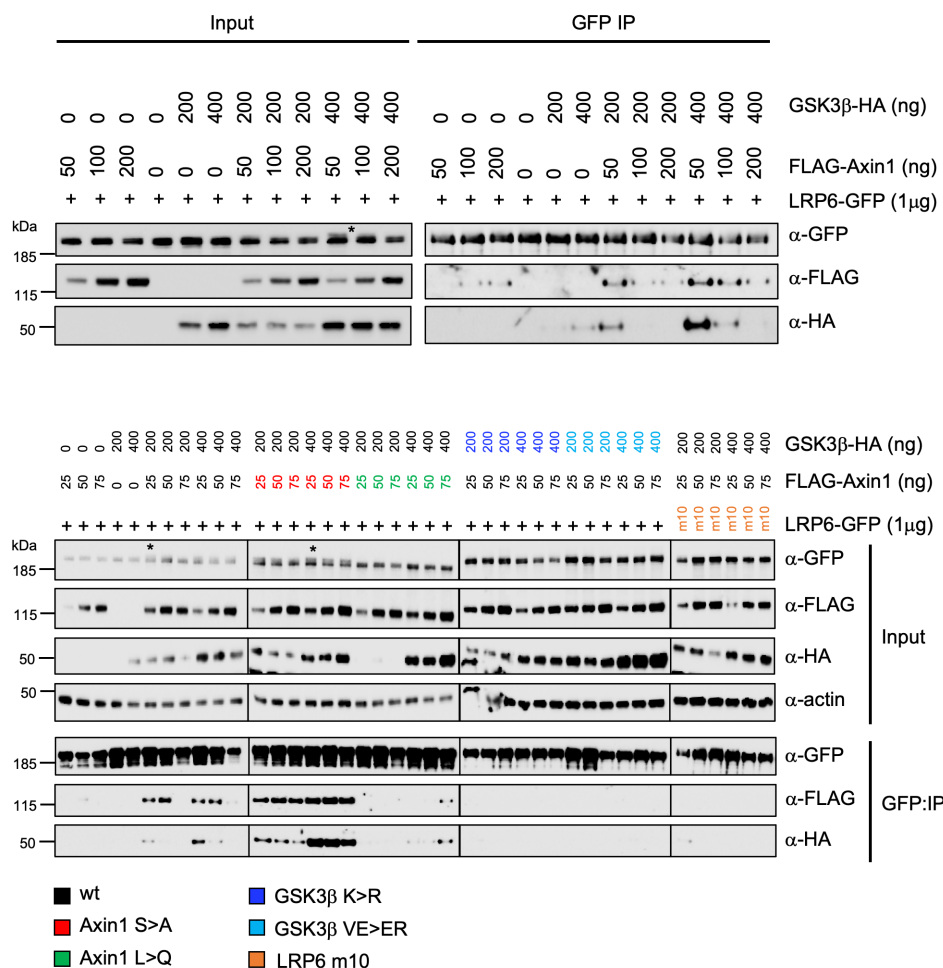
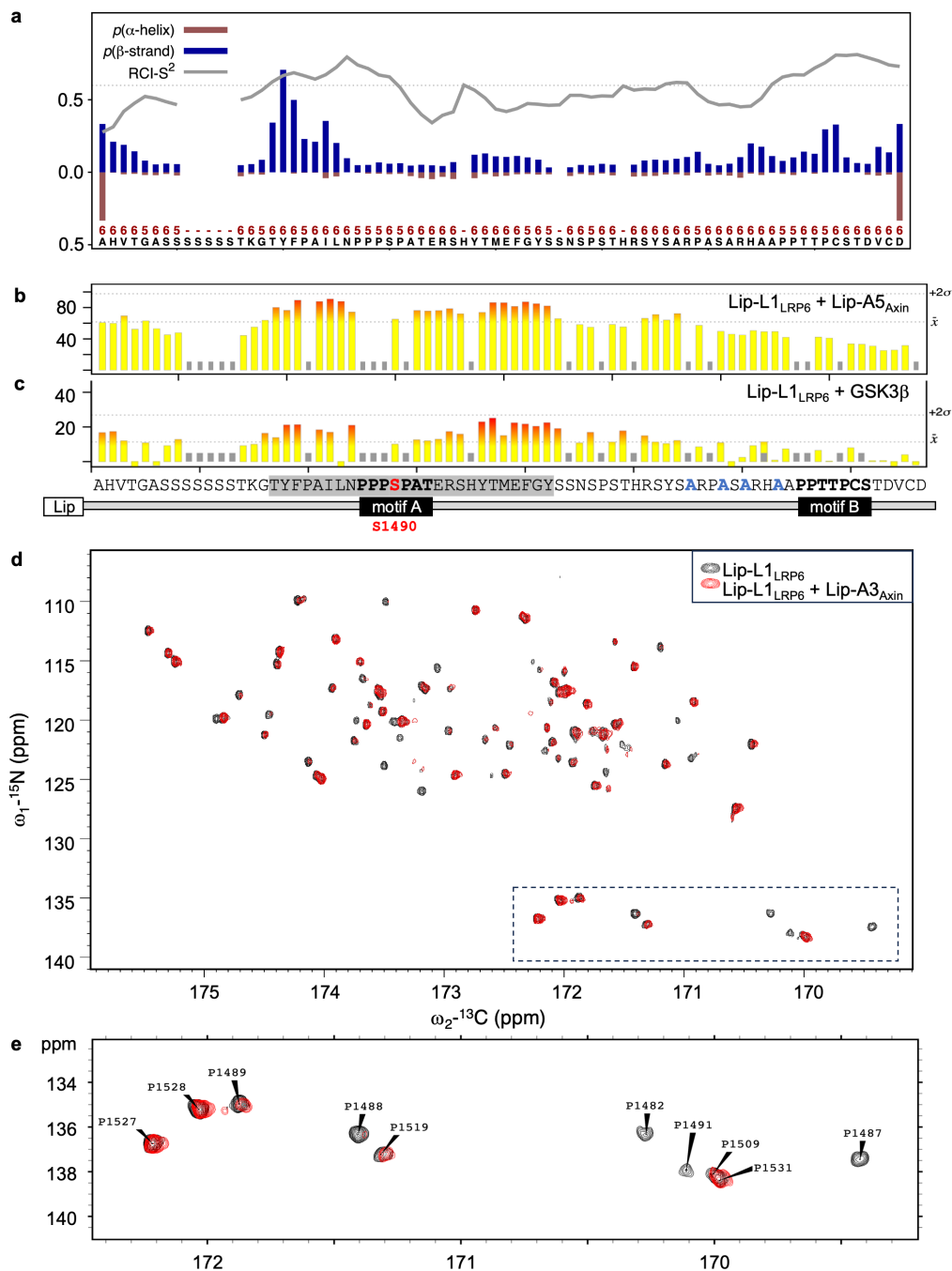




