

## Supplementary Information for

### *ASTN2 modulates synaptic strength by trafficking and degradation of surface proteins*

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#### **Other supplementary materials for this manuscript include the following:**

Datasets S1 to S2

## Supplementary Materials and Methods

*RT-PCR and qRT-PCR:* mRNA was extracted using the AllPrep DNA/RNA/Protein Mini Kit (Qiagen) and cDNA transcribed with the Transcription First Strand cDNA Synthesis Kit (Roche) according to the manufacturer's description. RT-PCR was carried out according to the manufacturer's descriptions using the HotStarTaq *PLUS* DNA Polymerase kit (Qiagen) with the following primers (*ASTN2*: forward, 5'-TACTGGTGCTCCAGGGAAAGG, reverse, 5'-CCCAATAGCTGGCTGAACAT,  $\beta$ -*ACTIN*: forward, 5'-AAACTGGAACGGTGAAGGTG, reverse, 5'-AGAGAAGTGGGGTGGCTTTT). qRT-PCR was performed with TaqMan primer/probe sets (*ASTN2* TaqMan gene expression assay (#Hs01024740\_m1) which detects exon boundary 18-19, Human *GUSB* (Beta Glucuronidase) Endogenous Control (#4333767T, Applied Biosystems) and TaqMan Fast Advanced Master Mix, all according to the manufacturers' descriptions on a Roche LightCycler 480 (Roche).

*Primary cell and cell line culture:* PBMCs from human subjects were isolated using BD Vacutainer™ CPT™ Tubes (BD Biosciences #362753).  $10 \times 10^6$  cells were first plated in T cell medium (RPMI-1640, 10% FBS, 2 mM Glutamine, 100 U/ml Penicillin/Streptomycin, 10  $\mu$ M HEPES) for 3 hrs, allowing monocytes to attach to the plate and be discarded. CD4+ cells were then sorted by MACS Separation using CD4 MicroBeads (human) and MS columns (Miltenyi Biotec) according to the manufacturer's descriptions and expanded in T cell medium containing 30 U/ml IL2 (Peprotech #200-02) and CD3/CD28 beads (Dynabeads #11161D) according to the manufacturer's description. Media change (T cell medium containing IL2) was performed every two days and cells were collected at DIV 8 for mRNA and protein analysis.

*Immunohisto/cytochemistry:* Briefly, vibratome sections were blocked with 15% normal horse serum (Gibco), 0.1% saponin in PBS overnight and then incubated with primary antibodies overnight at 4°C and with Alexa Fluor® secondary antibodies for 2 hours to overnight at room temperature and 4°C respectively. *In vitro* cultured cells were blocked in 1% normal horse serum, 0.05% Triton, incubated in primary antibodies overnight at 4°C followed by secondary Alexa Fluor® antibodies for 1 hour at room temperature. Sections/cells were mounted with ProLong® Gold anti-fade mounting media and sections were covered with 1.5 thickness Fisherbrand cover glass.

*Antibodies used for immunohistochemistry, immunoprecipitation and Western blot:* Primary antibodies, Rabbit: anti-ASTN2 (1:1000-2000 on sections, and 1:500 on cells, 1:200 for Western Blot (1), anti-Calbindin D28-k (1:500, Swant #CB38), anti-GFP (1:500, Invitrogen #A11122). Mouse: anti-Calbindin (1:500, Swant #300), anti-Flag (1:1000, Sigma #1804), anti-HA (1:500, Roche #1 583 816 001), anti-cMYC (1:50, Calbiochem #OP10), anti-AP-2 (1:250, BD Transduction Laboratories #611350), anti-NLG2 (1:100, Synaptic Systems #129 511), anti-GAPDH (1:10,000, Chemicon #mab374) anti-ROCK2 (1:1000, BD Transduction Laboratories #610623), anti-Rab4 (1:100, BD Transduction Laboratories #610888), anti-Rab5 (1:200, Synaptic Systems #108011), anti-Rab7 (1:100, Santa Cruz #sc-376362). Goat: anti-GluD2 (1:100, Santa Cruz #sc-26118). Secondary antibodies: Donkey anti-mouse, -rabbit, and -goat IgG conjugated to Alexa 405 (abcam), 555, 633, and 647 (Molecular Probes), all used at 1:300. HRP-conjugated secondary antibodies (Jackson ImmunoResearch) were used at 1:8000 (anti-mouse, # 515-035-062) or 1:3000 (anti-rabbit #111-035-144) for Western blots.

