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SUPPLEMENTARY INFORMATION

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Crystal structure of a mammalian Wnt–frizzled complex

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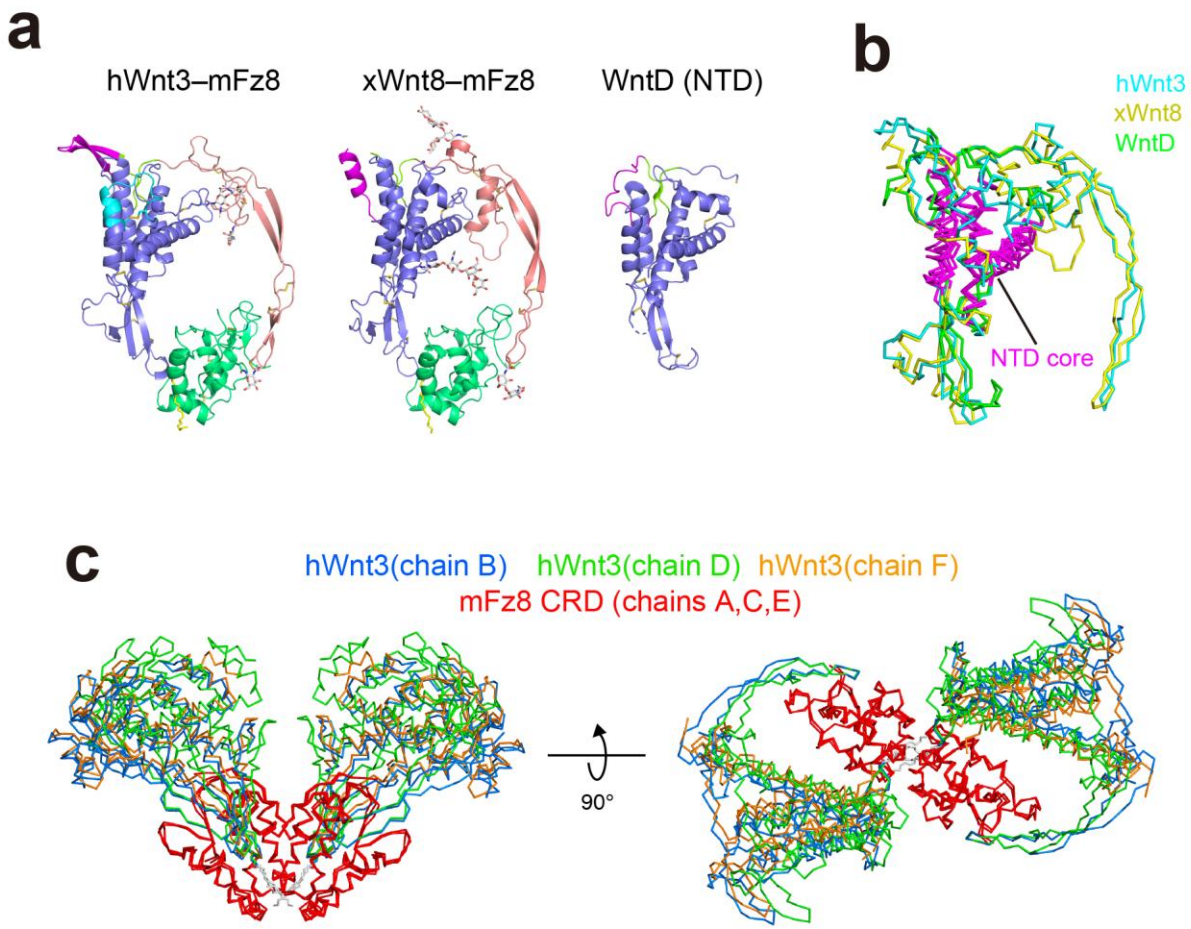
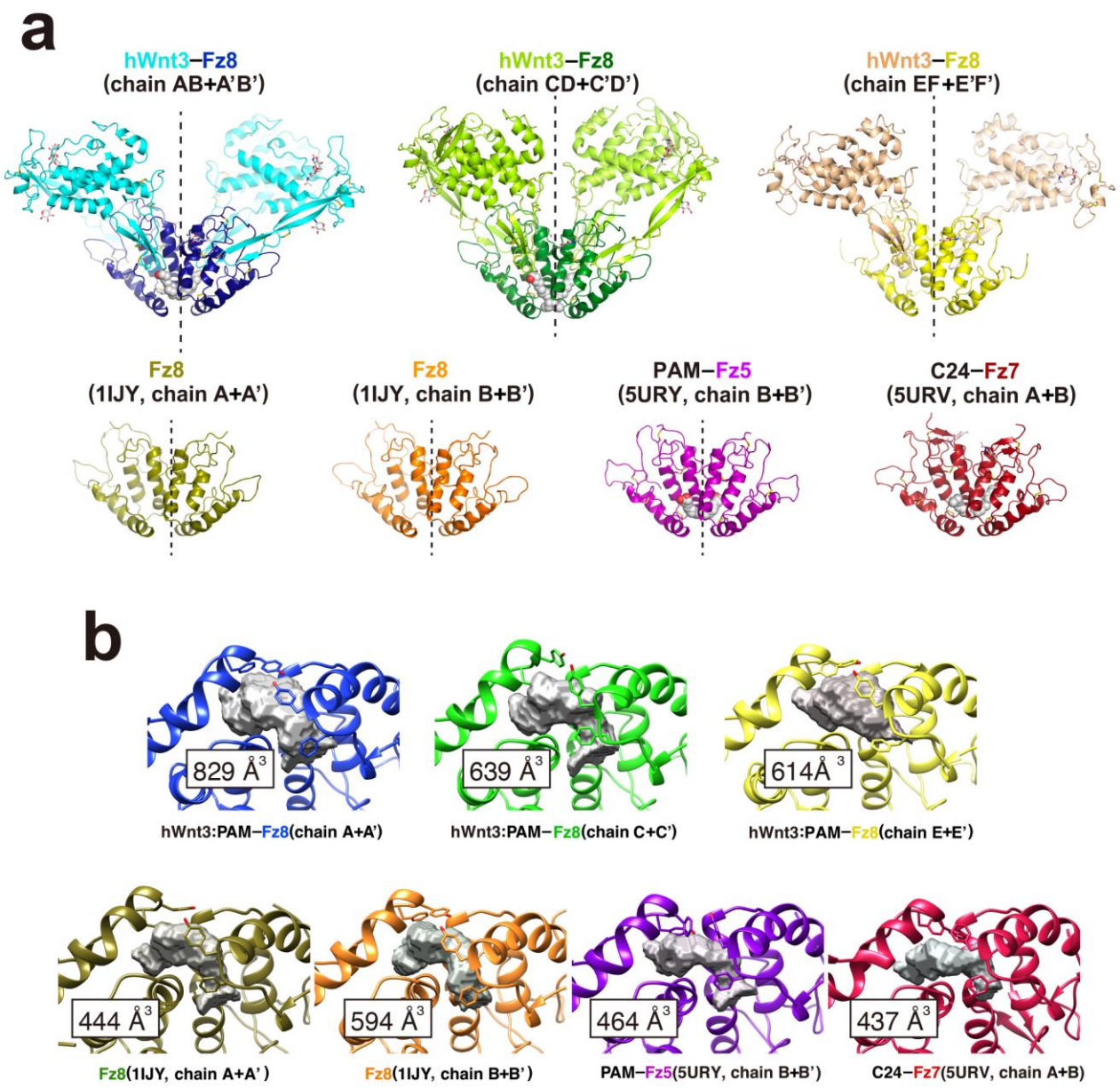