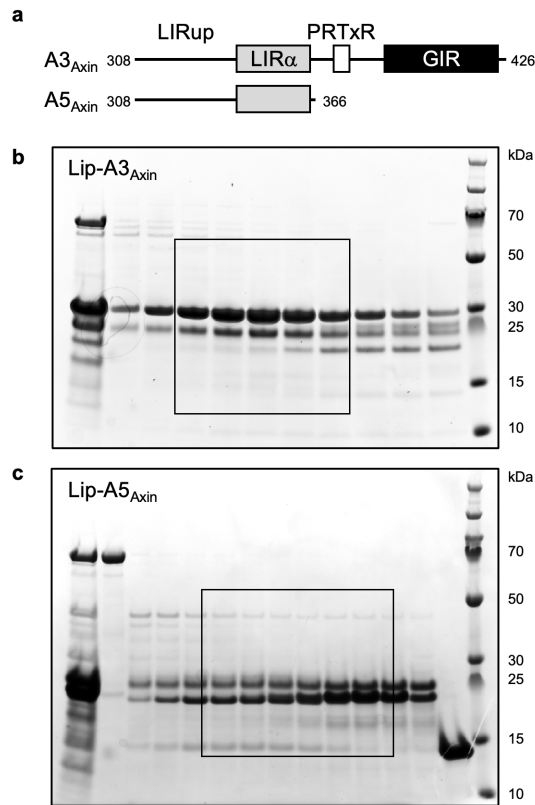
### Supplementary Fig. 1. Optimization of coIP assays

Optimization of coIP assays, by titrating the amounts of the co-transfected wt (*black*) and mutant (*colored*) plasmids in the transfection mixtures, as indicated (color key below panel); shown are Western blots probed with antibodies ( $\alpha$ ) as indicated on the right following immunoprecipitation (IP); positions of molecular weight markers indicated on the left (in kDa).



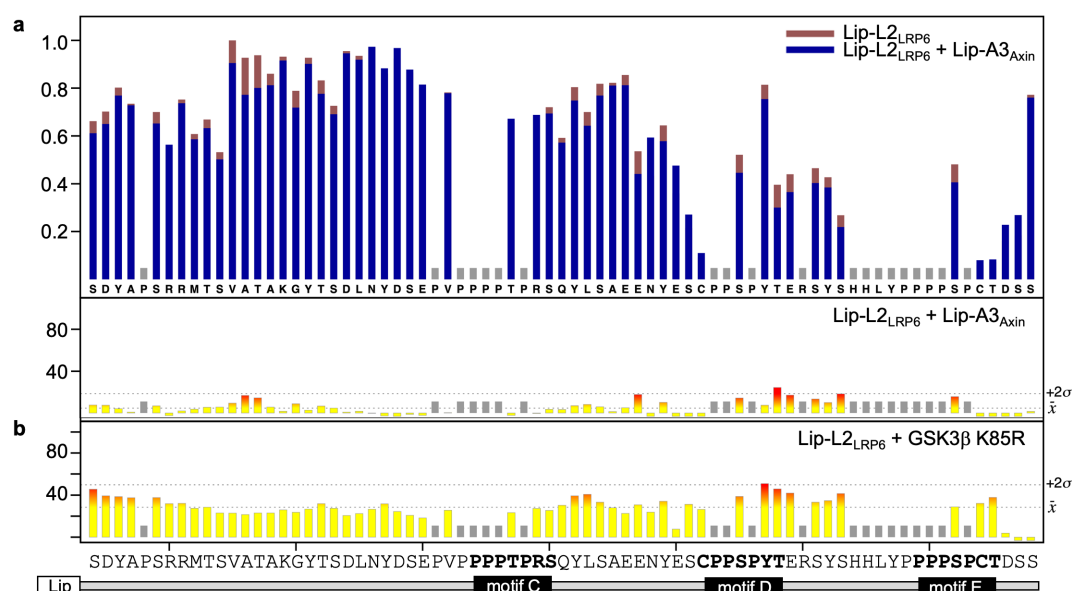
**Supplementary Fig. 2. Binding sites for Axin- and GSK3 in the proximal LRP6 ctail**

