (Bang et al., 2018).

Previous protocols for purifying Ndp in our lab used Tween-20 in the purification buffers, since Ndp, although not as hydrophobic as Wnts, still tends to aggregate in solution. The presence of Tween-20 in the final sample buffer might interfere with the stability of Fzd4, so, to overcome this, I created an Ndp construct containing an N-terminal MBP tag (construct referred to as Ndp3). This tag is known to increase protein solubility, while several other papers had used a similar strategy to produce Ndp in detergentless buffers.

The purification of Ndp3 following expression in a stable HEK293S GnTI⁻ TetR cell line and amylose resin purification resulted in high yields of soluble protein, which exhibited a sharp, clean peak on the final SEC profile (Fig. 59.A). It also contained a

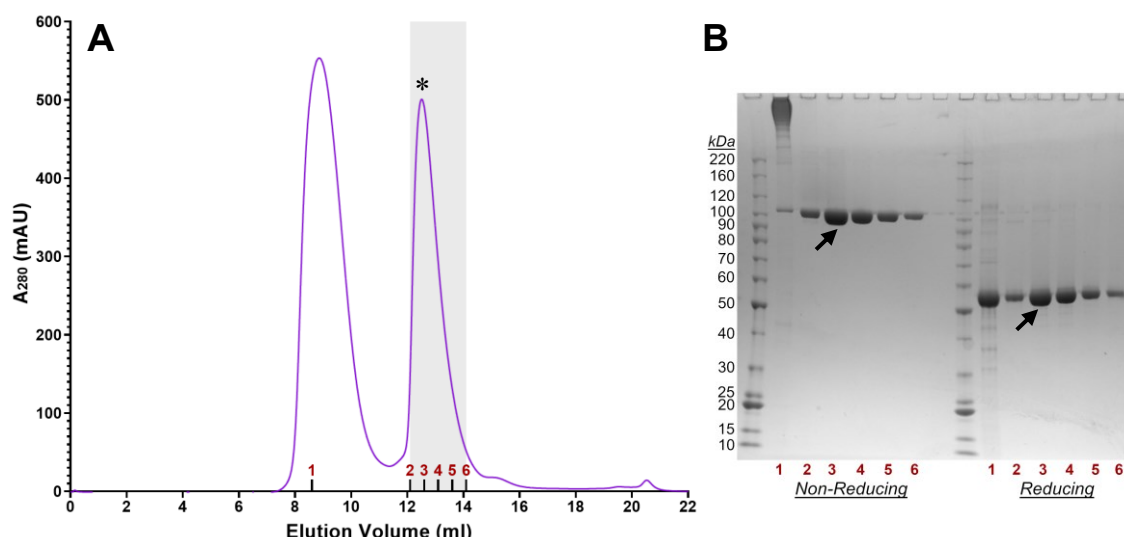


Fig. 59. Purification of Ndp3. **A** – SEC. Asterisk denotes peak containing the protein of interest. SEC performed using a Superose 6 Increase 10/300 GL column. **B** – *non-reducing and reducing SDS-PAGE*. Predicted molecular weight of an Ndp3 monomer is 55 kDa. Arrows denote bands corresponding to the protein of interest.

very high aggregate peak eluting at void volume, indicating that Ndp3 has a tendency to aggregate in solution. Reducing and non-reducing SDS-PAGE (Fig. 59.B) confirmed that the final sample contains a cys-linked Ndp dimer, indicating that the purification was successful.

Two things became apparent shortly after purifying Ndp. The first one was that upon removal of the MBP tag by 3C protease treatment, the protein immediately aggregates, with precipitation being present in a matter of minutes. This was to be expected, since MBP is thought to maintain its solubility. The second thing was that following purification, Ndp needs to be used for further experimentation immediately – it starts to aggregate almost immediately following purification, with severe precipitation becoming apparent in the samples after only 48 h of storage at 4° C, as well as after thawing samples flash-frozen in liquid nitrogen in the presence or absence of 10% glycerol. This meant that for each attempt at complex formation, fresh protein would have to be produced.

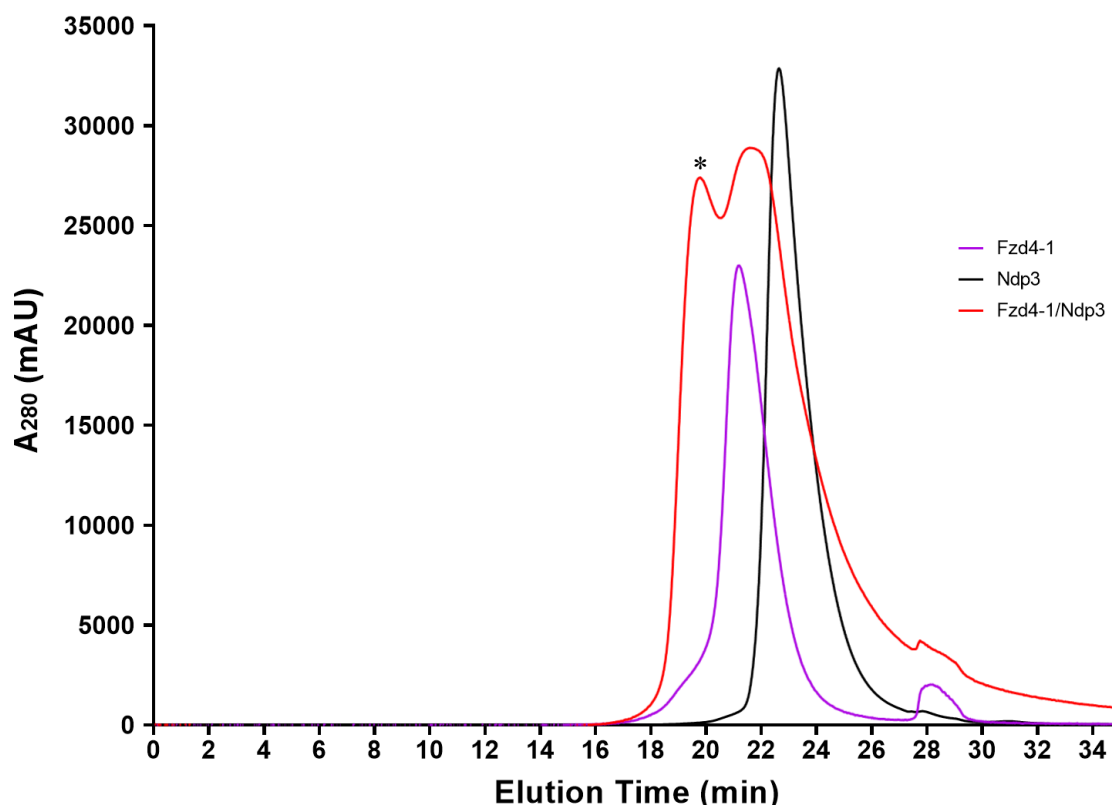


Fig. 60. Fzd4-1 and Ndp3 complex formation. Individual samples were mixed at a 1 : 0.75 Fzd4-1 : Ndp3 (dimer) molar ratio and incubated overnight at 4° C. SEC performed using a Superose 6 Increase 3.2/300 GL column at a flow rate of 0.08 ml/min. Asterisk denotes peak suspected to contain Fzd4-1 homodimers bound to Ndp3. The mVenus tag present on Fzd4-1 was removed beforehand via 3C cleavage.

To assess the ability of Ndp3 to dimerize detergent-solubilized Fzd4-1, I incubated cleaved Fzd4-1 with Ndp3 dimer (1:0.75 molar ratio) overnight, followed by SEC (Fig. 60). The resulting profile consisted of two overlapping peaks, one of them eluting slightly earlier than Fzd4-1, although the shift was not as large as I would expect upon the formation of a Fzd4-1 dimer in complex with Ndp3 (using a Superose 6 Increase 3.2/300 GL column). The other peak had a broad appearance, eluting at a volume between the peaks produced by Fzd4-1 and Ndp3 alone. These results indicated that Ndp3 was not able to induce Fzd4-1 dimerization using the sample conditions used.

To optimize Fzd4-1 dimer formation in the presence of Ndp3, I repeated the experiment while varying various parameters. Since the ability of Fzd4 to dimerize in the presence of Ndp3 would directly depend on Ndp3 concentration, I tried using higher Fzd4-1 :

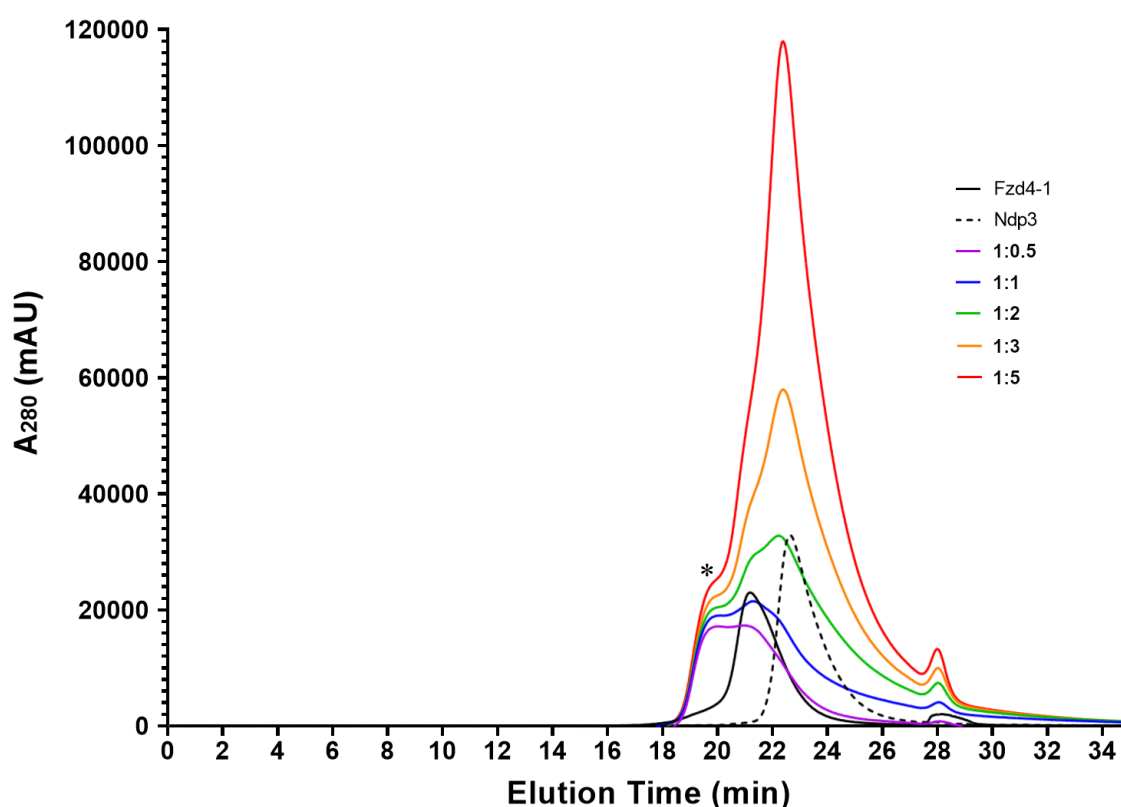


Fig. 61. Fzd4-1 and Ndp3 complex formation at different Fzd4-1 : Ndp3 (dimer) molar ratios. Individual samples were mixed at the specified molar ratio and incubated overnight at 4° C. SEC performed using a Superose 6 Increase 3.2/300 GL column at a flow rate of 0.08 ml/min. Asterisk denotes peak suspected to contain Fzd4-1 homodimers bound to Ndp3. The mVenus tag present on Fzd4-1 was removed beforehand via 3C cleavage.

Ndp3 molar ratios in the complexation mixtures (Fig. 61). Using a ratio of 1:0.5 resulted in two overlapping peaks, which eluted at volumes similar to Fzd4-1 and a species with a slightly higher Stokes radius. As the amount of Ndp3 was increased, the later-eluting peak became overshadowed by a peak belonging to the Ndp3 dimer, with no significant changes present in the elution volume of the earlier-eluting peak.

The SEC curve produced using a 1:0.5 Fzd4-1/Ndp3 (dimer) molar ratio suggested that the resulting protein complex might contain a mixture of monomeric Fzd4-1 either alone or binding Ndp3, still as a monomeric species. I then considered several possibilities. First, since Ndp3 had a propensity to aggregate over time, it could be that an overnight incubation of Fzd4-1 with Ndp3 was too long, leading to levels of Ndp3 degradation that would prevent detection of Fzd4-1 dimers within the final samples.

Second, it could be that a rapid addition of an excessive amount of Ndp3 to the Fzd4-1 sample could saturate the Ndp3 binding sites on individual Fzd4-1 monomers, thus preventing dimerization. Finally, it could be the presence of MBP tag on Ndp3 sterically limits the ability for Ndp3 to bind two molecules of Fzd4-1 at once.

To test these ideas, I prepared a new set of mixtures containing Fzd4-1 and Ndp3 at a 1:0.5 molar ratio, with some of the samples being injected onto a SEC column immediately after their preparation to limit the effects of Ndp3 degradation, while for others the total amount of Ndp3 was added more slowly - in 4 batches with 30 min incubation periods between each addition to limit the possibility of Ndp3 binding site saturation on Fzd4-1, while also permitting a slower, more controlled dimer formation.

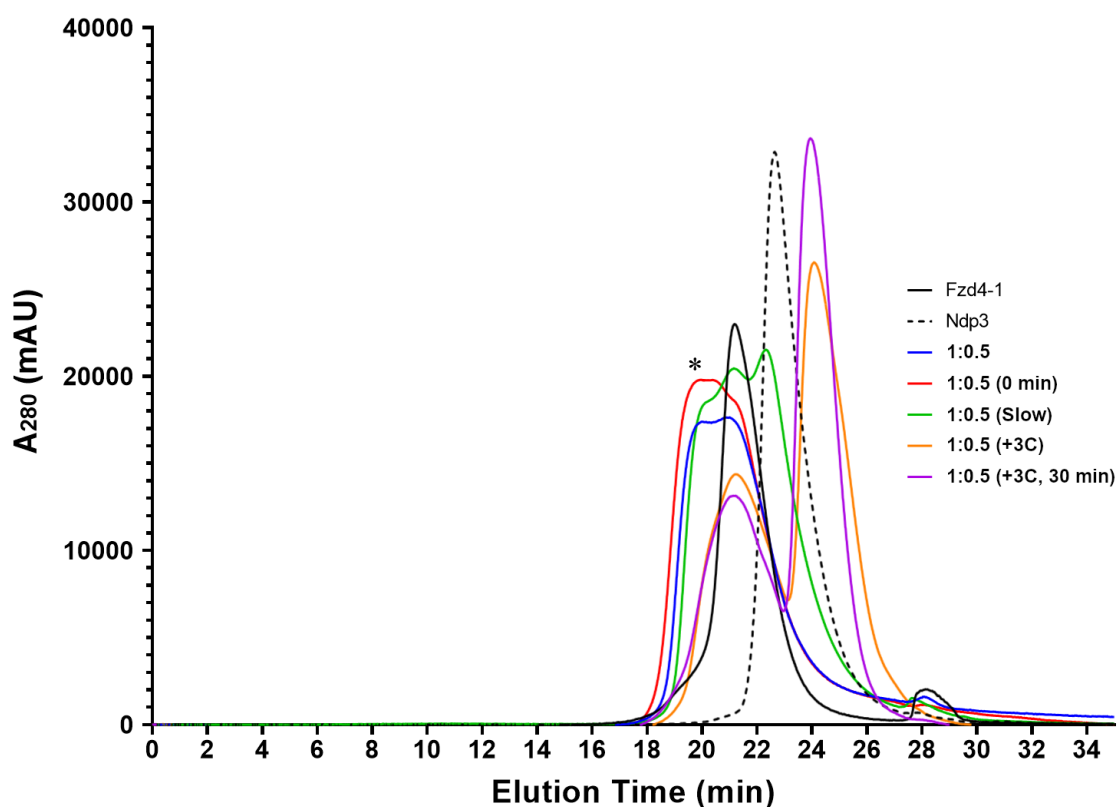


Fig. 62. Further optimisation of Fzd4-1 and Ndp3 complex formation. Unless otherwise specified, samples were mixed at the specified ratios (1:0.5, Ndp3 treated as a dimer) and incubated overnight at 4° C. “+3C” indicates co-incubation with 3C protease at a 1:200 w/w ratio. “Slow” indicates that the total amount of Ndp3 was added in 4 batches with 30 min incubation periods between each addition. “0 min” and “30 min” indicate the amount of time for which samples were incubated on ice before injection on the SEC column (instead of performing an overnight incubation). SEC performed using a Superose 6 Increase 3.2/300 GL column at a flow rate of 0.08 ml/min. Asterisk denotes peak suspected to contain Fzd4-1 homodimers bound to Ndp3. The mVenus tag present on Fzd4-1 was removed beforehand via 3C cleavage.

Finally, some of the samples were co-incubated with 3C protease to remove the MBP tags present on Ndp3 – although purified Ndp3 aggregated upon MBP removal, its association with Fzd4-1 might stabilize it, while also removing any steric effects that would limit dimerization.

The results of these experiments are shown in Fig. 62. The injection of the Fzd4-1/Ndp3 mixture immediately after its preparation did not result in any major differences between the resulting SEC profiles, although the main complex peak was slightly higher, potentially due to reduced Ndp3 degradation. Because of this, all future complexation experiments using Ndp3 were immediately used for further experimentation without any incubation steps, unless otherwise noted. Adding Ndp3 to Fzd4-1 more slowly resulted in a more heterogenous sample in comparison to a regular 1:0.5 overnight incubation, as indicated by a SEC profile containing three overlapping peaks instead of two. Finally, inclusion of 3C protease within the complexation mixtures resulted in the removal of Ndp3 from the sample, with the resulting two peaks being formed by Fzd4-1 and cleaved MBP tag. The height of the peak belonging to Fzd4-1 was reduced in comparison to a control, meaning that in the process of degradation, Ndp3 also leads to the degradation of Fzd4-1 present in the sample.

After performing all of the aforementioned optimization trials, it became clear that the reasons for not obtaining properly formed Fzd4-1 dimers upon Ndp3 addition were most likely related to the intrinsic properties of the reagents present. So far, I had used DDM for purification of detergent-solubilized Fzd4-1. The DDM solutions also contained cholesteryl hemisuccinate (CHS) added at a 5:1 DDM:CHS molar ratio. This cholesterol derivative is often included in detergent solutions when working with metazoan membrane proteins in order to make the detergent micelles more planar and

rigid, thus improving the stability of the transmembrane proteins within them, while also making their environment more native-like. Although CHS has benefits when it comes to studying transmembrane proteins in isolation, its properties might inhibit the ability of Fzd4-containing detergent micelles to fuse upon Ndp3 addition.

To test this, I repeated the Ndp3 complexation experiments using Fzd4-1 purified in the absence of CHS (Fig. 63). The resulting peak found within the SEC profiles exhibited a sharp, well-defined character, although it appeared that some of the Fzd4-1 was still present in a monomeric state. This indicated that a slight excess of Ndp3 could be used in future complexation experiments. The elution volume was clearly shifted from the peak formed by samples containing Fzd4-1, thus suggesting that Fzd4-1 and Ndp3 complexation, as well as the associated Fzd4-1 micelle fusion and dimerization was successful.

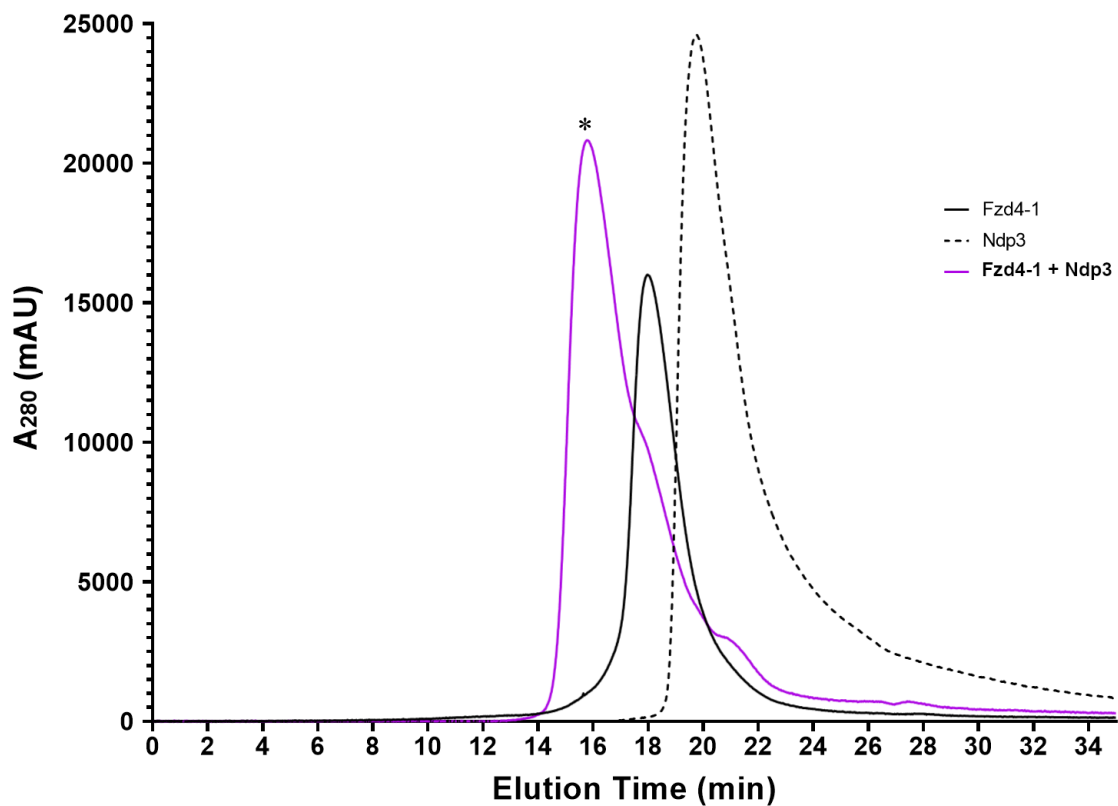


Fig. 63. Fzd4-1/Ndp3 complex formation in the absence of CHS. Fzd4-1 and Ndp3 were mixed at a 1:0.5 molar ratio (treating Ndp3 as a dimer) and injected on a Superdex 200 Increase 3.2/300 GL column using a flow rate of 0.08 ml/min. Asterisk denotes peak suspected to contain Fzd4-1 homodimers bound to Ndp3. The mVenus tag present on Fzd4-1 was removed beforehand via 3C cleavage.

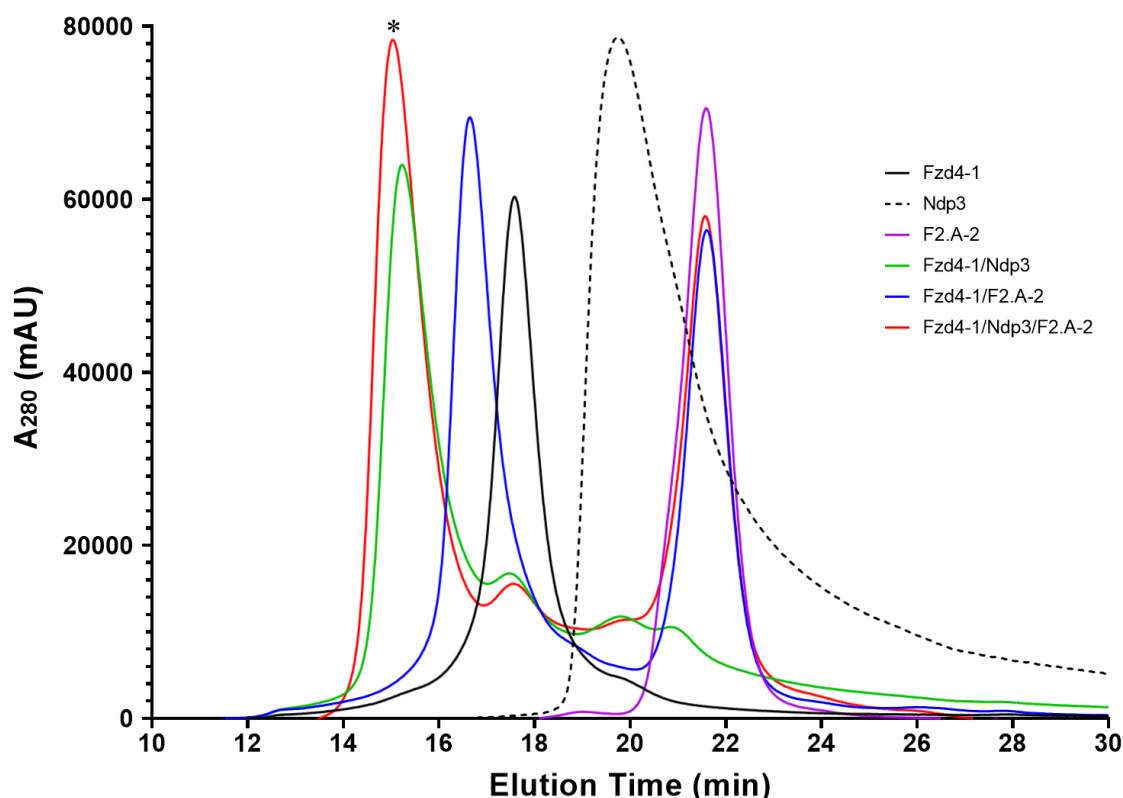


Fig. 64. Fzd4-1/Ndp3/F2.A-2 complex formation. A 1:0.75:2 molar ratio of Fzd4-1, Ndp3 (dimer), and F2.A-2 was used. SEC was performed using a Superdex 200 Increase 3.2/300 GL column and a flow rate of 0.08 ml/min. Asterisk denotes peak containing the complex of interest. No CHS was present in any of the buffers used. The mVenus tag present on Fzd4-1 was removed beforehand via 3C cleavage.

I then proceeded to assess the formation of the Fzd4-1/Ndp3/F2.A-2 complex following a similar strategy, except that F2.A-2 was added 30 min following the Fzd4-1 incubation with Ndp3 (Fig. 64). The resulting SEC peak eluted slightly earlier than the sample produced in the absence of F2.A-2, thus confirming that F2.A was capable of binding the Fzd4-1/Ndp3 complex.

