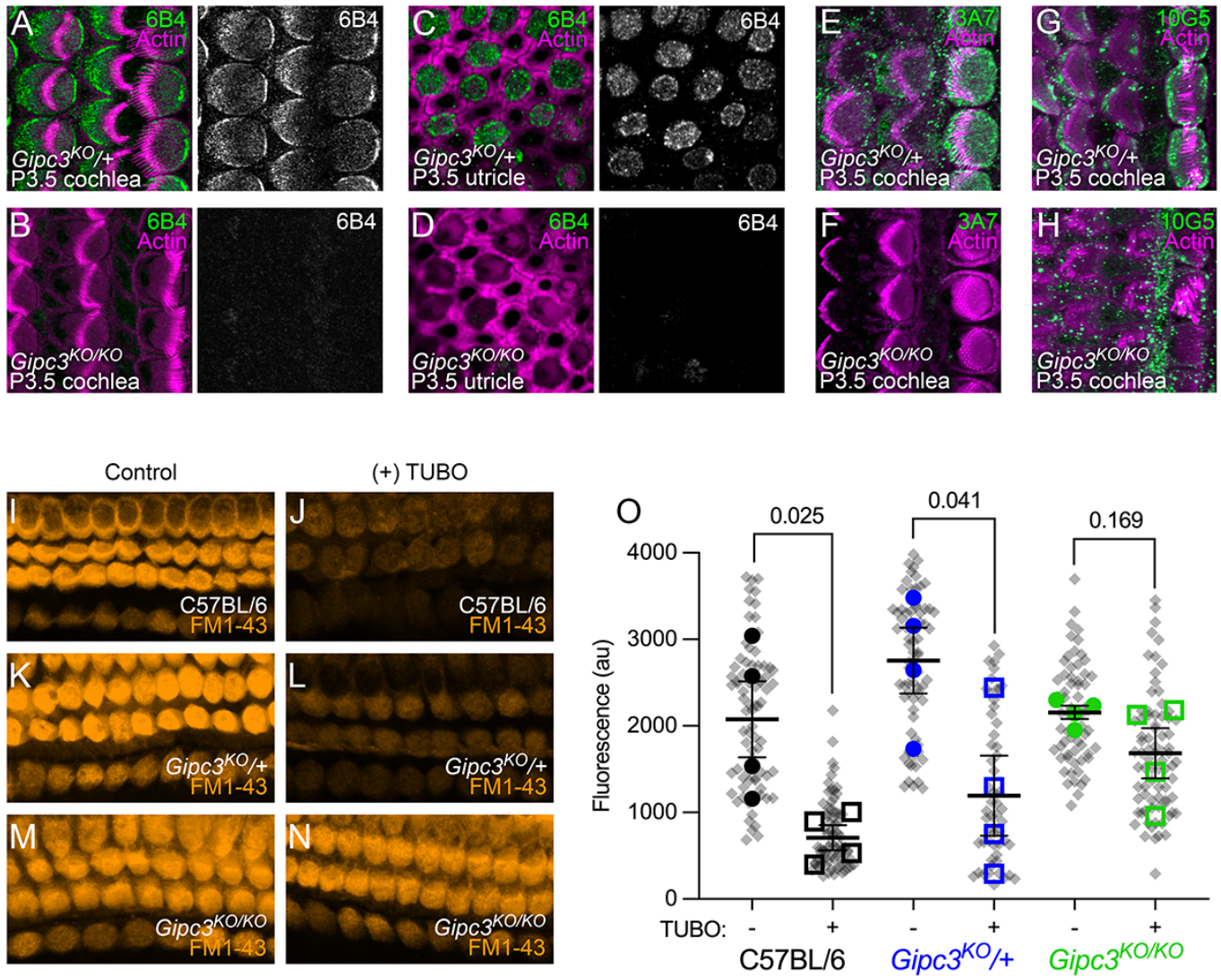

Fig. S1. Anti-GIPC3 and *Gipc3*^{KO} characterization. ***A-H*****, Characterization of anti- GIPC3 monoclonal** antibodies on mouse inner ear tissues. ***A-B***, 6B4 antibody on *Gipc3*^{KO}/+ and *Gipc3*$^{KO/KO}$ cochlea. ***C-D***, 6B4 antibody on *Gipc3*^{KO}/+ and *Gipc3*$^{KO/KO}$ utricle. ***E-F***, 3A7 antibody on *Gipc3*^{KO}/+ and *Gipc3*$^{KO/KO}$ cochlea. ***G-H***, 10G5 antibody on *Gipc3*^{KO}/+ and *Gipc3*$^{KO/KO}$ cochlea. ***I-N***, Images from FM1-43 labeling experiments. ***O***, FM1-43 signals determined for each hair cell (gray symbols). These cell measurements were averaged for each cochlea (11-29 cells per cochlea), and the averages were plotted with colored symbols (4 cochleas per condition) (Lord et al., 2020). Nested t-tests were used to compare the cochlea values for each genotype (Eisner, 2021); P values are indicated. Panel widths: A-H, 25 µm; I-N, 67 µm.