a
hWnt3–mFz8
xWnt8–mFz8
WntD (NTD)
b
hWnt3
xWnt8
WntD
NTD core
c
hWnt3(chain B)
hWnt3(chain D)
hWnt3(chain F)
mFz8 CRD (chains A,C,E)
90°

Supplementary Figure 1

Overall structure of Wnt-Fz CRD complex.

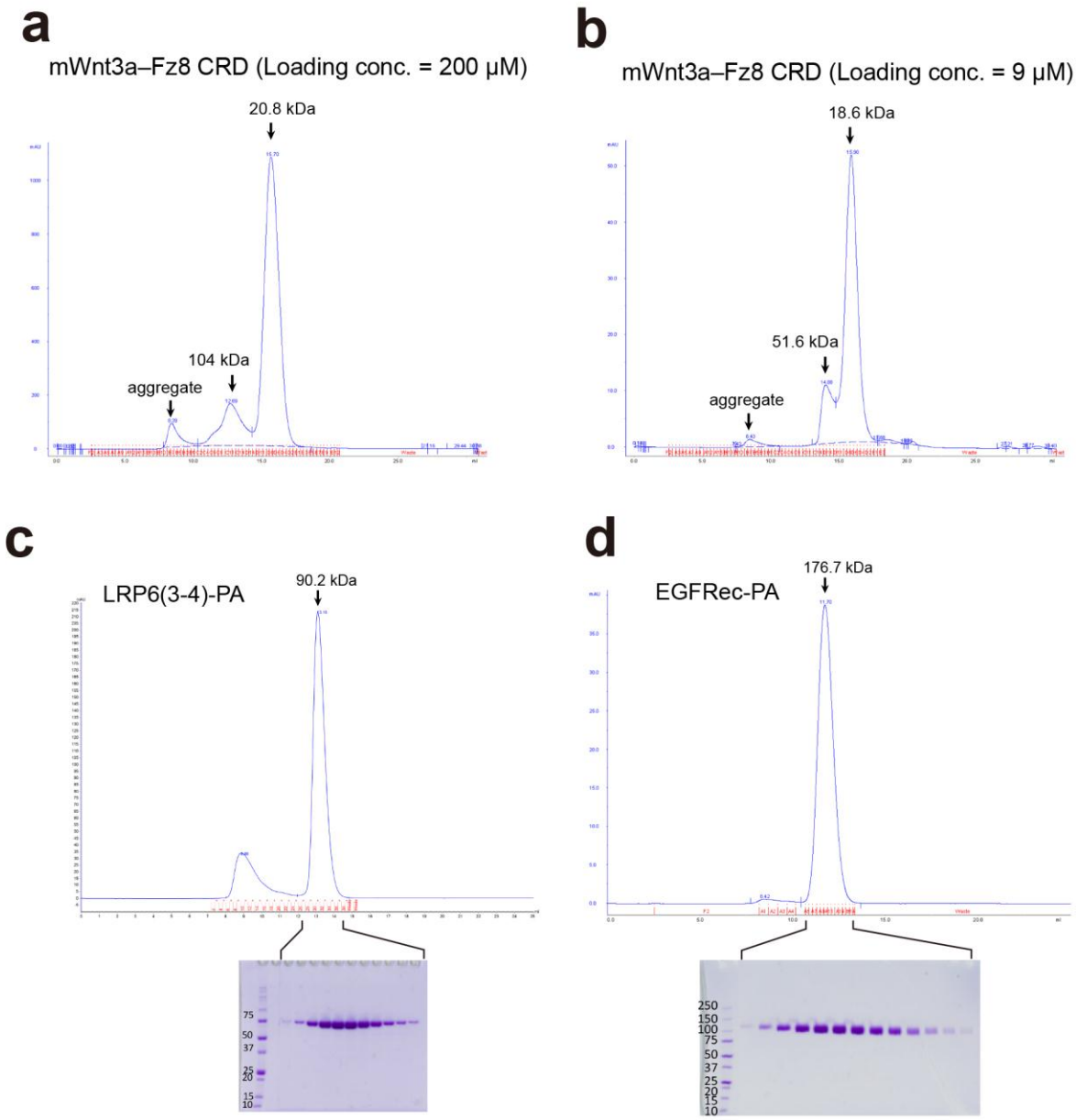
a, Structural comparison among the three Wnt proteins. Structures of Xenopus Wnt8/mFz8 CRD complex (PDB ID: 4F0A) and Drosophila WntD N-terminal domain (PDB ID: 4KRR) are shown in ribbon presentation after superposition with the hWnt3–mFz8 CRD structure at NTD, using the same color code as in Fig. 1a. **b,** Structural superposition of three Wnt proteins. Three Wnt structures are superposed at the core of the NTD comprised by five α-helices (magenta) and shown in Cα tracing models. **c,** Mobility of Wnt ligands upon the binding to Fz CRD. Three independent hWnt3 structures (chain B, skyblue; chain D, green; chain F, orange) in complex with mFz8 CRD are superposed at the Fz8 CRD (red) and shown as Cα tracings and viewed from two orientations.



Supplementary Figure 2

Fz–Fz dimer formation.

a, The 2:2 symmetric dimer found in the hWnt3–mFz8 CRD crystal. The upper panel shows three independent hWnt3/mFz8 CRD units (chains AB, CD, and EF) shown with their respective packing mates (chains A'B', C'D', and E'F'). The bottom panel shows the crystal structure of apo-form of Fz8 CRD (chains A and B, PDB ID: 1IJY) drawn with their packing mate (chains A' and B'), PAM-bound Fz5 CRD (chain B, PDB ID: 5URY) drawn with its packing mate (chain B'), as well as a pseudosymmetric dimer of Fz7 CRD bound by a C24 fatty acid (chains A and B, PDB ID: 5URV). The 2-fold symmetry axis is denoted by dotted lines. Lipid molecules are drawn as sphere models. **b,** Lipophilic cavity at the Fz-Fz interface. The volume of the cavity formed at the Fz-Fz symmetric dimer interface in the 7 dimeric structures in (a) are calculated by using the ChannelFinder program in the 3V server (http://3vee.molmovdb.org/) with the outer and inner probe radius set to 4.0 and 1.4 Å, respectively. The volume map files (.mrc format) produced by the server are opened in UCSF Chimera 1.7.0 and drawn with Fz CRD structures as in (a). The volume of the PAM chain moiety taken from chain B was calculated by the VolumeCalculation program in the same server, resulting in a value of 298 $Å^3$.



Supplementary Figure 3

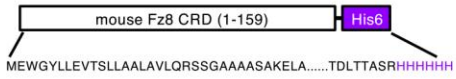
Gel filtration profiles of various protein preparations.

a,b, Mouse Wnt3a in complexwith mFz8 CRD prepared exactly as hWnt3–mFz8 CRD complexused in the crystallization experiments was loaded to a Superdex 200 gel filtration column at either 200 μM (a, 11 mg/ml) or 9 μM (b, 0.5 mg/ml) and eluted with HBS. The estimated molecular sizes for each peak were calculated from the elution positions of standard marker proteins. **c,d,** Purity and the monodispersityof the LRP6(3-4) (c) and EGFRec (d) preparations used in the SPR experiments were confirmed by gel filtration on a Superdex 200 column (upper panels) and the SDS-PAGE analysis (lower panels).