3.4.2. Structural analysis of Fzd4-1/Ndp3/F2.A-2 complex

Following optimization, I performed a large-scale preparation of the Fzd4-1/Ndp3/F2.A-2 complex. The SEC profile resulted in a distinctive peak (Fig. 65.A), which contained all components of the complex as indicated by SDS-PAGE (Fig. 65.B). The sample behaved well on cryo-EM grids, while the most of the resulting 2D class averages showed a detergent micelle containing additional density within its centre (Fig. 65.C). Extending from it was a central stalk-shaped density, which was adjacent to

two globular densities, each connected to a density resembling a Fab fragment. These 2D classes matched the predicted appearance of a Fzd4-1/Ndp3/F2.A-2 complex in a detergent micelle. Some classes also contained complexes where the Fzd4-1 monomer detergent micelles were not fused, suggesting that the process had not proceeded to completion. Furthermore, the 2D classification software appeared to focus on either the detergent micelle or the extracellular domains of the complex for each class, as indicated by an apparent blurriness of either the detergent micelle or the densities

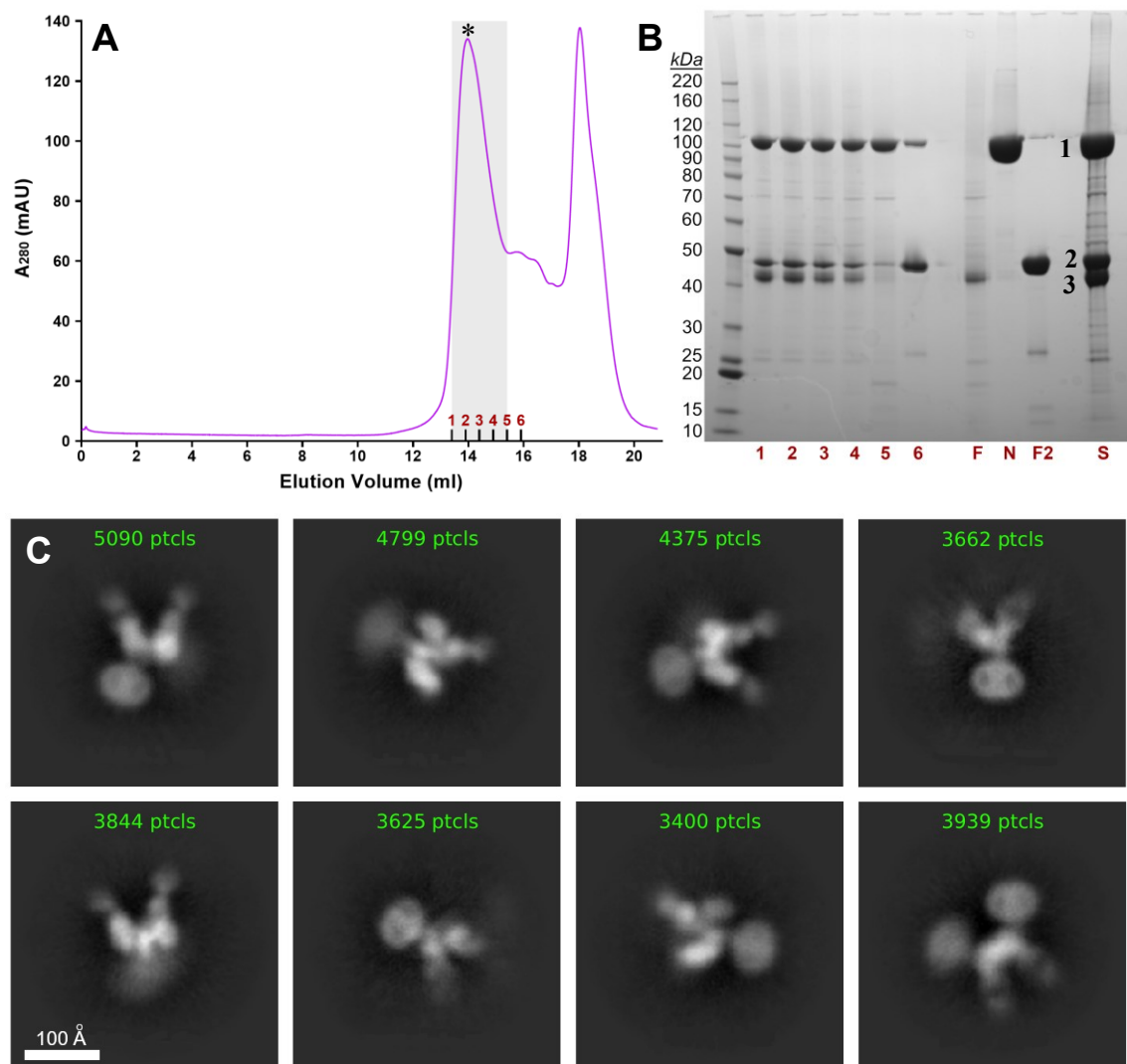


Fig. 65. Large-scale purification of Fzd4-1/Ndp3-F2.A-2 complex. **A** – SEC. A 1:0.75:2 molar ratio of Fzd4-1, Ndp3 (dimer), and F2.A-2 was used for complex formation. SEC performed using a Superose 6 Increase 10/300 GL column. Asterisk denotes peak containing the complex of interest. **B** – Non-reducing SDS-PAGE. F and 3 – Fzd4-1; N and 1 – Ndp3 dimer; F2 and 2 – F2.A-2; S – final sample. **C** – Cryo-EM 2D class averages. Movies recorded using a Glacios cryo-TEM (FEI) operated at 200 kV, with a Falcon 3 camera at a 92,000 x magnification in linear mode. Number of particles (ptcls) within each class shown above each class. No CHS was present in any of the buffers used. The mVenus tag present on Fzd4-1 was removed beforehand via 3C cleavage. For details, see S. Table 1, S. fig. 16, S. fig. 17, S. fig. 18.

corresponding to F2.A-2 broadened main peak. This is usually caused by considerable flexibility or heterogeneity within a localized region of the particles examined. Further 3D processing of the cryo-EM data did not lead to reconstructions of the full complex, with only one Fab present (S. fig. 18).

In an attempt to reduce the heterogeneity in the vicinity of the Fzd4-1/Ndp3/F2.A-2 extracellular regions, I prepared a large-scale sample of the complex and attempted the removal of the MBP tag present on Ndp3 via 3C protease cleavage once more.

Following a brief 30 min incubation on ice, the sample had completely precipitated, suggesting that even when bound to Fzd4-1 dimers, Ndp3 is still prone to precipitation upon removal of the MBP tag. I tried overcoming this by reducing the amount of 3C protease used and incubating the sample for longer periods of time, as well as increasing the salt concentration within the sample buffers, as well as performing cleavage with lower concentrations of complex present, but none of these changes prevented Ndp3 aggregation upon MBP tag removal.

Finally, I set up another cleavage reaction in the presence of 50 mM arginine and 300 mM NaCl, which prevented precipitation. The SEC profile showed a single, distinctive peak at the expected elution volume, which was overshadowed in height by the cleaved MBP peak eluting at 18 ml (Fig. 66.A). When preparing samples for SDS-PAGE, the fractions 1 - 4 forming the main complex peak aggregated upon addition of the SDS loading buffer. The band corresponding to cleaved Ndp was extremely faint (Fig. 66.B.e), whereas a high molecular weight band was present (Fig. 66.B.a). Since the peak examined by SDS-PAGE did not elute at excessively early volumes, this suggested that cleaved Ndp might aggregate in the presence of SDS similarly to Wnt5A (Fig. 53). In addition, fractions 1-4 also contained faint bands at a molecular weight

corresponding to uncleaved Ndp3 (Fig. 66.B.b), indicating that small amounts of it were still present in the final sample.

The sample was subsequently examined by cryo-EM, revealing that the particles were well dispersed (Fig. 67.A). 2D classification (Fig. 67.B) resulted in classes that were sharper and better defined than for the uncleaved sample (Fig. 65.C), indicating increased homogeneity of the sample. In comparison to the uncleaved sample, the two distinct lobes of density in the vicinity of the detergent micelle were replaced by a central F2.A-2 binding density. The previously observed blurring of either the micelle or the F2.A-2 regions within the 2D classes was still observed, while the increased quality of the classes also showed that the central density of the extracellular particle regions was located at a variable distance away from the detergent micelle. This suggested that the extracellular and transmembrane regions of Fzd4-1 might move independently of one another. This was further supported by generation of *ab initio* 3D volumes from the collected data, which consisted either of only the extracellular regions

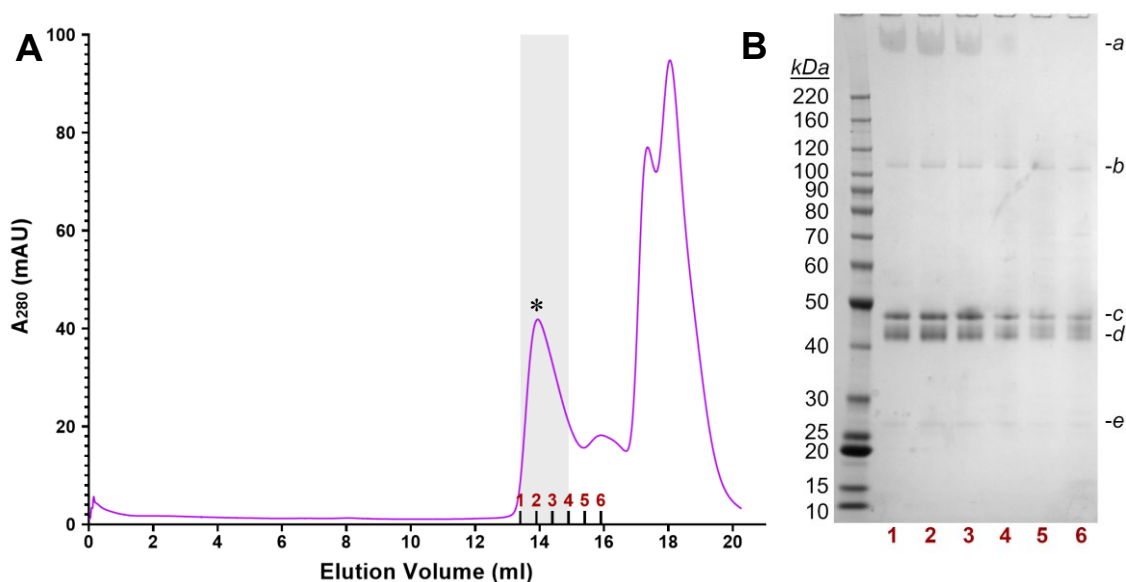


Fig. 66. Large-scale purification of cleaved Fzd4-1/Ndp3/F2.A-2 complex. **A** – SEC. A 1:0.75:2 molar ratio of Fzd4-1, Ndp3 (dimer), and F2.A-2 was used for complex formation. The mixture was then supplemented with 50 mM arginine, 300 mM NaCl, as well as 3C protease added at a 1:200 w/w ratio. The mixture was incubated for 30 min on ice and injected on a Superose 6 Increase 10/300 GL column. Main peak shaded grey, as well as denoted with an asterisk. **B** – non-reducing SDS-PAGE. *a* – high molecular weight band; *b* – uncleaved Ndp3; *c* – F2.A-2; *d* – Fzd4-1; *e* – cleaved Ndp3.

of the complex (Fig. 68.A), or one of the halves of the extracellular region missing from the complete particle (Fig. 68.B).

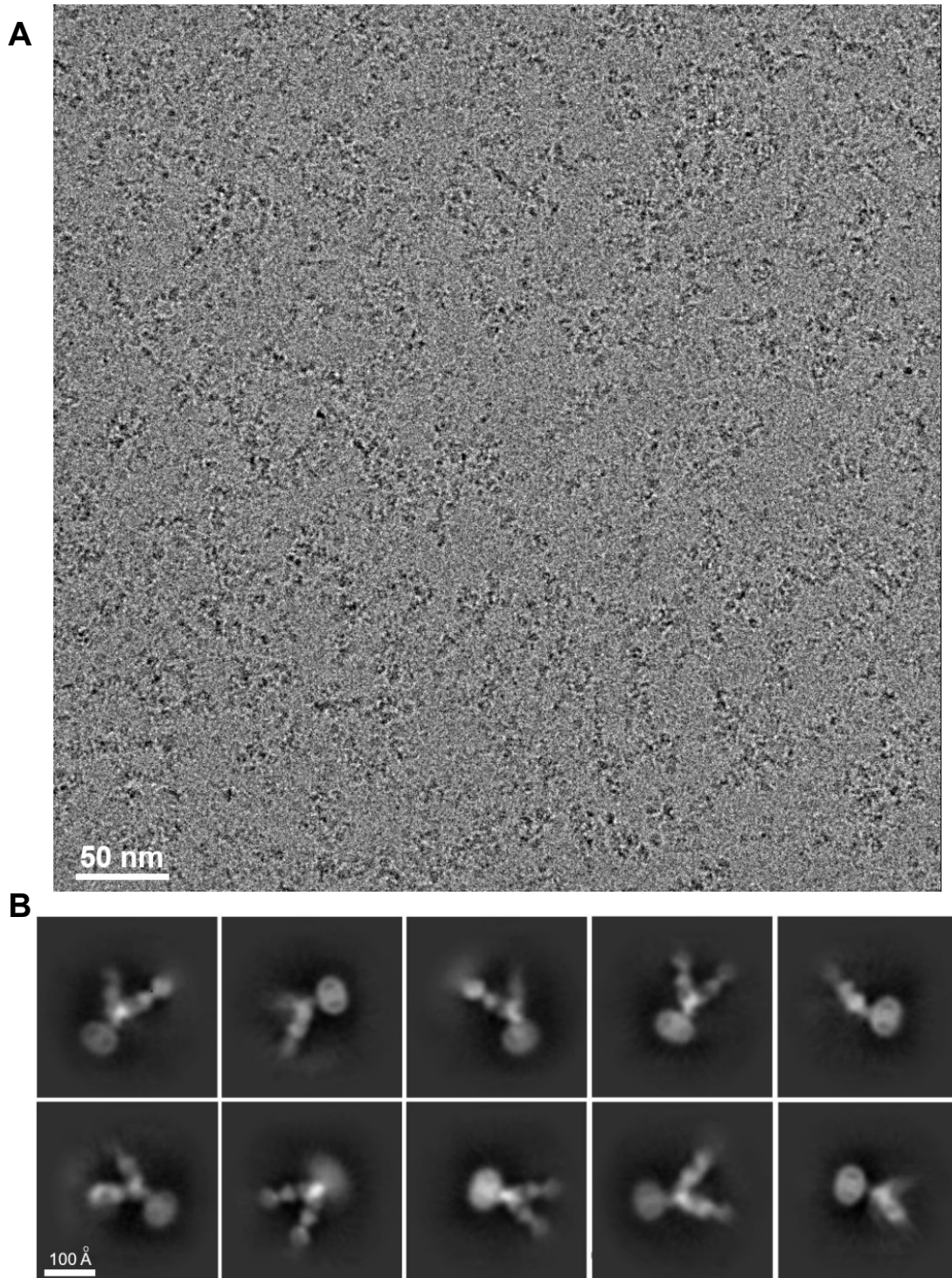


Fig. 67. Cryo-EM of cleaved Fzd4-1/Ndp3/F2.A-2 complex. **A** – *representative micrograph*. Grid prepared using sample at a 8.7 mg/ml concentration. Micrograph recorded using a Glacios cryo-TEM (FEI) operated at 200 kV, with a Falcon 3 camera at a 92,000 x magnification in linear mode. **B** – *Representative 2D class averages*. Each class contains approximately 2,000 particles. Movies recorded using a Titan Krios cryo-TEM (FEI) operated at 300 kV, with a Falcon 3 camera at a 59,000 x magnification in counting mode. For details, see S. Table 1, S. fig. 19.

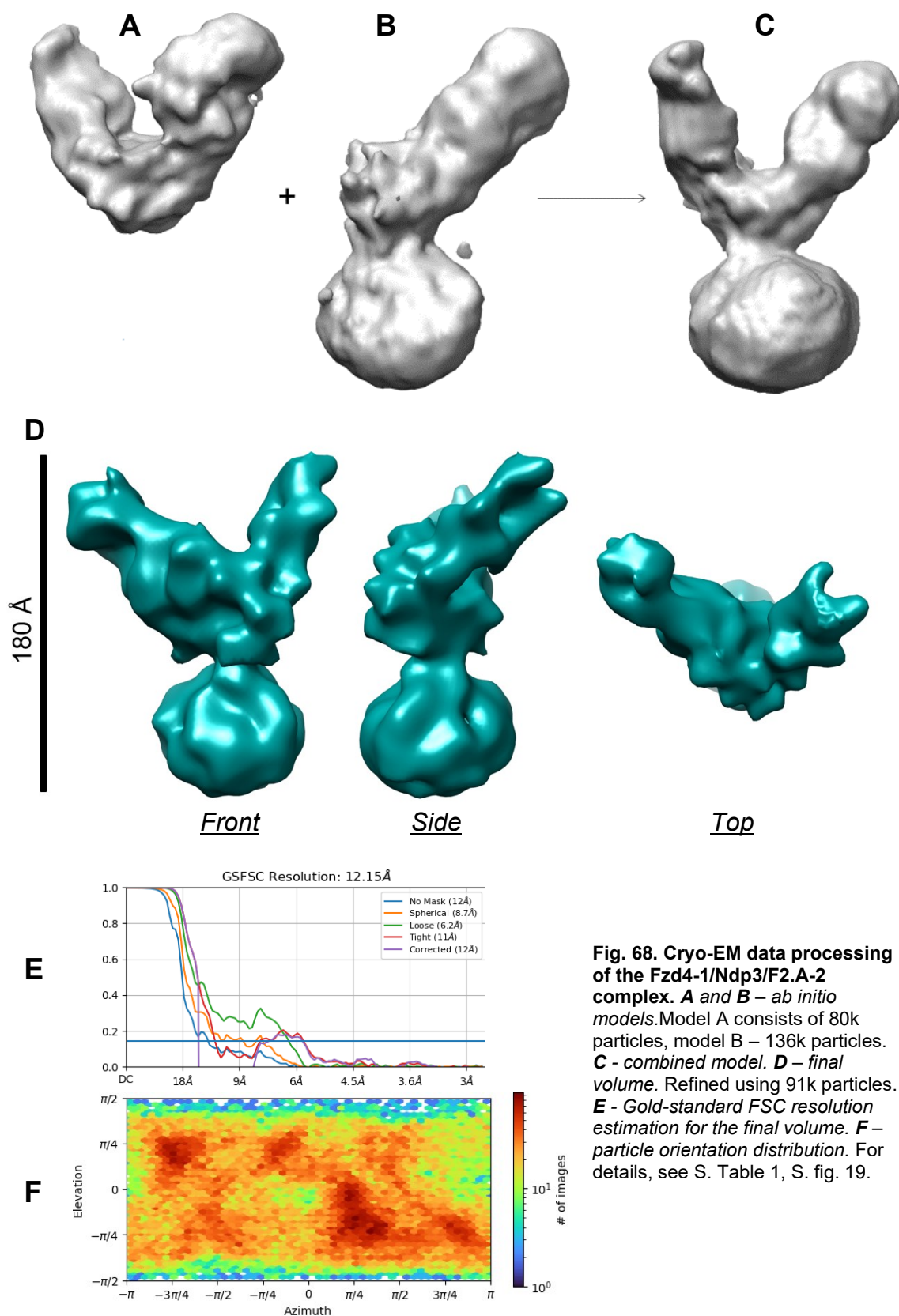


Fig. 68. Cryo-EM data processing of the Fzd4-1/Ndp3/F2.A-2 complex. A and B – *ab initio* models. Model A consists of 80k particles, model B – 136k particles. C – combined model. D – final volume. Refined using 91k particles. E – Gold-standard FSC resolution estimation for the final volume. F – particle orientation distribution. For details, see S. Table 1, S. fig. 19.

In an attempt to obtain the structure of the entire complex, these predominant *ab initio* models were then combined in a single model (Fig. 68.C), which was subsequently used for 3D volume refinement. Unfortunately, the best model obtained using this strategy

had a final FSC resolution of 12.2 Å (Fig. 68.D-F), which was not sufficient to reveal the inner architecture of the complex or distinguish between different protein components present. Additional heterogenous refinements performed on this volume showed variable distance between the detergent micelle and the presumed extracellular regions of the protein (S. fig. 21), thus further supporting the idea that the particles exhibit high levels of flexibility.

I then tried to obtain a model of the complex by using the predominant *ab initio* model containing only a single Fab molecule (Fig. 68.B). Although FSC resolution estimation of the final volume produced a value of 6.3 Å (Fig. 69.A/C), local resolution estimation showed that the inner parts of the volume reach a resolution of only 12 Å (Fig. 69.B).

Other optimization strategies for data processing, such as the repicking of particles using the final models, additional rounds of heterogenous refinement, particle subtraction, and localized refinement focusing only on the extracellular regions of the particles, as well as non-uniform refinements did not lead to any improvements in the final resolution. In addition, fresh sample of both the cleaved and uncleaved Fzd4-1/Ndp3/F2.A-2 complex was prepared multiple times, and various grid preparation conditions such as ice thickness, sample concentration and grid types were explored to improve the quality of the resulting data, but none of them led to an improved quality of the final particles.

Taken together, the localized blur of either the detergent micelle or the extracellular regions of the Fzd4-1/Ndp3/F2.A-2 particles during 2D classification, the variable distance between them present both in 2D classes and 3D reconstruction, as well as poor local resolution values within the core of the complex indicate that there is considerable

flexibility present between the TMD and CRD of Fzd4-1, making it unsuitable for determination of high resolution structures by cryo-EM.

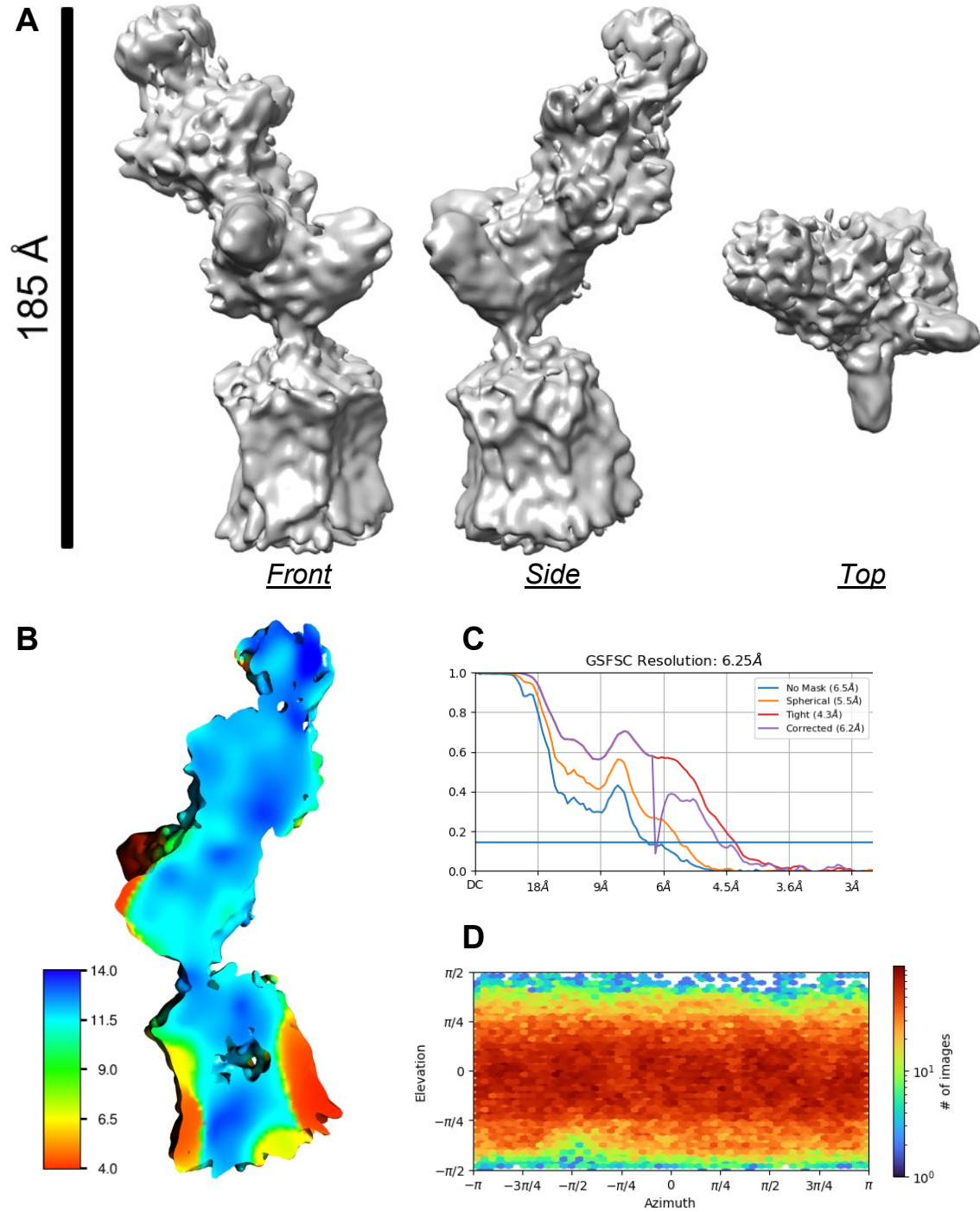


Fig. 69. Cryo-EM data processing of the Fzd4-1/Ndp3/F2.A-2 complex. **A** – final volume. Starting point for refinement was the *ab initio* model shown in Fig. 68.B. Refined using 107k particles. **B** – final volume cross-section showing local resolution. **C** - Gold-standard FSC resolution estimation for the final volume. **D** – particle orientation distribution. For details, see S. Table 1, S. fig. 19.

3.5. Discussion

Expression and purification of Fzd presented considerable challenges. After trying numerous optimizations and approaches, I have successfully expressed and purified sufficient quantities of protein to perform initial structural characterization and enable further experiments to elucidate Fzd structure. One key issue for my work and the field in general is the problem of obtaining sufficient amounts of functional ligands. The experiments presented here show that both Wnt3A and Wnt5A, the main ligands that could be used for the formation of Fzd signalling complexes, are expressed well in cells, but their isolation remains an issue, even when co-expressed with the carrier protein afamin. Since Wnts are thought to bind various glypicans on the cell surface following secretion, as well as associate with different extracellular structures, such as lipoprotein particles and exocytic vesicles, it could be possible that carrier proteins are only able to bind Wnts found within one or few of these subpopulations. As for the atypical Fzd4 agonist Ndp, the rapid aggregation of MBP-tagged Ndp constructs following purification and upon MBP-tag cleavage makes work with this protein very difficult. Previous research has suggested that its aggregation can be limited by the presence of detergent (Chang et al., 2015), although that was not the case when using DDM in the work presented here. This indicates that Ndp exhibits considerable hydrophobic character, which might limit its ability to freely diffuse in the extracellular space, similarly to Wnts. This aspect of Ndp signalling has not yet been addressed by scientific research, thus requiring further study.