(a) TALOS-N plot for L1<sub>LRP6</sub> (LRP6<sub>1463-1538</sub> bearing YYYY>A; see Fig. 2, bottom), depicting the probability ( $p$ ) of secondary structure ( $\alpha$ -helix, brown;  $\beta$ -strand, blue) and the Random Coil Index order parameter ( $\text{RCI-S}^2$ , gray) which scales between 0 (total disorder) and 1 (fully structured); numbers above L1<sub>LRP6</sub> sequence (shown at the bottom) indicate numbers of chemical shift values for (HN,N,CA,CB,CO,HA). Of note,  $\text{RCI-S}^2$  values  $<0.6$  (indicated by dotted line) are considered dynamic, while values for stable  $\alpha$ -helices are typically  $>0.9$ . (b-c) Bleach maps generated from BEST-TROSY spectra from 80  $\mu\text{M}$   $^{15}\text{N}$ -labeled Lip-L1<sub>LRP6</sub> incubated with 80  $\mu\text{M}$  (b) Lip-A5<sub>Axin</sub> or (c) GSK3 $\beta$ ; bleach maps depict percentage attenuation of peak height by incubation with ligand ranging from strong (red) to weak (yellow); mean attenuations ( $\bar{x}$ ) and means + 2 standard deviations ( $+2\sigma$ ) are given on the right in this and subsequent bleach maps; gray bars indicate residues for which NMR peaks are not assigned or obscured by overlap. (d, e) Overlay of  $^{13}\text{C}$ -detected 2D-CON spectra of 150  $\mu\text{M}$   $^{15}\text{N}$ - $^{13}\text{C}$  double-labeled Lip-L1<sub>LRP6</sub> by itself (black) or incubated with 150  $\mu\text{M}$  Lip-A3<sub>Axin</sub> (red); see main Fig. 2e, for corresponding bleach map; color key above panels. (e) Expansion of the 134-138 ppm  $^{15}\text{N}$  region of spectrum in (d), visualizing bleaching of proline residues which are not observable by BEST-TROSY.



### Supplementary Fig. 3. Sample preparations of A3<sub>Axin</sub> and A5<sub>Axin</sub> proteins

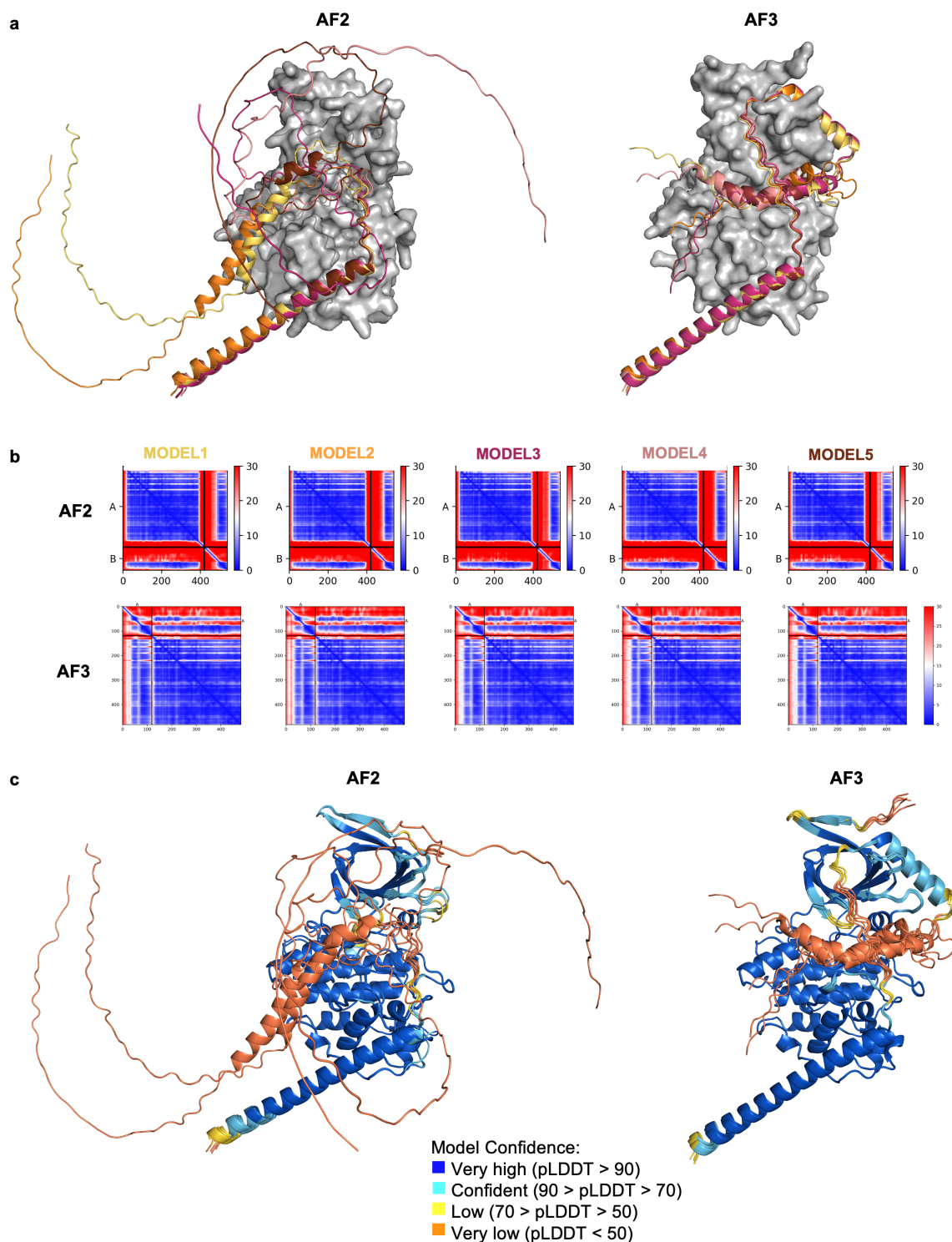
(a) Cartoons of Axin Lip-A3<sub>Axin</sub> and Lip-A5<sub>Axin</sub> (with numbers indicating 5' and 3' amino acids of human Axin1), with LRP6- and GSK3-interacting regions indicated (LIRup, LIRα, bipartite LRP6-interacting region; GIR, GSK3-binding region; PRTxR, contributes to multipronged interaction with GSK3). (b, c) SDS-PAGE analysis of representative sample preparations of (b) Lip-A3<sub>Axin</sub> and (c) Lip-A5<sub>Axin</sub>; shown are total cleared lysates after elution from Ni-NTA agarose (*left*) and fractions obtained after subsequent size exclusion chromatography with a HiLoad 26/600 Superdex 75 pg column (see Methods); fractions pooled for subsequent analysis by NMR or ITC are boxed; *right*, molecular weight markers (in kDa). Note the partial degradation of samples which inevitably occurred during protein purification, with full-length protein typically representing <50% of the total protein in the pooled fractions of Lip-A3<sub>Axin</sub>, and <30% in those of Lip-A5<sub>Axin</sub>. This explains the low (<1) molar ratios observed during ITC (see Fig. 3 and accompanying text).