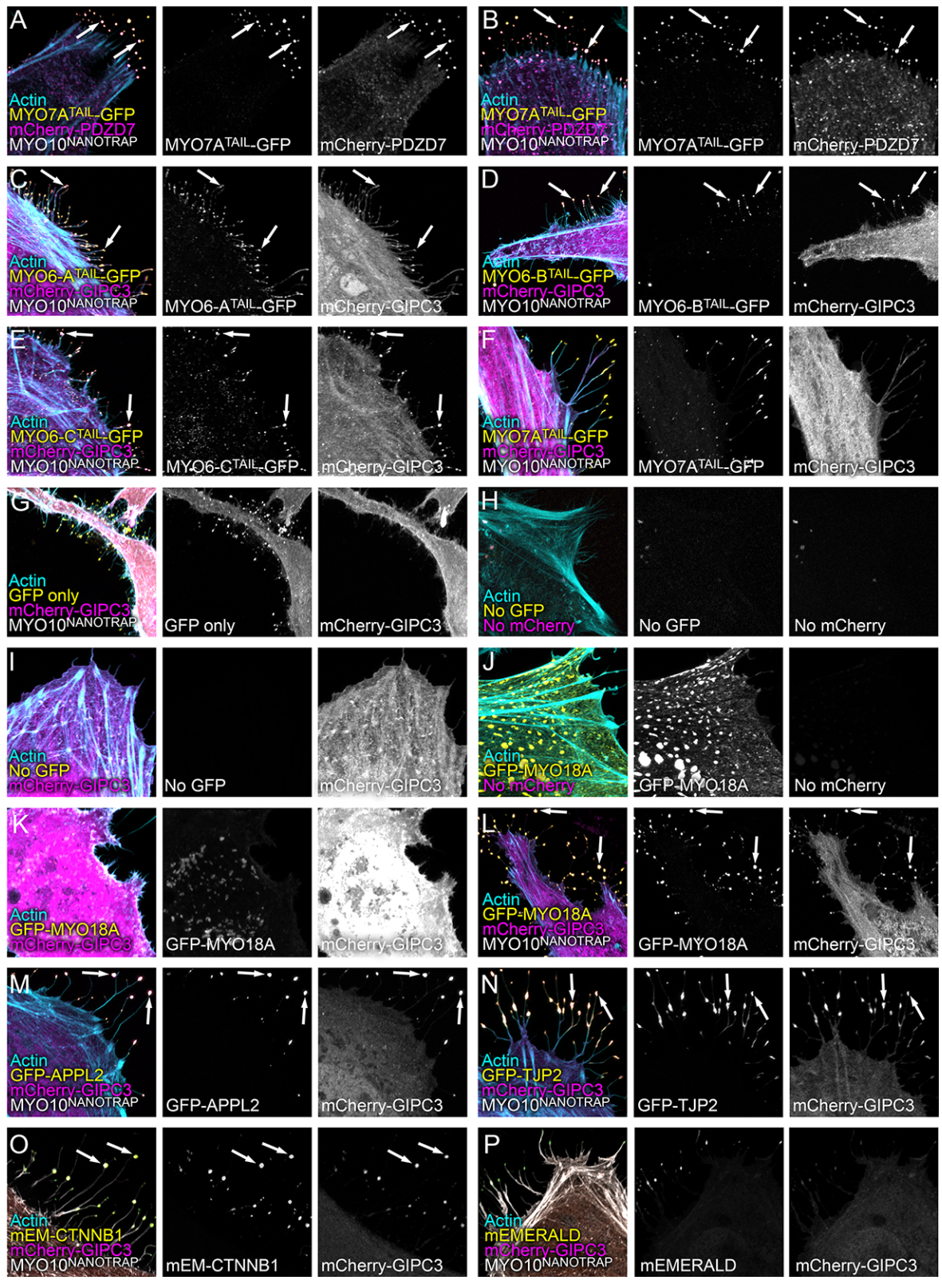

Fig. S2. NanoSPD controls. ***A-B***, Robust interaction (filopodial tip co-localization) between MYO7A^{TAIL}-GFP and mCherry-PDZD7 (two examples). ***C-E***, Robust interaction between A, B, or C splice forms of MYO6^{TAIL}-GFP and mCherry-GIPC3. ***F***, No interaction between MYO7A^{TAIL}-GFP and mCherry-GIPC3. ***G***, No interaction between GFP alone and mCherry-GIPC3. ***H***, No filopodia signal with negative control (no transfection). ***I,*** Cytoplasmic localization of mCherry-GIPC3 alone. ***J***, Cytoplasmic

aggregates with GFP-MYO18A alone. ***K***, Cytoplasmic localization of GFP-MYO18A and mCherry-GIPC3 (no MYO10^{NANOTRAP}). Aggregates were present for GFP-MYO18A but the signal was reduced compared to the mCherry signal. ***L***, Robust interaction between GFP-MYO18A and mCherry-GIPC3. ***M***, Robust interaction between GFP-APPL2 and mCherry-GIPC3. ***N***, Robust interaction between GFP-TJP2 (ZO2) and mCherry-GIPC3. ***O***, Robust interaction between mEMERALD-CTNNB1 (mEM-CTNNB1) and mCherry-GIPC3. ***P***, Very weak interaction between mEMERALD and mCherry-GIPC3. For appropriate panels, arrows indicate filopodial tip co-localization. Panel full widths: 30 µm.

