Supporting Information

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SI Materials and Methods

Protein Preparation. GST-Pins^{25–444}: Insc^{252–263} was purified by affinity and anion-exchange. All Pins and LGN mutants were purified similarly. Human NuMA^{1,807–1,987} was purified by nickel affinity and cation-exchange. To map the minimal interacting regions, Pins^{25–444}: Insc^{252–263} was incubated with trypsin in a 30:1 wt:wt ratio for 1 h at 4 °C, and analyzed by ESI-MS (ProMiFa). Human LGN: $G\alpha i^{GDP}$ was produced from insect cells by colysis with $G\alpha i^{26–354}$, and purified as reported (1). Human Insc: LGN: $G\alpha i^{GDP}$ complex was generated by coinfection with two baculoviruses. For crystallization studies, the proteolyzed Pins^{25–406}: Insc^{303–340} was concentrated up to 10 mg/mL in 10 mM Hepes pH 7.5, 0.1 M NaCl and 1 mM DTT.

Crystallization and Crystal Structure Determination. $Pins^{25-406}$: $Insc^{303-340}$ was crystallized by hanging-drop vapor diffusion at 20 °C with a reservoir containing 0.1 M Tris-HCl pH 8.5, 0.2 M Magnesium chloride hexahydrate and 15% PEG 4000. For data collection, crystals were transferred to a cryo buffer (reservoir buffer supplemented with 20% ethylene glycol), and flash-frozen in liquid nitrogen. Crystallization experiments were performed at the Crystallography Unit of the IFOM-IEO Campus (Milan). X-ray diffraction data were collected at beamline ID23-1 at European Synchrotron Radiation Facility (Grenoble). Data were processed with HKL2000 (2). Initial phases were derived using SHELX in hkl2map (3). Model building was initiated with helical fragments placed into the electron density by Phenix (4), and completed using iterative cycles of manual model building in

- Tall GG, Gilman AG (2005) Resistance to inhibitors of cholinesterase 8A catalyzes release of Galphai-GTP and nuclear mitotic apparatus protein (NuMA) from NuMA/LGN/ Galphai-GDP complexes. Proc Natl Acad Sci USA 102:16584–16589.
- Minor ZOaW (1997) Processing of X-ray diffraction data collected in oscillation mode. Methods in Enzymology Volume 276:307–326.
- 3. Pape T, Schneider TR (2004) HKL2MAP: a graphical user interface for phasing with SHELX programs. J Appl Cryst 37:843–844.

COOT (5) and restrained refinement. The final model contains residues 39–386 of Pins^{TPR}, and residues 307 to 335 of dInsc^{PEPT}. The loop 369–374 of Pins^{TPR} connecting the α B8 helix of the last TPR with the capping helix α C is missing as the density in this region is rather poor. For analogous reasons, only a poly-Ala model could be built for the terminal α C helix. Data statistics are shown in Table S1.

In Vitro Binding Assays. To test the effect of point mutations, GST-dInsc^{303–340}, GST-hInsc^{24–58}, and GST-NuMA^{1,886–1,914} (0.2 μ M) adsorbed on GSH-beads were incubated for 1 h at 4 °C with 0.2–0.4 μ M of the chosen TPR construct in a buffer containing 10 mM Hepes pH 7.5, 0.1 M NaCl, 5% glycerol, 0,1% Triton X-100, 0,1% Tween20 and 0,1% Na-deoxycholate.

ITC. Lyophilized dInsc³⁰³⁻³⁴⁰, hInsc²⁴⁻⁵⁸, and NuMA^{1,886-1,914} peptides and TPR domains were dialyzed against 10 mM Hepes pH 7.5, 0.15 M NaCl and 5% glycerol. ITC measurements were performed on a MicroCal VP-ITC (MicroCal, Inc).

Fluorescence Polarization. Fluorescence polarization measurements were performed on an Infinite F200 (Tecan). Fluoresceinlabeled NuMA^{PEPT} (15 nM) was incubated with 250 nM LGN^{TPR} in the presence of increasing concentration of unlabeled hInsc^{PEPT} in 10 mM Hepes pH 7.5, 0.15 M NaCl, 0.5 mM EDTA and 1 mM DTT. A similar experiment was performed titrating unlabeled NuMA^{PEPT} into a mixture of fluorescein-hInsc^{PEPT}.