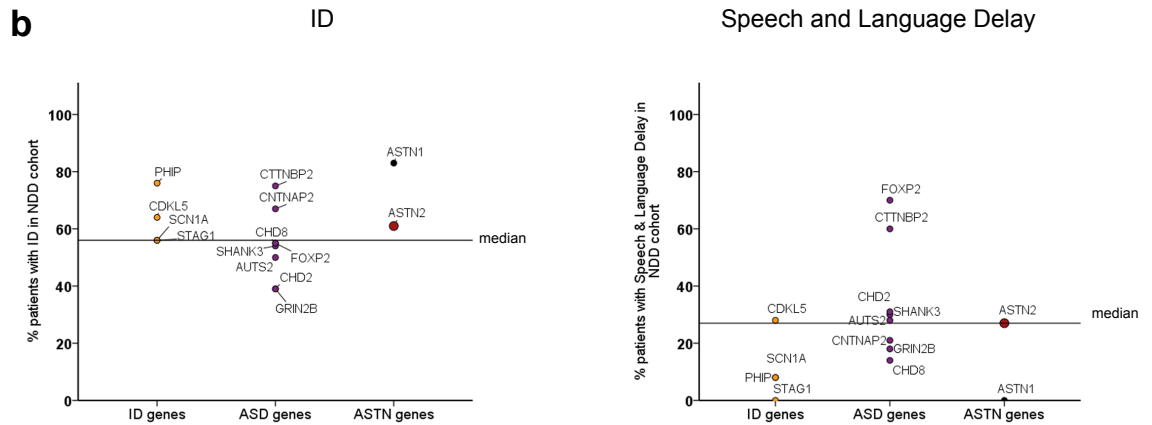
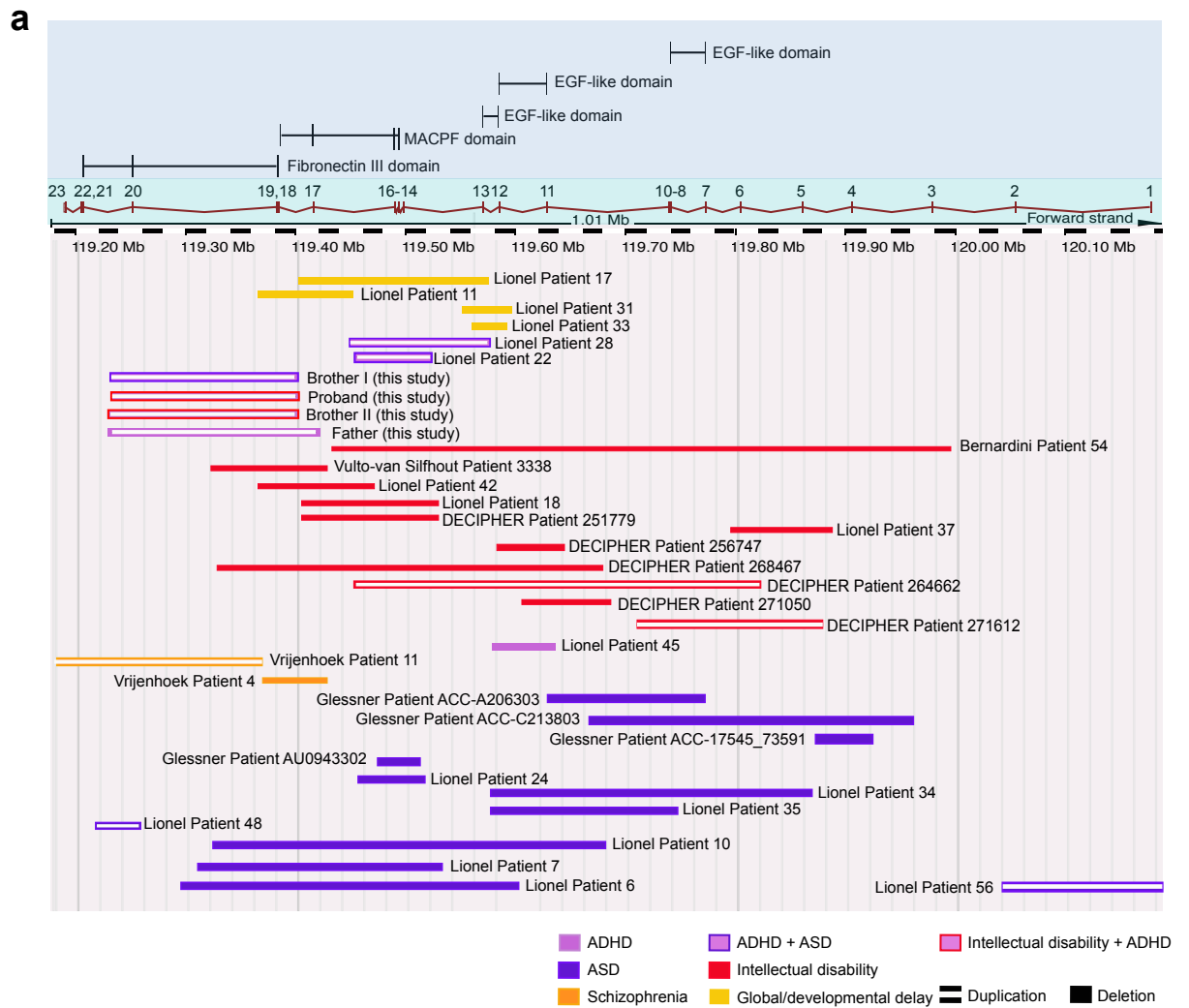
*cDNA/ShRNA constructs:* The following plasmids were used: pGIPZ lentiviral plasmids containing *Astn2* shRNA (# V3LMM440320) and scramble shRNA (Thermo Scientific Open Biosystems), OLFM1-MYC-Flag (Origene #MR207779), AP2s-MYC-Flag (Origene #MR200768), Clqc-MYC-Flag (Origene #MR203092), pCAG:GPI-GFP (Addgene #32601), pMES-SLC12a5-HA (2), pNice-NLGN1-CFP, pNice-NLGN2-CFP, pNice-NLGN3-YFP, pNice-NLGN4-YFP (3, 4), pNice-NLGN1-HA-YFP (gift from Dr Peter Scheiffele), pCdh2-CFP (Cdh2 cDNA gift from Dr. Richard Huganir, 5), pRK5-MYC and pMSCX $\beta$ -Venus- $\alpha$ -tubulin (used for flow cytometry control) were provided by Dr David Solecki. ASTN2 and JDUP containing constructs were created as follows: the sequence between the XbaI/BamHI sites of the pFU-cMVIIA-PE lentiviral plasmid (6) was removed including the DsRed/LoxP sequences. The full length *Astn2* mouse cDNA was amplified from previously reported plasmids (1) and modified to include the 5' region of the gene, using a 5' primer with an XbaI restriction site and a 3' primer with a BamHI site. This sequence (full length ASTN2 splice variant 201, www.ensemble.org) was inserted in frame into the pFU backbone at XbaI/BamHI, creating pFU-Astn2-EGFP. To create the conditional construct, pFU-cAstn2-EGFP, a LoxP-dsRED-LoxP sequence (synthesized as a gblock fragment by Integrated DNA Technologies, IDT) was inserted into the XbaI site, upstream of the *Astn2* sequence. pFU-cJDUP-EGFP was created by excising the sequence between the BsiW1/BlnI sites of pFU-cAstn2-EGFP and replacing it with a synthesized sequence representing a FNIII domain deleted version (gblock, IDT). The non-conditional version (pFU-JDUP-EGFP) was created by excising the LoxP-DsRED-LoxP sequence in pFU-cJDUP-EGFP with XbaI and re-ligating the plasmid. ASTN2-HA-FLAG and JDUP-HA-FLAG plasmids were made by replacing the EGFP sequence in pFU-ASTN2-EGFP and pFU-JDUP-EGFP with in-frame HA-FLAG sequences.