#### Supplementary Fig. 4. Interactions of Axin and GSK3 with the distal LRP6 ctail

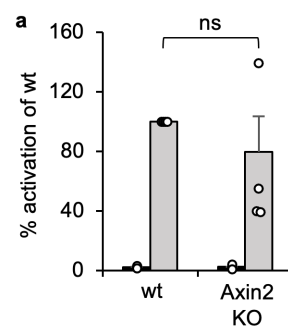
(a) Relative peak heights in a BEST-TROSY spectrum of 80  $\mu\text{M}$   $^{15}\text{N}$ -labeled Lip-L2<sub>LRP6</sub> incubated with 80  $\mu\text{M}$  Lip-A3<sub>Axin</sub> and corresponding bleach map. (b) Bleach map generated from BEST-TROSY spectra of 80  $\mu\text{M}$   $^{15}\text{N}$ -labeled Lip-L2<sub>LRP6</sub> incubated with 80  $\mu\text{M}$  GSK3 $\beta$ -K85R. *Bottom*, sequence of distal L2 fragment of LRP6 spanning PPPSPxS motifs C-E; colors and graph labeling as in main Fig. 2. The bleach maps indicate very weak interactions of both proteins with the distal LRP6 ctail (around PPPSPxS motif D) that are however not measurable by ITC (see also main text).





**Supplementary Fig. 5. Models of Axin-GSK3 complexes predicted by AlphaFold**

(a) Predicted models of Axin-GSK3 complexes generated by AF2 (*left*) or AF3 (*right*); GSK3 is shown in a light grey surface representation, while the top five Axin models predicted by each AlphaFold version are displayed in distinct colors, as indicated in the figure. (b) Predicted Aligned Error (PAE) plots for the top-5 models generated by AF2 or AF3; *blue*, regions of low error; *red*, regions of high error; colors in (a) correspond to those of top-5 models in (b). (c) Predicted Local Distance Difference (pLDDT) plots for the top-5 models generated by AF2 or AF3; colored according to confidence levels, as indicated in key.



**Supplementary Fig. 6. Testing Axin1 mutants in a complementation assay of Axin2 KO mutant HEK293T cells**

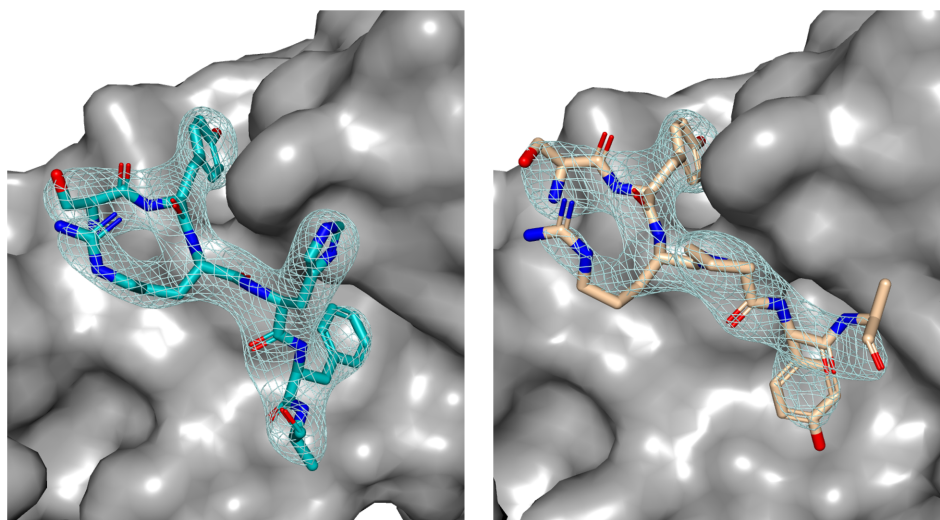
(a) SuperTOP assays in wt and Axin2 KO HEK293T cells, showing that these Axin2 KO cells are fully Wnt-responsive; *circles*, values obtained from each independent experiment (n = 3); One-way ANOVA with multiple comparisons.