1. mFz8 CRD-hingeless_Fc-His (crystallization construct)

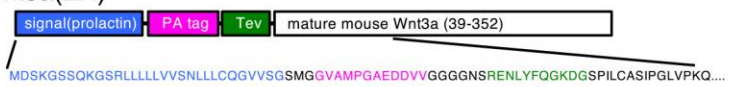
2. Tg-mFz8 CRD

3. mFz8 CRD-His

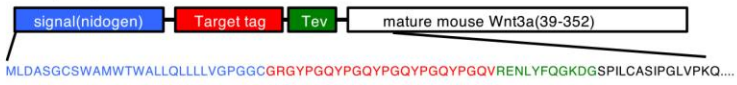


4. PA-hWnt3(ΔN) (crystallization construct)

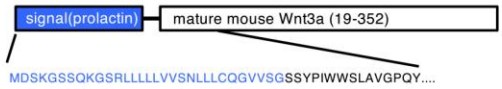
5. PA-mWnt3a(ΔN)



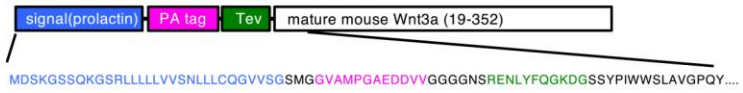
6. Tg-mWnt3a(ΔN)



7. mWnt3a(FL_tagfree)



8. mWnt3a(FL_NterPA)



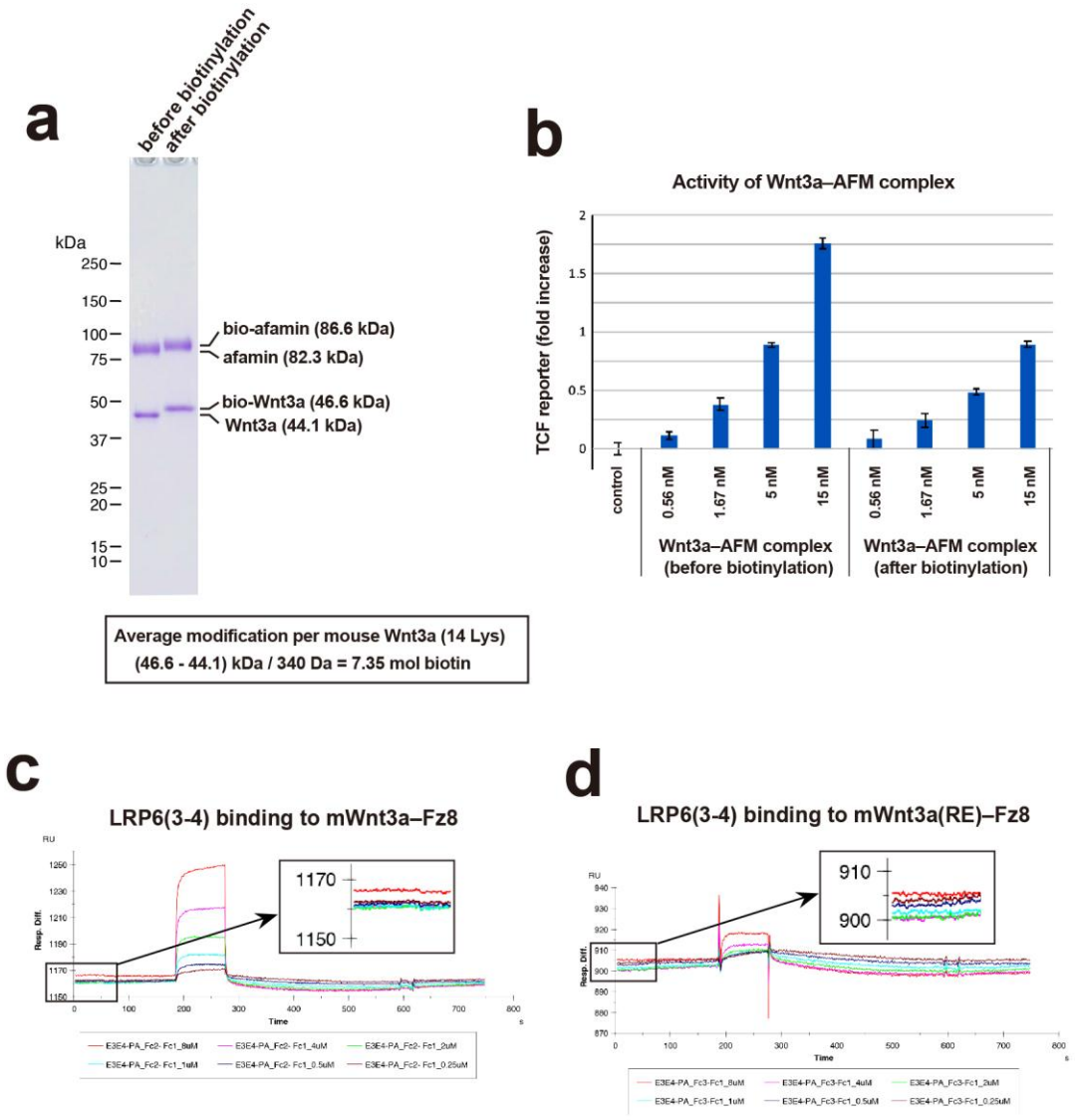
*All mWnt3a PA insertion mutant constructs

9. mWnt3a(FL_Q38PA) ....PQYSSLSTQ(38)GVAMPGAEDDVV(39)PILCASIPGLVPKQ....
10. mWnt3a(FL_iPA1) ....AVTRSCAEG(131)GVAMPGAEDDVV(132)SAAICGCSSRLQ....
11. mWnt3a(FL_iPA2) ....CGCSSRLQG(144)GVAMPGAEDDVV(145)SPGEGWKWGG....
12. mWnt3a(FL_iPA3) ....EFADARENR(176)GVAMPGAEDDVV(177)PDARSAMNRH....
13. mWnt3a(FL_iPA4) ....MVVEKHRES(249)GVAMPGAEDDVV(250)RGWVETLRPR....
14. mWnt3a(FL_iPA5) ....FCEPNPETG(288)GVAMPGAEDDVV(289)SFGTRDRTCNV....
15. mWnt3a(FL_iPA6) ....TRDRTCNVS(300)GVAMPGAEDDVV(301)SHGIDGCDLLC....