*Flow cytometry:* Transfected HEK 293T cells were harvested in 1 mM EDTA in PBS. The surface fraction of YFP-linked surface proteins was immuno-labeled (live) with rabbit anti-GFP followed by Alexa-647 anti-rabbit and cells were stained with Propidium Iodide (Sigma-Aldrich) for dead cell exclusion. Flow cytometry analysis (BD Accuri C6, BD Biosciences) was carried out using the 488 nm and 640 nm lasers and the CFlow Sampler software (BD Biosciences). A total of 20,000 single viable cells, identified by size and lack of Propidium Iodide staining, were analyzed per condition (resulted in approximately 100,000 events per condition). Gates were set using non-transfected control cells and cells expressing cytosolic GFP ( $\alpha$ -tubulin-GFP), which were processed for live GFP labeling as described above. Data were analyzed by FlowJo v.9.3.3 (TreeStar Inc., Ashland, OR).

*Knockdown of ASTN2 in HEK cells and neurons:* HEK cells were transfected using Lipofectamine 2000, with plasmids expressing the *Astn2* cDNA alone or together with shRNA and scrambled constructs. Two days later, cells were processed for immunohistochemistry and Western blot as described earlier. For knockdown in neurons, mixed cerebellar neurons isolated at P7 were nucleofected (Amaxa Nucleofector II) with shRNA or scrambled constructs using the mouse neuron nucleofector kit (Lonza) according to the manufacturer's description. Cells were cultured as described previously for six days and then processed for Western blot. In a second experiment, GFP+ cells indicative of shRNA construct expression were sorted (BD FACSAria) from GFP-negative cells and then processed for Western blot.

*Imaging:* Images were acquired using an inverted Zeiss LSM 880 NLO laser scanning confocal microscope with a Plan-Apochromat 40x/1.4 NA objective oil immersion lens and 2.8 x digital zoom. For the lower power image in Fig. 2a a Plan-Apochromat 10x/0.45 NA lens was used. Images were acquired by setting the same gain and offset thresholds for all images per experiment and over/underexposure of signal was avoided. Images were quantified in ImageJ (version 2.0.0-rc-38/1.50b) unless stated otherwise.

Fig. S1



**Figure S1. Genomic representation of *ASTN2* CNVs and comparison of the occurrence of ID and speech and language impairment in patients**

**(a)** Schematic of CNVs (pink box) along the *ASTN2* genomic sequence (green box, showing exons 1-23 of the largest transcript, GRCh 37) with *ASTN2* protein domains encoded by each exon depicted at the top (blue box). The CNVs are color-coded according to the diagnosis of the patient (stated in the key at the bottom) with deletions represented by solid boxes and duplications by lined boxes. The CNVs represented were gathered from ref. 7–11, the DECIPHER database, and this study. Only cases with CNVs affecting exons restricted to *ASTN2* (no additional genetic lesions) and a clear diagnosis of ADHD, ASD, Schizophrenia, developmental delay, or ID are depicted. For additional cases with *ASTN2* CNVs plus other genomic lesions or patients without the above diagnoses, please refer to Lionel et al., 2014, Vulto-van Silfhout et al., 2013 and the DECIPHER database. **(b)** Comparison of the occurrence rate of ID and speech and language delay in patients with various genetic lesions; 4 genes with strong association with ID, 8 genes with strong association with ASD, versus patients with *ASTN2* or *ASTN1* CNVs (DECIPHER database: <https://decipher.sanger.ac.uk>). *ASTN2* CNVs fall above the median for ID, *ASTN1* is the highest scoring for ID, while *ASTN2* but not *ASTN1* is among the highest for enrichment in speech and language delay, excluding the classical speech and language genes *FOXP2* and *CNTNAP2* (12).