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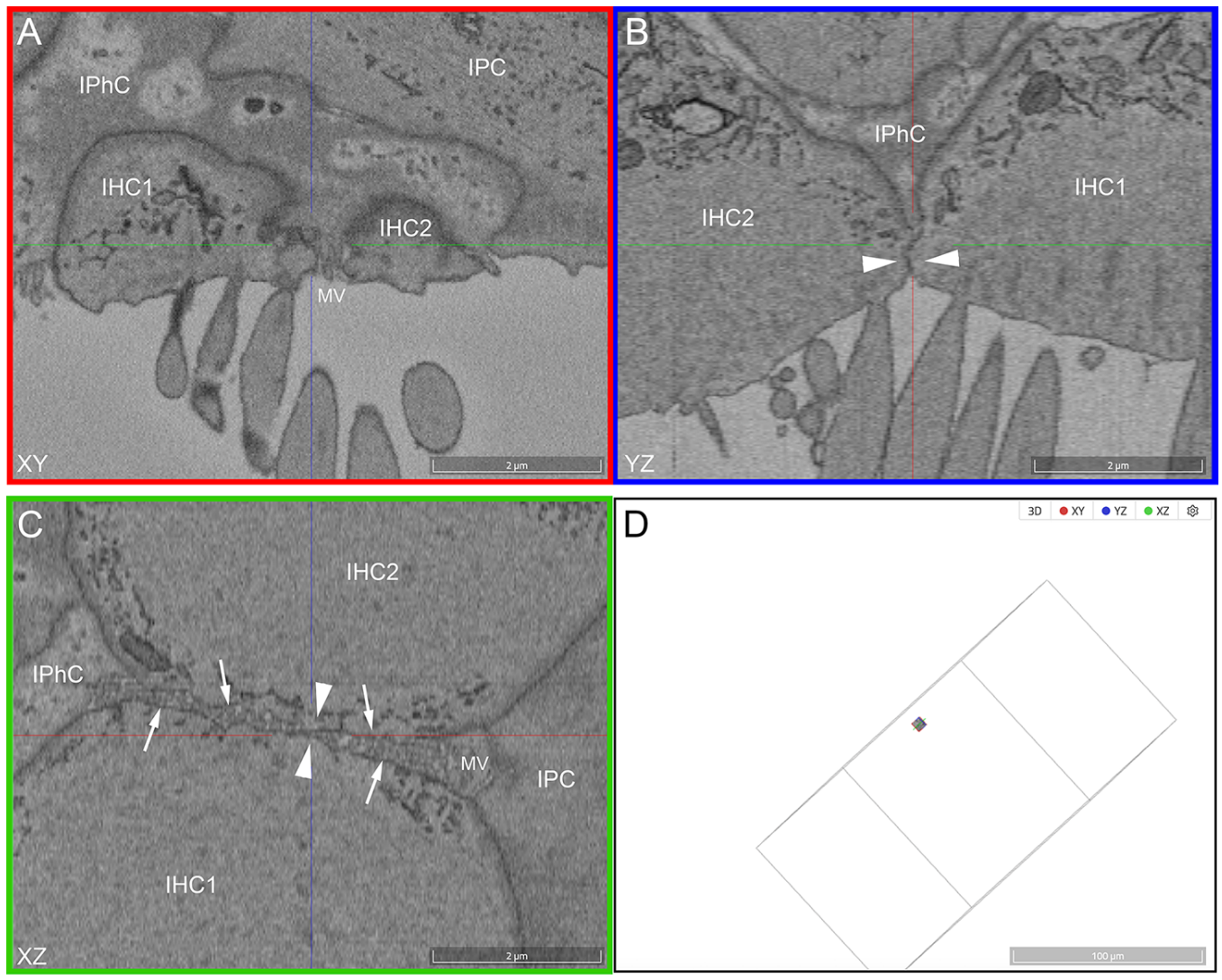

Data from: EM Reconstruction of Neural Circuitry in the Cochlea.
Y Hua, X Ding, H Wang, F Wang, Y Lu, J Neef, Y Gao, T Moser, H Wu.
Cell Reports. 5 January 2021. 10.1016/j.celrep.2020.108551

Fig. S3. Relationship between apical domains of inner hair cells and inner phalangeal cells. ***A***, X-Y plane. ***B***, Y-Z plane. ***C***, X-Z plane. ***D***, Location of the data presented within the entire dataset. Thin green, blue, and red lines show the transects for other image axes. Arrows indicate microvilli of inner phalangeal cells. Arrowheads indicate close IHC-IHC contact. The dataset used for this figure has been published (Hua et al., 2021) and is accessible at https://webknossos.org/datasets/b2275d664e4c2a96/HuaLab-CBA-mice-cochlea-mid-7w/#3954,4819,1586,0,37. Key: IHC, inner hair cell; IPC, inner pillar cell; IPhC, inner phalangeal cell; MV, microvilli.