Supplementary Figure 4

Fz and Wnt expression constructs used in this study.

Construct design of variously tagged Fz8 (3 versions) and Wnt (12 versions) proteins are schematically shown, with each amino acid sequence of the N-terminal regions starting at the initiation Met indicated at the bottom.



Supplementary Figure 5

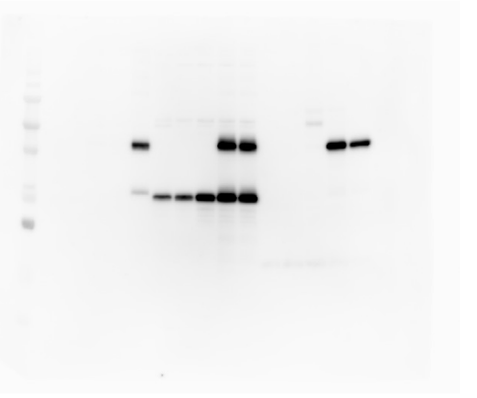
Verification of the Wnt3a protein preparations used in the study.

a, SDS-PAGE analysis of the purified afamin-Wnt3a complex sample before and after the biotinylation. Note that the Wnt3a protein used here retains the N-terminal extra 29-residue tag sequence, appearing as larger size than the mature protein (37 kDa). The number of biotin molecules incorporated in one Wnt3a molecule (=number of Lys modification) was estimated to be 7.35 mol/mol. **b,** TCF reporter assay to evaluate the signaling activity of the afamin–Wnt3a complex. Unmodified and biotinylated samples were added to the TCF reporter cells at indicated concentrations and the signaling activities were evaluated and expressed as in Fig. 3. **c, d,** Lack of dissociation of Wnt3a from the surface-captured Fz8 CRD-Fc during the SPR analysis. Shown are the same sensorgrams used to draw Fig. 4d (for c) and e (for d) but are before the Y-axis alignment at the time of injection (time=190 sec). The baseline value at the beginning of each binding cycle (see an expanded view in the inset) remained nearly constant with the total decline of less than 7 RU over one set of experiments, indicating the minimum dissociation of Wnt3a during this period.