Other proteins that could be used for Fzd signalling complex assembly are the LRP5/6 and ROR2 co-receptors, as well as the intracellular protein Dvl. ROR2 constructs containing its transmembrane domain appeared to express well, therefore making ROR2

a good candidate for the assembly and co-purification of Fzd and its co-receptor complexes. As for Dvl, it is still unclear whether it interacts with Fzd via its PDZ or DEP domains, but it has been shown to do so even in the absence of ligand stimulation (Tauriello et al., 2012b; Wong et al., 2003). BLI experiments showed that the DEP domain of Dvl2 exhibits higher affinity for Fzd6 than PDZ, although the interaction was very weak, forcing me to use high concentrations of Dvl2 constructs to detect any signal. This could be either due to cysteine removal from the Dvl2 constructs, or due to the presence of detergent, which has been shown to alter the structure of Fzd helix VIII containing the KTXXXW motif thought to be the main determinant of Dvl binding (Gayen et al., 2013). To address this, BLI experiments using detergent-free Fzd samples (e.g. using SMALPs) should be performed in the future. Another possibility is that a high-affinity interaction between Dvl and Fzd relies either primarily on Dvl polymerization during canonical Wnt signalling, or the presence of other signalling complex components. In this case, the importance of each Dvl domain for binding Fzd signalling complexes should be thoroughly examined within their cellular context by performing Förster resonance energy transfer-based experiments following stimulation of Wnt signalling.

There is considerable enthusiasm and hope that detergentless systems, such as nanodiscs and SMALPs, may help solve some general issues in membrane protein structural biology. Despite these systems being well characterized, their optimization is still very difficult and different for each protein or protein complex of interest. My experience with Fzd was that the optimization of the nanodisc reconstitution process was time-consuming, required a lot of material, and its efficiency was very low. The reason for this appeared to be the presence of transmembrane protein within the reaction mixture, indicating that Fzd might not be suitable for reconstitution within nanodiscs.

The lipid that I used for these experiments was POPC, and it is possible that the use of other lipids might yield better results, but this was not explored further due to SMALPs initially working much better. Despite this, the nanodisc reconstitution optimization procedures presented in this work illustrate the importance of evaluating different nanodisc scaffolds for reconstituting each target protein. It also underlines the necessity to explore variables that might affect nanodisc reconstitution kinetics, such as the rate of detergent removal, which is often overlooked when working with nanodiscs.

Solubilization of transmembrane proteins via SMA polymers is very appealing for cryo-EM studies due to their ease of use and a supposedly more native state of the target protein within the SMALP particles, while not requiring the presence of detergents at any point of the solubilization procedure. Despite this, the inability to perform SEC reproducibly complicated the assessment of final sample quality considerably.

Literature review and correspondence with other research groups has indicated that while some proteins behave well on SEC when solubilized using SMALPs, a lot of them do not (Qiu et al., 2018). Because of this, studies usually omit SEC for SMALP purification altogether (Damian et al., 2018; Karlova et al., 2019; Paulin et al., 2014). This severely limits the ability to troubleshoot complex formation when using this system, which, combined with the strange behaviour on microscopy grids, made progress impossible when using SMA-solubilized Fzd. SMA polymer used for SMALP formation is amenable to chemical modification, and there has been considerable interest in creating new polymers that could be used for the formation of lipid particles with altered characteristics (Chen et al., 2020). The next generation of polymers could therefore avoid the aforementioned problems, making them better suited for the determination of transmembrane protein complex structures.

The highest-quality cryo-EM data that was obtained during this project was for the Fzd4-1/Ndp3/F2.A-2 complex, which was solubilized using CHS-free DDM. The experiments conducted as a part of this work indicated that CHS is detrimental for achieving micelle fusion, therefore it should be noted any experiment attempting to form a complex between detergent-solubilized transmembrane proteins should reduce or omit CHS from their buffers. This also applies to any future experiments aiming to reconstitute Fzd and its co-receptor complexes *in vitro*.

Detergent-solubilized Fzd4-1/Ndp3/F2.A-2 complex behaved well on microscope grids, and the particles were large, with a predicted molecular weight of 230 kDa, which should make the complex amenable to structure determination. However, 2D classification software tended to focus particle alignment either on the detergent micelle or the extracellular regions of the complex, and the resulting 2D class averages suggested that the distance between the Fzd4-1 transmembrane domain and its CRD is variable. This may indicate that there was considerable flexibility in the particle, most likely in the linker region between Fzd4 TMD and CRD domains. It has been previously reported that this linker region undergoes rearrangements upon formation of the Fzd4/Ndp complex (Bang et al., 2018). The flexibility observed in Fzd4-1/Ndp3/F2.A-2 particles suggests that a stable interaction between Fzd4 TMD and CRD is not present upon receptor activation, therefore the reported changes in the linker architecture might arise due to Fzd4 dimerization via Ndp. The absence of rigid contacts between ligand-bound Fzd4 CRD and its TMD indicates that Fzd4 probably does not function similarly to class A GPCRs, which propagate agonist-induced signals across the membrane via allosteric rearrangement of their transmembrane helices. This is further supported by recently published cryo-EM data. Structural characterization of the Fzd5/XWnt8 complex has also shown severe flexibility between Fzd5 CRD and TMD domains

despite being in an active state (Tsutsumi et al., 2020), whereas the CRD was completely absent from structures of an active Fzd7/heterotrimeric G_s complex (Xu et al., 2021). In conclusion, currently available structural biology approaches explored in this work do not appear to be suitable for determining the functional mechanisms behind the activation of Fzd signalling upon agonist stimulation. This is due to the flexibility of the Fzd CRD, which, in the case of Fzd4, does not appear to be stabilized relative to the Fzd transmembrane domain upon its interaction with Ndp.

All of this supports the possibility that the role of Fzd in β -catenin and PCP signalling is to act as a passive scaffold that brings Dvl in close proximity to the co-receptor intracellular domains upon stimulation by Wnt or Ndp. It is not clear how this might alter the ability of Dvl to interact with downstream effectors, since direct interactions between Dvl and co-receptors have not been reported. One possibility is that downstream effector recruitment to Fzd signalling complexes depends on several weak interactions with both Dvl and co-receptor intracellular domains, therefore enabling binding and downstream signalling only upon Fzd signalling complex assembly.

The assumption that Wnt or Ndp binding to the Fzd CRD does not result in conformational changes within the TMD does not explain how Fzd could mediate heterotrimeric G protein activation required for Ca²⁺ pathway activation. A recent publication has shown that Fzd7 can constitutively activate heterotrimeric G_s proteins, thus indicating that ligand binding might not be a requirement for this process (Xu et al., 2021). Then again, constitutive activity does not seem to be responsible for Ca²⁺ pathway activation, since it usually does require Wnt stimulation across many Fzds assessed (Schulte and Wright, 2018). Further research is therefore required to elucidate the mechanisms of heterotrimeric G protein activation by Fzd.

Interactions between Fzd and heterotrimeric G proteins appear to result in complexes that are more easily stabilised in comparison to complexes involving the Fzd CRD, as illustrated by recent publications (Tsutsumi et al., 2020, Xu et al., 2021). The same might also apply to other interactions involving the intracellular side of Fzd, therefore future structural studies aiming to elucidate the molecular mechanisms behind Fzd signalling should explore the interactions between the transmembrane domain of Fzd with its intracellular binding partners, particularly Dvl. As previously mentioned, the interaction between Dvl and Fzd potentially relies on the assembly of Fzd and its co-receptor complexes, which would need to be reconstituted *in vitro* for structural examination. Fzd complex extraction using SMALPs, Fzd dimer reconstitution from detergent-solubilised monomers, and preliminary experiments with ROR2 and Dvl presented in this work thus serve as a starting point for future research aiming to achieve this.

4. Structural characterization of Notum inhibitors

4.1 Thienopyrimidine-derived Notum inhibitors

The work presented here was conducted in collaboration with the group of Dr Paul Fish (University College London, UK). Their lab had carried out Notum inhibitor screens with the aim of identifying novel compounds targeting this protein that could be used as a starting point for the synthesis of potential drug candidates. The primary assay used for IC₅₀ determination of potential inhibitors was the OPTS (trisodium 8-octanoyloxy pyrene-1,3,6-trisulfonate) assay. OPTS is a fluorogenic esterase substrate that enables quantification of Notum activity. In the presence of a Notum inhibitor, the rates of fluorescent product generation upon OPTS cleavage by Notum are decreased, thus allowing the determination of its IC₅₀ value (for details, see Atkinson et al., 2019). Our role in this project was to determine the structures of Notum in complex with various inhibitors identified via these assays. This would allow to rationalize the importance of different chemical features present on the compounds tested in relation to their IC₅₀ values, thus guiding further development of Notum inhibitors.

We decided to start by determining the structure of Notum in complex with a previously characterized thienopyrimidine-derived molecule, referred to as '67'. This compound has been reported to bind Notum with high affinity, having an IC₅₀ value of 2 nM. The structure of this complex would help to explain previously observed structure-activity relationships within this class of molecules (Fig. 3), while giving us insight into the molecular features required for binding the active site of Notum with high affinity.

Human Notum construct containing a 6xHis-tag, a C330S mutation, and lacking the flexible N- and C-terminal loop regions (hNot-Cryst., Fig. 16) was expressed via transient transfection of HEK293T cells. The purification procedure resulted in a single peak (Fig. 70.A), which migrated as a major and minor band at 43 and 40 kDa on SDS-PAGE (Fig. 70.B). Since the predicted molecular weight of hNot-Cryst. is 42 kDa, the purification was considered a success, although the presence of two closely migrating bands on SDS-PAGE suggested the presence of various glycosylation states. Because of

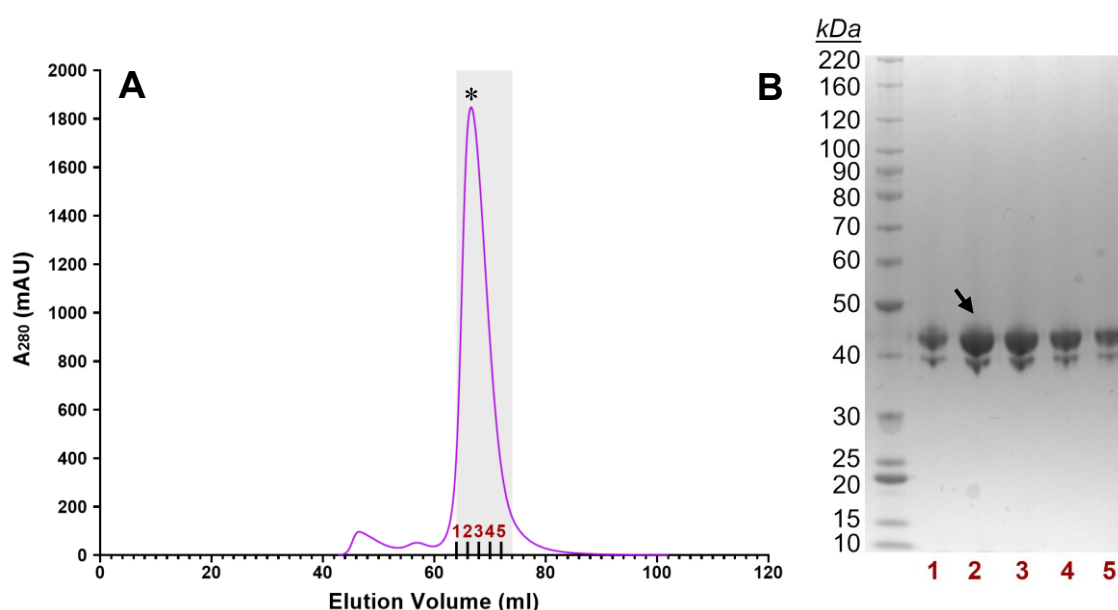


Fig. 70. Purification of hNot-Cryst. **A** – SEC profile. Performed using a HiLoad 16/60 Superdex 75 pg column. Pooled fractions shaded grey, asterisk denotes peak containing the protein of interest. **B** – reducing SDS-PAGE. Arrow denotes bands corresponding to the protein of interest.

this, before use in crystallization, the sample was treated with endoglycosidase H and SEC repeated to obtain a homogenous sample.

The hNot-Cryst. sample was then used to produce crystals using previously reported crystallization conditions (Atkinson et al., 2019). The crystals were soaked overnight with compound 67 or other compounds of interest. The crystals were then flash-frozen and the X-ray diffraction patterns collected (S. Table 2, S. Table 3). All crystals exhibited $P2_12_12_1$ symmetry, with one protein molecule per asymmetric unit. Data was processed using molecular replacement with a previously determined hNot-Cryst.

structure (PDB ID: 4UZ1). A full description of the crystallization and data processing procedure can be found in the “Methods” section.

As expected, the previously reported high-affinity Notum inhibitor ‘67’ bound in the active site of Notum, and its carboxyl group aligned well with the one found in Notum-bound palmitoleic acid structures (Fig. 71). It was in close proximity to the catalytic S232 and H389, thus suggesting interactions with their sidechains. In addition, the position of the carboxyl group was further stabilized via interactions with the backbone of the loop consisting of residues 125-127, which form the top part of the entrance to the active site. The aromatic thienopyrimidine rings were found to be in close proximity to the F268 sidechain, thus hinting at a possible π - π stacking interaction.

Since no other significant or obvious interactions were observed deeper within the cavity, it appeared that the main determinant for high-affinity interaction between ‘67’ and Notum was the presence of the carboxyl group within the ligand. In regards to the

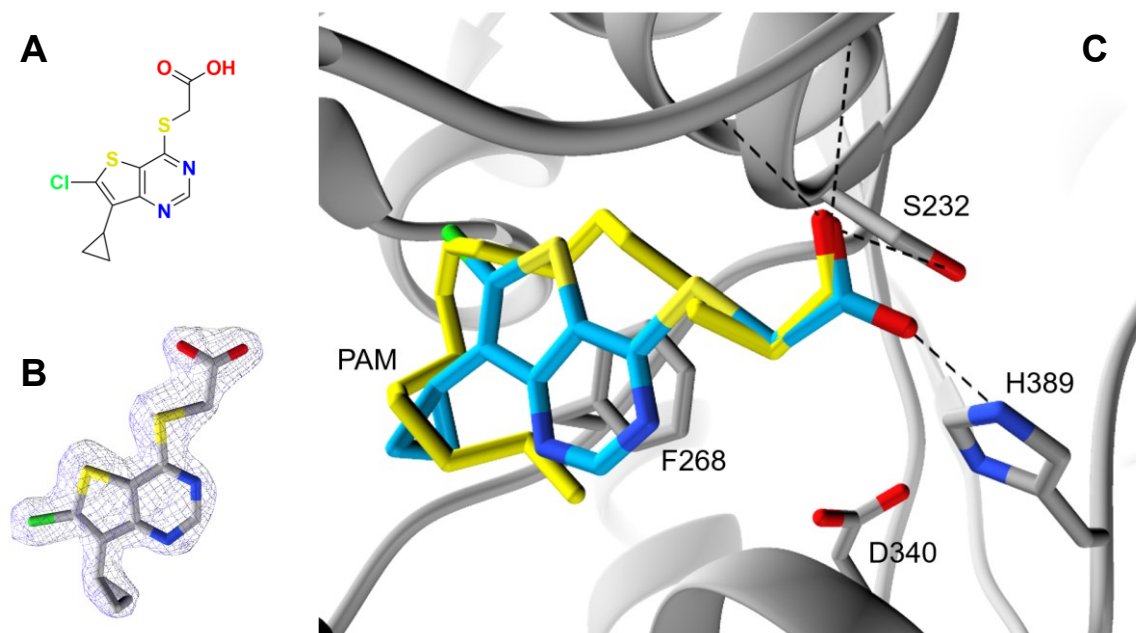


Fig. 71. Inhibitor ‘67’ in complex with Notum. **A** - inhibitor ‘67’. **B** – Final 2Fo-Fc electron density of ligand. Countoured at 1.5 σ . **C** -Inhibitor ‘67’ within the active site of Notum. Carbon atoms of ‘67’ coloured teal. Dashed lines indicate potential interactions. Palmitoleic acid (PAM, carbon atoms coloured yellow) within the active site shown for comparison (based on Kakugawa et al., 2015, PDB ID: 4UZQ). Structure refined at 2.0 Å resolution.

previously reported structure-activity relationships of thienopyrimide-based inhibitors of Notum (Fig. 3), this would also explain why modifications of this group resulted in reductions of inhibitor potency. As for the linker region between the carboxyl group and the thienopyrimidine rings, it is positioned in the more narrow entrance to the ligand binding cavity (Fig. 2.B), therefore the modifications to this linker could result in steric hindrance that would prevent the inhibitor from binding. The same is also true for the six-membered ring of thienopyrimidine. In contrast, its five-membered ring is positioned deeper within the cavity, where there appears to be more unoccupied space that could accommodate aliphatic modifications. This would result in a better molecular fit and thus higher inhibitor affinity due to additional van der Waals forces.

The importance of the carboxyl group within '67' indicated that it would be quite difficult to develop inhibitors of Notum that could be used as potential treatments of neurodegenerative disease. Although necessary for high-affinity interaction with the catalytic residues of Notum, the negative charge of this group would also reduce the ability of inhibitors to pass the blood-brain barrier (BBB) (Manallack et al., 2012). Because of this, future work focused on characterization of Notum inhibitors with either a modified carboxyl group, or it being absent altogether.

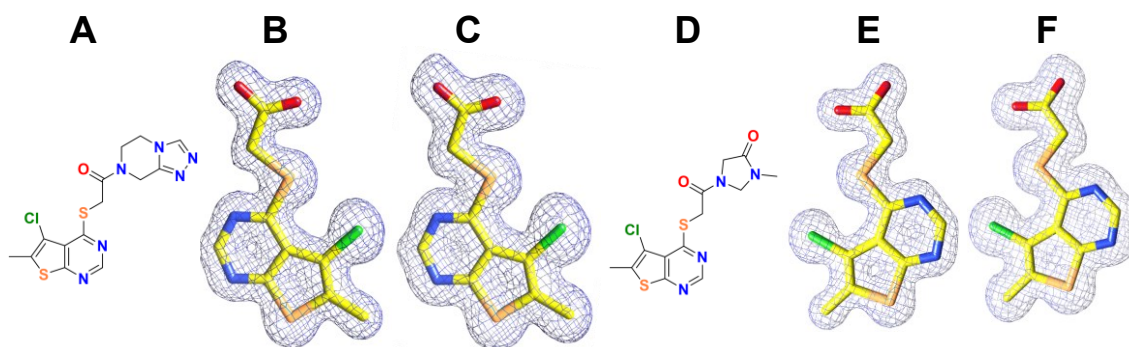


Fig. 72. Electron density of Notum inhibitors '1128' and '1147'. **A** – '1128'. **B** – Final '1128' 2Fo-Fc electron density at 1 σ . **C** – Final '1128' 2Fo-Fc electron density at 0.5 σ . **D** – '1147'. **E** – Final '1147' 2Fo-Fc electron density at 1 σ . **F** – Final '1147' 2Fo-Fc electron density at 0.3 σ . Both structures refined at 1.4 Å resolution.

Previous reports indicated that the modification of the Notum inhibitor carboxyl group via amidation was tolerated for potency (Tarver et al., 2016). It was predicted that these additional modifications would make additional contacts with Notum outside of its active site, so, to identify these interactions, we determined the structures of Notum in complex with two such molecules '1128' and '1147', which had IC₅₀ values of 3 and 2 nM respectively.

Upon processing of X-ray data, it became evident that the amide modifications of '1128' and '1147' were absent in the final structure, even when examined at lower electron density contouring levels (Fig. 72). The integrity of the ligands was confirmed by high-performance liquid chromatography, and purified Notum was not able to catalyse the cleavage of the amide bond under the crystallization conditions used (both experiments performed by the Fish group). Despite this, Notum might be able to cleave the amide bonds of both compounds given the prolonged, relatively uninterrupted exposure of the inhibitor to its active site, that could occur in soaked crystals. This suggests that the determination of the structures of these compounds in complex with Notum via X-ray crystallography is impossible.

4.2. Heteroaryl inhibitors of Notum

Since the amidation of the carboxyl group present within thienopyrimidine-based inhibitors of Notum did not yield to structural characterization, we decided to focus on a set of heteroaryl compounds that were identified as inhibitors of Notum (Fig. 73).

Although their IC₅₀ values were higher in comparison to thienopyrimidines, they were still in the nanomolar range. More importantly, they did not contain a carboxyl group, which could potentially correspond to increased BBB permeability. The heteroaryl group was modified with either hydroxyl or hydroxymethyl groups on one side, as well as a chlorine- or trifluoromethyl-modified benzyl ring on the other. Within this set of inhibitors were pairs of molecules that differed from one another by modifications present on either their heteroaryl or benzyl groups, thus allowing us to evaluate and compare their importance for Notum binding. hNot-Cryst. crystals soaked with these inhibitors diffracted to a resolution between 1.8 and 1.4 Å, producing well-defined ligand densities within the Notum active site that allowed confident molecule placement within the structures.

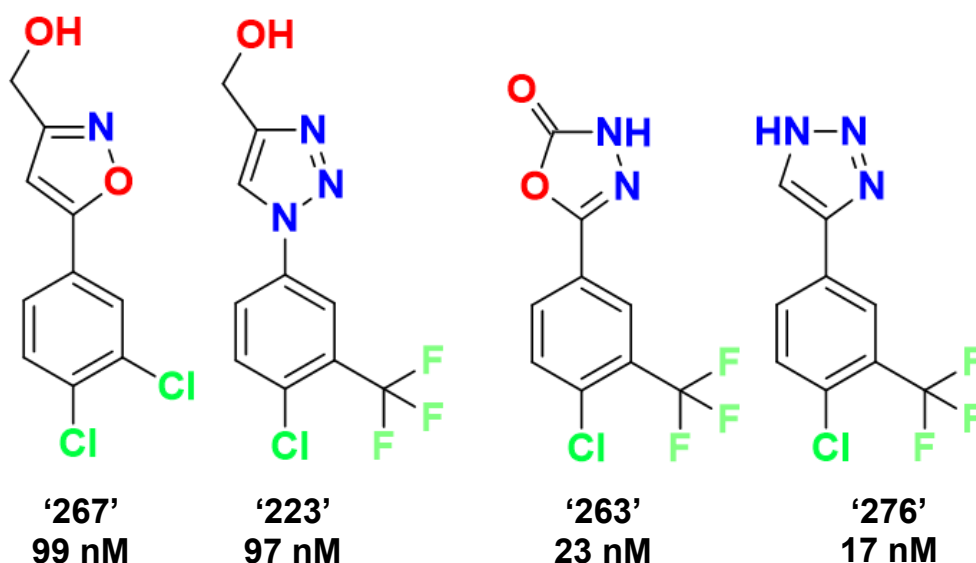


Fig. 73. Heteroaryl inhibitors of Notum. Values below each molecule indicate IC₅₀ values of Notum inhibition.

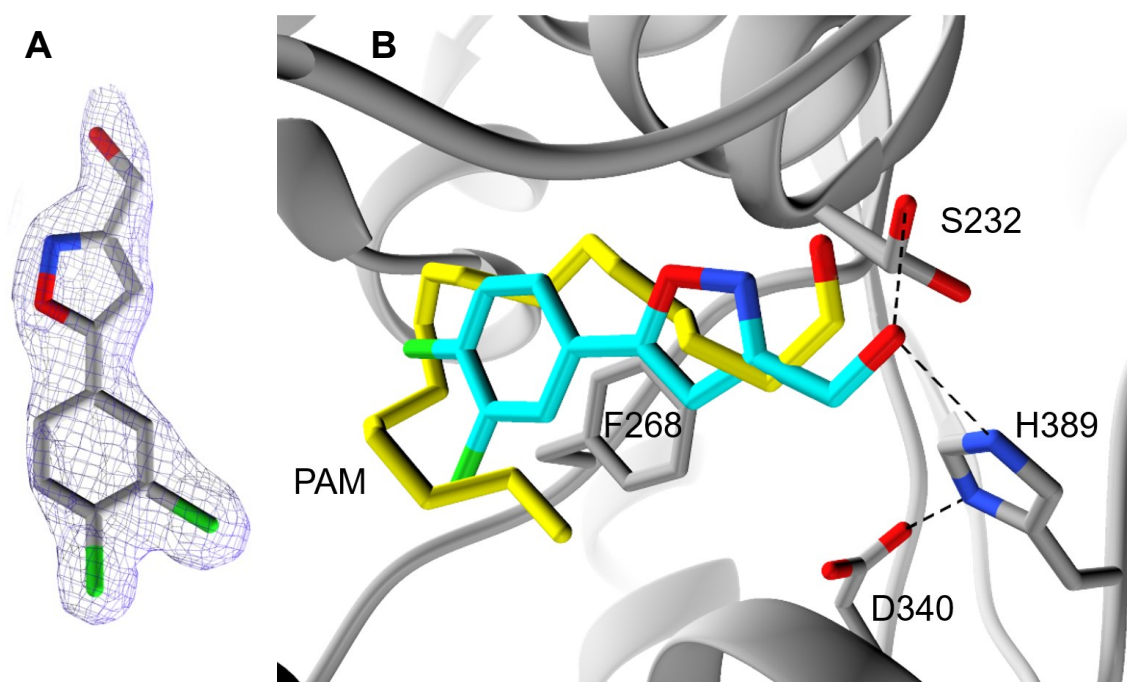


Fig. 74. Inhibitor '267' in complex with Notum. **A** – Final 2Fo-Fc electron density of '267'. Contoured at 1.5 σ . **B** -Inhibitor '267' within the active site of Notum. Carbon atoms of '267' coloured cyan. Dashed lines indicate potential interactions. Palmitoleic acid (PAM, carbon atoms coloured yellow) within the active site shown for comparison (based on Kakugawa et al., 2015, PDB ID: 4UZQ). S232 sidechain was found in two potential orientations. Structure refined at 1.8 Å resolution.

The molecule '267', which showed the lowest affinity for Notum across this set of compounds (Fig. 73), appeared not to fully enter the cavity, with its hydroxyl group being located much further outside of the cavity as for the native substrate (Fig. 74.B). The benzyl group still appeared to potentially participate in a π - π stacking interaction with F268. Despite this, the hydroxyl group remained in close vicinity of S232 and H389, while the interactions with the backbone were absent in comparison to inhibitor '67'.