- Adams PD, Grosse-Kunstleve RW, Hung LW, loerger TR, McCoy AJ, Moriarty NW, Read RJ, Sacchettini JC, Sauter NK, Terwilliger TC (2002) PHENIX: building new software for automated crystallographic structure determination. *Acta Crystallogr D Biol Crystallogr* 58:1948–1954.
- 5. Emsley P, Cowtan K (2004) COOT: Model-building tools for molecular graphics. Acta Cryst D60:2126–2132.



Fig. S1. SDS-PAGE of the untreated and trypsinized sample that was submitted to Mass Spectrometry analysis.



			αΑ	insertion	α	B	
Hs LGN		$-\infty$		<u> </u>			<u>~</u>
Repeat	Begin						End
TPR1	17	CLEL	ALEGERLCKSGD		CRAGVSFFE	AAVQVGTE	D 50
TPR2	55	SAIY	SQLGNAYFYLHD-		YAKALEYHE	HDLTLART	I 88
TPR3	95	AKAS	GNLGNTLKVLGN-		FDEAIVCCQ	RHLDISRE	L 128
TPR4	135	ARAL	YNLGNVYHAKGK:	SFGCPGPQDVGEFPI	EEVRDALQAAVDFYE	ENLSLVTA	L 188
TPR5	195	GRAF	GNLGNTHYLLGN-		FRDAVIAHE	QRLLIAKE	F 228
TPR6	235	RRAY	SNLGNAYIFLGE		FETASEYYF	KTLLLARQ	L 268
TPR7	275	AQSC	YSLGNTYTLLQD-		YEKAIDYHI	KHLAIAQE	L 308
TPR8	315	GRAC	WSLGNAYTALGN-		HDQ <mark>A</mark> MHFAB	KHLEISRE	V 348
TPR positi	on	1 4	6789 11		20	3	4
Dm PINS	3						
Repeat	Begin						End
TPR1	43	CLEL	ALEGERLCKAGD		CRAGVAFFQAAI	QAGTED	76
TPR2	81	SAIY	SQLGNAYFYLGD-		YNKAMQYHKHDI	JTLAKSM	114
TPR3	121	AKSS	GNLGNTLKVMGR-		FDEAAICCERHI	TLARQL	154
TPR4	161	GRAL	Y nlgn V y hakgki	HLGQRNPGKFGDDVI	KEALTR A VEFYQENI	KLMRDL	211
TPR5	218	GRAC	GNLGNTYYLLGD-		FQAAIEHHQERI	RIAREF	251
TPR6	258	RRAN	IS NLGN S H IFLGQ-		FEDAAEHYKRTI	ALAVEL	291
TPR7	298	AQSC	YSLGNTYTLLHE		FNTAIEYHNRHI	AIAQEL	331
TPR8	338	ARAC	WSLGNAHSAIGG-		HER <mark>A</mark> LKYAEQHI	QLAKEL	371
TPR position		1	6789 11		20	34	

Fig. S2. Top: cartoon drawing of the Pins^{TPR} architecture showing the TPRs. Invariant Asn are shown in sticks. Bottom: alignment of the TPR motifs of LGN and Pins. The structure-based alignment of TPR sequences of LGN and Pins highlights the presence of NLGN motifs in the αA helix (in purple), and of conserved hydrophobic residues (in red) conforming to the canonical TPR consensus (shown at the bottom). The presence of a 17-20 residues insertion between helix αA and αB of TPR4 is visible in both proteins.



Fig. S3. Structural comparison of Pins^{TPR} and OGT. The topological features of the Pins^{TPR} superhelical arrangements were compared with OGT using the server Rapido, which revealed the presence of an invariant rigid body consisting of the first TPR of Pins^{TPR} (residues 42 to 70) and the third TPR of OGT (residues 112 to 140). Superposition of Pins^{TPR} (in gold) and OGT (in light gray) on this rigid body shows an outward displacement of the helical axis of Pins^{TPR} caused by the TPR4 insertion, which would otherwise clash on the following helical turn. Two orthogonal views are shown.