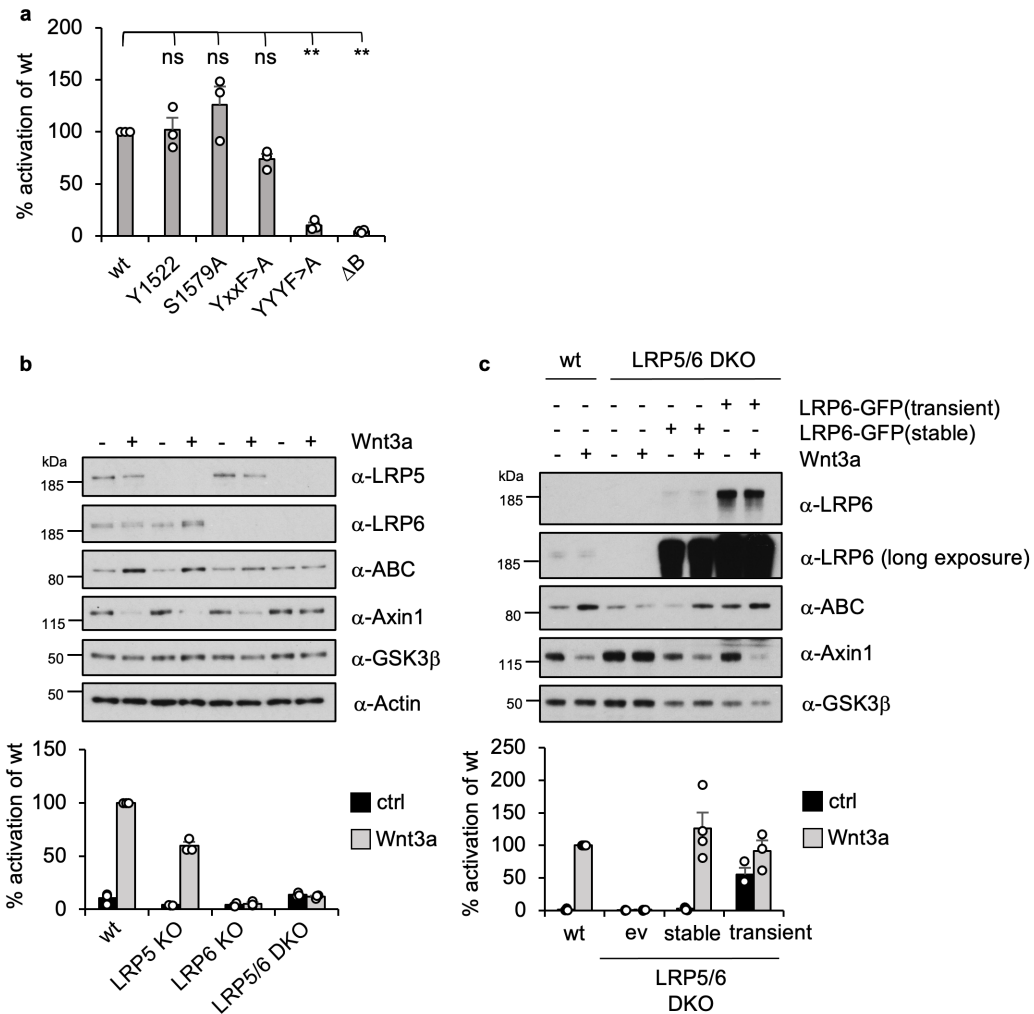
**Supplementary Fig. 7. Evolutionary conservation of Axin fragment spanning Lip-A3**

Sequence alignment of Axin orthologs, indicating conservation of residues in key regulatory elements (LIRup, LIRα, PRTxR motif; GIR, GSK3 interacting region; BIR, β-catenin interacting region; S1-S4 serines are colored and underlined (see also main Fig. 4).





