

Supporting Information

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SI Materials and Methods

Plasmids. Eukaryotic plasmids expressing FLAG- or V5-tagged full-length Munc18 variants were obtained by shuttling the full-length Munc18 insert from the entry vector into plasmids pFLAG-CMV-2 (MDC Berlin) or pcDNA3.1-nV5-DEST (Invitrogen), respectively. The plasmid expressing full-length EmGFP-FEZ1 was obtained by shuttling the insert from an entry vector containing full-length FEZ1 human cDNA to pcDNA6.2/N-EmGFP-DEST. The cDNAs for various truncations of FEZ1 were generated by PCR and directly inserted into pEGFP-C1 (Clontech) by conventional cloning (1). Alternatively, the PCR products were first cloned into pENTR/D-TOPO and subsequently shuttled into pcDNA6.2/N-EmGFP-DEST. The plasmid expressing rat V5-tagged KIF5C was obtained by amplifying full-length rat KIF5C cDNA from pBlueScript-KHC-rat KIF5C (a kind gift from Eckhard Mandelkow, Max-Planck-Unit for Structural Molecular Biology, Hamburg, Germany). The insert was subcloned into pENTR/D-TOPO and subsequently shuttled into pcDNA3.1-nV5-DEST.

Antibodies. The following monoclonal antibodies (mAbs) were commercially purchased: M2-FLAG (Stratagene), anti-V5 (Invitrogen), and anti-Kinesin heavy chain (Millipore). mAbs against Stx (clone 78.3), α -tubulin, and Munc18 (clone 131.1) were obtained from Synaptic Systems. Polyclonal antibodies recognizing green fluorescent protein were obtained from Synaptic Systems. FEZ1 antibody was generated using purified recombinant protein corresponding to amino acids 1–91.

Automated Y2H Screening. Automated Y2H screening was carried out as published previously with minor modifications (2). For the construction of bait ORFs for Y2H, selected presynaptic proteins were subdivided into established domains according to data obtained from protein information databases. Inserts corresponding to each domain were amplified from their respective full-length cDNA by PCR and subcloned into the entry vector pENTR/D-TOPO (Invitrogen) using topoisomerase-mediated ligation. Inserts were subsequently shuttled into the Y2H plasmids pBTM116-D9 or pACT4_DM by recombinational cloning.

To create a matrix for interaction mating, the L40cc α MAT α yeast strain was individually transformed with prey plasmids (coding Gal4 activation domain fusions); the resulting yeast clones were arrayed in 96-well microtiter plates (MTPs). Simultaneously, the bait plasmids (coding LexA DNA-binding domain fusions) were introduced into a L40ccU MAT α strain and assembled in 96-well plates. Baits, which activated the *HIS3*, *URA3*, or *lacZ* reporter genes after mating with a MAT α strain carrying the prey plasmid without insert, were excluded from the Y2H analysis. For the domain-based Y2H screen, 5- μ L liquid cultures of the MAT α yeast strains were replicated in 96-well MTPs, grown, mixed with 40- μ L MAT α strains and then transferred onto YPD agar plates. For the screen of the point-mutated FEZ1 baits, MAT α bait spots were resuspended directly in 30 mL of –AT/HUL medium, grown, and transferred to 384-well MTPs. In a second step, MAT α prey strains were scraped off from agar, transferred to the 384-well MTPs containing the bait culture, mixed, and stamped onto YPD agar plates. In each case, the YPD agar plates were incubated for 36 h at 30 °C. After mating, the clones were transferred into 96-well MTPs containing synthetic drop-out (SD) II (–Leu-Trp) liquid medium. For selection of protein-protein interactions, diploid yeasts were spotted onto SDIV (–Leu-Trp-Ura-His) agar plates, as well as

nylon membranes placed on SDIV agar plates. After 5–6 d of incubation at 30 °C, digitized images of the agar plates and nylon membranes were scored for growth and β -galactosidase activity using the software Visual Grid (GPC Biotech).