The compound '223', which had an almost identical affinity to '267', showed a placement of the hydroxyl group (Fig. 75) that was slightly closer to the native substrate position in comparison to '267' (Fig. 74). It was in close proximity to S232 sidechains in both of its possible orientations, while also potentially being stabilized by a coordinated water molecule within a short distance of H389. The benzyl group of '223' was modified with a trifluoromethyl group instead of one of the chlorine groups found

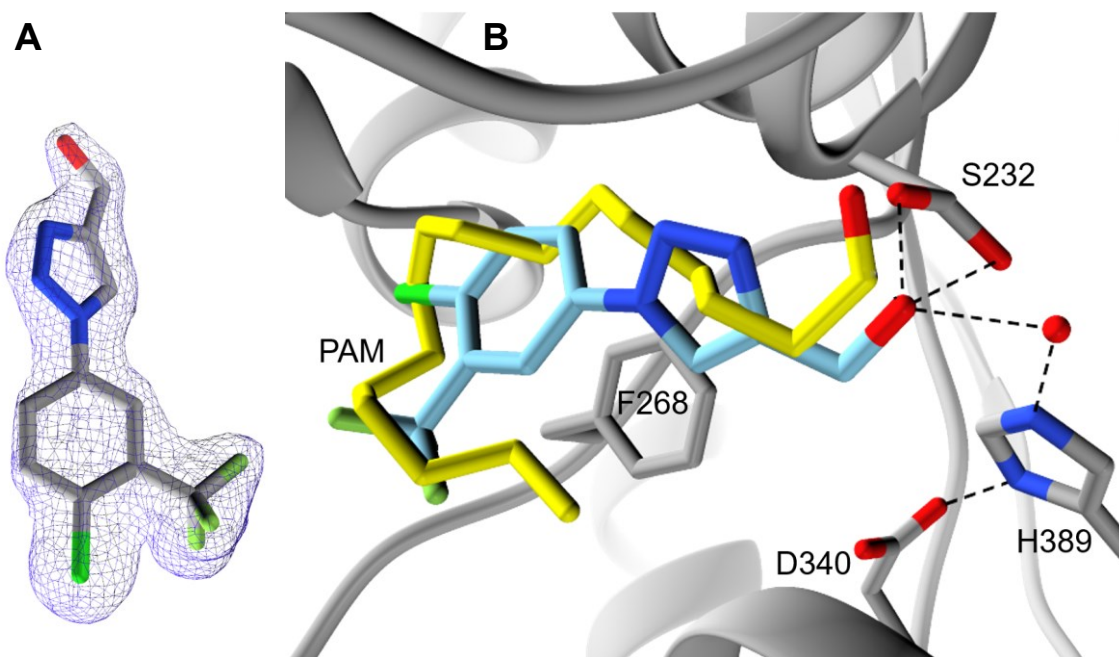


Fig. 75. Inhibitor '223' in complex with Notum. **A** – Final 2Fo-Fc electron density of '223'. Countoured at 1.5 σ . **B** -Inhibitor '223' within the active site of Notum. Carbon atoms of '223' coloured teal. Dashed lines indicate potential interactions. Palmitoleic acid (PAM, carbon atoms coloured yellow) within the active site shown for comparison (based on Kakugawa et al., 2015, PDB ID: 4UZQ). S232 sidechain was found in two potential orientations. Water molecule shown as red sphere. Structure refined at 1.7 Å resolution.

in '267'. This led to a rotation of the benzyl group, thus disrupting its potential stacking interaction with F268, while also enabling the trifluoromethyl group to extend deeper within the hydrophobic cavity.

The slight difference in IC₅₀ values for '267' (99 nM) and '223' (97 nM) could be rationalized by the presence of the trifluoromethyl group instead of a chlorine group on its benzyl ring, since the heteroaryl headgroups and their modifications are very similar. Based on the ability of the trifluoromethyl group to alter the orientation of the '223' benzyl ring, it appears that its interaction with the sidechains forming the hydrophobic pocket of Notum is stronger than the potential π - π stacking interaction of its benzyl ring with F268. The loss of the latter could therefore diminish any gains of potency gained by this modification.

The oxadiazole group of inhibitor '263' was modified with a hydroxyl group. Due to its reduced length in comparison to hydroxymethyl groups, it was positioned deeper within

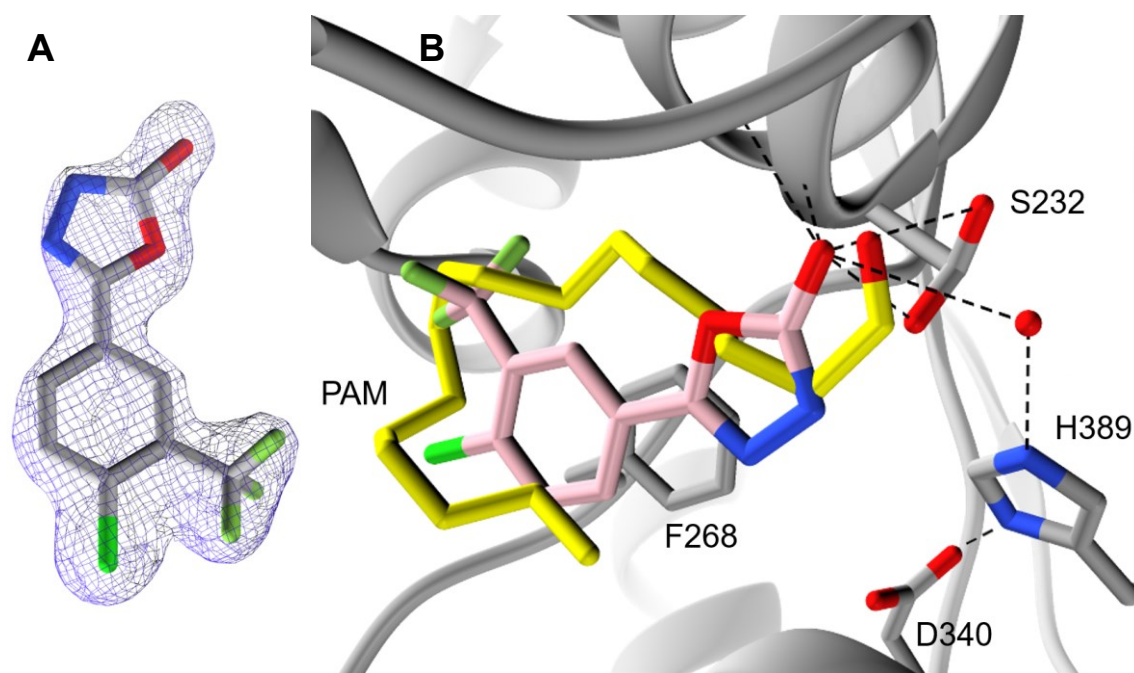


Fig. 76. Inhibitor '263' in complex with Notum. **A** – Final 2Fo-Fc electron density of '263'. Countoured at 1.5 σ . **B** -Inhibitor '263' within the active site of Notum. Carbon atoms of '263' coloured pink. Dashed lines indicate potential interactions. Palmitoleic acid (PAM, carbon atoms coloured yellow) within the active site shown for comparison (based on Kakugawa et al., 2015, PDB ID: 4UZQ). S232 sidechain was found in two potential orientations. Water molecule shown as red sphere. Structure refined at 1.4 Å resolution.

the hydrophobic cavity than both '223' and the native substrate of Notum (Fig. 76).

This hydroxyl was positioned within an interaction distance to both possible orientations of S232, while also being in close proximity of the residue 125-127 and S232 backbone carbonyls, similarly to inhibitor '67'. The distance to the coordinated water molecule previously observed in the '223' structure was increased, thus potentially weakening its contribution to '263' affinity for Notum. It appeared that in order to adopt this position, the molecule was slightly rotated relative to the binding pocket, with its benzyl group located more 'downwards', closer to F268, whereas its trifluoromethyl group was now facing 'up'.

'276', the last compound within the set of heteroaryl molecules tested, contained an unmodified triazole group. It has a nitrogen-bound hydrogen atom, thus allowing the triazole group to act as an efficient hydrogen bond donor. The triazole group was positioned deeper in the Notum binding cavity than its native substrate similarly to

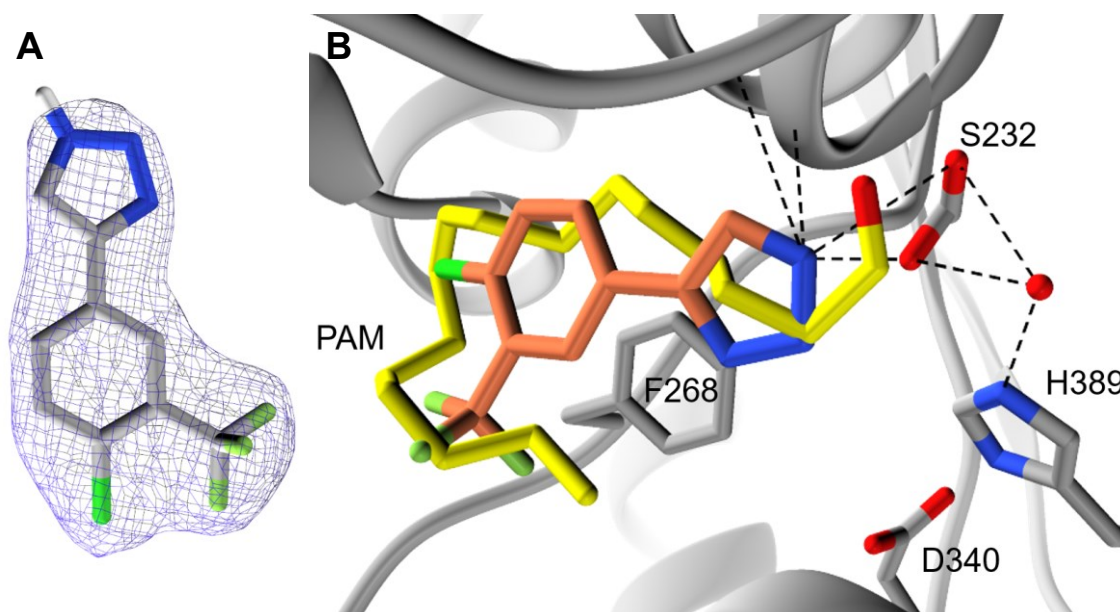


Fig. 77. Inhibitor '276' in complex with Notum. **A** - Final 2Fo-Fc electron density of '276'. Countoured at 1.5 σ . **B** -Inhibitor '276' within the active site of Notum. Carbon atoms of '276' coloured orange. Dashed lines indicate potential interactions. Palmitoleic acid (PAM, carbon atoms coloured yellow) within the active site shown for comparison (based on Kakugawa et al., 2015, PDB ID: 4UZQ). S232 sidechain was found in two potential orientations. Water molecule shown as red sphere. Structure refined at 1.8 Å resolution.

'263' (Fig. 77), also potentially forming similar interactions with S232 and the backbone carbonyls in its vicinity. The main difference appeared to be the orientation of the benzyl group, the trifluoromethyl group of which was pointing 'down' within the binding pocket. This could be due to the smaller space occupied by the triazole group in comparison to the modified oxadiazole group in '263', thus suggesting that the 'down' position is the preferred orientation of the trifluoromethyl group if enough space is available. It should be noted that it was not possible to unambiguously place the nitrogen atoms of the triazole ring within the ligand electron density observed, although the orientation of the ligand presented here resulted in improved R/R_{free} values during data processing.

In conclusion, the results indicate heteroaryl compounds containing a modified benzene ring exhibit higher affinities for Notum if their heteroaryl groups are unmodified, or the length of the modifications is kept short. This permits the heteroaryl group to position itself deeper within the hydrophobic pocket of Notum, thus allowing it to form more

optimal interactions with the catalytic S232, as well as the adjacent Notum backbone carbonyls. The structure and IC₅₀ values of '276' also suggest that the presence of amines within Notum inhibitors could lead to higher affinities for Notum, due to them being better hydrogen bond donors. Finally, the variety of different orientations of the benzyl group within the hydrophobic pocket suggests that there might still be unoccupied space within the pocket. This means that further modifications to the benzyl ring could be made in order to increase the affinity of the inhibitors even further.

4.3. Optimized heteroaryl inhibitors

Notum inhibition screens performed by the Fish group revealed that an additional chlorine modification present on the benzyl ring of the previously tested '276' compound resulted in a more than 5-fold decrease of IC₅₀ values (compound '1241'). We therefore selected a new set of heteroaryl compounds for structure determination by X-ray crystallography (Fig. 78). Within the set, we included '1233', which contained an amine group on its oxadiazole ring, as well as '1185', where the modified benzyl group was attached to the triazole ring via C-N linkage, thus eliminating the hydrogen atom present in '1241'. Determination of these structures would allow us to characterize the effects of the additional chlorine modification on Notum inhibitor binding modes, as

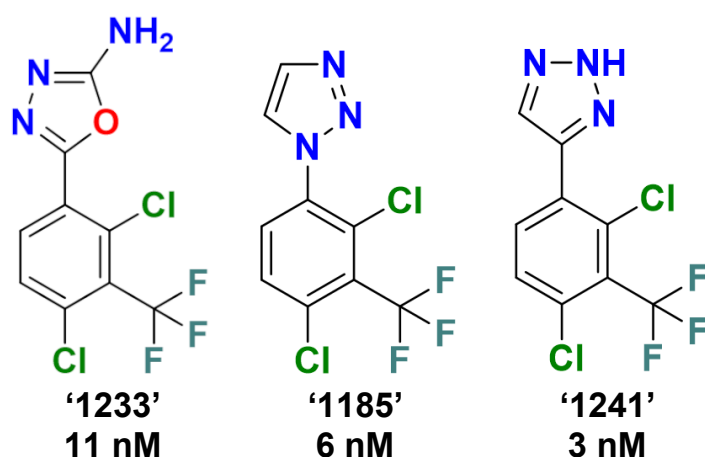


Fig. 78. Improved heteroaryl inhibitors of Notum. Values below each molecule indicate IC₅₀ values of Notum inhibition.

well as evaluate the importance of the nitrogen-bound hydrogen atom present on '276' and '1241'.

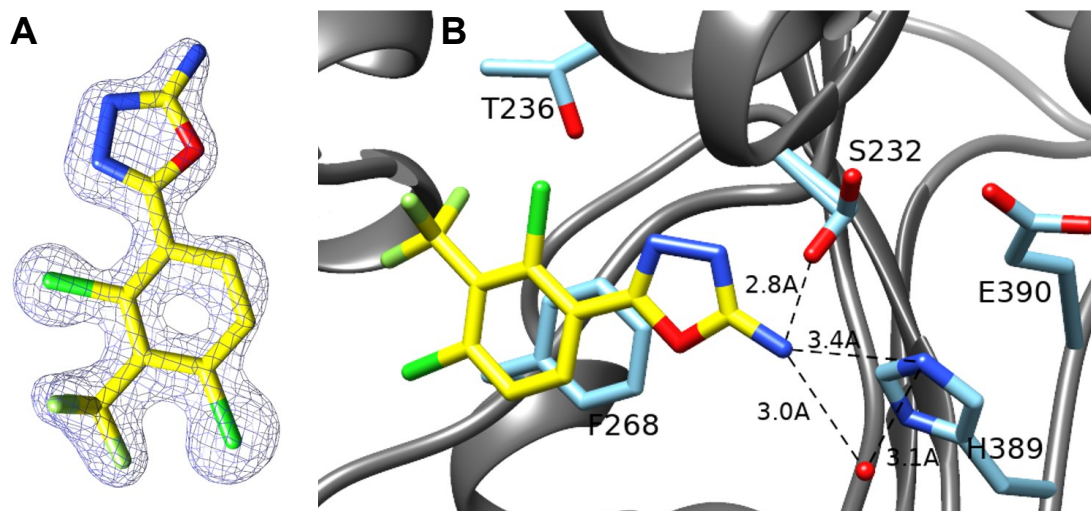


Fig. 79. Inhibitor '1233' in complex with Notum. **A** - Final 2Fo-Fc electron density of '1233'. Contoured at 2.0 σ . **B** - Inhibitor '1233' within the active site of Notum. Carbon atoms of '1233' coloured orange. Dashed lines indicate potential interactions. S232 sidechain was found in two potential orientations. Water molecule shown as red sphere. Structure refined at 1.4 Å resolution.

Similarly to the hydroxyoxadiazole containing '263' compound (Fig. 76), '1233' was slightly rotated relative to the hydrophobic pocket of Notum, with its benzyl ring in close proximity of F268 and its modifications pointing 'up' (Fig. 79). The additional chlorine group had enough space to avoid clashes with the surrounding residues, thus resulting in a better fit of the inhibitor within the pocket. In contrast to the '263' hydroxyl group, the amine of '1233' was pointing downwards, in close vicinity of one of the possible orientations of the S232 sidechain, H389, and a coordinated water molecule.

Inhibitors '1185' and '1241' had almost identical binding modes within the active site of Notum (Fig. 80). The modifications of the benzene ring were in a 'down' position in contrast to '1233', in which the benzyl group is tilted downwards to allow the positioning of the amine group in the vicinity of S232 and H389 (Fig. 79). Since in the absence of these restraints, the chlorine and trifluoromethyl groups were pointing

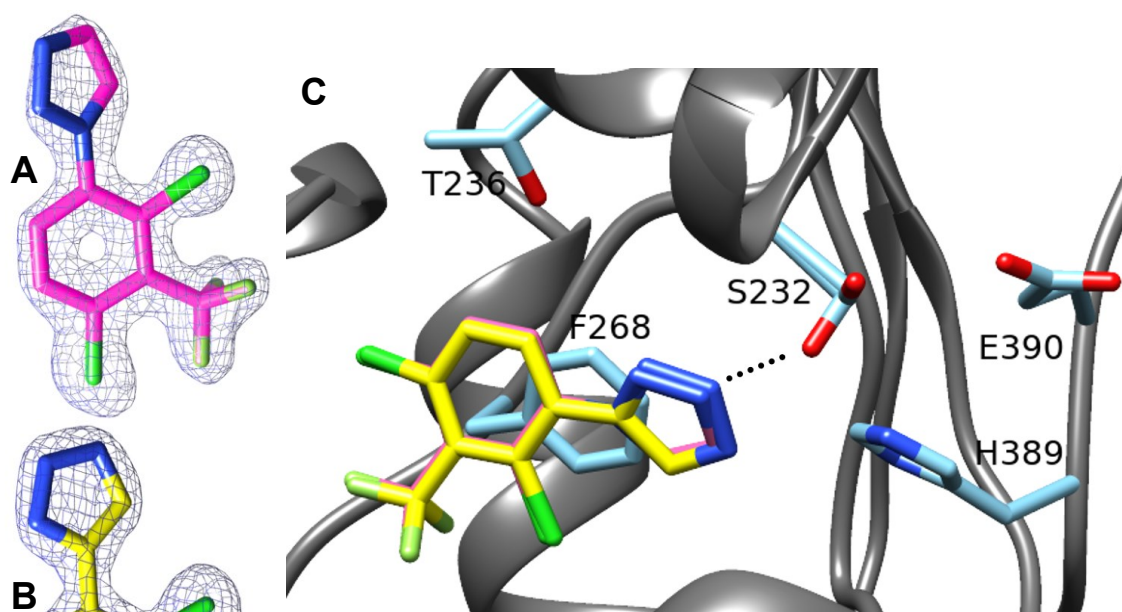


Fig. 80. Inhibitors '1185' and '1241' in complex with Notum. **A** - Final 2Fo-Fc electron density of '1185'. Contoured at 2.0 σ . **B** - Final 2Fo-Fc electron density of '1241'. Contoured at 2.2 σ . **C** - Inhibitors binding within the active site of Notum. Carbon atoms of '1185' and '1241' coloured magenta and yellow respectively. Dashed lines indicate potential interactions. S232 sidechain was found in two potential orientations. Structures refined at 1.5 and 1.4 Å resolution for '1185' and '1241' respectively.

'down', it can be assumed that it is their preferred binding mode that results in a higher affinity interaction with Notum.

The only difference between '1185' and '1241' is the N-C or C-C linkage between their triazole and benzyl groups, therefore it must be responsible for the 2-fold decrease in IC_{50} values for '1241'. As indicated previously, the C-C linked triazole within '1241' contains an N-linked hydrogen atom, allowing it to act as a good hydrogen bond donor, which would result in higher affinity for Notum. Despite this, the N-C linked triazole compound '1185' still produced a reasonably low IC_{50} value of 6 nM when tested *in vitro*. This is still almost 3-fold lower than that of '276' (Fig. 77), which contains a C-C linked triazole, but lacks the additional chlorine modification of the benzyl ring.

To confirm the role of this chlorine modification, the previously characterized Notum inhibitor '223' was modified by an additional chlorine group on its benzyl ring ('1043',

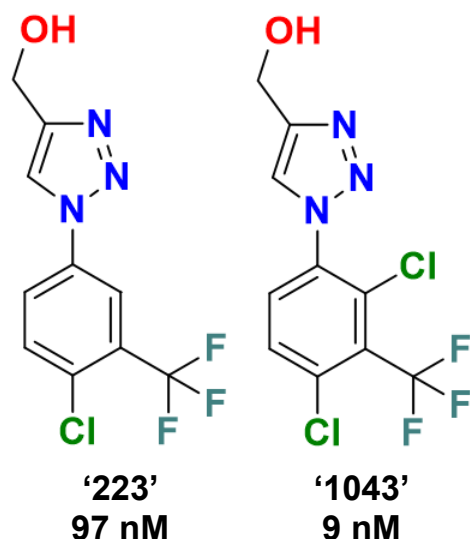


Fig. 81. Notum inhibitors '223' and '1043'.
 Values below each molecule indicate IC₅₀ values of Notum inhibition.

Fig. 81). As determined by the Notum inhibition assays (performed by the Fish group), this reduced the IC₅₀ values of the compound by more than 10-fold (Fig. 81). In contrast, switching the N-C linked hydroxymethyltriazole of '223' for a C-C linked triazole led to only ~6-fold decrease (Fig. 73). In conclusion, it appears that the modifications present on the benzyl ring and the overall fit of the molecule within the

hydrophobic pocket is a more important determinant for binding Notum with high affinity than the identity and interactions formed by the heteroaryl headgroup.

4.4. Discussion

The development of novel medications targeting Notum for the treatment of NDDs presents a major challenge of fine-tuning the chemical properties of small-molecule inhibitors to result in good pharmacokinetic profiles and BBB penetration, while also maintaining high potency. The work presented here attempts to rationalize how different modifications of small-molecule Notum inhibitors affect their ability to interact with the active site of Notum, thus aiding future drug development process aimed at improving the properties of Notum inhibitors *in vivo*.

Structural analysis of several Notum inhibitors indicates that their affinity for Notum depends on the interaction networks formed between the molecule and the residues found at the entrance of the Notum catalytic pocket, as well as the overall molecular fit of the inhibitor aliphatic groups within the Notum hydrophobic cavity. Increased

polarity of the functional groups present on the inhibitor molecules appears to result in stronger interactions with catalytic S232 and other residues in its vicinity. The displacement of the inhibitor polar groups relative to the reported position of the palmitoleate carbonyl group results in reductions of affinity, thus illustrating how interactions between Notum and its native substrate are optimal for the reaction to proceed. Despite this, Notum inhibitors with smaller polar groups appear to be bound deeper within the catalytic site, allowing them to form more interactions with the backbone residues forming the entrance of the hydrophobic pocket, thus resulting in higher affinity.

The molecular fit of Notum inhibitor aliphatic groups within the enzyme hydrophobic pocket appears to be the main determinant of high affinity interaction with Notum. Although it was not possible to pinpoint individual residues within the hydrophobic cavity that would be important for inhibitor binding, the comparison of '223' and '1043' IC₅₀ values with structural data (Fig. 81) indicates that this cavity is crucial for inhibitor binding. Furthermore, it is possible to significantly increase the affinity of small molecules for this site by simply ensuring a better fit of their aliphatic groups within the hydrophobic pocket. This is most likely due to the compounding effects of multiple van der Waals interactions possible within this site. The modifications present on the benzyl group of the heteroaryl compounds tested were found in both 'up' and 'down' orientations within the Notum hydrophobic cavity (Fig. 79, Fig. 80). This suggests that even with the two chlorine and one trifluoromethyl modifications present on the benzyl ring within the last set of molecules tested, the Notum hydrophobic pocket was still not fully occupied. Therefore, further improvements in Notum inhibitor potency could be made by improving the molecular fit between the inhibitor aliphatic groups and the hydrophobic cavity of Notum.

The data presented here provides an insight into the principles governing small-molecule interactions with the active site of Notum. Unfortunately, structural data does not provide information about the behaviour of drugs *in vivo*. This work has led to the identification of several heteroaryl inhibitors of Notum exhibiting good ADME profiles and pharmacokinetic behaviour in mice (Mahy et al., 2020a, 2020b), thus demonstrating the usefulness of structural biology approaches in drug development. However, identification of molecules exhibiting high BBB penetration remains a considerable challenge, since it is difficult to rationalize. For instance, Notum inhibitor ‘263’ (Fig. 77) exhibited physicochemical properties that should make it suitable for targeting the brain, while *in vitro* MDCK-MDR1 assays showed ‘263’ has good cell permeability in the presence of multidrug resistance protein 1 (MDR1) (Mahy et al., 2020a; Rankovic, 2015; Wager et al., 2010). Despite this, the concentrations of free inhibitor in the brain upon oral dosing of mice remained low, probably due to the action of other multidrug efflux transporters besides MDR1. Therefore, further studies in animal models will be required for identification of Notum inhibitors that could be useful for NDD treatment.

5. References

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6. Appendix I – Structure of SARS-CoV-2

Spike protein in complex with H11-D4

nanobodies

6.1. Introduction

In late 2019, a novel coronavirus, now called SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2), emerged in China (Hu et al., 2020). Human infection with this virus, termed COVID-19, results in symptoms ranging from headaches, cough, and fever to pneumonia, hypoxia, or even multiorgan dysfunction and respiratory failure. SARS-CoV-2 rapidly spread across the globe, leading to an estimated number of 4 million deaths as of July 2021.

Since no vaccines or treatments against COVID-19 were available at the onset of this pandemic, their development was urgently needed. One of the ways to treat COVID-19 would be the use antibodies against the SARS-CoV-2 Spike protein. This homotrimeric class I viral membrane fusion protein is responsible for recognizing the angiotensin-converting enzyme ACE2 on the surface of human cells via its receptor binding domain (RBD), subsequently leading to viral entry (Huang et al., 2020). Binding of the Spike RBD by antibodies would prevent SARS-CoV-2 from entering human cells, thus limiting progression of the disease.