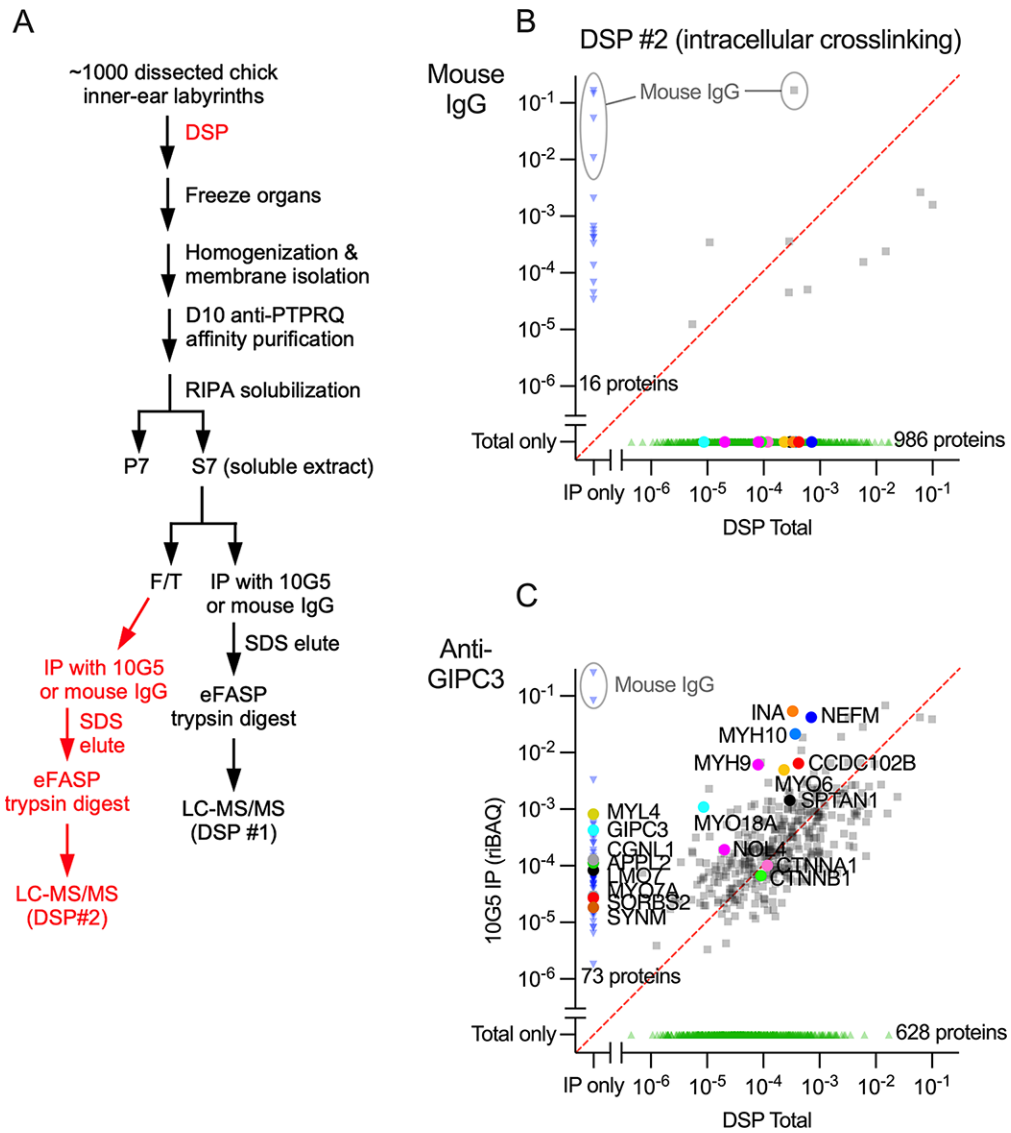

Fig. S4. Anti-GIPC3 immunoaffinity chromatography from chick stereocilia extracts using DSP #2 extract. ***A***, Flow chart for immunoaffinity chromatography, with new steps highlighted in red. The DSP #2 extract corresponds to the flow-through from the first DSP immunoaffinity chromatography step, rerun on either mouse IgG or 10G5. ***B-C***, Comparison of abundance (riBAQ) of proteins detected in total (starting DSP #2 S7 extract; plotted on x-axis) compared to the immunoprecipitates (y-axis) in mouse IgG control (B) and 10G5 anti-GIPC3 (C) experiments. Red dashed line is the unity line (equal riBAQ in total and IP). Key proteins are called out. Mouse IgG from immunoprecipitation is highlighted in gray.

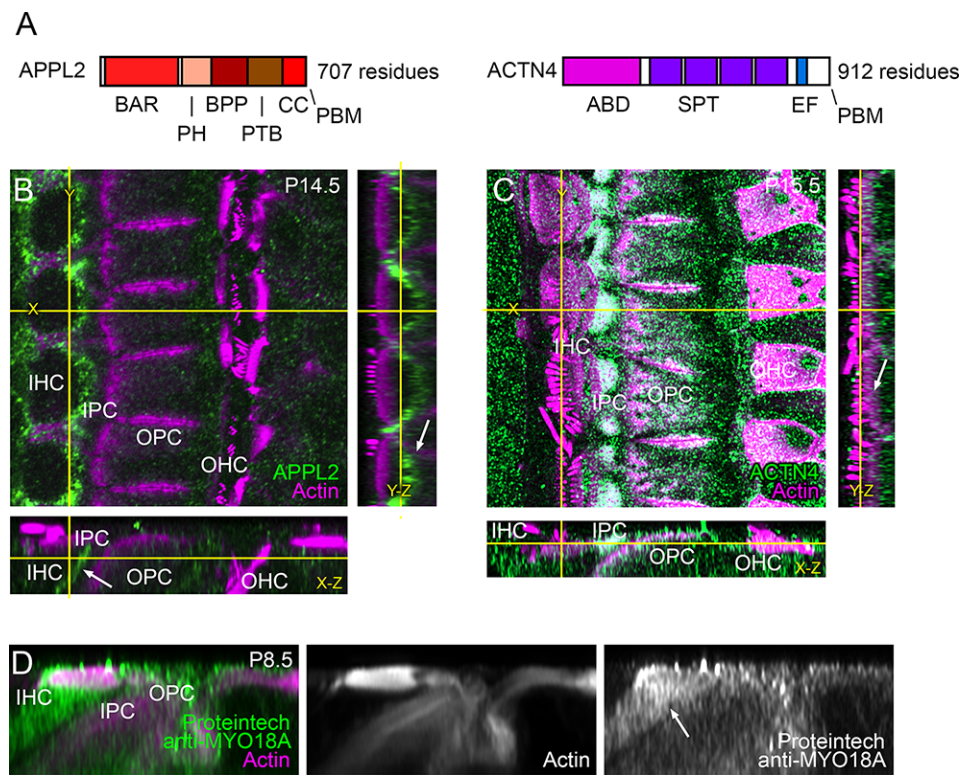

Fig. S5. Localization of APPL2, ACTN4, and MYO18A in mouse cochlea. ***A***, Domain structure of APPL2 and ACTN4. Key: BAR, Bin1/amphiphysin/rvs167; PH, pleckstrin homology; BPP, "region between PH and PTB domains;" PTB, phosphotyrosine binding; CC, coiled coil; PBD, PDZ- binding motif; ABD, actin-binding domain; SPT, spectrin domains; EF, EF hand. ***B***, Immunolocalization of APPL2 in P14.5 mouse cochlea; slices from a three-dimensional image stack. Transects for other image axes are shown in yellow; the X and Y transects in the main X-Y image show the locations for the Y-Z and X-Z images. Arrow indicates concentration of APPL2 immunoreactivity. Key: IHC, inner hair cell; IPC, inner pillar cell; OPC, outer pillar cell; OHC, outer hair cell. ***C***, Immunolocalization of ACTN4 in P15.5 mouse cochlea. Arrow indicates modest ACTN4 in IHC cuticular plates; the strongest labeling is in the head of the inner pillar cells and the sides of the outer pillar cells. immunoreactivity. ***D***, Labeling of IHC with Proteintech anti-MYO18A antibody, showing MYO18A distribution in the cuticular plate region. Key: IHC, inner hair cell; IPC, inner pillar cell; OPC, outer pillar cell; OHC, outer hair cell. Panel widths: X-Y, 37.5 µm (same scale applies to Y-Z and X-Z panels).