Immunoprecipitation of endogenous FEZ1 and Kinesin-1 complexes. Rat brain cortices were homogenized in ice-cold sucrose buffer (5 mM Hepes, pH 7.4, 320 mM sucrose) and centrifuged for 2 min at $2,988 \times g$. The ensuing supernatant was re-centrifuged for 12 min at $14,461 \times g$. The resultant supernatant (S2) was then mixed with an equal volume of 2 \times PBS/BSA and used as starting material for immunoisolation of FEZ1 transport complexes. Antibodies recognizing FEZ1 or Kinesin-1 were added to the mixture and incubated overnight at 4 °C. To isolate the immune-complexes, Dynabeads Protein A (Invitrogen) was added to the mixture and incubation continued for an additional hour. After 3 washes with PBS plus 0.3% BSA, the complexes were resuspended in 2 \times LDS buffer and analyzed by immunoblotting.

Primary Hippocampal Neurons and Cell Culture. Primary hippocampal neurons were prepared from newborn rats as described previously (3). Human embryonic kidney (HEK) 293 cells were maintained in DMEM supplemented with 10% FCS. For transfection using Lipofectamine reagent (Invitrogen), cells were seeded at a density of $\sim 90\%$ and transfected with various plasmids 1 d after plating following the recommended protocol.

C. elegans Strains and Generation of Transgenic Animals. *C. elegans* strains were cultured at 20 °C as described previously (4). The strains *unc-116(e2310)* and *unc-76(e911)* were obtained from C. elegans Genetic Center. Wild-type animals were Bristol strain N2 (4).

The rab-3::GFP-Gateway and rab-3::mCherry-Gateway vectors were constructed by replacing the mig-13 promoter with the 1.2-kb rab-3 promoter in the mig-13::GFP-GW and mig-13::mCherry-GW vectors (kind gifts from Kang Shen, Howard Hughes Medical Institute, Department of Biology, Stanford University, Stanford, CA), respectively. For worm expression, *unc-18c* was amplified from genomic DNA by PCR and subcloned into pDONR201 vector (Invitrogen). cDNAs encoding *unc-64a* and *unc-76a* were amplified from cDNA library and subcloned into pDONR201 vector (Invitrogen). The cDNAs were then shuttled into either rab-3::GFP-Gateway or rab-3::mCherry-Gateway destination vectors.

Transgenic strains were generated by microinjection (5) of the plasmids *prab3::gfp-unc-64* (10 ng/ μ L) and *prab-3::gfp-unc-18* (20 ng/ μ L) in combination with the markers pRF4 (*rol-6(su1006)*) (100 ng/ μ L) and odr-1-RFP (50 ng/ μ L). Crosses were performed using classical genetic approaches. To obtain rescue, *unc-76(e911)* Ex [rab3::gfp-unc-64] were microinjected with *prab3::mCherry-unc-76*, *prab3::mCherry-FEZ1* wild-type or *prab3::mCherry-FEZ1-S58A* at 30 ng/ μ L.

To image *C. elegans*, live animals were placed on 2% (wt/vol) agarose pads and immobilized with 5 mM Levamisol in M9 buffer. Confocal images were acquired using a Zeiss Axiovert 200M microscope equipped with 100 \times objective, spinning disk confocal head and Andor Xion EM-CCD camera.

Analyses of GFP-UNC64 and GFP-UNC18 Clustering. Metamorph imaging software was used to analyze axonal aggregations in the *C. elegans* experiments. For each image, we randomly placed line scans (100 pixels long) along the axonal bundles of wild-type, mutant, or transgenic worms. Corresponding background values

were measured near outside of each axon bundle analyzed. Average intensities and SDs were exported into SigmaPlot 9.0 software (Systat Software). The aggregation index was calculated by dividing the SD of each line scan against the background corrected average intensity of each measurement (6). The average of eight to nine measurements were calculated for each strain and used to plot the charts in Figs. 4 and 6. Values are given as means \pm SE. Statistical significance was evaluated by Student *t* test.

Electron Microscopy. Wild-type, *unc-76*, or *unc-116* mutant worms were transferred into 100- μ m-deep aluminum platelets (Microscopy Services, Flintbek) filled with *E. coli* OP50 suspension and high pressure frozen using a BalTec HPM 10. Freeze substitution was carried out in a Leica EM AFS at -90°C for 100 h in 0.1% tannic acid followed by another 40 h in 2% OsO_4 (each wt/vol in dry acetone), with the temperature raised slowly to 4°C according to Rostaing et al. (7). Infiltration and thin embedding in Epoxy Resin (Agar Scientific; R1140) was performed at room temperature.