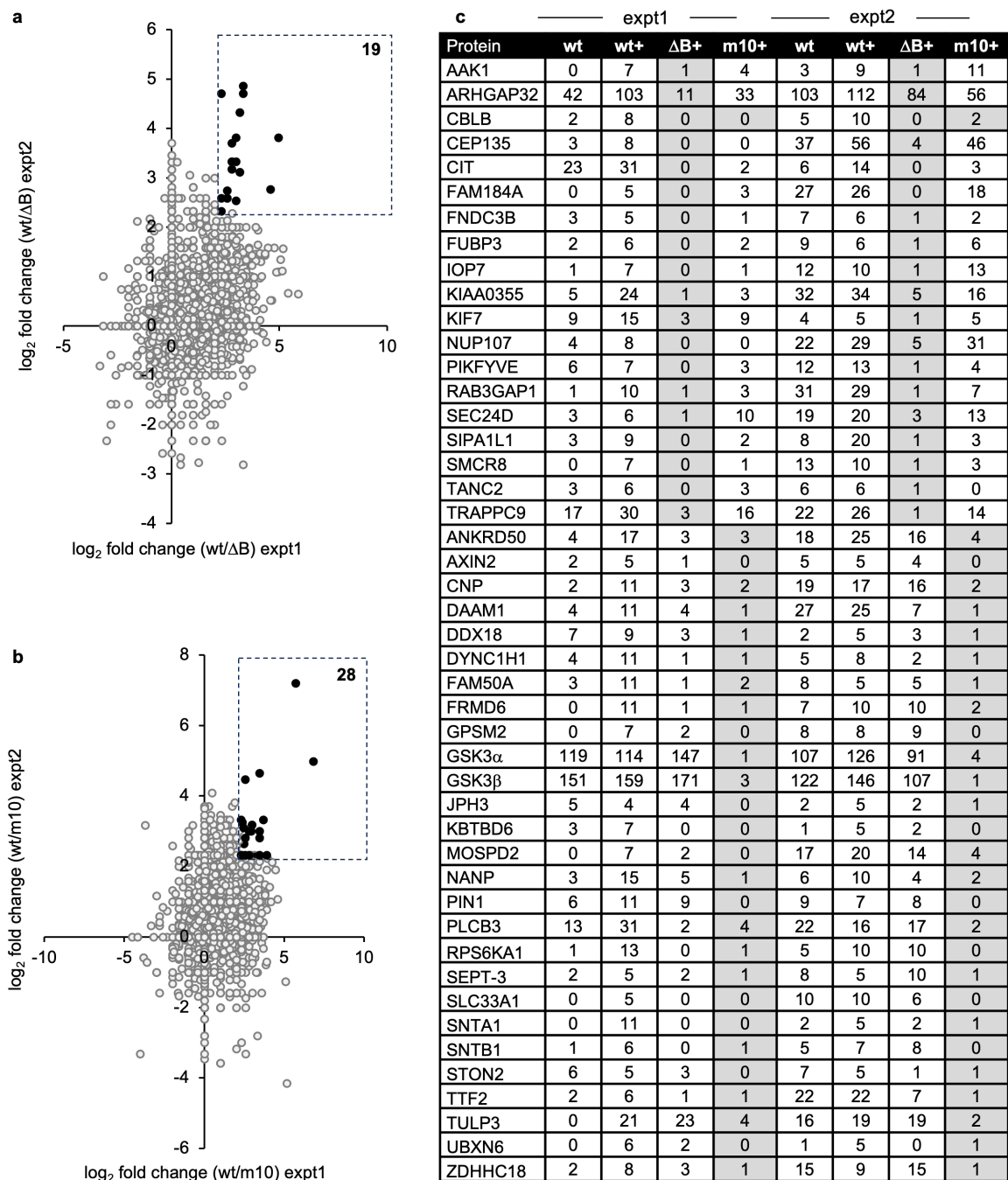
**Supplementary Fig. 9. Contour maps corresponding to LRP6 YxxY and YxxF motifs bound to AP2 $\mu$**   
2F<sub>c</sub>-F<sub>o</sub> maps (contoured at 1.6 $\sigma$ ) for YRHF (*teal*) and YRPY (*wheat*) in stick representation binding to the cargo-binding domain of AP2 $\mu$  in surface representation (*gray*).



**Supplementary Fig. 10. A complementation assay based on LRP5/6 DKO null mutant HEK293T cells**

(a) SuperTOP assays in HEK293T cells transiently transfected with wt or mutant LRP6-GFP (as indicated); one-way ANOVA with repeated measures, statistical significance \*\*,  $p < 0.01$ ; *circles*, values obtained from each independent experiment ( $n = \text{at least } 3$ ) in this and subsequent panels. (b) SuperTOP assays in wt and LRP5 KO, LRP6 KO and LRP5/6 DKO HEK293T cells +/- Wnt3a, and corresponding Western blots probed with antibodies as indicated on the right, revealing that LRP6 is critical for the Wnt response of these cells whereas LRP5 is dispensable. (c) SuperTOP assays in wt and LRP5/6 DKO HEK293T cells +/- Wnt3a, transiently (transient) or stably (stable) transfected with LRP6-GFP based on the pBABE vector (see Methods) and corresponding Western blots. In both cases, the Wnt response of the DKO cells is fully restored by the exogenous LRP6, however, this response is only Wnt-dependent in the stably transfected cells, likely because LRP6 is expressed at much lower levels in these cells than after transient transfection; positions of molecular weight markers indicated on the left (in kDa).