Uncropped raw images relevant to Fig. 2b

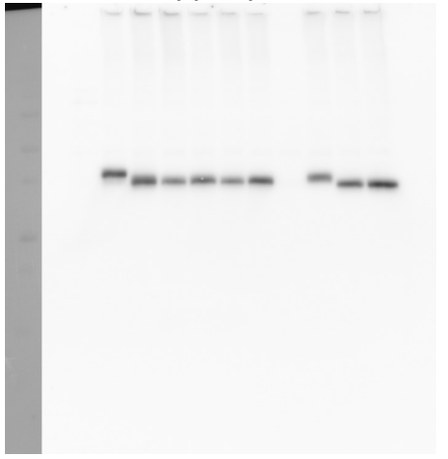
upper panel

lower panel

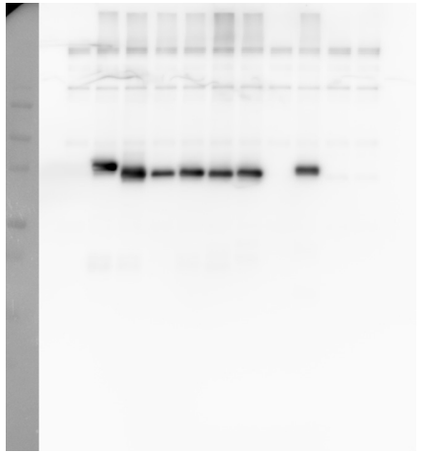


Uncropped raw images relevant to Fig. 3b

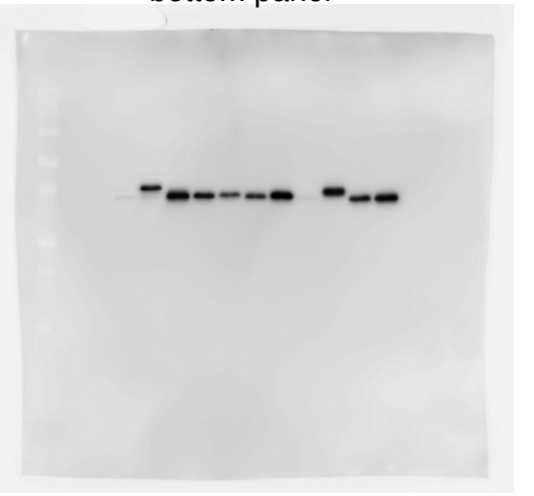
upper panel



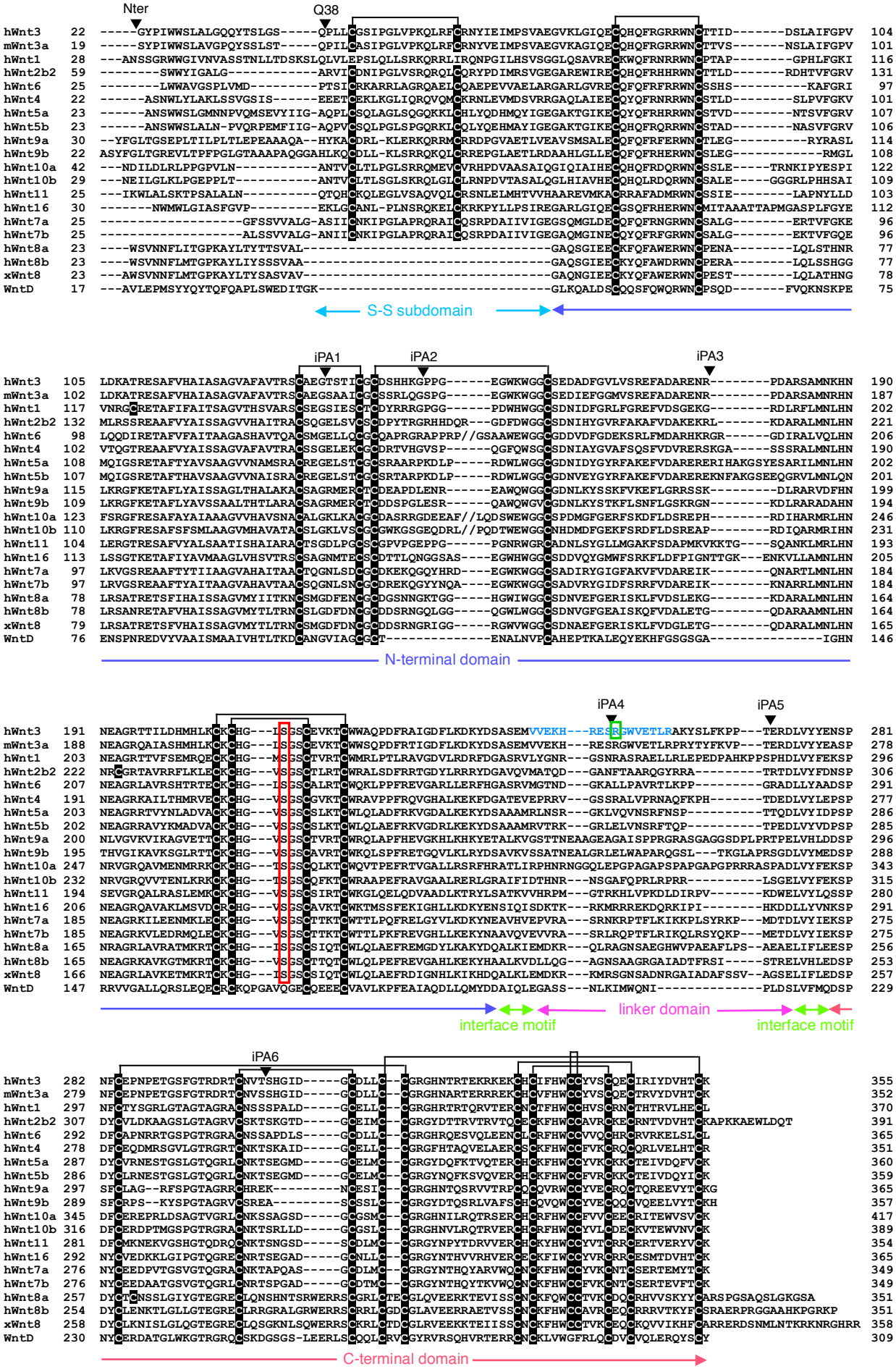
middle panel



bottom panel



Supplementary Data Set 1


Nter
Q38
S-S subdomain
iPA1
iPA2
iPA3
N-terminal domain
iPA4
iPA5
linker domain
interface motif
interface motif
iPA6
C-terminal domain

Supplementary Note 1. Sequence alignment of all Wnt proteins. Sequences are for mature (i.e., after signal sequence removal) proteins, with the residue numbering starting from the predicted N-terminal Met. For mammalian Wnts, sequences for human orthologues are shown except for mouse Wnt3a. Each subdomain region is indicated at the bottom using the color scheme used in Fig. 1a. Insertion positions for the PA tag in mouse Wnt3a used in the experiments shown in Fig. 3 are indicated by the black triangles and labeled. The PAM-modified Ser residues are boxed in red. The segment corresponding to the LRP6-binding LP3 peptide (identical between hWnt3 and mWnt3a) is colored in blue, with the critical Arg residue marked by a green box.