Sections of 50 nm in thickness were cut using a Leica UC6 ultramicrotome. Ribbons of sections were transferred on Formvar-coated copper slot-grids. The grids were placed for 30 min on drops of 4% (wt/vol) uranyl acetate in water and then washed in distilled water. After air drying, the grids were placed on drops of lead citrate for 2 min in a CO_2 -free chamber and washed in distilled water (8). Micrographs were taken with a 1024×1024 pixel CCD detector (Proscan CCD HSS 512/1024; Proscan Electronic Systems) attached to a Zeiss EM 902A transmission electron microscope that was operated in the bright field mode.

Analyses of Phosphorylation Sites by Mass Spectrometry. HEK 293 or bacterially expressed FEZ1 was fractionated by 1D gel electrophoresis using a NuPAGE 4–12% gradient gel (Invitrogen). The protein band corresponding to FEZ1 was excised from the gel and digested by trypsin (9). The tryptic peptides were subsequently analyzed by nanoLC-MS/MS using a LTQ XL Orbitrap (Thermo Fisher Scientific) coupled to an Agilent 1100 series LC-system. Peptides were separated at a flow rate of 200 nL/min on a reversed-phase column (C18; Reprosil; Dr. Maisch). Elution of the peptides was done with a 54-min gradient from 7 to 45% mobile phase B [80% (vol/vol) acetonitrile, 0.15% formic acid]. Peak lists were searched against NCBI RefSeq database using Mascot v.2.2 as search engine. Mass accuracy was 10 ppm for the parent ion and 0.5 Da for the fragment ions. The peptides were constrained to be tryptic with a maximum of two missed cleavages. Carbamidomethylation of cysteines considered a fixed modification, whereas oxidations of methionine and phosphorylation of serine, threonine, and tyrosine residues were considered as variable modification.

Alkaline Phosphatase Treatment. Cells were lysed in ice-cold HNM buffer [50 mM Hepes (pH 7.2), 150 mM NaCl, 1.5 mM MgCl_2 , 1% (vol/vol) Triton X-100] containing Complete EDTA-free protease inhibitor mixture. After centrifugation, the clarified supernatant was divided into two. One half was treated with 30 units of AP (Roche) for 30 min at 30°C . The other half served as the control. Following AP treatment, samples were immunoprecipitated as described, and proteins were eluted with $2\times$ LDS buffer for analysis by immunoblotting. Blot quantification was done using ImageJ software.

1. Sambrook J, Russell DW (2006) *The condensed protocols from molecular cloning: A laboratory manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.), pp v, 800 pp.
2. Stelzl U, et al. (2005) A human protein-protein interaction network: a resource for annotating the proteome. *Cell* 122:957–968.
3. Rosenmund C, Stevens CF (1997) The rate of aldehyde fixation of the exocytotic machinery in cultured hippocampal synapses. *J Neurosci Methods* 76:1–5.
4. Brenner S (1974) The genetics of *Caenorhabditis elegans*. *Genetics* 77:71–94.
5. Mello C, Fire A (1995) DNA transformation. *Methods Cell Biol* 48:451–482.

6. Barszczewski M, et al. (2008) A novel site of action for alpha-SNAP in the SNARE conformational cycle controlling membrane fusion. *Mol Biol Cell* 19:776–784.
7. Rostaing P, et al. (2006) Analysis of synaptic ultrastructure without fixative using high-pressure freezing and tomography. *Eur J Neurosci* 24:3463–3474.
8. Reynolds ES (1963) The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J Cell Biol* 17:208–212.
9. Shevchenko A, Tomas H, Havlis J, Olsen JV, Mann M (2006) In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nat Protoc* 1: 2856–2860.