An alternative to therapeutic antibodies would be nanobodies – V_HH fragments engineered from heavy-chain antibodies found in camelids. Although the camelid immunization process requires a few months, selection from artificially generated nanobody libraries combined with affinity maturation could be used to quickly obtain

high-affinity binders against a protein of interest. Other advantages of nanobodies also include selection for specific epitopes, a quick and simple nanobody production procedure, as well as their small size and concave shape, which enables them to recognize epitopes that might be inaccessible to regular antibodies (Pardon et al., 2014).

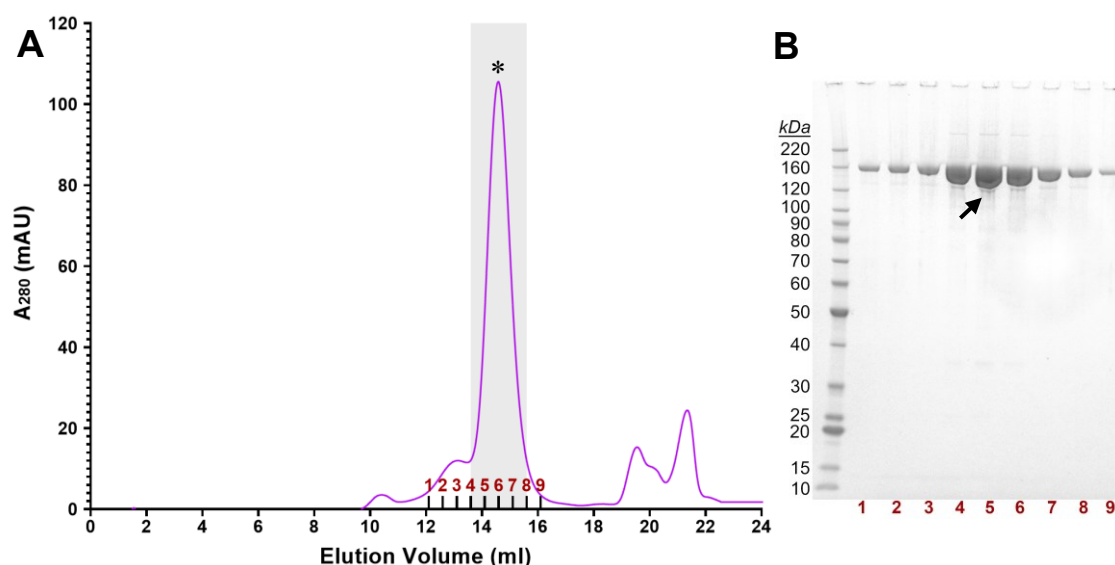
We therefore decided to characterize the interaction of SARS-CoV-2 Spike protein with nanobody H11-D4, which was obtained by screening a naive llama and alpaca nanobody phage library against the RBD of Spike (residues 332-529, with P527 omitted), as well as affinity maturation. An H11-D4-Fc fusion construct was reported to neutralize live wild-type virus with an ND_{50} value of 6 nM, therefore having potential to be used as a treatment against SARS-CoV-2 (Huo et al., 2020).

6.2. Expression and purification of SARS-CoV-2 Spike protein

SARS-CoV-2 Spike protein (Uniprot ID: P0DTC2) was produced similarly to Notum, with slight differences. Conditioned media was obtained from HEK293T cell cultures in roller bottles transiently transfected with a pHLsec vector encoding the SARS-CoV-2 Spike ectodomain (residues 1-1208). The construct contained mutations of RRAR > GSAS (furin cleavage site) at residues 682-685, and KV > PP at residues 986-987. A T4 trimerization domain, an HRV 3C cleavage site, an 8xHis- and Twin-Strep-tags were also present at the C-terminus of the construct.

The conditioned media was diafiltrated against buffer S (10 mM HEPES, pH 7.5, 150 mM NaCl) using a 10 kDa MWCO QuixStand cartridge. The diafiltrate was loaded onto a 5 ml HisTrap FF column (pre-equilibrated with buffer S) using a flow rate of 3 ml/min. The column was then washed two times with 15 CV buffer S, which was supplemented with 50 mM imidazole for the second wash. The protein was eluted with buffer S supplemented with 500 mM imidazole.

The eluate was then concentrated using a 100 kDa MWCO Vivaspin column, centrifuged at 26,000 x g for 15 min, and injected onto a Superdex 200 10/300 GL column equilibrated with buffer S using an Äkta pure (GE) operating at 8° C. The final sample was stored at 15° C.



A. fig. 1. Purification of SARS-CoV-2 Spike protein. **A** – SEC profile. SEC performed using a Superose 6 Increase 10/300 GL column. Asterisk denotes peak containing the protein of interest. Fractions pooled for cryo-EM shaded grey. **B** – Reducing SDS-PAGE. Arrow denotes bands corresponding to the protein of interest.

6.3. SARS-CoV-2 Spike/H11-D4 cryo-EM data collection, processing, and modelling

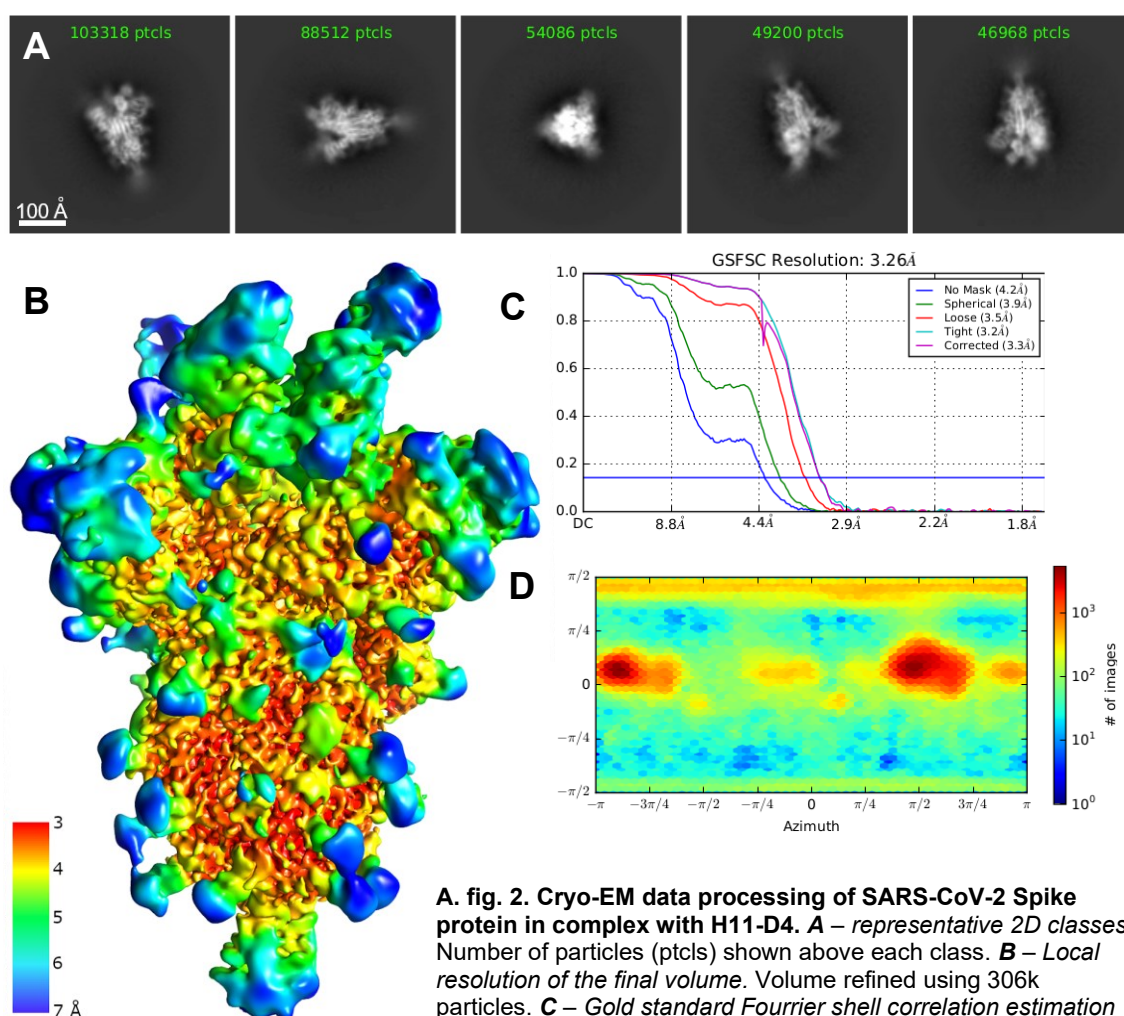
Following a brief incubation of purified Spike (A. fig. 1) with H11-D4 at a 1: 3.6 spike trimer:nanobody molar ratio, the sample was used to prepare cryo-EM grids. Purified H11-D4 nanobody sample was provided by Dr James Naismith (The Rosalind Franklin Institute, UK). Pre-screened grids were sent to the electron Bio-Imaging Centre (eBIC) at Diamon Light Source, where they were imaged using a Titan Krios G2 electron microscope operating at 300 kV, equipped with a K3 camera (Gatan) and a GIF Quantum energy filter (Gatan) with a 20 eV slit. Data were collected in super-resolution counting mode using a custom EPU version 2.5 (Thermo Fisher Scientific). The resulting movies (40 frames each) were 2x-binned and motion-corrected in Relion v3.1

(Zivanov et al., 2018). The final volume was obtained using a data processing strategy described previously (see ‘Cryo-EM data processing’), which also included 3D variability analysis for particle selection.

A previously obtained model of SARS-CoV-2 Spike was used as a starting model (PDB ID: 6VXX). Subdomains of the Spike monomer (residues 27–307; 308–321; 322–333; 334–528; 529–590; 591–700; 701–1147) were individually rigid body fitted within the cryo-EM map using PHENIX (Adams et al., 2011). Due to the low local resolution of the cryo-EM map in the region corresponding to H11-D4, a crystal structure of Spike receptor binding domain (RBD) in complex with H11-D4 (PDB ID: 6YZ5) was superimposed onto the structure and checked for fit in the map. Hydrogen atoms were added to the model using ReadySet (Liebschner et al., 2019). The model was then refined by several rounds of manual inspection in COOT (Emsley et al., 2010) and real-space refinement with PHENIX. Visual representations of the final model were generated using Chimera 1.14 (Pettersen et al., 2004).

6.4. Results

The Spike/H11-D4 particles exhibited preferential orientation in ice (A. fig. 2.D), with most 2D classes containing either sideviews or topviews of the complex (A. fig. 2.A). Despite this, 3D processing resulted in a high resolution reconstruction with a gold standard FSC resolution of 3.3 Å (A. fig. 2.C). The highest local resolutions of 2.8 to 3 Å were observed within the core of the trimer, whereas its N-terminal domains (NTDs) and RBDs had average local resolutions of 5 Å (A. fig. 2.B). The lowest local resolutions of 7 Å were found in densities belonging to glycosylation sites, the outer part of NTDs, as well as H11-D4. 3D variability analysis of the data indicated that this



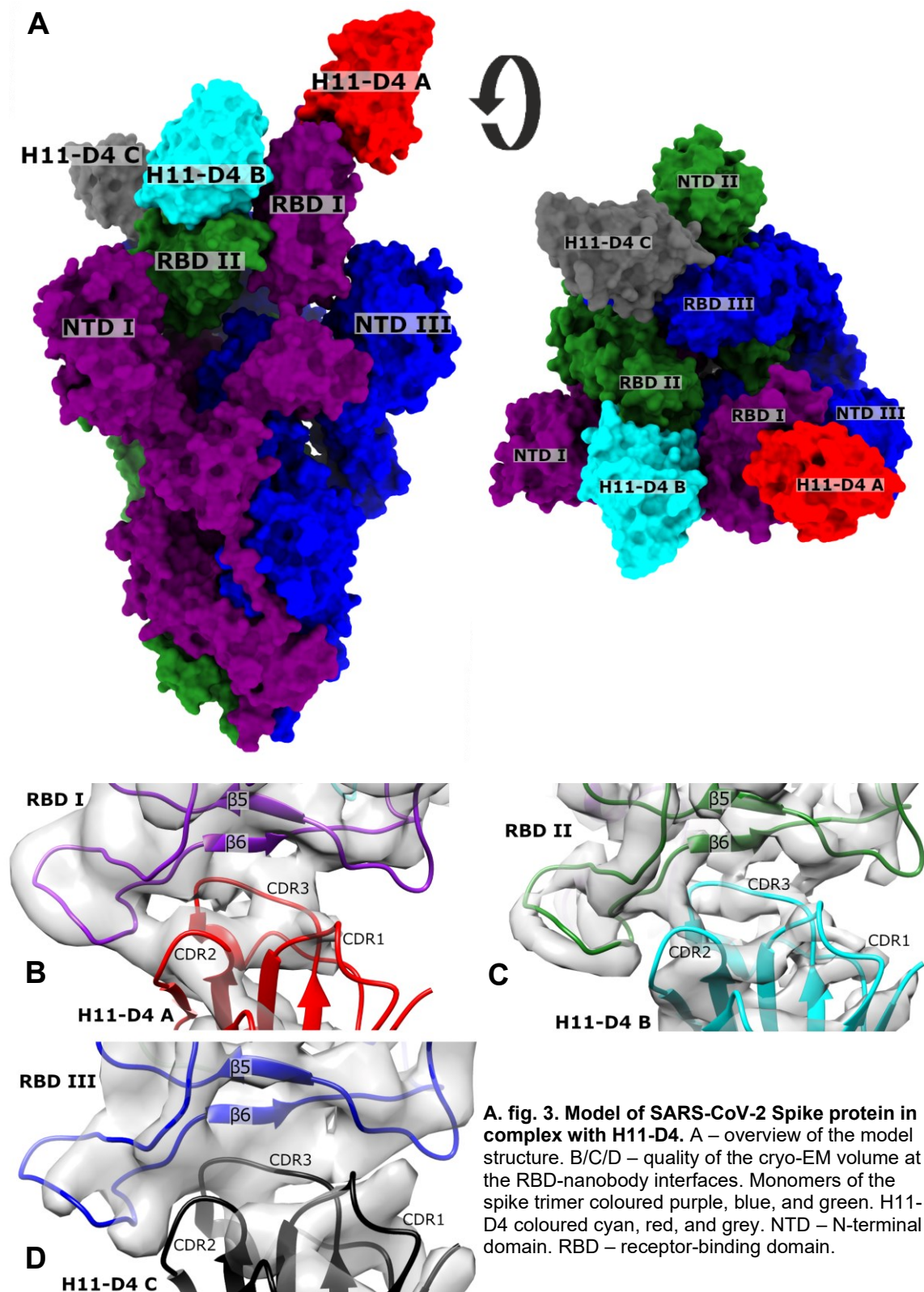
A. fig. 2. Cryo-EM data processing of SARS-CoV-2 Spike protein in complex with H11-D4. *A* – representative 2D classes. Number of particles (ptcls) shown above each class. *B* – Local resolution of the final volume. Volume refined using 306k particles. *C* – Gold standard Fourier shell correlation estimation of the final resolution (3.26 Å). *D* – distribution of particle orientation on cryo-EM grids. For details, see S. Table 4, S. fig. 23, S. fig. 24.

was due to the ‘breathing’ motions of the Spike NTD and RBD domains previously observed in other viral fusion proteins (Wrapp et al., 2020).

The final volume was then used to generate an atomic model of spike in complex with H11-D4, using a previously reported model of Spike in a closed state as a starting point for refinement (PDB ID: 6VXX). Due to the low local resolutions in the vicinity of the RBD-nanobody interface (A. fig. 3.B/C/D), crystal structures of RBD-H11-D4 were rigid-body-fitted inside the cryo-EM volumes.

The model showed that Spike was in a closed state, with one of its RBDs in an ‘up’ position (RBD I, A. fig. 3.A). This has been frequently observed in previously

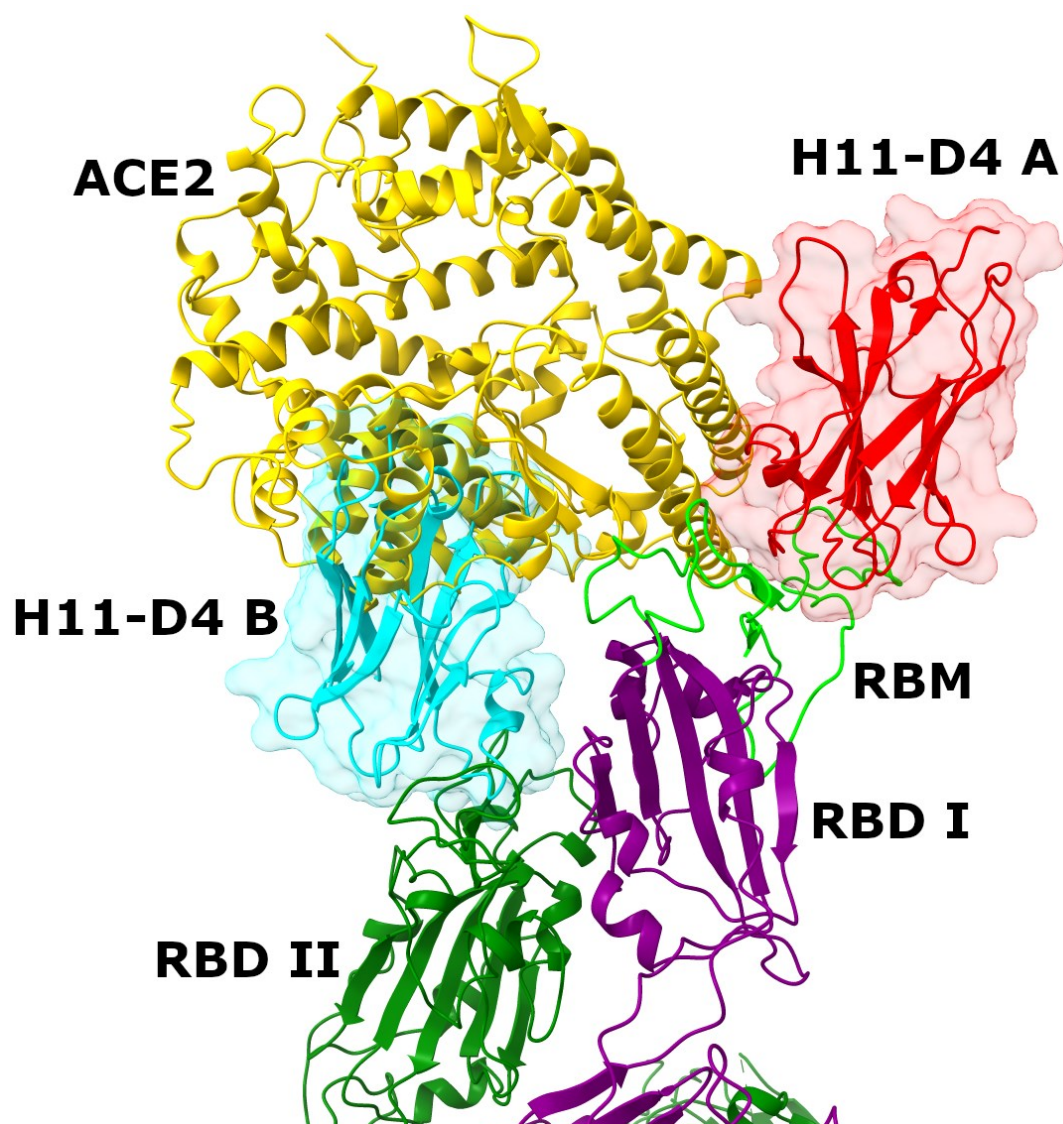
published structures of this protein. Judging from the Coulomb potential maps, the main interactions between RBD and H11-D4 were found between the nanobody CDR3 and the loop region of RBD containing the β -strand 6 (formed by residues 489-496), while



CDR2 also bound to the same loop. The region of RBD recognized by H11-D4 forms a part of the receptor binding motif (residues 438-503), containing 16 residues reported to interact with ACE2 upon interaction (Lan et al., 2020). The interface between H11-D4 and RBD contains 4 of these residues (G446, Y449, Q493, Y489), thus indicating that the ability of Spike to recognize ACE2 in the presence of H11-D4 would be limited.

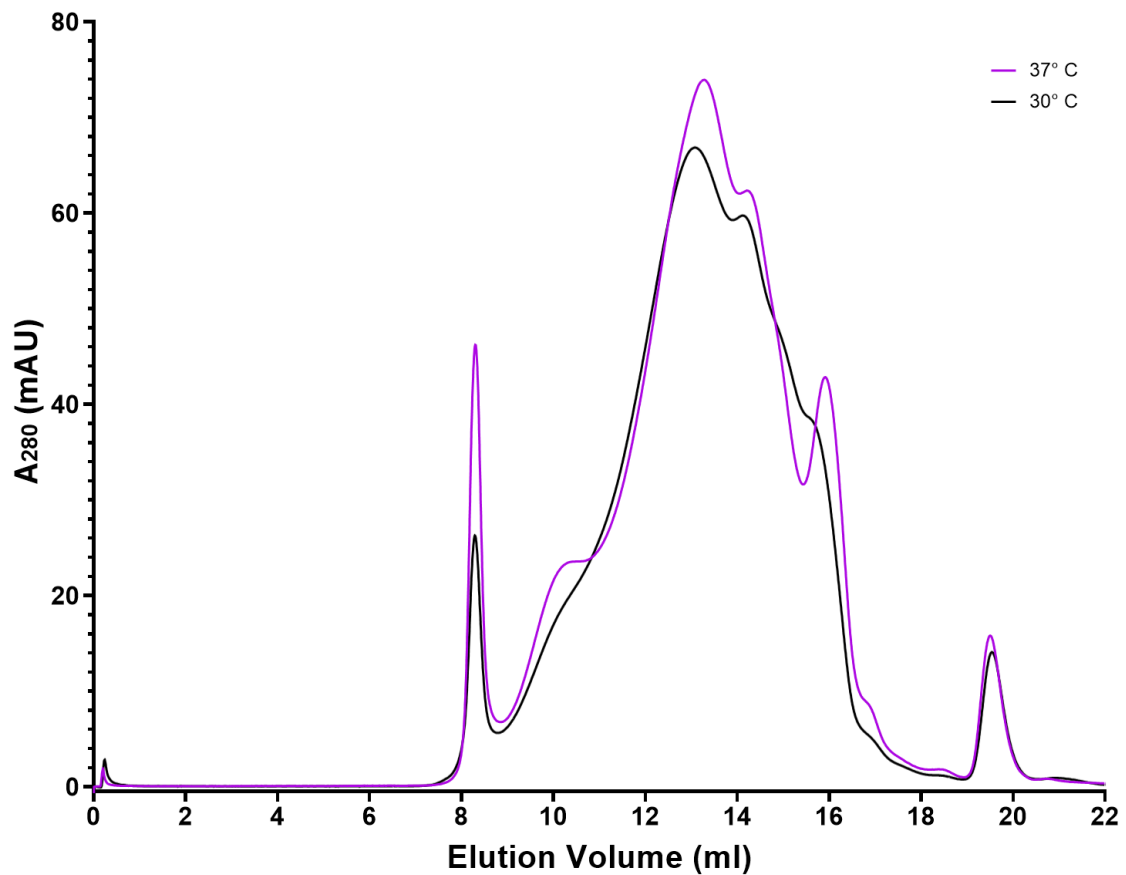
If previously obtained structures of SARS-CoV-2 Spike RBD in complex with ACE2 are aligned with RBD-‘up’ in our structure (Lan et al., 2020), it can be seen that H11-D4 binds on the side of RBM that is facing away from the RBD-ACE2 interface (A. fig. 4). Although the nanobody binding to RBD-‘up’ does slightly overlap with ACE2 within the alignment, it appears that the most important factor limiting ACE2 binding to H11-D4 bound Spike is the nanobody binding to an adjacent RBD found in a ‘down’ position, since almost half of its volume overlaps with ACE2.

Since the RBM of SARS-CoV-2 Spike is only accessible to ACE2 binding in an ‘up’ conformation, and because cryo-EM examination of Spike proteins usually shows a mixture of ‘up’/‘down’ positions of its RBD domains, it is thought that the Spike RBD tend to stochastically switch between the two possible orientations, thus enabling ACE2 binding. Based on the results presented here, H11-D4 bound to RBD-‘down’ would prevent the interaction between any RBD domains within the Spike protein that enter an ‘up’ orientation, thus neutralizing the virus.

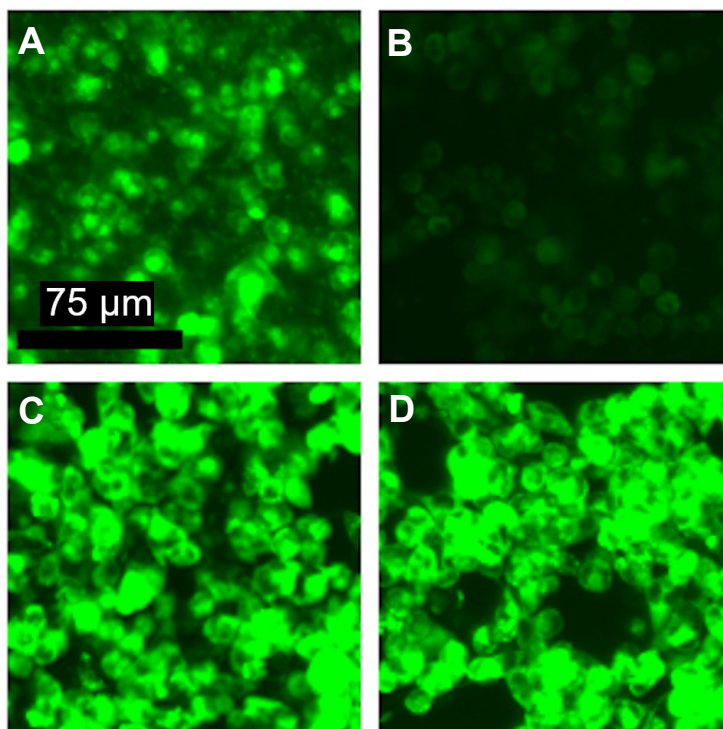


A. fig. 4. Alignment of SARS-CoV-2 Spike/H11-D4 with a SARS-CoV-2 RBD/ACE2 complex. RBD I and RBD II are in 'up' and 'down' conformations respectively. Alignment was performed using 6M0J (Lan et al., 2020), with an RMSD value of 0.65 Å. RBM of RBD I coloured lime.