Supplementary Note 2

Optimization of crystallization constructs

We have previously discovered that a serum protein afamin can solubilize Wnt proteins[1]. When coexpressed and complexed with afamin, Wnt3a and Wnt3 can be purified to homogeneity and remain monodispersed and water-soluble in the absence of detergents, which should be an ideal condition for crystallization. Despite repeated trials, however, we could not obtain diffraction-quality crystals of Wnt3a(or Wnt3)-afamin complex. We then turned to the coexpression with the mouse Fz8 (mFz8) CRD after the success of Janda *et al*[2]. Although Janda *et al* reported that xWnt8–mFz8 CRD complex was freely soluble in aqueous buffer due to the masking of the lipid moiety by the Fz8 CRD, we found that purified Wnt3a–Fz8 CRD complex still remained hydrophobic and required the presence of detergents during the concentration, which hampered the crystallization. We next tried N-terminal truncation of Wnts to mimic the cleavage of the N-terminal peptide by a metalloprotease Tiki, which has been reported to reduce the overall hydrophobicity[3]. Thus N-terminal 20 residues were removed from the human Wnt3 or mouse Wnt3a constructs (Supplementary Note 1), and the resultant proteins (PA-hWnt3(ΔN) or PA-mWnt3a(ΔN), Supplementary Fig. 4) were co-expressed with mFz8 CRD C-terminally fused with modified human Fc. It was confirmed that the Wnt3/3a-Fz8 CRD complexes thus obtained were fully soluble in aqueous buffer and could be concentrated to >5 mg/ml in the absence of any detergents. Expression construct for Fz8 CRD also needed optimization. Initially, we followed the construct design of Janda *et al* and attached normal (i.e., with hinge region) human IgG1 Fc to the C-terminal of mouse Fz8 CRD, intervened by a TEV protease cleavage sequence for the later Fc removal. Although the coexpression with PA-Wnt3(ΔN) resulted in the formation of stable complex with high yield, the resultant complex could not be cleaved by TEV protease treatment at all, even with very high concentration of the enzyme. Several constructs with different linker lengths were tested and showed no improvements. We then decided to use IdeS protease to remove Fc portion. This bacterial protease is reported to cleave the lower hinge region of human IgG at very high specificity[4]. Since IdeS cleavage occurs downstream of the inter-chain disulfide bonds connecting two Fc chains, we removed upper hinge hinge region from the construct[5], resulting in a construct mFz8 CRD-hingeless-Fc-His (Supplementary Fig. 4). Transient transfection of this construct with PA-hWnt3(ΔN) at a DNA ratio of 19:1 in Expi293F cells resulted in the production of maximum amount of Wnt3-containing complex with a large excess of mFz8 CRD-Fc protein.

Effect of lysine modification on the biological activity of Wnt3a

Since our Wn3–Fz8 CRD complex structure was derived from a sample after lysine methylation, we checked if the chemical modification of lysines may have impacted the functional integrity of Wnt. As a mean to modify surface-exposed lysine residues, we used biotinylation using NHS-biotin instead of methylation, due to the ability to evaluate the chemical modification state. Biologically active mWnt3a–afamin complex was purified from the stable cell line producing this complex[1], dialyzed against phosphate-buffered saline, pH7.5, and treated with 100-fold molar excess EZ-Link NHS-biotin reagent (Thermo Scientific, #21336) at room temperature for 1h. After the termination of the reaction by adding 50 mM Tris (final concentration), the sample was re-dialysed against HBS. SDS-PAGE analysis of the modified sample indicated both afamin and Wnt3a were converted to uniformly modified molecular species with the relative molecular masses increased by 4.3 and 2.5 kDa, respectively (Supplementary Fig. 5a). Based on the uniform band-shift, we estimate that 100% of the Wnt3a protein in the sample was labeled and the average number of lysine residues modified was ~50% (7.35/14 total Lys in Wnt3a sequence). The biological activity of this complex, before and after the modification, were evaluated by the TCF reporter assay at different concentrations (Supplementary Fig. 5b). It was revealed that the Lys-modified Wnt3a retained ~50% of its activity, suggesting that Lys residues that are susceptible to chemical modifications are not directly involved in the protein-protein interaction processes critical for the signaling.

Supplementary references

1. Mihara, E. et al. Active and water-soluble form of lipidated wnt protein is maintained by a serum glycoprotein afamin/alpha-albumin. *Elife* **5**, e11621 (2016).

2. Janda, C.Y., Waghray, D., Levin, A.M., Thomas, C. & Garcia, K.C. Structural basis of Wnt recognition by Frizzled. *Science* **337**, 59-64 (2012).

3. Zhang, X. et al. Tiki1 is required for head formation via Wnt cleavage-oxidation and inactivation. *Cell* **149**, 1565-1577 (2012).

4. von Pawel-Rammingen, U., Johansson, B.P. & Bjorck, L. IdeS, a novel streptococcal cysteine proteinase with unique specificity for immunoglobulin G. *EMBO J.* **21**, 1607-1615 (2002).