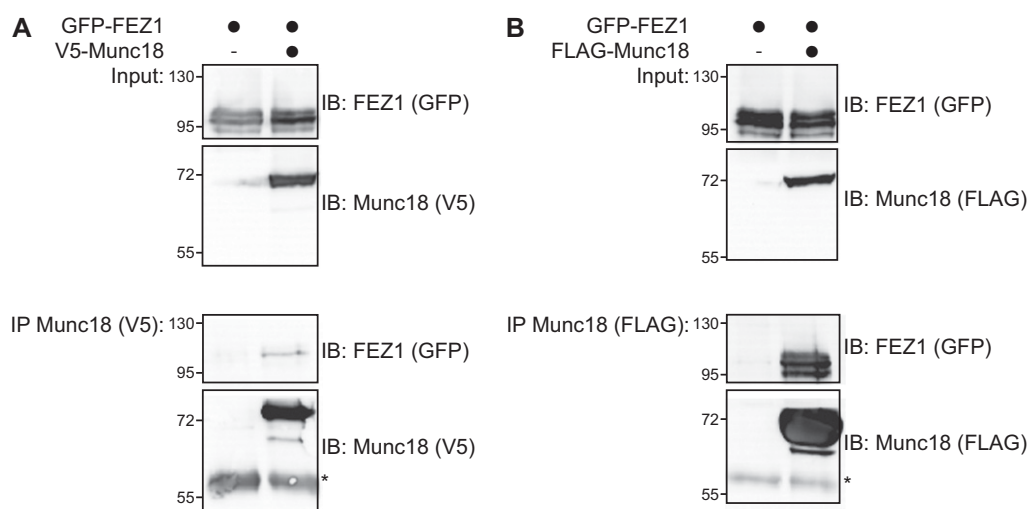


Fig. S1. Additional coimmunoprecipitations of FEZ1 and Munc18 using a different epitope tag or antibody. HEK 293 cells expressing combinations of tagged FEZ1 and Munc18 proteins (black circles on top of the lanes) were immunoprecipitated (IP) using tag-specific antibodies (anti-V5 or anti-FLAG) and analyzed by immunoblotting (IB) for the presence of GFP-FEZ1, V5-Munc18, or FLAG-Munc18, respectively. Molecular mass markers indicated are in kilodaltons. *Ig heavy chain.



Fig. S2. FEZ1 coiled-coil domain mediates binding to Munc18. (A) Diagram of the FEZ1 constructs used in the domain mapping studies. The coiled-coil domain of FEZ1 is indicated. (B and C) HEK 293 cells expressing combinations of tagged versions of FEZ1 peptides and Munc18 or Stx (black circles on top of the lanes) were immunoprecipitated (IP) using tag-specific antibody (anti-GFP) and analyzed by immunoblotting (IB). (B) Sequential deletion of either the N- or C terminus of FEZ1 reduced binding of Munc18. (C) In addition to the coiled-coil domain, a region immediately before this region also contributes to the binding of FEZ1 to Stx. Only background binding was observed when an even shorter FEZ1 was used in the coimmunoprecipitation. Coimmunoprecipitation of GFP-Munc18 and Myc-Stx was included as a positive control. Molecular mass markers indicated are in kilodaltons. *Ig heavy chain.

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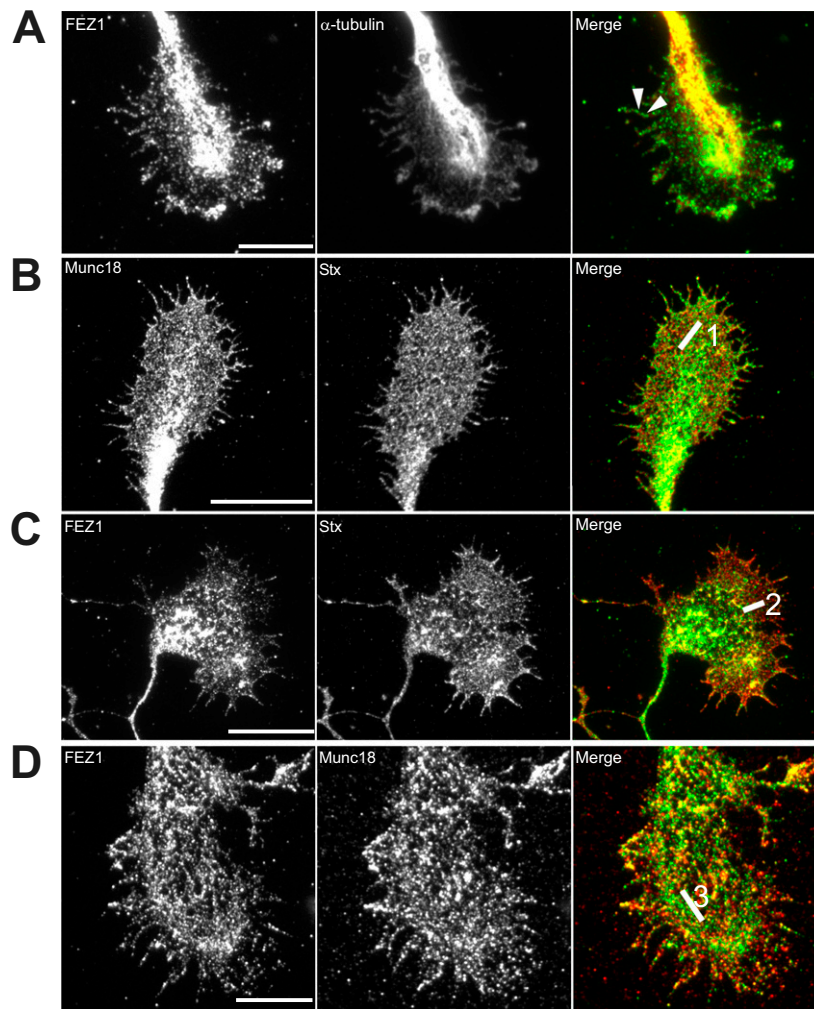