Fig. S4. Surface views of Pins^{TPR} colored by electrostatic potential. The EPE^{Insc} triplet is shown in sticks.

Dm_Insc Hs_Insc Mm_Insc Gg_Insc Ol_Insc	250 1 19 1	GE AP PP Y NNE TVTF GATE QDE S L QQQP S PL RVRS L RF TAS TS TP KS GS K I AKRGKK HPE P VAS WAS E QR WAGE PE V MCT L QH KS I AQE AY K NYT I TT S AV CKL VR MMAL P GGR HL DS VTL P GQR L HL	354 71 71 89 62
Dm_Insc Hs_Insc Mm_Insc Gg_Insc Ol_Insc	355 72 72 90 63	QLQQQALSLQVHFERSERVLSGLQASSLPEALAGATQLLSHLDDFTATLERRGVFFNDAKIERRRYEQHLEQIRTVSKDTRYSLERQHYINLESLLDDVQLLKRH DPLQLLLKRGWVISTELRRIGQKLAQDRWARVHSMSVRLTCHARSMVSEVSAVSRNSLKEMGEIEKLLMEKCSELSAVTER-CLQVENEHVLKSMKAC DPLQLLLKRGWVISTELRRIGQKLAQDRWARVHSMSVRLTCHARSMVSEYSTISRTASQEMGQAEKLLMEKCSELSAVTER-CLQVENEHVLKSMKAC DNLQLLLKRAWVISTELRRIGQKLAQDRWARVHSMSVRLTCHARSMVSEYSTISRTASQEMGQAEKLLMEKCSELSAVTER-CLQVENEHVLKSMKAC DNLQLLLKRAWVISTELTRIAQKLEKNRWQRVHSMTVRVNCHVRSMINEYNAFTRSSSEEMNQLEKLLIDKCSEFTAFTER-CIQIEDEQMLRSMKSC DHVARNNLQTLLRRALVVSTELGKMFQRLEKGRWQRVHSTAVRANCHVRSLVHEYGAA-RSIPPEMQKYEKSLLEKCMELTNITER-CLHTDDEFFLKSMREA	459 168 168 186 163
Dm_Insc Hs_Insc Mm_Insc Gg_Insc Ol_Insc	460 169 169 187 164	T L I TL RL I F E R L V R V L V I S I E QS QCD L L RAN I NMVAT L MN I DY DGF AS L S DAF V QNE AV R T L L V V V D H K QS S V RAL AL RAL AT L C CAP QA VS E TL S ML GQHF GQL L E LAL TRE V Q	551 230 230 248 258
Dm_Insc Hs_Insc Mm_Insc Gg_Insc Ol_Insc	552 231 231 249 259	INQLGS CGG I E I VRD I LQVES AGE RGA I E RRE AVS LLAQ I TAAWHGS E HRVPGLRDCAE SLVAGLAAL LQPE CCT OT LLLCAAAL NNLSR ME ATS HYS I MS NEA I KE GGVVAL FKVCRODS FRCLYP QAL RTLAS I CCVE E GVHQLE KVDGVLCLADILTDNS HSEATRAE AAAVVAQVTS PHLPVTQHLSS FLES ME I VTALVKL KE GGVVAL FKVCRODS FRCLYP QAL RTLAS I CCVE E GVHQLE KVDG I LCLADILTDES HSEATRAE AAAVVAQVTS PHLSFTQHLTS FLENMEE I VTALVKL KE GGVVAL FK I CRODY FRCLYP QTL RTLAS I CCVE E GMHQLE KVDG I LCLADILTDNT HSEATHAE AAAVVAQVTS PHLFT QHLSS FLENMEE I VTALVKL KE GGVVAL FK I CRODY FRCLYP QTL RTLAS I CCVE E GMHQLE KVDG I LCLADILTDNT HSEATHAE AAAVVAQ VTS PHLTFTQHLSS FLENMEE I VTALVKL KE GAVVAL FK I CRODY FRCLYP QTL RTLAS I CCVE E GMHQLE KVDG I LCLADILTDNT HSEATHAE AAAVVAQ I TS PHLTFTQHLSS FLENMEE I VTALVKL KE GAVVAL FK I CRODY FRCLYP QTL RTLAS I CSVE E GINQL DKVDG I LCLADILTD SSVEAARAE AAAVVAQ I TS PHLTSTQHLASFLES MHDI VTALIKL	656 332 332 350 360