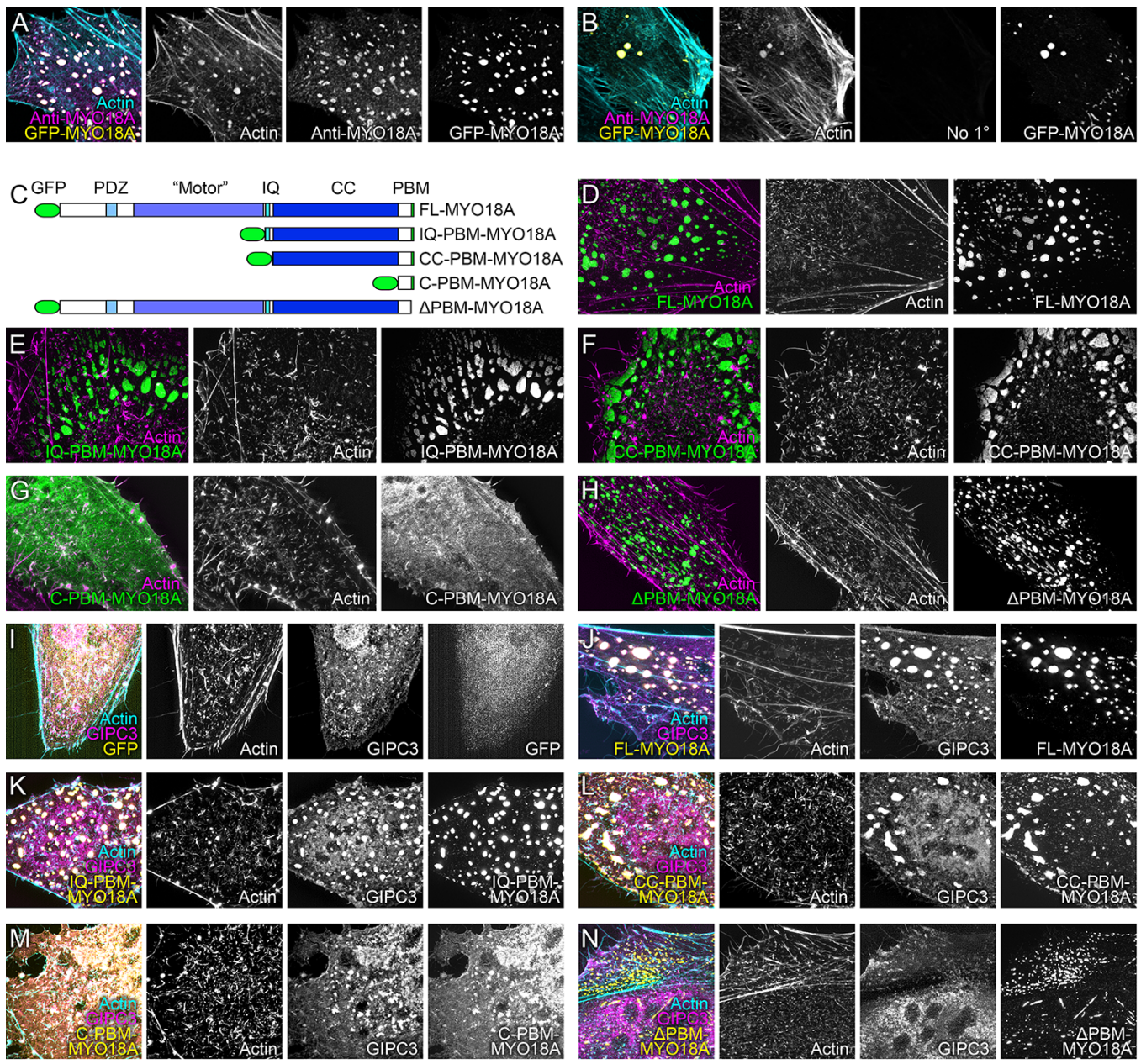

Fig. S6. MYO18A forms aggregates and interacts with GIPC3 in the HeLa cell cytoplasm. ***A-B***, Anti-MYO18A antibody specificity. ***A***, Anti-MYO18A detects the outer shell of GFP- MYO18A aggregates, but cannot penetrate into the core. ***B***, No signal in the absence of primary antibody. ***C***, Diagram of MYO18A expression constructs. The GFP fusion and MYO18A domains are indicated. Key: FL, full-length; IQ, calmodulin-binding IQ domain; CC, coiled-coil domain; C, C-terminal domain; PBM, PDZ-binding motif. ***D-H***, Expression of GFP-MYO18A constructs in HeLa cells. Co-labeled with phalloidin to show F-actin. ***I***, Co-expression of GFP and mCherry-GIPC3 in HeLa cells. ***J-N***, Co- expression of GFP-MYO18A constructs and mCherry-GIPC3 in HeLa cells. Panel full widths: A-B and I- N, 30 μm; D-H, 40 μm.