Fig. S4. FEZ1 colocalizes with Stx, Munc18, and α -tubulin in neuronal growth cones. Images for growth cones from 2–3 DIV neurons in Fig. 3 shown here in their entirety. (A) Numerous FEZ1 puncta are observed in neuronal growth cones that are strongly microtubule-associated (e.g., arrowheads). (B) Stx and Munc18 colocalizes in growth cones as expected. FEZ1 colocalizes with Stx (C) and Munc18 (D) in growth cones. Line scans of regions of interest indicated in B–D are identical to that shown in Fig. 3. Refer to main text for details. [Scale bars: 10 μ m (A); 20 μ m (B, C, and D).]

Fig. S5. Interactions of UNC-64, UNC-18, and UNC-76 are conserved in *C. elegans*. UNC-64 and UNC-64 coprecipitates with UNC-76. HEK 293 cells expressing combinations of tagged versions of UNC-76, UNC-64, and/or UNC-18 were immunoprecipitated (IP) using anti-GFP antibodies and immunoblotted (IB) using tag-specific antibodies (anti-FLAG or anti-GFP). Interactions between FEZ1 (UNC-76), Stx (UNC-64), and Munc18 (UNC-18) are conserved in *C. elegans*. Molecular mass markers are indicated in kilodaltons.