Dm_Insc Hs_Insc Mm_Insc Gg_Insc Ol_Insc	657 333 333 351 361	FRLIDTLE HQTCGTS VFLYEQ IVGMLHNMSLNKKCHS HLANGVIINFITS VYQTEFYQTYGS RAESDAQR RTIKTILHTLTRLASDS PTLGAELLEQWHLPDL CQEASSGEVFLLASAALANITFFDT MACE MLLQLNA IRVLLEACSDKQRVDTPYTRDQ IVTILANMSVLEQCASDIIQENG VQLIMGMLSEKPRSGTPAEV CQEASSGEVFLLASAALANITFFDK MACE MLLQLNA IRVLLEACGDKQRVDTPYTRDQ IVTILANMSVLEQCASDIIQENG VQLIMGMLSEKPRSGTPAEV CQEASSGEVFLLASAALANITFFDT MACE MLLQLNA IRVLLEACGDKQRVDTPYTRDQ - VVTILANMSVLEQCASDIIQENG VQLIMGMLSEKPRSGTPAEV CQEASSGEVFLLASAALANITFFDT MACE MLLQLNA IRVLLEACGDKQRVDTPYSRDQ - VVTILANMSVLDQCASEIIQCNG - VQLIMEMLFERSSSGNSAEV CENASCGEVFLLASAALANITFFDT MACE MLLQLNAWKI - LLAACSDKHIVDTPYSRDQ - VVTILANLSVLEPCASEVMQEQG VERLLLLGEKPSSPSFG	759 433 433 451 461
Dm_Insc Hs_Insc Mm_Insc Gg_Insc Ol_Insc	760 434 434 452 462	L RQS L A L KP ANS QQQQHL DS S Y GGD I S QL AR QL L RAHGQE QQL L QATPAP GGS F S S GHQT PE AQTQT NS S GMS S S GS KTKAKS S A S P LL KF NL TRQ <mark>E S F V</mark> AACE R V QQKAAVT L AR L S RD – P DVARE AVR L S CMS R L I – E L CRS P S E R NS S DAVL VAC L AAL RR L AGVC PE GL QDS DF QQL V Q P R L VDS F LL C S N ME E S F V AACE R V QQKAAVT L AR L C RD – P DVAQE AVR L S CMS R L I – E L CRS P S E R NS S DAVL VAC L AAL RR L AGVC PE GL QDS DF QQL V Q P R L VDS F LL C S N ME E S F V AACE R V QQKAAVT L AR L C RD – P DVAQE AVR L S CMS R L I – E L C RS P S E R NS S DAVL VAC L AAL RR L AGVC PE GL QDS DF QQL V Q P R L VDS F LL C S N ME E S F V AACE R V QQKAAVT L AR L S RD – P E V ADAAVK L S C I P R L I – E L C RS P T E R NNS DS V L VAC L AAL RR L AVMS PE GL E DS DF QQL V K P R L VDS F LL C S N ME E S F V AACE R V QQKAAVT L AR L S RD – P D V AQT AI Q L KAVP R L I – C L C RS P T E R NNS DS V L VAC L AAL RR L AGC P S S I S V ADHQQL I K P R L VDS F LL C S N ME E S F V	859 532 532 550 560
		• Residues of Insc required for binding to Pins/LGN	