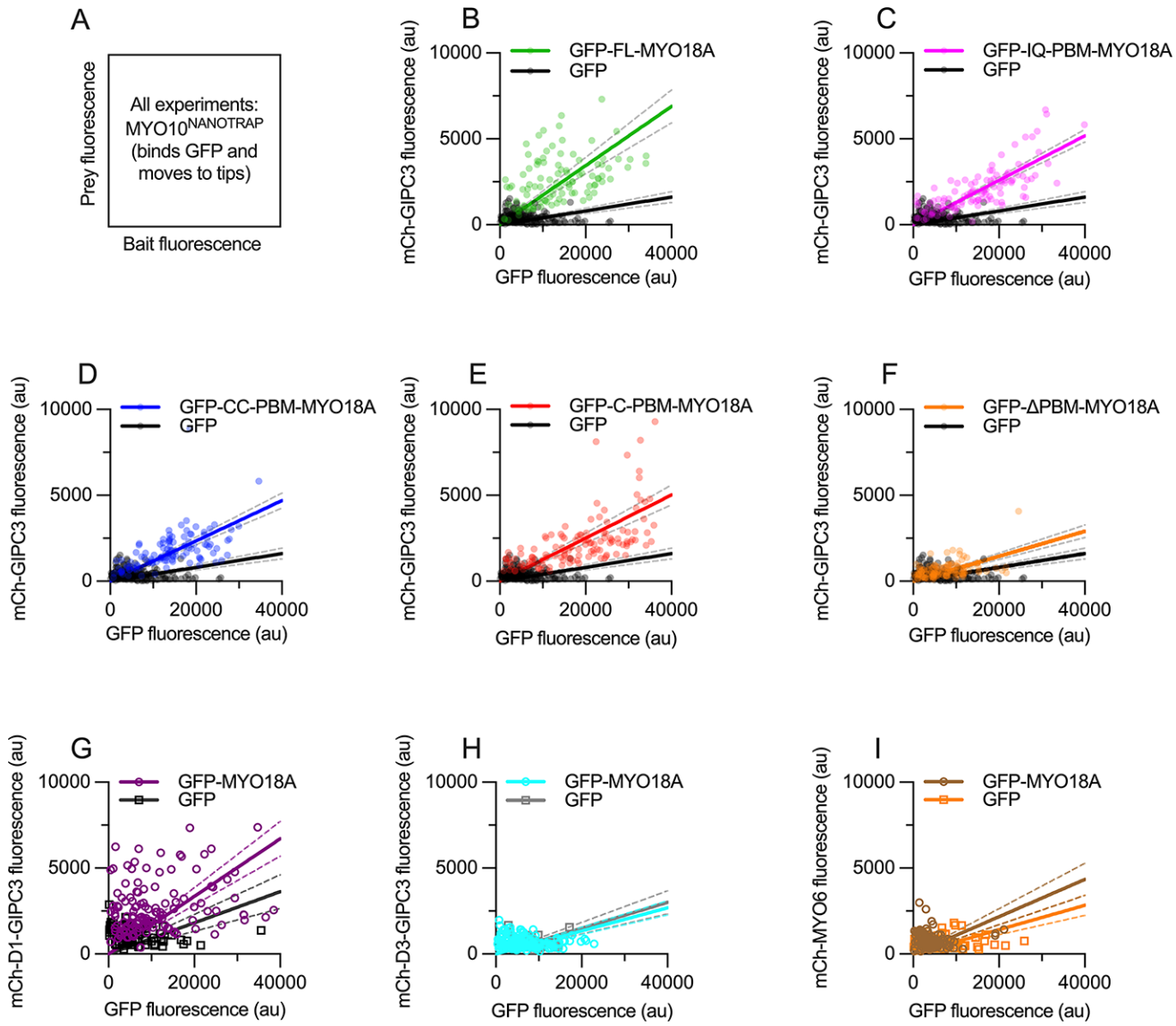

Fig. S7. Intensity correlation analysis for MYO18A domain mapping by NanoSPD. ***A***, Key for experiments and plots. ***B-F***, Intensity correlation analysis for interaction of mCh-GIPC3 with GFP-MYO18A constructs, using scatter plot of bait (x-axis) and prey (y-axis) fluorescence at individual filopodia tips (from three independent determinations). Dashed lines are 95% confidence intervals. ***G-H***, Intensity correlation analysis for interaction of GFP-MYO18A with mCh-GIPC3 constructs. ***I***, Intensity correlation analysis for interaction of GFP-MYO18A with mCh-MYO6 (negative control). Sample sizes were the same as in Fig. 8.

Table S1. Plasmids used in nanoSPD experiments.

Plasmid name	Source	Vendor	Catalog #	Sequence info
pcDNA3.1-MYO10-HMM-Nanotrap	Commercial	Addgene	87255	https://www.addgene.org/87255/
pEGFP-MYO7A-TAIL	Commercial	Addgene	89585	https://www.addgene.org/89585/
EGFP-Myo6 Tail A	In-house	N/A	N/A	MYO6 tail domain (aa 901-1294): Splice variant A (delta aa 1147-1156)
EGFP-Myo6 Tail B	In-house	N/A	N/A	MYO6 tail domain (aa 901-1294): Splice variant B (delta aa 1036-1067)
EGFP-Myo6 Tail C	In-house	N/A	N/A	MYO6 tail domain (aa 901-1294): Splice variant C (delta aa 1036-1067 & aa 1147-1156)
mCherry-Gipc3	Commercial	GeneCopoeia	Ex-Mn14117-M55	Full length mouse GIPC3
mCherry-Gipc3 Domain 3 (D3)	In-house; derived from GeneCopoeia Gipc3 plasmid	N/A	N/A	GIPC3 amino acids 221-291
mCherry-Gipc3 Domain 1 (D1)	In-house; derived from GeneCopoeia Gipc3 plasmid	N/A	N/A	GIPC3 amino acids 92-297
mEmerald-Beta-Catenin-20	Commercial	Addgene	54017	Full length mouse CTNNB1
mEmerald-control	In-house; derived from Addgene Beta-Catenin plasmid	N/A	N/A	mEMERALD tag
EGFP-Appl2	Commercial	VectorBuilder	VB211013-1283jsd	Full length mouse APPL2
EGFP-control	Commercial	Thermo Fisher Scientific	12288015	N-terminal GFP tag
mCherry-Myo6-A	In-house; derived from Myo6 Tail A	N/A	N/A	MYO6 tail domain (aa 901-1294): Splice variant A (delta aa 1147-1156)
EGFP-Myo18a	Commercial	VectorBuilder	VB211220-1232ekc	Full length MYO18A
EGFP-Myo18a-IQ tail	In-house; derived from VectorBuilder Myo18a plasmid	N/A	N/A	IQ domain of MYO18A
EGFP-Myo18a-CC tail	In-house; derived from VectorBuilder Myo18a plasmid	N/A	N/A	Coiled-coil domain of MYO18A
EGFP-Myo18a-c term	In-house; derived from VectorBuilder Myo18a plasmid	N/A	N/A	C-terminal end of MYO18A
EGFP-Myo18adelta4AA	In-house; derived from VectorBuilder Myo18a plasmid	N/A	N/A	MYO18A last 4 amino acids deleted
mCherry-PDZD7	Gift from Mhamed Grati	N/A	N/A	Full length mouse PDZD7
pEGFP-TJP2	Commercial	Addgene	27422	Full length mouse TJP2

Table S2. Genotype-phenotype comparison for G0 i-GONAD experiments.