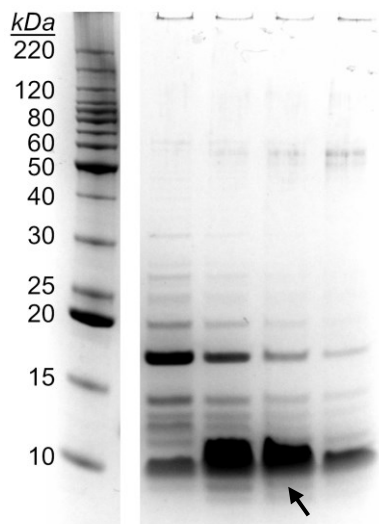
7. Appendix II – Supplementary data



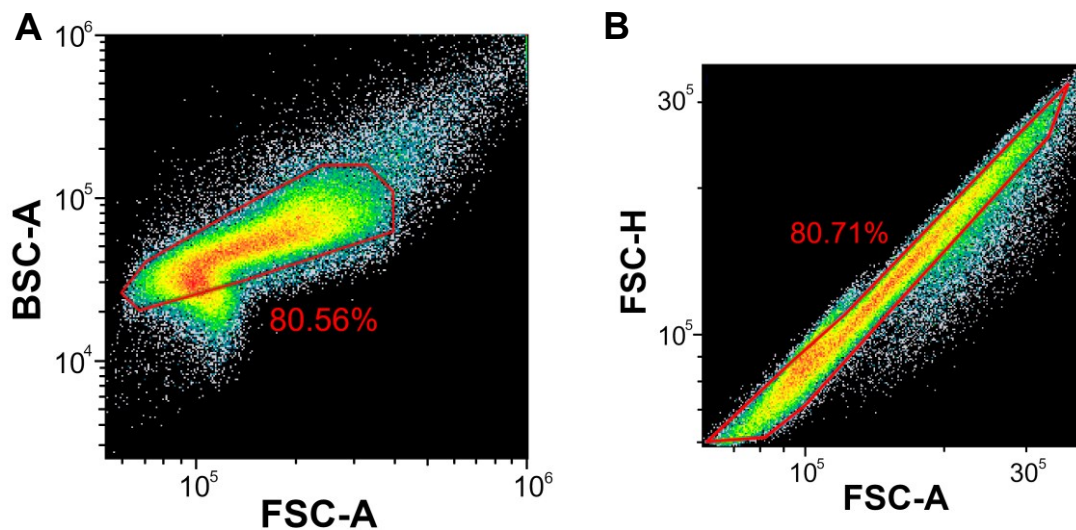
S. fig. 1. SEC of Fzd6-5(M237C/A348C) expressed at 30° C. SEC curve for protein expressed at 37° C shown for comparison.



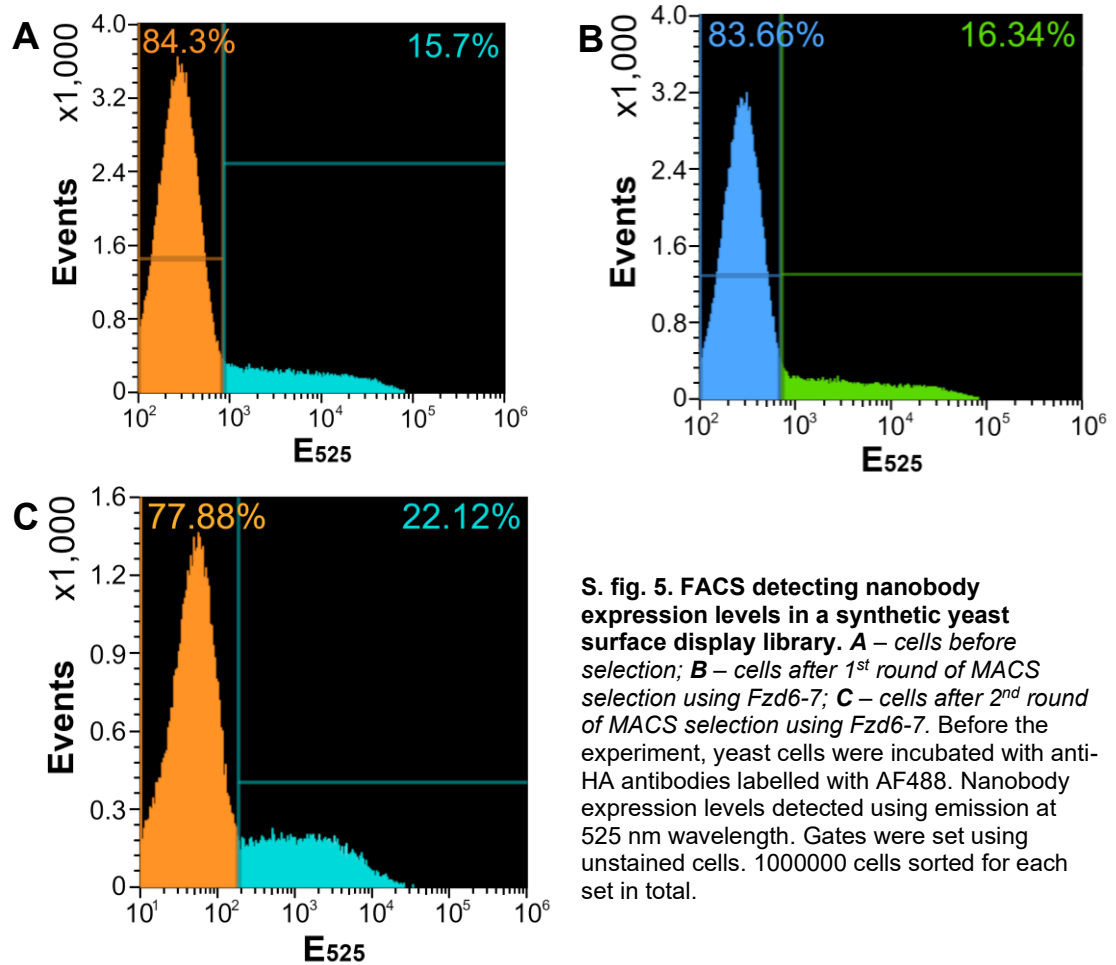
S. fig. 2. Localization of different proteins expressed in HEK293S GnTI⁻ TetR cells. **A** – ROR2-2; **B** – LRP6-3; **C** – Wnt3A + AFM; **D** – Wnt5A + AFM. All cells were imaged following induction of protein expression for 72 h with 10 μg/ml doxycycline. 600 ms exposure.



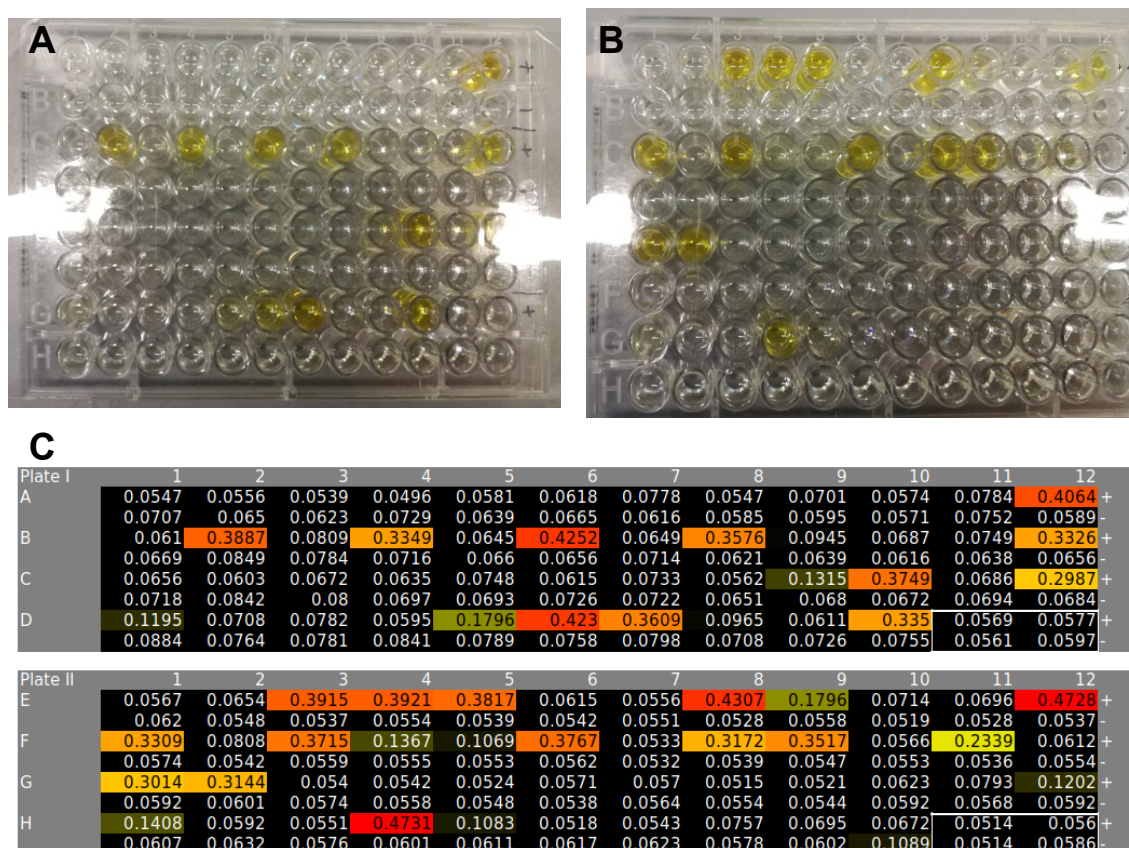
S. fig. 3. Reducing SDS-PAGE of Dvl1-PDZ without sample heating. Lanes contain samples taken from SEC fractions eluting at 86-92 ml (see Fig. 62.A). Arrow denotes bands corresponding to the protein of interest.



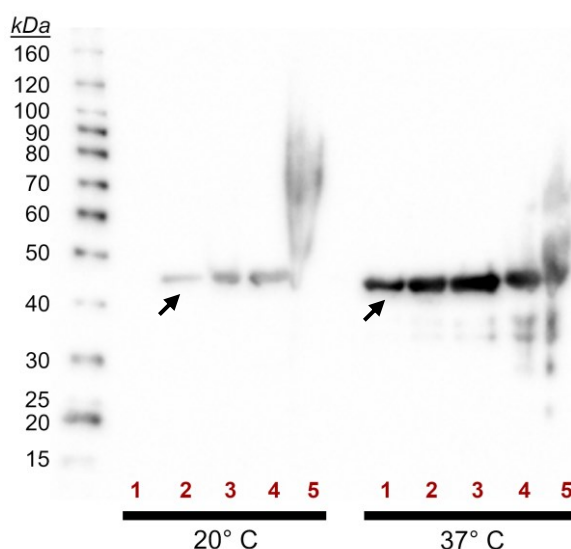
S. fig. 4. Selection of yeast cells for FACS. **A** – Front scatter (FSC) versus back scatter (BSC) plot. **B** – FSC height (FSC-A) versus FSC height (FSC-H) plot. Cells selected for FSC-A/FSC-H analysis are gated with a red line in the FSC/BSC plot. Cells selected for fluorescence analysis are gated with a red line in the FSC-A/FSC-H plot. FSC correlates to cell size, BSC inversely correlates to cell granularity and density. FSC signal area and height are compared to remove cell doublets from the final analysis.



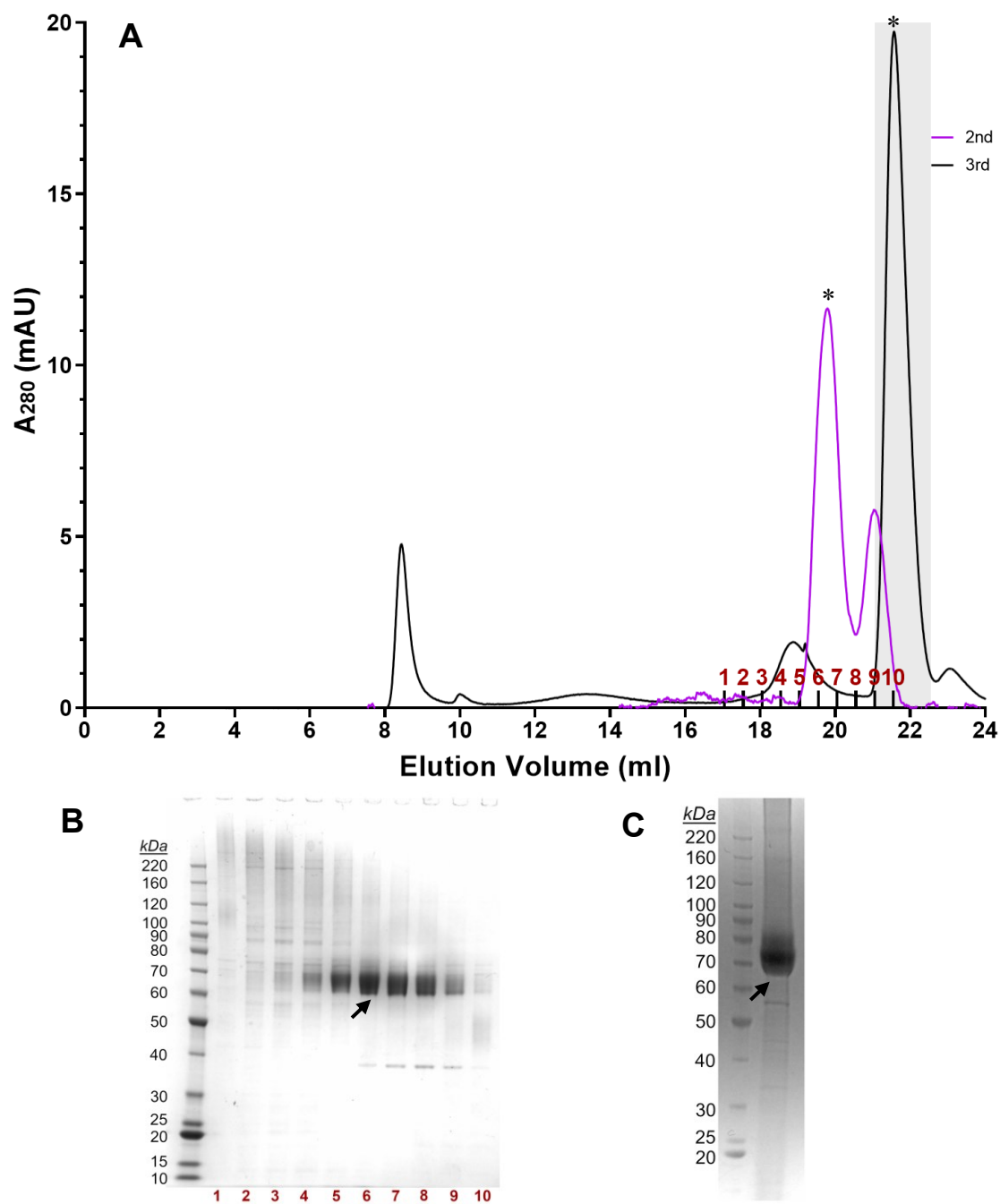
S. fig. 5. FACS detecting nanobody expression levels in a synthetic yeast surface display library. A – cells before selection; B – cells after 1st round of MACS selection using Fzd6-7; C – cells after 2nd round of MACS selection using Fzd6-7. Before the experiment, yeast cells were incubated with anti-HA antibodies labelled with AF488. Nanobody expression levels detected using emission at 525 nm wavelength. Gates were set using unstained cells. 1000000 cells sorted for each set in total.



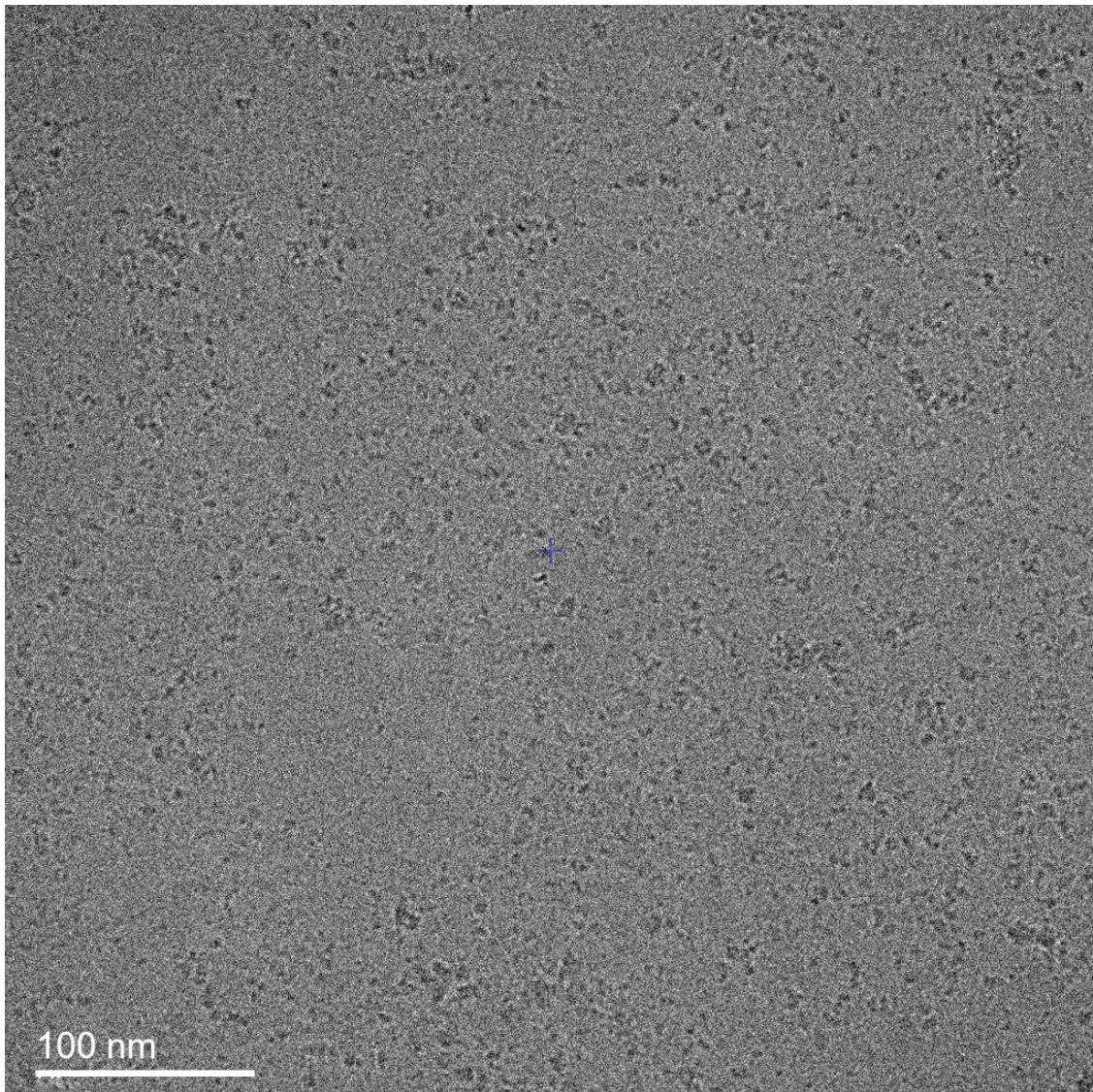
S. fig. 6. ELISA selection of nanobodies from a yeast surface display library. A – plate I; B – plate II; C – 430 nm absorbance of each well from plates I and II. Following FACS selection, the genes encoding nanobodies were isolated from yeast cells and subcloned into a bacterial expression vector. The nanobodies were expressed, and the cells lysed. Each plate contains 48 different lysates. Every other row starting with the first was coated with biotinylated Fzd6-7, whereas the remaining contained only streptavidin (negative control, see '+' and '-' on the right of C). The negative control for each lysate was located below the Fzd6-7-coated well (e.g. 'A1+' and 'A1-' in C).



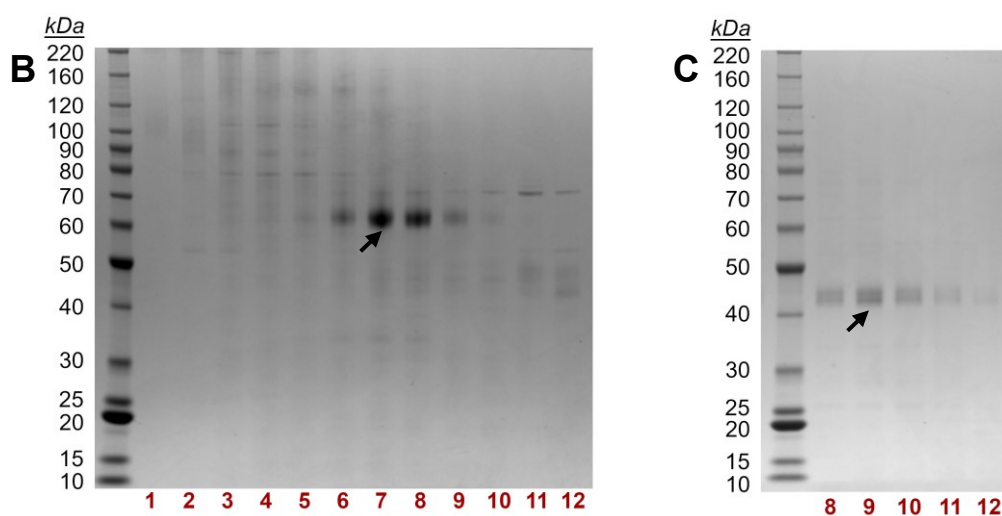
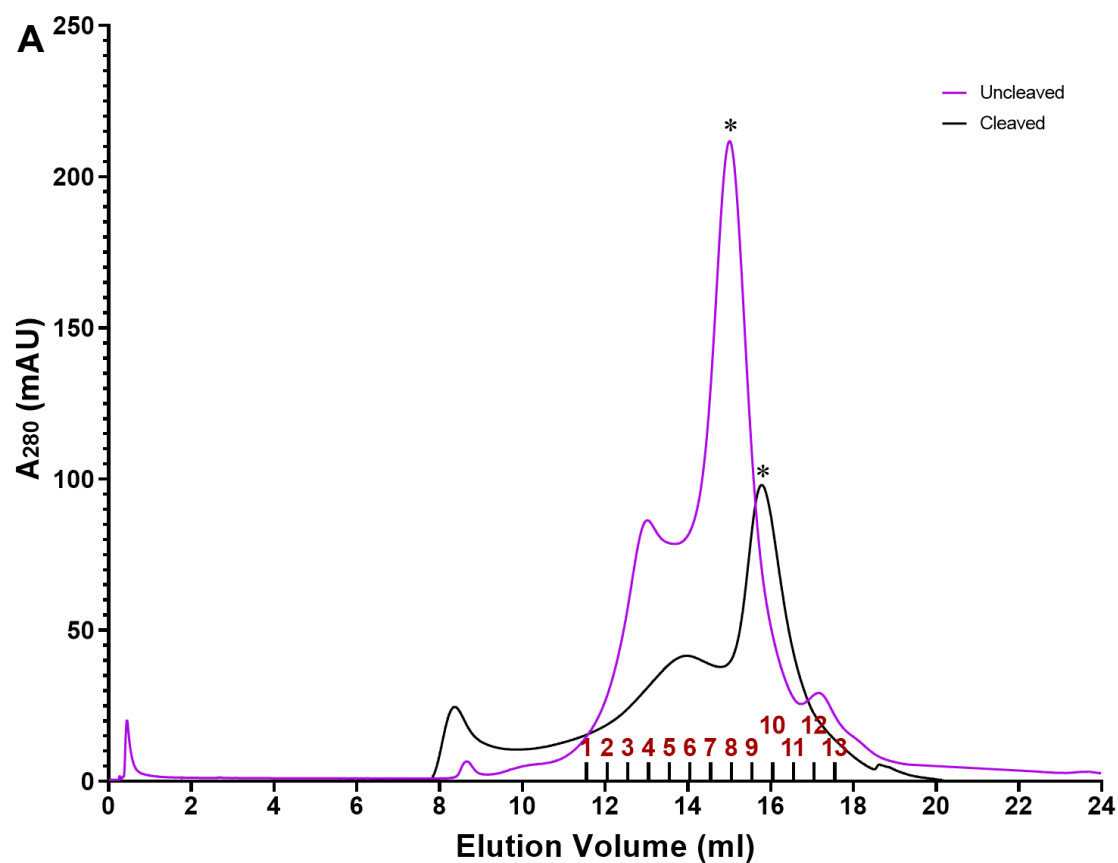
S. fig. 7. MSP2N2 expression test. MSP2N2 was expressed for 1 h in *E. coli* Rosetta 2(DE3) cells at either 20° or 37° C. Protein induction was performed at various OD₆₀₀ values: (1) - 0.3; (2) - 0.8; (3) - 1.2; (4) - 2.0; (5) - 2.5. 1 ml of cell culture was then used for cell lysis, and the resulting lysates used to perform a western blot using anti-6xHis primary antibody. Since degradation bands (below 40 kDa) are only present when higher amounts of protein were loaded, the results were inconclusive. Arrows denote bands corresponding to the proteins of interest. Experiments performed under non-reducing conditions.



S. fig. 8. Second and third purification of Fzd6-5 SMALPs. **A** – SEC profiles. SEC performed using a Superose 6 Increase 10/300 GL column. Asterisks denote peaks containing SMALPs. Shaded area indicates the fractions of the third purification used for SDS-PAGE. **B** – Non-reducing SDS-PAGE of second purification SEC fractions. **C** – Non-reducing SDS-PAGE of the sample obtained from the third purification. Arrows denote bands corresponding to the proteins of interest.



S. fig. 9. Examination of Fzd6-5/Wnt5A SMALPs by cryo-EM. Grid (Quantifoil R2/2) prepared using sample at 0.73 mg/ml concentration. Micrograph recorded using a Glacios cryo-TEM (FEI) operated at 200 kV, with a Falcon 3 camera at a 92,000 x magnification in linear mode.