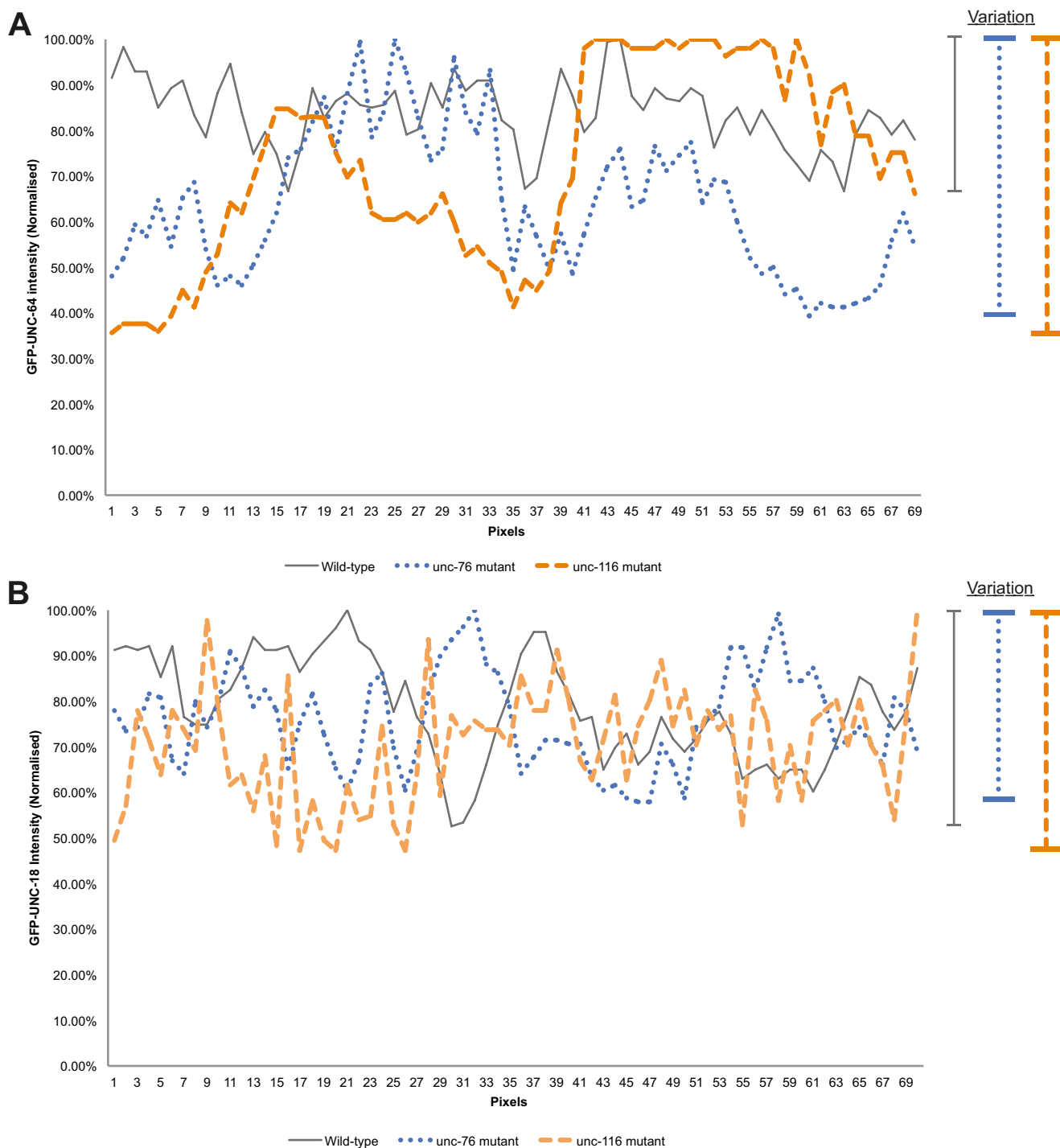


Fig. S6. Line scan analyses reveal significant increase in axonal UNC-64 aggregation in *unc-76* and *unc-116* mutants. Representative line scans showing signal intensities of GFP-UNC-64 (A) or GFP-UNC-18 (B) along VNC axonal bundles from wild-type and mutant worms. Intensities were normalized to the highest signal obtained in each line scan. Whereas similar variations of GFP-UNC-18 intensity along axons between wild-type and mutant worms are seen, significantly increased fluctuations of GFP-UNC-64 are observed in *unc-76* and *unc-116* mutant worms. Vertical lines on the right of each graph indicate the range of signal variation for their respective strains.