V Boundaries of the *Drosophila* 'asymmetric domain'

100 50 0% sequence identity

Fig. S5. Sequence alignment of Insc orthologs. Insc residues are colored according to their conservation, which was calculated based on alignment of five orthologs from *Drosophila melanogaster, Homo sapiens, Mus musculus, Gallus gallus* and *Oryzias latipes (Medaka)*. The secondary structure of the region corresponding to dInsc^{PEPT} as derived from structural analysis is displayed on top of the alignment, with the residues required for the interaction with Pins^{TPR} indicated by red circles. Purple triangles mark the boundaries of the *Drosophila* asymmetric domain.

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Fig. S6. Measurement of binding affinities between Pins^{TPR}:dlnsc^{PEPT}, LGN^{TPR}:hlnsc^{PEPT}, and LGN^{TPR}:NuMA^{PEPT}. Isothermal Titration Calorimetry experiments revealed that Pins^{TPR} binds dlnsc^{PEPT} with an affinity comparable to the one of LGN^{TPR} for hlnsc^{PEPT}, with dissociation constants of 5 nM and 13 nM respectively. NuMA^{PEPT} binds LGN^{TPR} with a lower affinity, with a K_D of 50 nM. All reactions are exothermic and exhibit a 1:1 stoichiometry.



Fig. S7 Fluorescence polarization measurement of the binding affinity between LGN^{TPR} and NuMA^{PEPT}. LGN^{TPR} was titrated into 15 nM of fluorescein-labeled NuMA^{1886–1914}. Fitting of the polarization curve yielded a dissociation constant of 52 nM, in agreement with the value obtained for the same reaction by ITC (Fig S6). The experiment established conditions for the fluorescence polarization-based competition assays presented in Fig. 4C.



Fig. S8 NuMA disrupts the LGN^{TPR}:LGN^{GoLoco} interaction. (A) A GST-LGN^{TPR} fusion protein (2 μ M) was adsorbed on GSH beads, and its ability to interact with LGN^{GoLoco} was analyzed in the presence of increasing amounts of NuMA^{1821–2001}. Upon addition of NuMA^{1807–1987} the retention of LGN^{GoLoco} on beads was proportionally reduced confirming that NuMA competes for the binding to LGN^{TPR}. Already at roughly equimolar NuMA^{1807–1987} and LGN^{GoLoco} concentrations, NuMA^{1807–1987} remained bound to LGN^{TPR}, indicating that it is a higher-affinity ligand, whose binding interferes with the closed state of LGN. (*B*) The ability of NuMA to open the LGN switch was tested by size exclusion chromatography (SEC). Upon NuMA^{1807–1987} addition, LGN^{GoLoco} is displaced from LGN^{TPR}.