		Phenotype				
Pup #	Genotype	Wild type	Ambig-uous	Mosaic	KO	Not exam-ined
12	Mosaic. Ex2, no sequence. Ex4, indel with no size estimate; large deletion, indel ≈8587bp.				X	
13	Wild type	X				
14	Wild type	X				
15	Mosaic. Ex2 and Ex4, no PCR bands. Large deletion, indel with 8610bp deletion plus a 47bp insertion.				X	
16	Wild type		X			
17	Wild type	X				
18	Wild type					X
19	Wild type	X				
20	Wild type					X
29	Wild type	X				
30	Wild type					X
31	Mosaic. Ex2 WT; Ex4, ≈5bp indel.			X		
32	Wild type					X
33	Wild type					X
34	Wild type					X
35	Mosaic. No Ex2 band. Ex4 mosaic with 7bp indel. Mosaic large deletions (~8515bp deletion and 8573bp deletion + 59bp insertion).			X		
36	Mosaic. Ex2, 378bp insertion. Ex4 PCR mosaic for WT and 199bp insert.			X		
37	Wild type	X				
38	Mosaic. Ex2 18bp deletion. Ex4 single T-to-A. Large deletion, an indel with 8560bp deletion and 40bp insertion.				X	
39	Wild type	X				
40	Mosaic. Ex2 17bp deletion. Ex4 single T insertion. Large deletion (8533bp).				X	
41	Wild type	X				
42	Mosaic. Ex2 50bp deletion. Ex4 single T-to-A, plus single T insertion. Large deletion (8560bp).				X	
43	Mosaic. Ex2 60bp deletion. Ex4 single T insertion. Mosaic large deletions (~8560bp, ~8660bp, and ~8660bp deletion plus a 45bp insertion.				X	
45	Wild type					X
46	Wild type					X
47	Wild type					X
48	Wild type	X				
49	Wild type					X
50	Wild type					X
51	Wild type					X
52	Wild type	X				
53	Mosaic. Ex2 indel (unknown size). Ex4 6bp deletion. Large deletion 8577bp.				X	
54	Mosaic. Ex2 7bp indel . Ex4 mosaic for large deletion (8574bp).				X	

Summary: 10 mice with wild-type genotypes were examined, and all 10 had wild-type phenotypes; 11 mice with mosaic genotypes were examined, and all 10 had mosaic or knockout phenotypes.

Table S3. Proteins enriched in 10G5 anti-GIPC3 immunoprecipitates relative to chicken inner extract. Only proteins detected in 2/2 technical replicates from one of the two experiments are displayed (35 total); proteins that were detected in the immunoprecipitates using control mouse IgG were excluded.

Protein characteristics			DSP experiment #1				DTSSP experiment			
Best protein identifier	Best protein description	Best protein symbol	Detected	riBAQ	Stoichiometry re GIPC3	IP/total	Detected	riBAQ	Stoichiometry re GIPC3	IP/total
ENSGALP00000043100	GIPC PDZ domain containing family, member 3	GIPC3	2/2	4.14E-04	1.0	IP only	2/2	2.33E-03	1.0	IP only
ENSGALP00000005475	Oncomodulin (parvalbumin 3)	OCM	2/2	2.22E-04	0.5	IP only	2/2	8.14E-04	0.3	1.7
ENSGALP00000019801	Septin 7	SEPTIN7	2/2	1.71E-04	0.4	IP only	2/2	2.51E-04	0.1	8.5
ENSGALP00000007822	Coronin 1C	CORO1C	2/2	1.49E-04	0.4	IP only	2/2	1.76E-05	0.0	2.2
ENSGALP00000015531	Dynein, cytoplasmic 1, intermediate chain 2	DYNC1I2	2/2	1.36E-04	0.3	IP only	2/2	2.41E-05	0.0	2.1
ENSGALP00000007644	Myosin phosphatase Rho interacting protein	MPRIP	2/2	1.14E-04	0.3	IP only	2/2	1.12E-05	0.0	2.6
ENSGALP00000029968	Ribosomal protein L3	RPL3	2/2	1.05E-04	0.3	IP only	2/2	1.88E-04	0.1	0.7
ENSGALP00000020645	Adaptor protein, phosphotyrosine interaction, PH domain and leucine zipper containing 2	APPL2	2/2	8.47E-05	0.2	IP only	2/2	3.16E-04	0.1	112.6
ENSGALP00000006826	Cingulin-like 1	CGNL1	2/2	6.72E-05	0.2	IP only	2/2	3.60E-05	0.0	IP only
ENSGALP00000001044	Myosin VIIA	MYO7A	2/2	6.36E-05	0.2	IP only	2/2	4.13E-04	0.2	15.5
ENSGALP00000027304	LIM domain 7	LMO7	2/2	2.74E-05	0.1	IP only	2/2	6.19E-06	0.0	1.0
ENSGALP00000033263	Synemin, intermediate filament protein	SYNM	2/2	2.63E-05	0.1	IP only	2/2	2.57E-05	0.0	IP only
ENSGALP00000021860	Sorbin and SH3 domain containing 2	SORBS2	2/2	1.61E-05	0.0	IP only	2/2	7.49E-05	0.0	17.1
ENSGALP00000023016	LIM and calponin homology domains 1	LIMCH1	2/2	1.16E-05	0.0	IP only	1/2	6.29E-06	0.0	2.6
ENSGALP00000042590	Keratin 18	KRT18	2/2	1.80E-03	4.4	166.3	2/2	1.92E-03	0.8	7.1
ENSGALP00000000419_family	Family[1] (Internexin neuronal intermediate filament protein, alpha)	INA	2/2	3.45E-02	83.2	104.0	2/2	1.14E-01	49.1	55.7
ENSGALP00000038853	Myosin XVIIIA	MYO18A	2/2	7.76E-04	1.9	89.1	2/2	4.17E-04	0.2	39.4