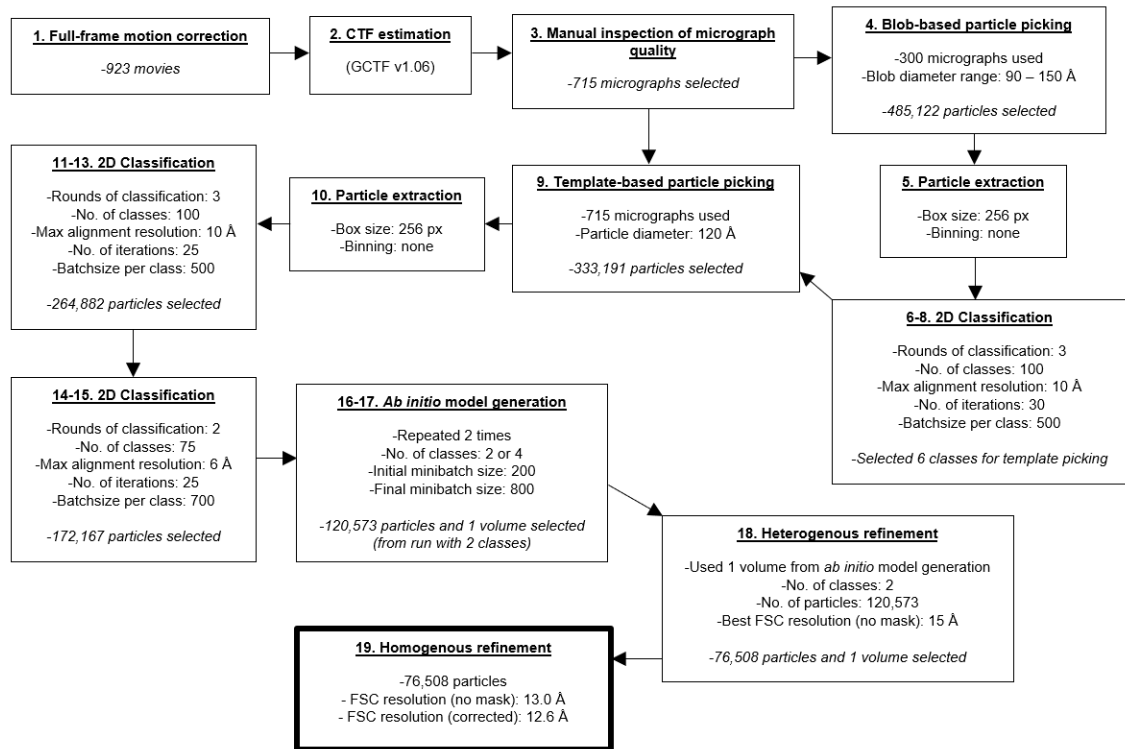
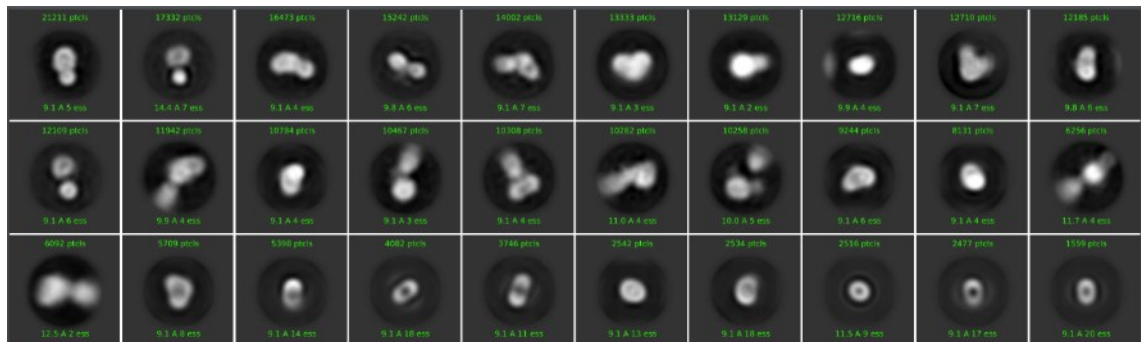
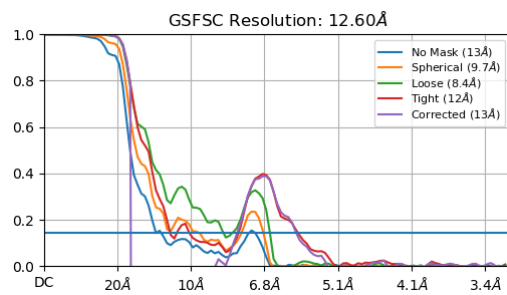
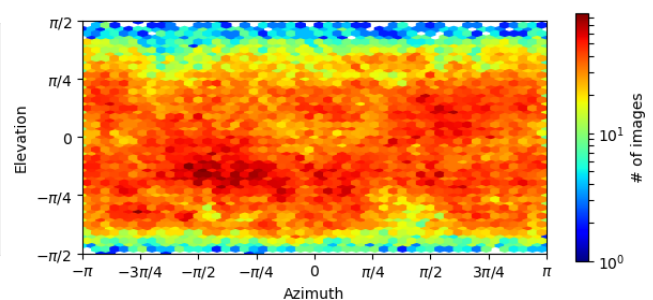


S. fig. 10. Purification of 3C-cleaved and uncleaved Fzd4-1 in DDM. A – SEC. Performed using a Superose 6 Increase 10/300 GL column. Asterisks denote peaks containing the proteins of interest. **B – SDS-PAGE of uncleaved Fzd4-1. C – SDS-PAGE of cleaved Fzd4-1.** Arrows denote bands corresponding to the proteins of interest. Experiments performed under non-reducing conditions.

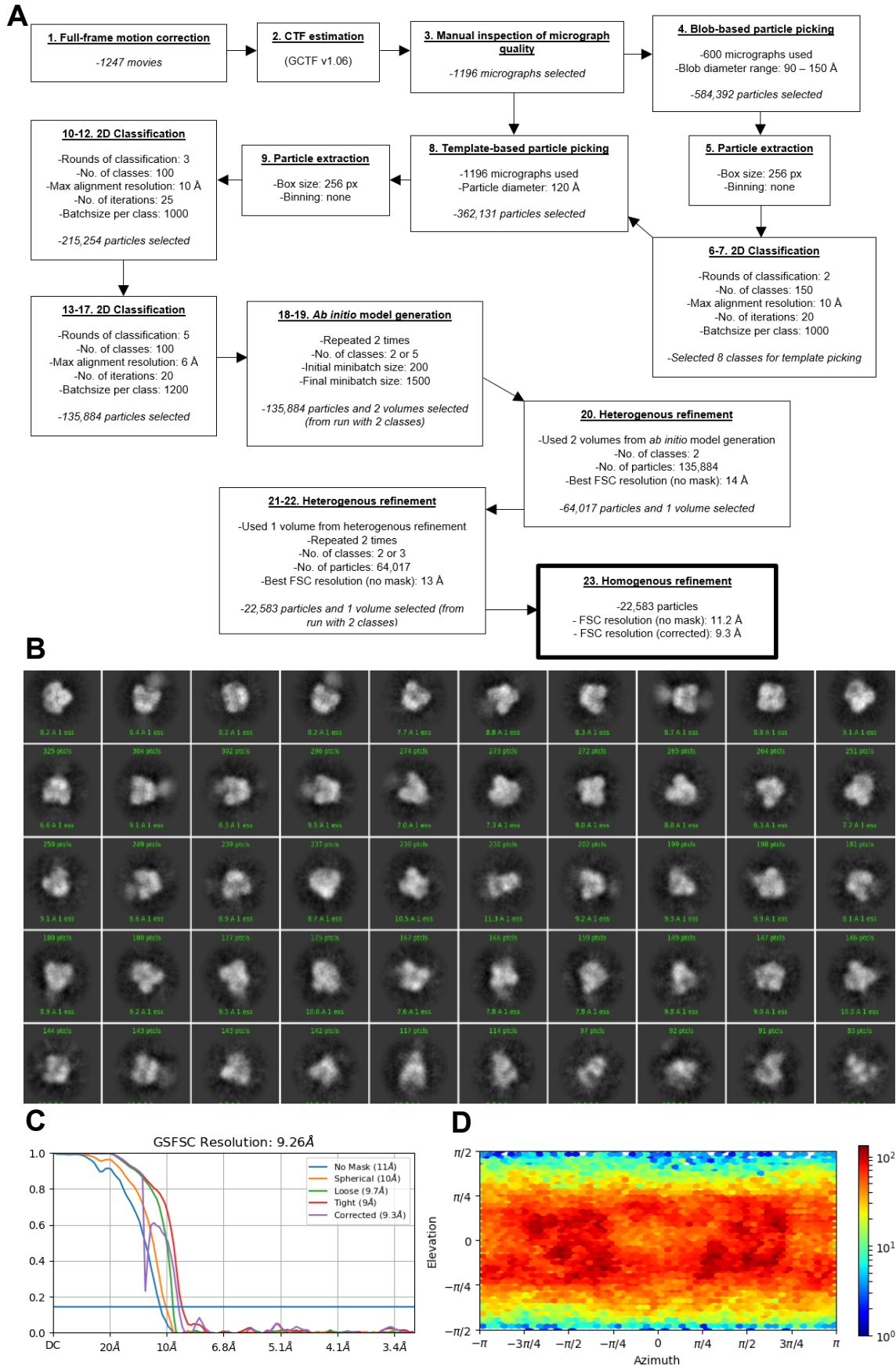
	Fzd6-5 in SMALPs	Fzd4-1/Ndp1 in SMALPs	Fzd4-1/Ndp1/F2.A-2 in SMALPs
Data collection and processing			
Magnification	76,000x	92,000x	92,000x
Voltage (kV)	300	200	200
Electron exposure (e-/Å ²)	31	58	58
Defocus range (μm)	0.3-1.0	1.2-2.7	1.2-2.7
Pixel size (Å/pixel)	1.08	1.55	1.55
Symmetry imposed	C1	C1	-
Initial particle images (no.)	333,191	362,131	-
Final particle images (no.)	76,508	22,583	-
Map resolution (Å)	12.6	9.3	-
FSC threshold	0.143	0.143	-

	Fzd4/F2.A in DDM	Fzd4-1/Ndp3/F2.A-2 in DDM	Fzd4-1/Ndp3/F2.A-2 in DDM (cleaved)
Data collection and processing			
Magnification	59,000x	92,000x	59,000x
Voltage (kV)	300	200	300
Electron exposure (e-/Å ²)	60	60	60
Defocus range (μm)	1.2-3.0	1.2-3.0	1.2-3.0
Pixel size (Å/pixel)	1.4	1.6	1.4
Symmetry imposed	C1	C1	C1
Initial particle images (no.)	1,208,793	1,916,316	3,798,854
Final particle images (no.)	34,885	83,135	106,842
Map resolution (Å)	16.0	8.0	6.2
FSC threshold	0.143	0.143	0.143

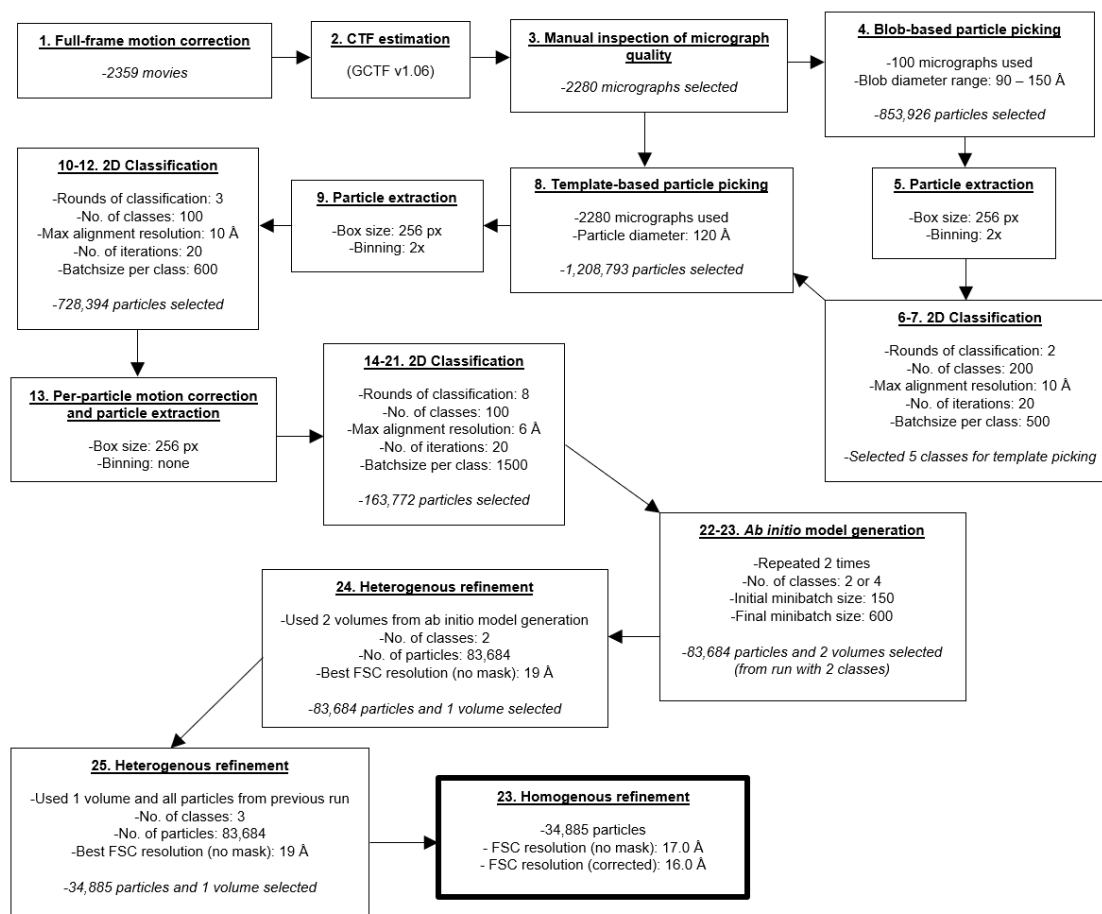
S. Table 1. Cryo-EM data collection and processing statistics. The number of initial particle images refers to the number of particles obtained following the first .

A**B****C****D**

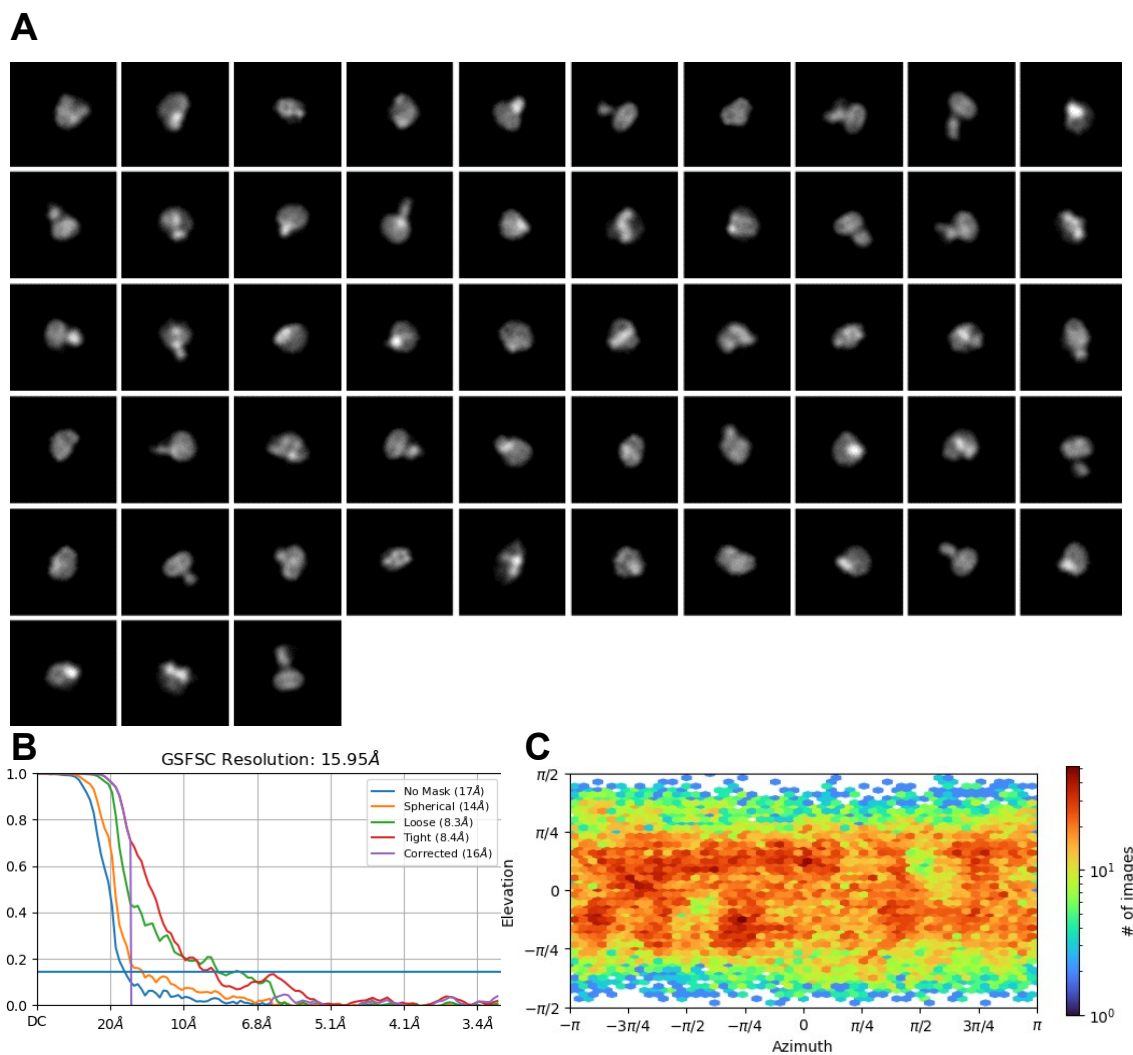
S. fig. 11. Cryo-EM processing of Fzd6-5 in SMALPs. **A** – Data processing. Every step performed in Cryosparc v3.2 using default settings, unless otherwise specified. **B** – Best 2D classes. From (A.15). **C** – Gold-standard FSC resolution estimation for the final volume. From (A.19). **D** – particle orientation distribution. From (A.19).



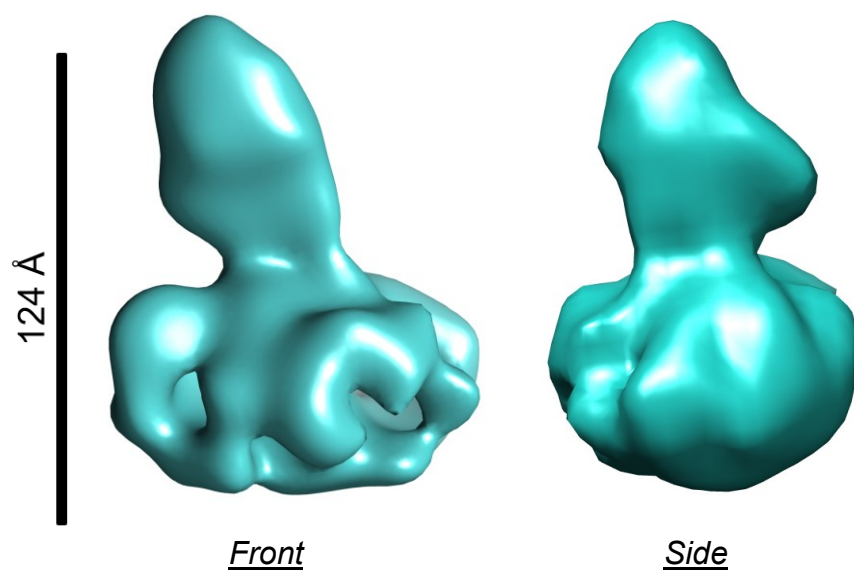
S. fig. 12. Cryo-EM processing of Fzd4-1/Ndp1 in SMALPs. A – Data processing. Every step performed in Cryosparc v3.2 using default settings, unless otherwise specified. **B – Best 2D classes.** From (A.17). **C – Gold-standard FSC resolution estimation for the final volume.** From (A.23). **D – particle orientation distribution.** From (A.23).



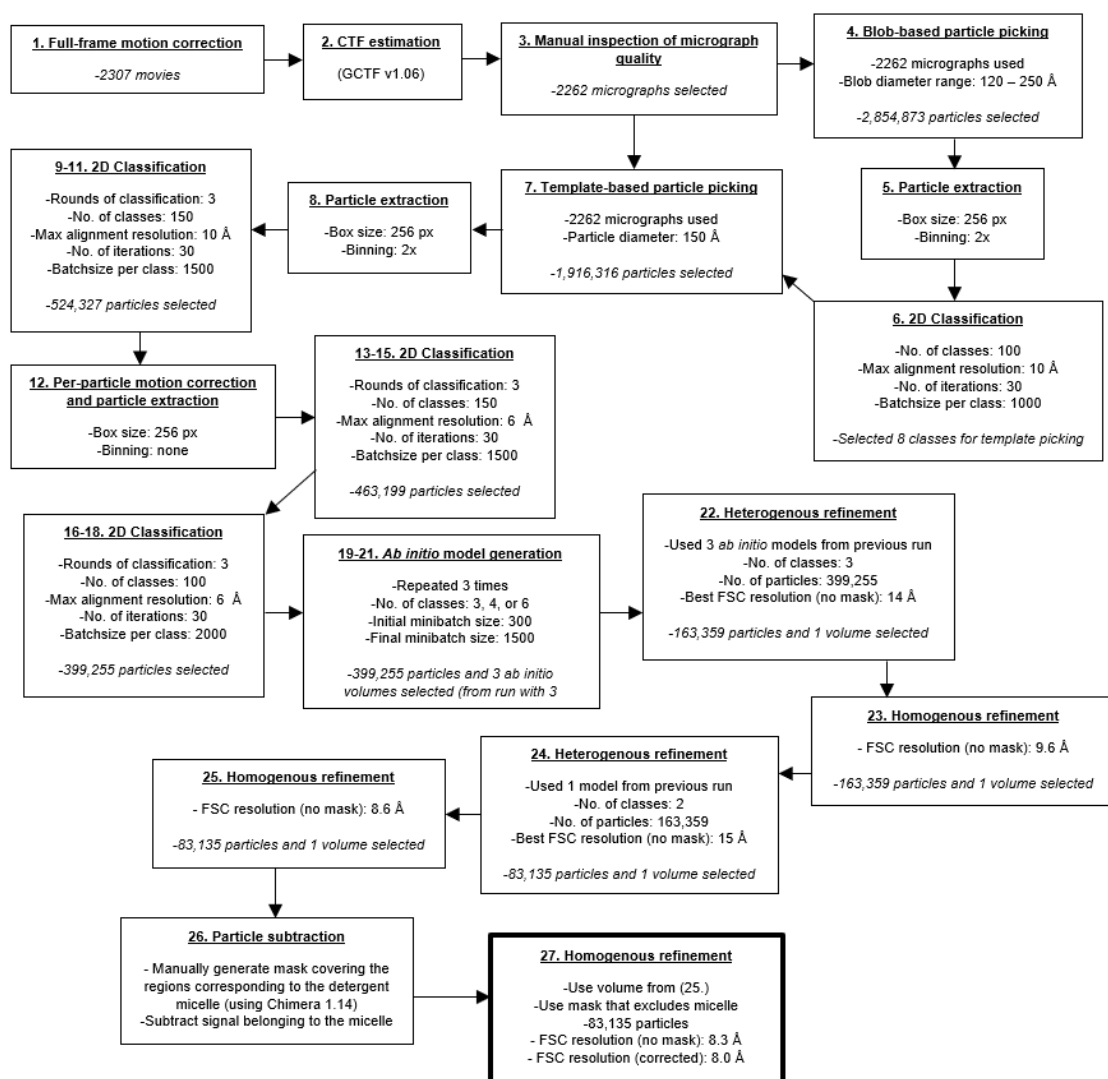
S. fig. 13. Cryo-EM processing of Fzd4-1/F2.A in DDM. Every step performed in Cryosparc v3.2 using default settings, unless otherwise specified.



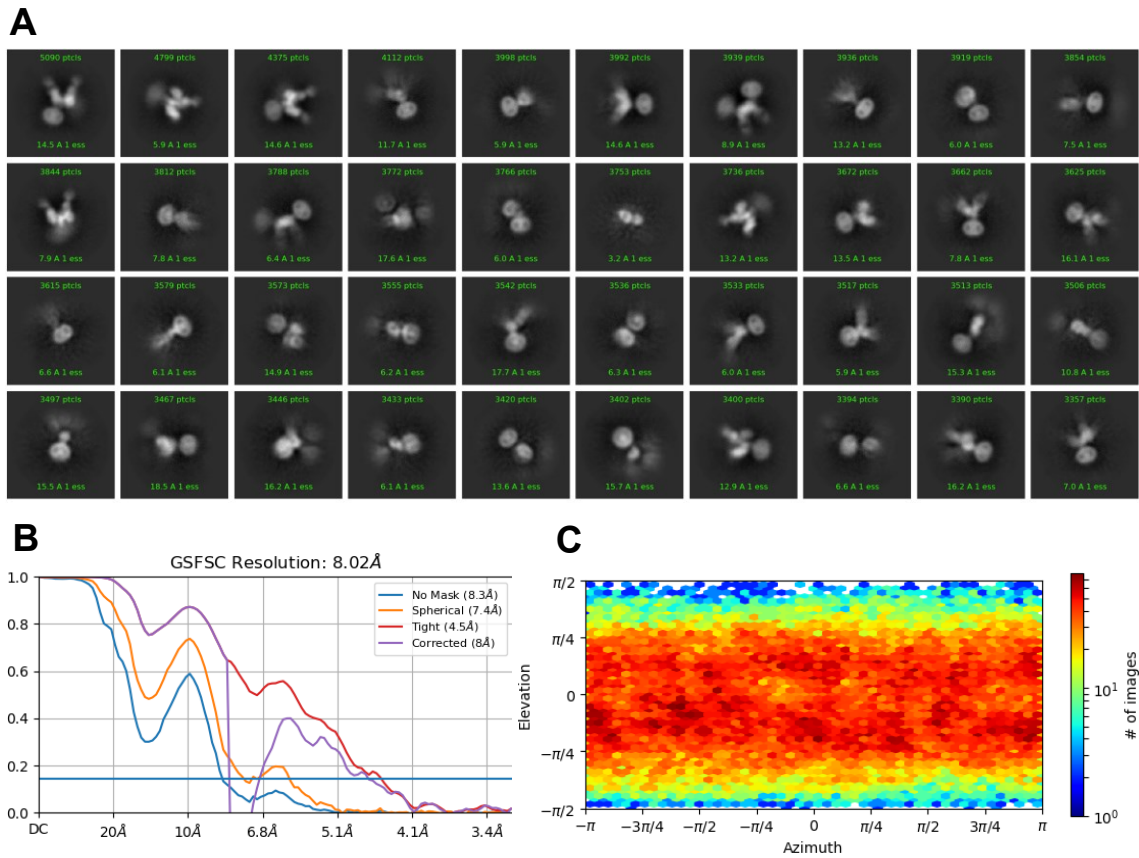
S. fig. 14. Cryo-EM processing of Fzd4-1/F2.A in DDM. A – Best 2D classes. From S. fig. 13.21. **B – Gold-standard FSC resolution estimation for the final volume.** From S. fig. 13.23. **C – particle orientation distribution.** From S. fig. 13.23.