Hs_NuMA Mm_NuMA Ga_NuMA	1778 1774 1817	S L GDVF L DS GRKTRS – ARRRTTQ I I N I TMTKK L D – VE E P DS ANS S F YS TRS – – – – AP AS QAS L RATS – – – – S TQS L AR L G – – – – S P DY – – GNS AL L S L P S L GDAF P DS GRKTRS – ARRTTQ I I N I TMTKK L E – L E E P DS ANS S F YS TQS – – – – AP AS QANL RATS – – – – – S TQS L AR L G – – – – S P DD – – GNS AL L S L P GROPPTOTI DI LINIT TWY KETTER C CADAGE SS LI S GADO WAR ADA DADI DE SADEL AS SADEL	1860 1856 1900
X1_NuMA	1902		1985
Dr_NuMA	2013	S I GDL VVDS AKKLTAS ARRRTT QVINITLAKKAQGSS E SEESF SSLHS ARSHPNLAVHQS RP I SI DF SE DGPATTLS KADTL QNLP	2099
Dm_Mud	1892	R L N E K I L E QQK QHA I I S T N L R H L QMQP I S E T K P S S T T L T V S S S S S A	1937
		1886 1914	
Hs NuMA	1861		1932
Mm NuMA	1857		1928
Ga NuMA	1901	GYRPATRSSL RRSQAGSSSSLGRSSLYL GTCODEPEOL-DDWNR LAFL OORNRACPPHLKTCYPLESRPSD	1970
X1_NuMA	1986	GYQHPTRRSARLSQTGGRSSFYMSTCQDEPDPQ-DDWTRIAELQARNKTCPPHLKTSYPLESRPSI	2050
Dr_NuMA	2100	GYRRS TVHS VGPPRATS TFC I GAE NEPE HAADDWMR I AE L QS RNKACLPHL KS SYPL E S RPS L G	2163
Dm_Mud	1938	PNDDWQPFKRPNVPSSNLAME DEEGEVFNNTYLTDLKLGRVPADMTAEELIYRNSLQPPHLKSTYAAQYDLGSQDEDLKDGPHSLDDSMS	2027
	1022		1092
Mm NuMA	1933		1962
Ga NuMA	1923		2034
X1 NuMA	2051	FSSTITDEEVKLGDPKETLRRATLLPGOIODSMTSTRROTLAVPGAEHLKGHNISTROOMKRVSEESHY	2119
Dr NuMA	2164	PSFTVTDEDLRLGDPSETIRRASMMPGQILESLNSRRFSLAPETSSSQAPARSQPORATMLPCQIQSSTAA-HRASQLTKTSSRSHTSENKRSPLAPKRP	2262
Dm_Mud	2028	ALLSSSSTGTRKKS MGTHYKRPGPPTPSKNGGRLSFGSSEPPRE ILREFGDHNNTSKTPARFKFLTQRFSVGSSGLPRDELPRRKRPNLLTGIHRRRLRHAVDIF	2132
	1983		2064
Mm NuMA	1979		2064
Ga NuMA	2035		2111
X1 NuMA	2120		2200
Dr NuMA	2263		2342
Dm_Mud	2133		2237
Hs_NuMA	2065	NTRS GTR RS PR- ATTTAS AATAAA GATP RAKGKAKH 2101	
Mm_NuMA	2061	NPRS GTRRSPR-IATTTGTATVATTPRAKGKVKH 2094	
Gg_NuMA	2112	ALKDTKF 2141	00/
XĪ_NuMA	2201	RGRGANGSTSSTSNKPSHLSLRKSPSQRSPR-VSTAKSPRASNKLFERKQQRNK- 2253	ر ۳
Dr_NuMA	2343	AVASRALRSAVSGKSPLDVTLRKSPRNKSPKTSNAKKGTQ- 2382 — Human NuMAPEPT	<u> </u>
Dm_Mud	2238	AGKRVQLRRKLRRDRIMGRFDEARHLDQMRLSHSAQAAKSPENNNYSLHNR2288 sequence ide	ntity

Fig. S9 Sequence alignment of the C-terminal portion of NuMA orthologs. Coloring of NuMA reflects the conservation among homologues from *Homo* sapiens, *Mus musculus, Gallus gallus, Xenopus laevis, Danio rerio,* and *Drosophila melanogaster*. The sequence of human NuMA^{PEPT} encompassing the LGN^{TPR} binding site is indicated in green on top of the alignment, with the invariant glutamic acid residues of the EPE motif marked as green circles. *Drosophila* Mud shares little sequence similarity with the other NuMA orthologs even in the Pins-binding region, where consecutive EPE-EGE triplets are present.

Data set	Pins-Insc native	Pins-Insc SeMet SAD
Beamline	ESRF ID23-1	ESRF ID23-1
Space group	C2	C2
Wavelength (Å)	0.979	0.979
Unit cell dimensions (Å)	160.2	159.29
	64.23	64.20
	107.60	107.20
	eta= 117.9°	eta= 118.2°
Resolution (Å) *	25.0–2.1 (2.18–2.1)	20.0–3.0 (3.1–3.0)
Total observations	472,231	733,917
Unique reflections	56,295	19,213
Data completeness (%)	98.7 (96.7)	99.8 (98.9)
Rsym (%) ⁺	5.6 (22.4)	11.4 (29.9)
Ι/σΙ	12.5 (2.3)	11.4 (3.5)
Refinement		
Resolution range (Å)	23.5–2.1	
Rwork / Rfree †	20.8/25.6	
Number of protein atoms	5,755	
Number of solvent atoms	487	
rmsd bond lengths (Å)	0.019	
rmsd bond angles (°)	1.59	
Mean B-factor protein (Å ²)	42.1	
Ramachandran values		
Favored (%)	98.1	
Allowed (%)	1.9	
Outliers (%)	0	

*Values in parentheses refer to the outer resolution shell.

 ${}^{\dagger}R_{\rm free}$ is equivalent to $R_{\rm conv}$ for a 5% subset of reflections not used in the refinement.

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