**Supplementary Fig. 11. Identification of ΔB- and LRP6m10-sensitive TurboID hits**

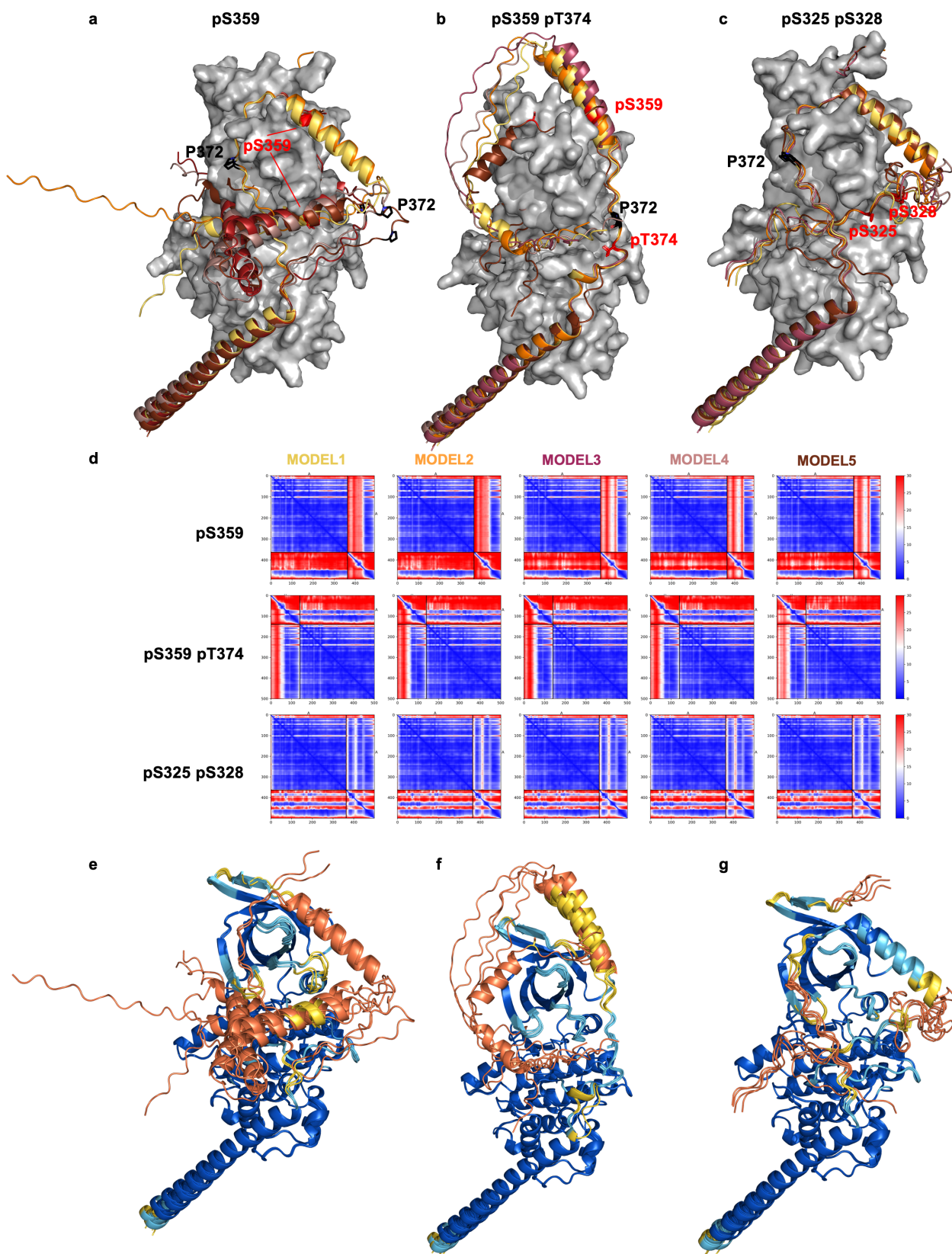
(a, b) Volcano plots showing additional (a) ΔB-sensitive or (b) LRP6m10-sensitive hits including those shown in main Fig. 8 (black circles in dashed boxes), reduced >5x compared to wt in two independent TurboID experiments; X axis, expt1; Y axis, expt2; in cases where no peptide counts were identified, the 0 value was replaced with 1 for calculation of ratios. (c) Total unweighted spectral counts (>95% probability) of ΔB- or LRP6m10-sensitive hits (shaded) from the two experiments shown in (a, b) with (+) or without 2 hours of Wnt stimulation.

| kinase | kinase group | log <sub>2</sub> (score) | site percentile | percentile rank |
|--------|--------------|--------------------------|-----------------|-----------------|
| GSK3B  | CMGC         | 2.864                    | 99.             | 1               |
| GSK3A  | CMGC         | 5.468                    | 99.787          | 2               |
| CDK8   | CMGC         | 6.082                    | 99.161          | 3               |
| CDK19  | CMGC         | 6.547                    | 99.144          | 4               |
| P38B   | CMGC         | 6.789                    | 99.096          | 5               |
| KIS    | Other        | 6.092                    | 98.950          | 6               |
| ERK5   | CMGC         | 3.707                    | 98.715          | 7               |
| CDK7   | CMGC         | 5.574                    | 98.515          | 8               |
| ERK1   | CMGC         | 6.230                    | 98.399          | 9               |
| P38D   | CMGC         | 6.413                    | 98.070          | 10              |
| CDK16  | CMGC         | 5.968                    | 97.944          | 11              |
| P38G   | CMGC         | 6.007                    | 97.873          | 12              |
| CDK18  | CMGC         | 6.178                    | 97.829          | 13              |
| CDK17  | CMGC         | 5.725                    | 97.719          | 14              |
| MPSK1  | Other        | 2.378                    | 97.604          | 15              |
| MAK    | CMGC         | 5.876                    | 97.372          | 16              |
| P38A   | CMGC         | 5.297                    | 97.133          | 17              |
| JNK2   | CMGC         | 5.290                    | 96.714          | 18              |
| CDK1   | CMGC         | 5.248                    | 96.471          | 19              |
| ICK    | CMGC         | 4.031                    | 96.431          | 20              |
| CLK3   | CMGC         | 4.503                    | 96.086          | 21              |
| JNK1   | CMGC         | 5.080                    | 95.953          | 22              |
| DYRK4  | CMGC         | 5.250                    | 95.936          | 23              |
| HIPK2  | CMGC         | 5.617                    | 95.610          | 24              |
| PASK   | CAMK         | 1.787                    | 95.424          | 25              |
| DYRK1A | CMGC         | 4.238                    | 95.063          | 26              |
| ERK2   | CMGC         | 4.079                    | 94.979          | 27              |
| HIPK4  | CMGC         | 4.232                    | 94.956          | 28              |
| JNK3   | CMGC         | 4.539                    | 94.840          | 29              |
| CDK3   | CMGC         | 4.573                    | 94.833          | 30              |
| CDK13  | CMGC         | 4.121                    | 94.716          | 31              |
| DYRK2  | CMGC         | 4.580                    | 94.711          | 32              |
| CDK12  | CMGC         | 4.138                    | 94.010          | 33              |
| DYRK1B | CMGC         | 4.360                    | 93.824          | 34              |
| MOK    | CMGC         | 3.459                    | 93.234          | 35              |
| CDK14  | CMGC         | 4.004                    | 92.831          | 36              |
| HIPK1  | CMGC         | 4.157                    | 92.426          | 37              |
| CDK5   | CMGC         | 3.909                    | 91.986          | 38              |
| GRK1   | AGC          | 1.655                    | 91.488          | 39              |
| CK1D   | CK1          | 0.584                    | 90.720          | 40              |
| CDK9   | CMGC         | 3.315                    | 90.664          | 41              |
| CK1E   | CK1          | 0.708                    | 89.947          | 42              |
| NDR2   | AGC          | 1.269                    | 89.561          | 43              |
| CDKL5  | CMGC         | 1.960                    | 89.234          | 44              |
| MASTL  | AGC          | -0.100                   | 88.547          | 45              |
| HIPK3  | CMGC         | 2.995                    | 87.251          | 46              |
| PRKD1  | CAMK         | 1.238                    | 87.131          | 47              |
| LATS1  | AGC          | 1.072                    | 87.005          | 48              |
| CDK4   | CMGC         | 2.852                    | 86.668          | 49              |
| NLK    | CMGC         | 2.267                    | 85.953          | 50              |
| CHK1   | CAMK         | 0.496                    | 85.319          | 51              |
| CDKL1  | CMGC         | 1.373                    | 84.383          | 52              |
| ATR    | PIKK         | 0.189                    | 83.869          | 53              |
| GRK7   | AGC          | 1.459                    | 83.822          | 54              |
| CDK6   | CMGC         | 2.418                    | 83.621          | 55              |
| DYRK3  | CMGC         | 1.774                    | 83.538          | 56              |
| CDK10  | CMGC         | 1.791                    | 83.347          | 57              |
| CLK2   | CMGC         | 1.859                    | 83.077          | 58              |
| SRPK1  | CMGC         | 1.197                    | 80.057          | 59              |