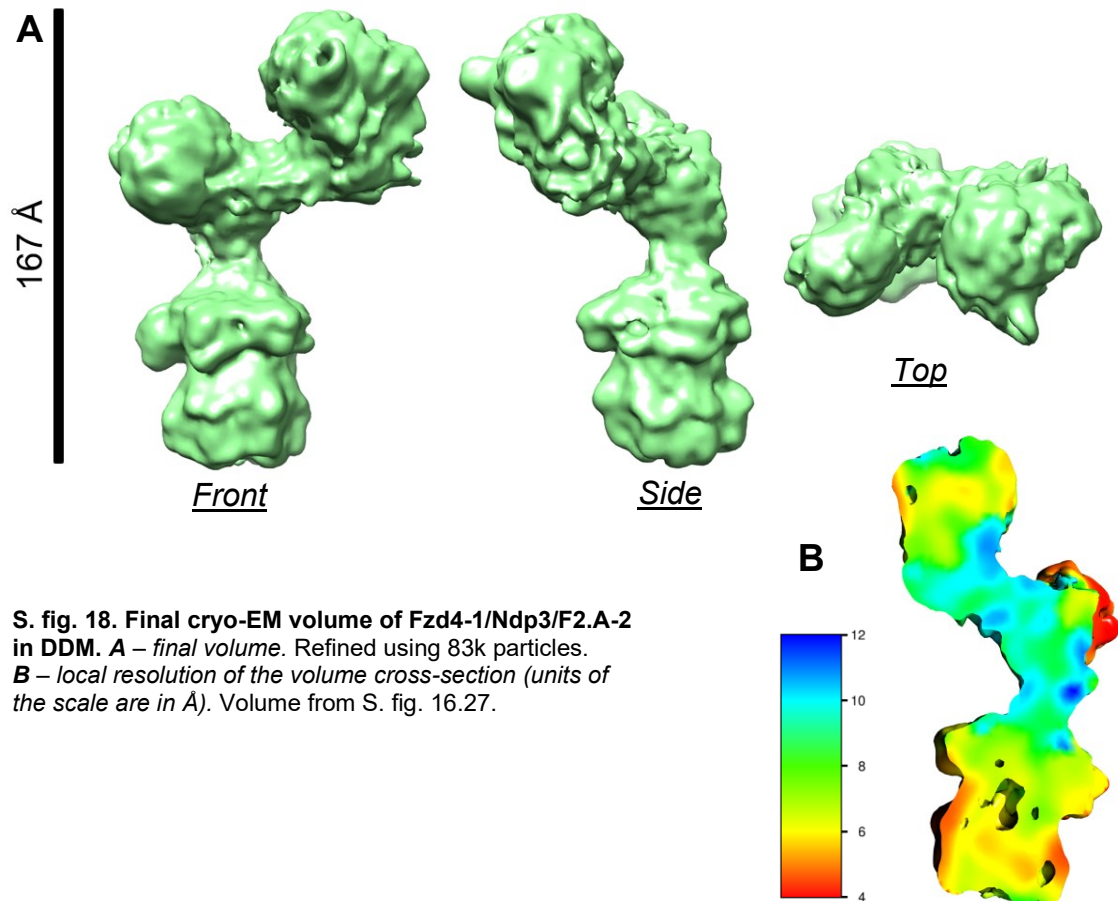
S. fig. 15. Final cryo-EM volume of Fzd4-1/F2.A in DDM. Refined using 35k particles. From S. fig. 13.23.



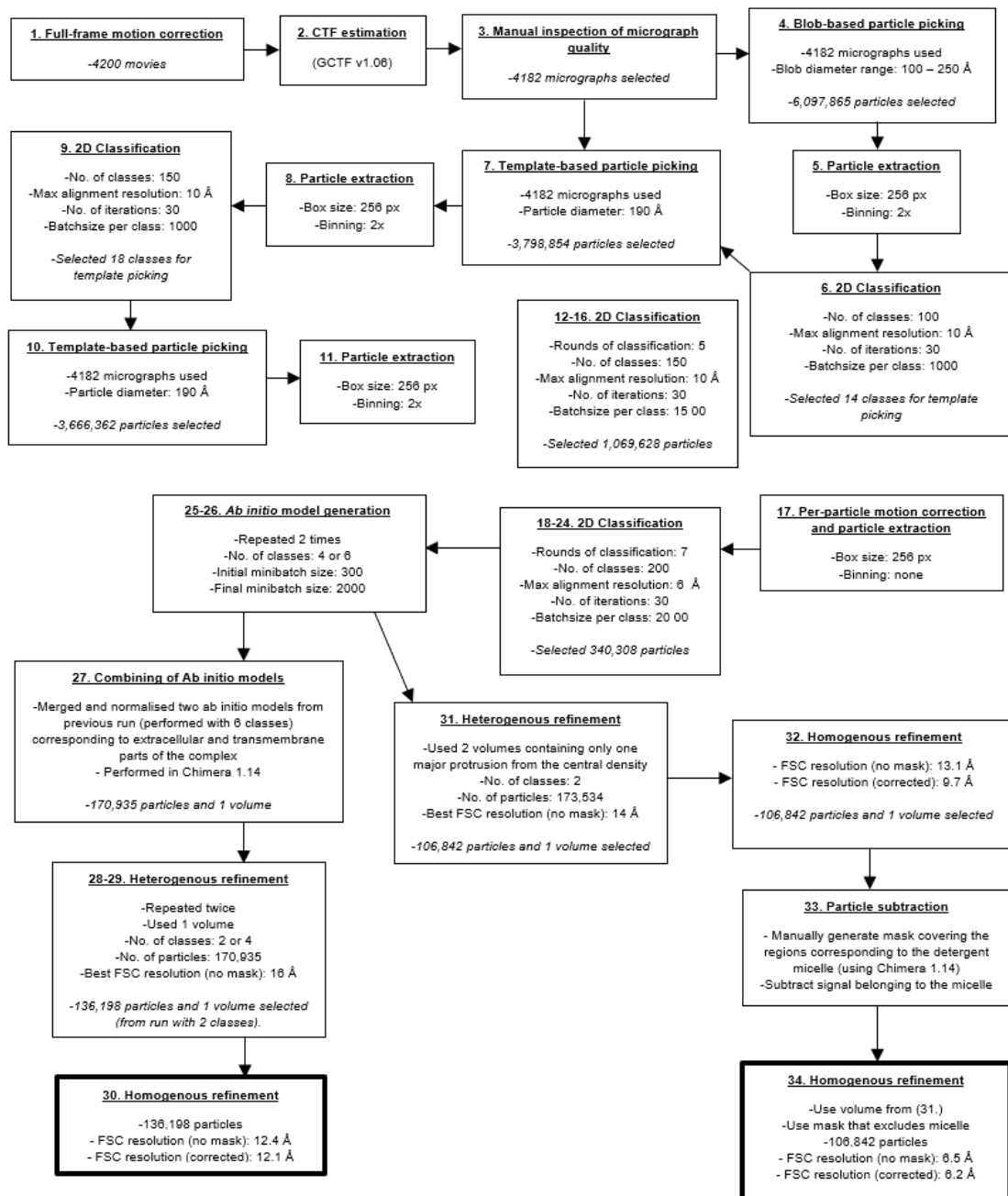
S. fig. 16. Cryo-EM processing of Fzd4-1/Ndp3/F2.A-2 in DDM. Every step performed in Cryosparc v3.2 using default settings, unless otherwise specified.



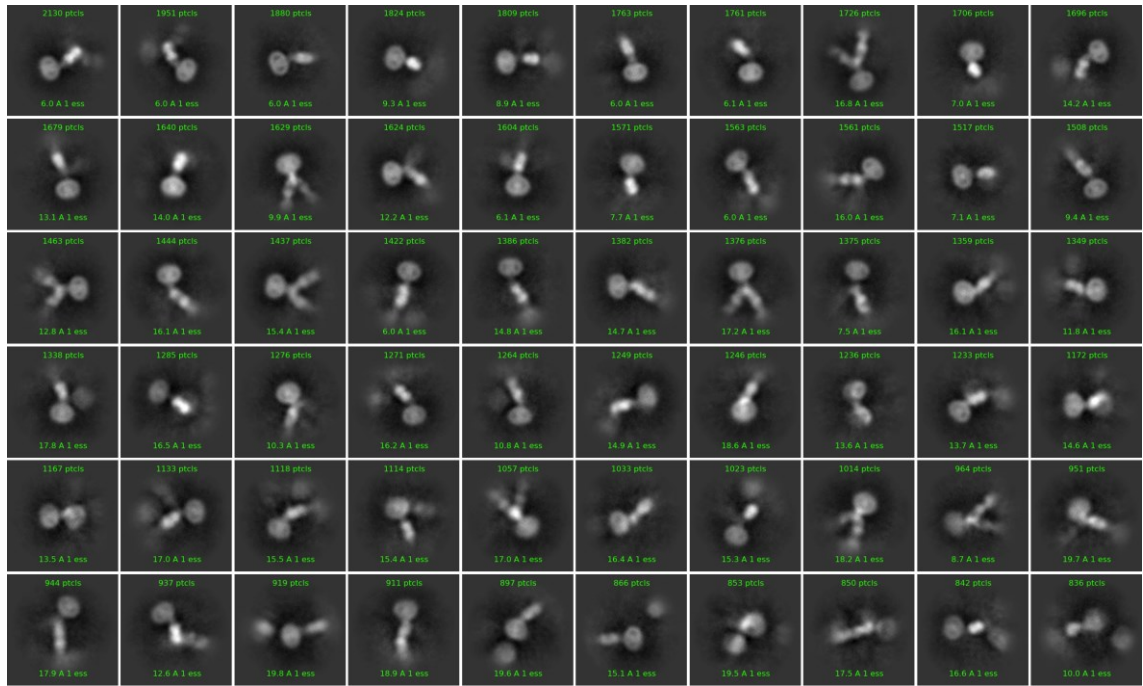
S. fig. 17. Cryo-EM processing of Fzd4-1/Ndp3/F2.A-2 in DDM. A – Final 2D classes. From S. fig. 16.18. **B – Gold-standard FSC resolution estimation for the final volume.** From S. fig. 16.27. **C – particle orientation distribution.** From S. fig. 16.27.



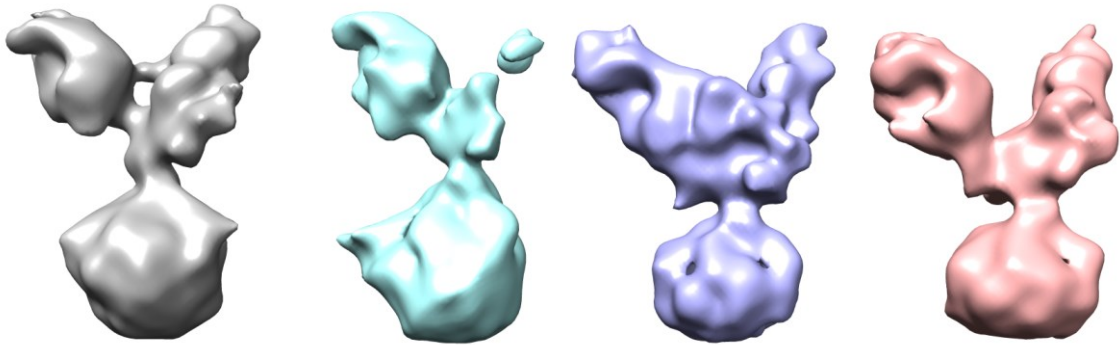
S. fig. 18. Final cryo-EM volume of Fzd4-1/Ndp3/F2.A-2 in DDM. A – final volume. Refined using 83k particles. **B – local resolution of the volume cross-section (units of the scale are in Å).** Volume from S. fig. 16.27.



S. fig. 19. Cryo-EM processing of Fzd4-1/Ndp3/F2.A-2 in DDM (cleaved). Every step performed in Cryosparc v3.2 using default settings, unless otherwise specified.



S. fig. 20. Cryo-EM 2D class averages of Fzd4-1/Ndp3/F2.A-2 in DDM (cleaved). From S. fig. 19.24.



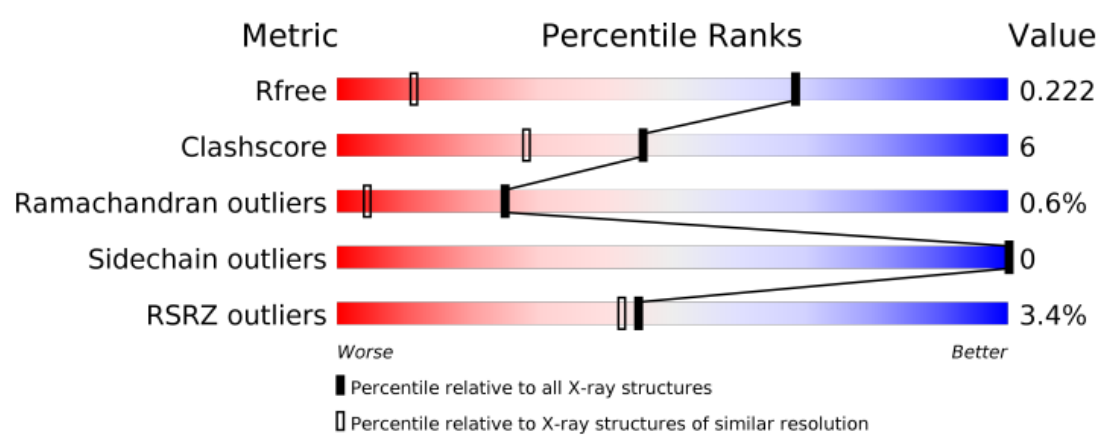
S. fig. 21. Heterogenous refinement of Fzd4-1/Ndp3/F2.A-2 complex (cleaved) in DDM. Original volume obtained by combining *ab initio* models (see Fig. 68). Note the differences in distance between the detergent micelle (bottom) and the extracellular domains. From S. fig. 19.29 (run with 4 classes). Approximately 33k particles per class.

	67	223	263 (6ZVL)	267	276
Data collection					
X-ray source (Diamond)	I04-1	I04-1	I04-1	I04-1	I04-1
Wavelength (Å)	0.9159	0.9159	0.9159	0.9159	0.9159
Space group	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁
Cell dimensions					
a, b, c (Å)	60.37, 71.77, 78.71	60.24, 71.91, 78.53	59.8, 71.80, 78.0	59.95, 71.29, 77.90	60.14, 71.86, 78.26
α, β, γ (°)	90, 90, 90	90, 90, 90	90, 90, 90	90, 90, 90	90, 90, 90
Resolution	2.00 (2.07 - 2.00)	1.70 (1.76-1.70)	1.30 (1.32 - 1.30)	1.80 (1.86 - 1.80)	1.75 (1.81 - 1.75)
R _{merge}	0.04 (0.39)	0.02 (0.46)	0.08 (0.92)	0.03 (0.44)	0.03 (0.56)
I / σI	11.11 (1.80)	16.27 (1.57)	14.3 (1.80)	13.14 (1.59)	13.70 (1.28)
CC _{0.5}	0.99 (0.73)	1.00 (0.69)	0.99 (0.69)	1.00 (0.75)	0.99 (0.67)
Completeness (%)	99.89 (99.66)	99.99 (99.97)	99.92 (96.6)	99.88 (99.94)	99.91 (99.97)
Redundancy	12.1 (12.7)	11.9 (8.3)	12.3 (10.0)	11.4 (10.3)	12.2 (12.8)
Refinement					
Resolution (Å)	39.35 - 2.00	47.80 - 1.70	52.80 - 1.30	24.40 - 1.80	24.22 - 1.75
No. Reflections	47415 (4632)	76431 (3758)	81769 (7674)	63188 (6242)	69740 (6832)
R _{work} / R _{free}	0.195 / 0.253	0.187 / 0.210	0.201 / 0.222	0.196 / 0.223	0.180 / 0.202
<i>No. Atoms</i>					
Protein	2891	2888	3000	2878	2897
Ligand / other	18 / 57	18 / 60	17 / 46	15 / 63	16 / 53
Water	121	147	120	193	156
<i>B factors (Å²)</i>					
All atoms	39	37	24	35	37
Ligand	34	38	46	42	59
<i>R.m.s. Deviations</i>					
Bond lengths (Å)	0.009	0.007	0.008	0.009	0.019
Bond angles (°)	1.18	1.11	1.46	1.41	1.74

S. Table 2. X-ray data collection and refinement statistics for Notum in complex with various compounds. Values in parentheses are for highest-resolution shell.

	1128	1147	1185	1233	1241
Data collection					
X-ray source (Diamond)	I04-1	I04-1	I04-1	I04-1	I04-1
Wavelength (Å)	0.9201	0.9201	0.9201	0.9201	0.9201
Space group	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁
Cell dimensions					
a, b, c (Å)	59.89, 71.63, 78.29	59.97, 71.57, 78.26	60.07, 71.50, 78.36	59.74, 71.76, 78.14	59.79, 71.73, 78.25
α, β, γ (°)	90, 90, 90	90, 90, 90	90, 90, 90	90, 90, 90	90, 90, 90
Resolution	1.40 (1.45-1.40)	1.40 (1.45 - 1.40)	1.41 (1.46 - 1.41)	1.40 (1.45-1.4)	1.40 (1.45-1.40)
R _{merge}	0.02 (0.31)	0.01 (0.23)	0.02 (0.56)	0.02 (0.34)	0.01 (0.33)
I / σI	18.76 (2.11)	19.37 (2.64)	12.94 (1.13)	15.59 (1.70)	21.12 (1.85)
CC _{0.5}	0.99 (0.85)	1.00 (0.88)	1.00 (0.54)	1.00 (0.81)	1.00 (0.82)
Completeness (%)	99.98 (99.98)	99.97 (99.97)	99.97 (99.95)	99.96 (99.88)	99.92 (99.98)
Redundancy	12.5 (13.1)	13.6 (12.5)	12.8 (11.3)	13.0 (13.2)	10.8 (10.2)
Refinement					
Resolution (Å)	52.81 - 1.40	52.82 - 1.40	47.67 - 1.41	47.46 - 1.40	52.88 - 1.40
No. Reflections	66908 (6811)	133932 (13206)	131438 (6490)	133592 (6588)	133743 (6615)
R _{work} / R _{free}	0.152 / 0.189	0.154 / 0.180	0.161 / 0.193	0.169 / 0.197	0.168 / 0.195
<i>No. Atoms</i>					
Protein	2895	2875	2897	2889	2878
Ligand / other	16 / 38	16 / 24	17 / 29	18 / 24	17 / 24
Water	254	270	223	240	243
<i>B factors (Å²)</i>					
All atoms	24	23	26	27	31
Ligand	15	15	29	51	26
<i>R.m.s. Deviations</i>					
Bond lengths (Å)	0.014	0.014	0.005	0.011	0.012
Bond angles (°)	1.40	1.57	0.85	1.50	1.47

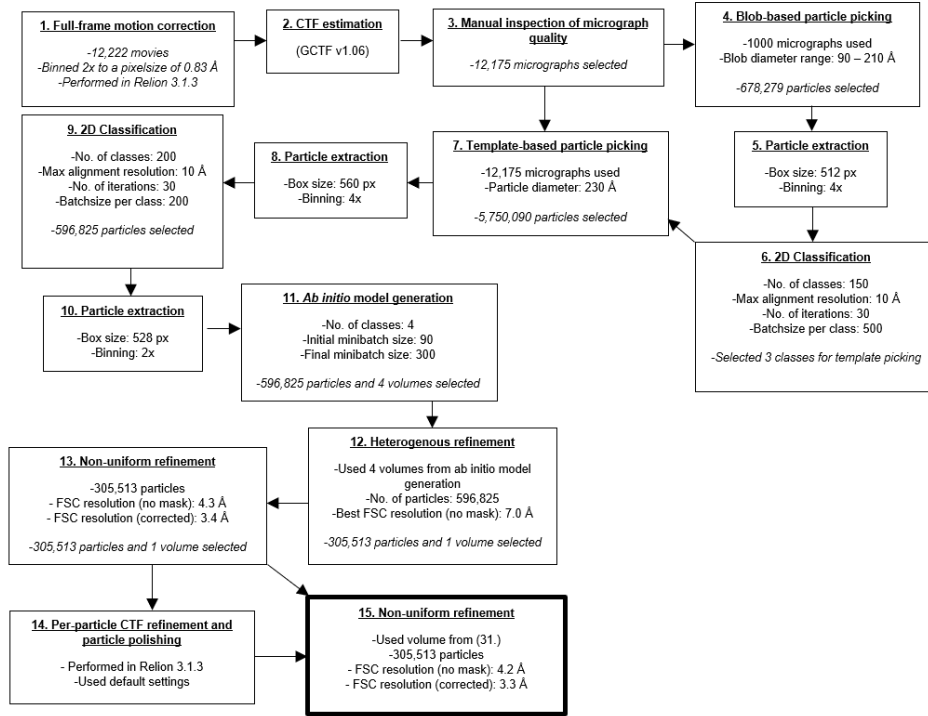
S. Table 3. X-ray data collection and refinement statistics for Notum in complex with various compounds (cont.). Values in parentheses are for highest-resolution shell.



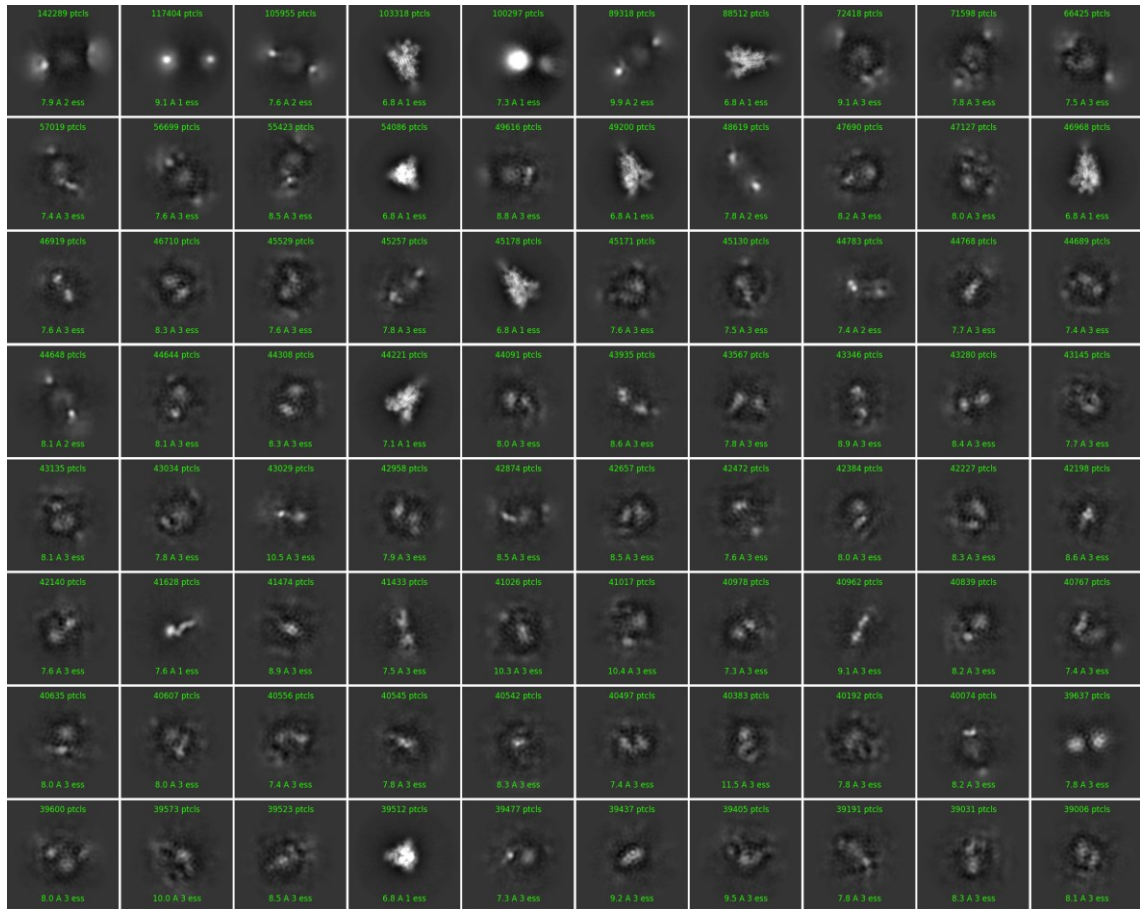
S. fig. 22. Validation of the structure of Notum in complex with compound 263. Validation performed using the wwPDB server. Structure available with PDB ID: 6ZVL.

SARS-CoV-2 Spike - H11-D4 (EMD-11068, PDB 6Z43)	
Data collection and processing	
Magnification	105,000
Voltage (kV)	300
Electron exposure (e ⁻ /Å ²)	43
Defocus range (μm)	0.8-2.6
Pixel size (Å/pixel)	0.415
Symmetry imposed	C1
Initial particle images (no.)	5,750,090
Final particle images (no.)	305,513
Map resolution (Å)	3.3
FSC threshold	0.143
Map resolution range (Å)	3.2-9.7
Refinement	
Initial model used	PDB 6VXX
Model resolution (Å)	3.4
FSC threshold	0.143
Model resolution range (Å)	3.3-6.0
Map sharpening B factor (Å ²)	-117
CC _{0.5}	0.83
<i>Model composition</i>	
Non-hydrogen atoms	26,725
Protein residues	3,351
<i>B factors (Å²)</i>	
Protein residues	119
<i>R.m.s. Deviations</i>	
Bond lengths (Å)	0.005
Bond angles (°)	0.58
Validation	
MolProbity score	1.43
Clashscore	8.7
Poor rotamers (%)	0.1
<i>Ramachandran plot</i>	
Favoured (%)	94.8
Allowed (%)	5.1
Disallowed (%)	0.1

S. Table 4. Cryo-EM data collection and refinement statistics for SARS-CoV-2 Spike protein in complex with H11-D4 nanobody.



S. fig. 23. Cryo-EM processing of SARS-CoV-2 Spike in complex with H11-D4 nanobody. Every step performed in Cryosparc v3.2 using default settings, unless otherwise specified.



S. fig. 24. Cryo-EM 2D classification of SARS-CoV-2 Spike in complex with H11-D4 nanobody. From S. fig. 23.9.