ENSGALP00000041153	Keratin X	KRTX	2/2	6.56E-03	15.8	72.4	2/2	1.07E-03	0.5	7.9
ENSGALP00000020445	Myosin, heavy chain 9, non-muscle	MYH9	2/2	5.11E-03	12.3	63.4	2/2	2.79E-03	1.2	18.9
ENSGALP00000023352	Myosin, heavy chain 10, non-muscle	MYH10	2/2	1.96E-02	47.3	52.6	2/2	1.01E-02	4.3	16.6
ENSGALP00000030296_family	Family[1] (Myosin, light chain 12A, regulatory, non-sarcomeric)	MYL12A; MYL12B; MYL9	2/2	5.30E-03	12.8	43.0	2/2	2.88E-03	1.2	6.3
ENSGALP00000000422	Neurofilament, medium polypeptide	NEFM	2/2	2.81E-02	67.9	39.3	2/2	9.53E-02	41.0	41.4
ENSGALP00000016648	Keratin 78	KRT78	2/2	1.74E-02	41.9	34.9	2/2	1.36E-02	5.8	5.7
ENSGALP00000025573	Myosin VI	MYO6	2/2	5.20E-03	12.5	22.3	2/2	8.29E-03	3.6	12.7
ENSGALP00000024476	Nucleolar protein 4 like	NOL4	2/2	4.37E-04	1.1	21.5	2/2	4.15E-04	0.2	IP only
ENSGALP00000040890	Sad1 and UNC84 domain containing 2	SUN2	2/2	5.91E-04	1.4	17.0	2/2	2.78E-05	0.0	0.1
ENSGALP00000014023	General transcription factor IIH, polypeptide 4, 52kDa	GTF2H4	2/2	1.91E-03	4.6	15.8	2/2	1.62E-04	0.1	1.8
ENSGALP00000023635	Lamin B1	LMNB1	2/2	6.45E-03	15.6	13.5	2/2	2.19E-04	0.1	0.7
ENSGALP00000009801	Lamin A/C	LMNA	2/2	8.38E-04	2.0	12.0	2/2	3.37E-05	0.0	0.3
ENSGALP00000023085	Actinin, alpha 4	ACTN4	2/2	6.49E-04	1.6	10.2	2/2	3.56E-04	0.2	2.0
ENSGALP00000015409	Actinin, alpha 1	ACTN1	2/2	2.61E-04	0.6	10.0	2/2	1.24E-04	0.1	1.9
ENSGALP00000030322	Coiled-coil domain containing 102B	CCDC102B	2/2	4.09E-03	9.9	9.7	2/2	3.81E-04	0.2	IP only
ENSGALP00000022004_family	Family[1] (Tropomyosin 3)	TPM3; TPM1; TPM2; TPM4	2/2	3.77E-04	0.9	8.8	2/2	4.13E-04	0.2	2.8
ENSGALP00000036906	Calcium/calmodulin-dependent protein kinase type II delta chain	CAMK2D	2/2	1.53E-04	0.4	8.7	2/2	6.75E-05	0.0	1.1
ENSGALP00000035478	Filamin B, beta	FLNB	2/2	4.48E-04	1.1	8.4	2/2	3.06E-04	0.1	3.8

[1] Protein families are defined as groups of proteins that share 20% or more of their identified peptides. The protein with the most evidence (unique peptides identified) is listed first.

Table S4. Proteins interacting with GIPC1 and their C-terminal sequences. The C-terminal ten amino acids of Ensembl splice forms of proteins identified as GIPC1 interactors in Katoh (2001) are listed. These sequences were used with Weblogo (https://weblogo.berkeley.edu/logo.cgi) to create the sequence logo in Fig 7.

Species and Ensembl splice form	C-terminal
Hs PLXND1-201	DNIYECYSEA
Hs LHCGR-204	LLDKTRYTEC
Hs RGS19-202	QGPSQSSSEA
Hs VANGL2-201	VMRLQSETSV
Hs FZD9-201	DPSLENPTHL
Hs FZD4-201	KPGKGSETVV
Hs FZD2-201	TNSRHGETTV
Hs APPL1-201	EGGKKRESEA
Hs ITGA5-201	QLKPPATSDA
Hs ITGA6-201	SDKERLTSDA
Hs ITGA8-201	QLTNDKTPEA
Hs NRP1-206	LNTQSTYSEA
Hs SLC2A1-203	FHPLGADSQV
Hs SEMA4C-201	PDSNPEESSV
Hs SDC4-201	KKAPTNEFYA
Hs IGF1R-217	ALPLPQSSTC
Hs ADRB1-201	RPGFASESKV
Hs CD93-201	QYSPTPGTDC
Hs DRD2-202	RKAFLKILHC
Hs ENG-202	STPCSTSSMA
Hs LRP1-201	PEDEIGDPLA
Hs LRP2-205	ANLVKEDSEV
Hs NTRK1-206	APPVYLDVLG
Hs TGFBR3-201	STPCSSSSTA
Hs TPBG-201	LTNLSSNSDV
Hs TYRP1-203	KLQNPNQSVV

Supplemental References

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