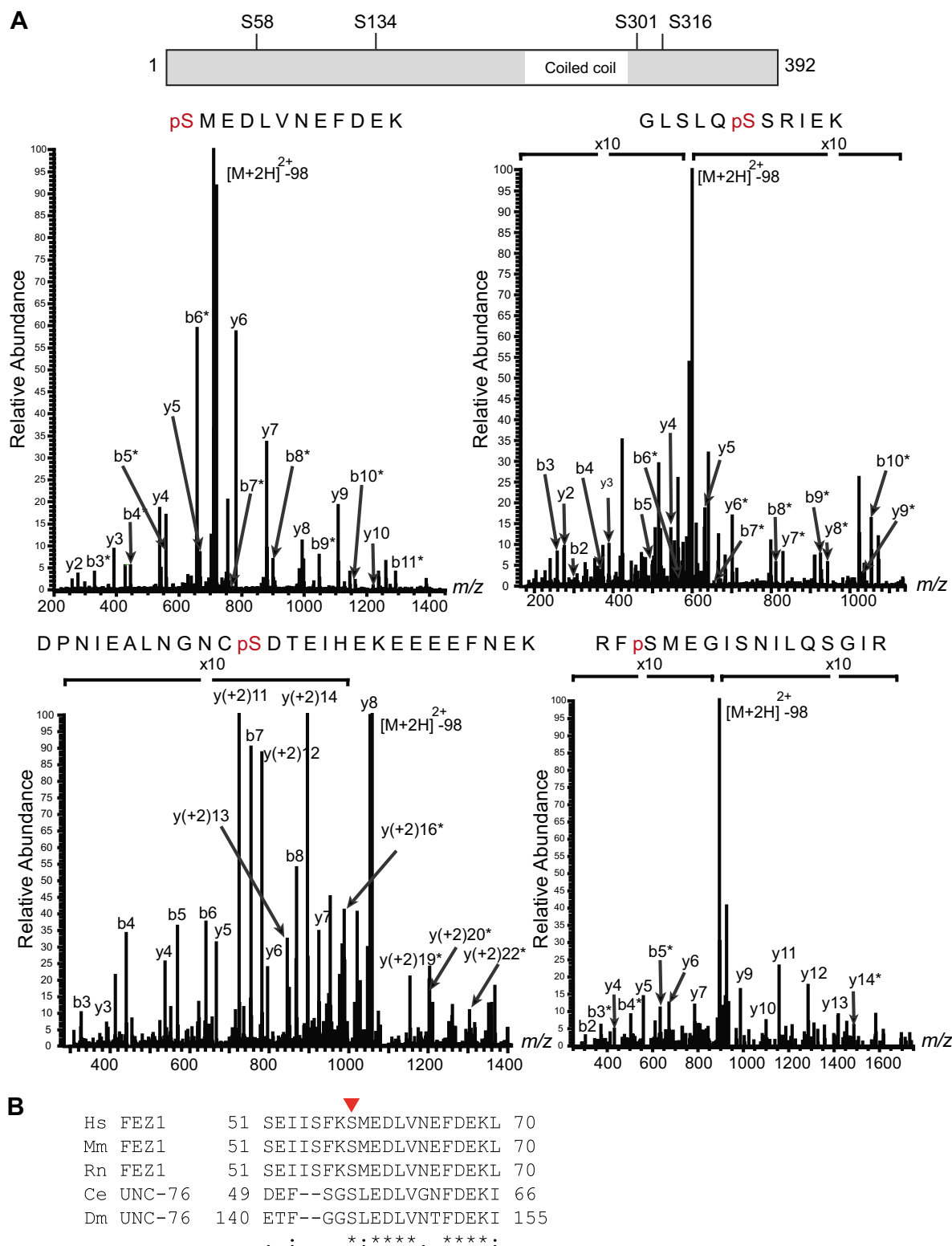


Fig. S7. FEZ1 is phosphorylated at multiple sites. (A) Identification of phosphorylation sites in FEZ1. Diagram of the four phospho-serine sites identified is shown at the top of the diagram. FEZ1-GFP was expressed in HEK 293 cells and immunoprecipitated using an anti-GFP antibody. Immunoprecipitated FEZ1 was resolved by 1D SDS/PAGE, and the protein band corresponding to FEZ1 protein was excised from the gel and subjected to digestion by trypsin. The tryptic peptides were subsequently analyzed by LC-MS/MS for identification of phosphorylation sites. S58, S134, S301, and S316 were found to be phosphorylated in FEZ1. MS/MS spectrum for identification of S58 (upper left corner), S134 (lower left corner), S301 (upper right left corner), and S316 (lower right corner). Ions marked with an asterisk (*) indicate loss of phosphoric acid attributable to the conversion of phospho-serine into dehydroalanine. For simplicity, only b- and y-ions are indicated. (B) FEZ1 S58 is homologous to S143 in dUNC76. In *Drosophila*, phosphorylation of S143 was shown previously to be important in regulating binding of dUNC-76 to synaptotagmin. Protein sequences used to produce the T-Coffee generated alignments are: human FEZ1, GI: 4826724; rat FEZ1, GI: 13994121; mouse FEZ1, GI: 148747561; *Drosophila* UNC-76, GI: 18543271; and *C. elegans* UNC-76, GI: 115534730.

Table S1. FEZ1 interactions are regulated by phosphorylation

Baits	Preys		
	KIF5A	KIF5C	Munc18-D3
FEZ1 (wild-type)	++	+	++
FEZ1 S58A	—	—	—
FEZ1 S134A	AA	AA	AA
FEZ1 S301A	—	—	++
FEZ1 S316A	—	—	++
FEZ1 S(58+134)A	—	—	—
FEZ1 S(134+316)A	—	—	++
FEZ1 S(301+316)A	—	—	++
FEZ1 S(58+134+316)A	—	—	—
FEZ1 S(134+301+316)A	—	—	++
FEZ1 S(58+134+301+316)A	—	—	—

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