5. Novarra, S. et al. A hingeless Fc fusion system for site-specific cleavage by IdeS. *MAbs* **8**, 1118-1125 (2016).

a. mFz8 CRD

```
                30        40        50        60        70        80        90        100       110       120       130
                |         |         |         |         |         |         |         |         |         |         |
construct  ASAKELACQEITVPLCKGIGYNYTYMPNQFNHDTQDEAGLEVHQFWPLVEIQCSPDLKFFLCSMYTPICLEDYKKPLPPCRSVCERAKAGCAPLMRQYGFAWPDRMR
chain A    .....LACQEITVPLCKGIGYNYTYMPNQFNHDTQDEAGLEVHQFWPLVEIQCSPDLKFFLCSMYTPICLEDYKKPLPPCRSVCERAKAGCAPLMRQYGFAWPDRMR
chain C    ...KELACQEITVPLCKGIGYNYTYMPNQFNHDTQDEAGLEVHQFWPLVEIQCSPDLKFFLCSMYTPICLEDYKKPLPPCRSVCERAKAGCAPLMRQYGFAWPDRMR
chain E    ......ACQEITVPLCKGIGYNYTYMPNQFNHDTQDEAGLEVHQFWPLVEIQCSPDLKFFLCSMYTPICLEDYKKPLPPCASVCARAAAGCAPLMAQYGFAWPDAMA

               140       150       160
                |         |         |
construct  CDRLPEQGNPDTLCMDYNRTDLTTASRPAPELLG
chain A    CDRLPEQGNPDTLCMDYN................
chain C    CDRLPEQGNPDTLCMDYN................
chain E    CDALPEQGNPD.......................
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b. hWnt3

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                     50        60        70        80        90        100       110       120       130       140
                      |         |         |         |         |         |         |         |         |         |
construct  GKDGSPLLCGSIPGLVPKQLRFCRNYIEIMPSVAEGVKLGIQECQHQFRGRRWNCTTIDDSLAIFGPVLDKATRESAFVHAIASAGVAFAVTRSCAEGTSTICGCDS
chain B    ......LLCGSIPGLVPKQLRFCRNYIEIMPSVAEGVKLGIQECQHQFRGRRWNCTTIDDSLAIFGPVLDKATRESAFVHAIASAGVAFAVTRSCAEGTSTICGCDS
chain D    .......LCGSIPGLVPKQLRFCRNYIEIMPSVAEGVKLGIQECQHQFRGRRWNCTTIDDSLAIF...LDKATRESAFVHAIASAGVAFAVTRSCAEGTSTICGCDS
chain F    ....SPLLCGSIPGLVPKQLRFCRNYIEIMPSVAEGVKLGIQECQHQFRGAAWNCTTIDDSLAIFGPVLDKATRESAFVHAIASAGVAFAVTRSCAEGTSTICGCDS

                150       160       170       180       190       200       210       220       230       240       250
                 |         |         |         |         |         |         |         |         |         |         |
construct  HHKGPPGEGWKWGGCSEDADFGVLVSREFADARENRPDARSAMNKHNNEAGRTTILDHMHLKCKCHGLSGSCEVKTCWWAQPDFRAIGDFLKDKYDSASEMVVEKHR
chain B    HHKGPPGEGWKWGGCSEDADFGVLVSREFADARENRPDARSAMNKHNNEAGRTTILDHMHLKCKCHGLSGSCEVKTCWWAQPDFRAIGDFLKDKYDSASEMVVEKHR
chain D    HHKGPPGEGWKWGGCSEDADFGVLVSREFADARENRPDARSAMNKHNNEAGRTTILDHMHLKCKCHGLSGSCEVKTCWWAQPDFRAIGDFLKDKYDSASEMVVEKHR
chain F    H...PPGEGWKWGGCSEDADFGVLVSREFADARENRPDARSAMNKHNNEAGRTTILDHMHLKCKCHGLSGSCEVKTCWWAQPDFRAIGDFLKDKYDSASEMVVEKHR

                    260       270       280       290       300       310       320       330       340       350
                     |         |         |         |         |         |         |         |         |         |
construct  ESRGWVETLRAKYSLFKPPTERDLVYYENSPNFCEPNPETGSFGTRDRTCNVTSHGIDGCDLLCCGRGHNTRTEKRKEKCHCIFHWCCYVSCQECIRIYDVHTCK
chain B    ESRGWVETLRAKYSLFKPPTERDLVYYENSPNFCEPNPETGSFGTRDRTCNVTSHGIDGCDLLCCGRGHNTRTEKRKEKCHCIFHWCCYVSCQECIRIYDVHTCK
chain D    ESRGWVETLRAKYSLFKPPTERDLVYYENSPNFCEPNPETGSFGTRDRTCNVTSHGIDGCDLLCCGRGHNTRTEKRKEKCHCIFHWCCYVSCQECIRIYDVHTCK
chain F    ESRGWVETLRAKYSLFKPPTERDLVYYENSPNFCEPNPETGSAGTAAAACAAAAAGAAGCAAACCGAG..................................
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Supplementary Note 3. Amino acid sequence of the final structural model. In the top row, amino acid sequence of the mouse Fz8 CRD (a) and human Wnt3 (b) constructs used in the crystallization experiment are shown, with the residue number in the original protein shown above. Amino acids derived from the vector and not present in the respective proteins are underlined. The rows “chain A~F” are the actual amino acid sequences included in the final structural models. The Lys residues assigned as methylated Lys are indicated by yellow background. The segments excluded in the models are shown as dots. Residues that did not show clear electron density for the side chain are modeled as Ala and shown in red.