**Supplementary Fig. 12. Wnt-priming kinases for LRP6 S1490**

List of kinases that can phosphorylate LRP6 S1490 based on Kinase Library (KL) scores >80.





**Supplementary Fig. 13. Models of phosphorylated Axin-GSK3 complexes predicted by AF3**

(a-c) AF3 models of GSK3 binding to Axin phosphorylated at distinct sites as indicated above models; GSK3 is shown in a light surface representation, while the top-5 Axin models are displayed in distinct colors; *red*, phosphorylated residues; *black*, P372. (d) Predicted Aligned Error (PAE) plots for the top-5 models; colors in (a-c) correspond to the top-5 models in (d); *blue*, regions of low error; *red*, regions of high error. (e-g) Predicted Local Distance Difference (pLDDT) plots for the models in (a-c); coloring reflects model confidence (pLDDT) as indicated in **Supplementary Fig. 5** (*dark blue*, >90; *light blue*, >70; *yellow*, >50; *orange*, <50).

| PDB ID                                | 9FIW                         | 9FIY                         |
|---------------------------------------|------------------------------|------------------------------|
| <b>Data collection</b>                |                              |                              |
| Space group                           | P 6 <sub>4</sub>             | P 6 <sub>4</sub>             |
| <b>Cell dimensions</b>                |                              |                              |
| a, b, c (Å)                           | 127.6, 127.6, 75.1           | 128.4, 128.4, 74.6           |
| α, β, γ (°)                           | 90, 90, 120                  | 90, 90, 120                  |
| Resolution (Å)                        | 48.6 – 2.81<br>(2.97 – 2.81) | 48.7 – 2.88<br>(3.04 – 2.88) |
| R <sub>merge</sub>                    | 0.070 (1.58)                 | 0.086 (1.63)                 |
| I / σ                                 | 30.1 (2.2)                   | 23.8 (1.9)                   |
| Completeness (%)                      | 99.9 (99.5)                  | 99.9 (99.6)                  |
| Redundancy                            | 20.3 (20.7)                  | 19.8 (20.1)                  |
| <b>Refinement</b>                     |                              |                              |
| Resolution (Å)                        | 48.6 – 2.81                  | 48.7 – 2.88                  |
| No. reflections                       | 16168                        | 15176                        |
| R <sub>work</sub> / R <sub>free</sub> | 0.192/0.246                  | 0.191/0.242                  |
| <b>No. atoms</b>                      |                              |                              |
| Protein                               | 2068                         | 2070                         |
| Ligand/ion                            | 0                            | 0                            |
| Water                                 | 0                            | 0                            |
| <b>B-factors</b>                      |                              |                              |
| Protein                               | 95.5                         | 100.6                        |
| Ligand/ion                            | n/a                          | n/a                          |
| Water                                 | n/a                          | n/a                          |
| <b>R.m.s. deviations</b>              |                              |                              |
| Bond lengths (Å)                      | 0.008                        | 0.007                        |
| Bond angles (°)                       | 1.90                         | 1.83                         |

**Supplementary Table 1. Crystal data collection and refinement statistics**

Structures were collected and solved from a single crystal, values in parentheses are for highest-resolution shell.