**Fig S2**

**Astn2-201 (mouse isoform 2, 1300 aa)**

MAAAGARRSPGRGLGLRGRPRLGFHPGPPPPPPPLLLLLFLLLLPPPPLLAGATAAAAASR  
EPDSPCRLKTVTVSTLPALRESDIGWSGARTGAAAGAGAGTGAGAGAAAAAASAASPGSA  
GSAGTAAESRLLLLFVRNELPGRIAVQDDLDNTELPFFFTLEMSGTAADISLVHWRQQWLEN  
GTLYFHVSMSSSSGQLAQATAPTLQEPSEIVEEQMHILHISVMGGLIALLLLLLVFTVALY  
AQRWQKRRRIPOKSASTEATHEIHYIPSVLLGPQARESFRSSRLQTHNSVIGVPIRETPI  
ILDDYDYEEEEPPRRANHVSREDEFGSQMTHALDSLGRPGEKVEFEKKGGISFGRTKG  
TSGSEADDETQLTFYTEQYRSRRRSKGLLKSPVNKTALTIAVSSCILAMVCGNQMSCPL  
TVKVTLHVPEHFIADGSSFVSEGSYLDISDWLNPAKLSLYYQINATSPWVRDLCGQRTT  
DACEQLCDPDTGECSCHEGYAPDPVHRHLCVRSWGWQSEGPWPYTTLERGYDLVTGEQAP  
EKILRSTFSLGQGLWLPVSKSFVPPVELSINPLASCKTDVLVTEPADVREEAMLSTYF  
ETINDLLSSFGPVRDCSRNNGGCTRNFKCVSDRQVDSSGCVCPPEELKPMKDGSGCYDHSK  
GIDCSDGFNGGCEQLCLOQTLPYDTSSTIFMFCGCVVEEYKLAPDGKSCMLSDVCEG  
PKCLKPD SKFNDTLFGEMLHG YNNRTQHVNQGQVFQMTFRENNFIKDFPQLADGLLVIPL  
PVVEEQCRGVLSEPLP DLQLLTGDIRYDEAMGYPMVQQWRVRSNLYRVKLSTITLSAGFTN  
VLKILTKESSRDELLSFIQHYGSHYIAEALYGSELTCTIIHFPSKKVQQQLWLQYQKETTE  
LGSKKELKSMFFITYLSGLLTAQMLSDDQLISGVEIRCEEKGRCPSTCHLCRRPGKEQLS  
PTPVLLEINRVVPLYTLIQDNGTKEAFKNALMSSYWC SGKGDVIDDWCRCDLSAFDASGL  
PNCSPLPQP VLRSLPTVEPSSTVVSLEWVDVQPAIGTKVSDYILQHKKVDEYTDTDLYTG  
EFLSFADDLLSGLGTSCVAAGRSHGEVPEVSIYSVIFKCLEPDGLYKFTLYAVDTRGRHS  
ELSTVTLRTACPLVDDNKAEEIADKIYNLYNGYTSGKEQQTAYNTLMEVVSASMLFRVQHH  
YNSHYEKFGDFVWRSEDELGPRKAHLILRRLERVSSHCSLLRSAYIQSRVDTIPLYFCR  
SEEVPRAGMVWYSILKDKITCEEKVMVSMARNTYGETKGR

**JDUP sequence (1103 aa)**

MAAAGARRSPGRGLGLRGRPRLGFHPGPPPPPPPLLLLLFLLLLPPPPLLAGATAAAAASRE  
PDSPCRLKTVTVSTLPALRESDIGWSGARTGAAAGAGAGTGAGAGAAAAAASAASPGSAGS  
AGTAAESRLLLLFVRNELPGRIAVQDDLDNTELPFFFTLEMSGTAADISLVHWRQQWLENGTL  
YFHVSMSSSSGQLAQATAPTLQEPSEIVEEQMHILHISVMGGLIALLLLLLVFTVALY AQR  
WQKRRRIPOKSASTEATHEIHYIPSVLLGPQARESFRSSRLQTHNSVIGVPIRETPIILDDY  
DYEEEEPPRRANHVSREDEFGSQMTHALDSLGRPGEKVEFEKKGGISFGRTKGTSGSEA  
DDETQLTFYTEQYRSRRRSKGLLKSPVNKTALTIAVSSCILAMVCGNQMSCPLTVKVTLH  
VPEHFIADGSSFVSEGSYLDISDWLNPAKLSLYYQINATSPWVRDLCGQRTTDACEQLCD  
PDTGECSCHEGYAPDPVHRHLCVRSWGWQSEGPWPYTTLERGYDLVTGEQAPEKILRSTFS  
LGQGLWLPVSKSFVPPVELSINPLASCKTDVLVTEPADVREEAMLSTYFETINDLLSSF  
GPVRDCSRNNGGCTRNFKCVSDRQVDSSGCVCPPEELKPMKDGSGCYDHSK GIDCSDGFNGG  
CEQLCLOQTLPYDTSSTIFMFCGCVVEEYKLAPDGKSCMLSDVCEGPKCLKPD SKFND  
TLFGEMLHG YNNRTQHVNQGQVFQMTFRENNFIKDFPQLADGLLVIPLPVVEEQCRGVLSE  
LP DLQLLTGDIRYDEAMGYPMVQQWRVRSNLYRVKLSTITLSAGFTNVLKILTKESSRDEL  
LSFIQHYGSHYIAEALYGSELTCTIIHFPSKKVQQQLWLQYQKETTELGSKKELKSMFFITY  
LSGLLTAQMLSDDQLISGVEIRCEEKGRCPSTCHLCRRPGKEQLSPTPVLLEINRVVPLYT

LIQDNGTKEAFKNALMSSYWCSGKGDVIDDWCRCDSLAFDASGLPNCSPLPQPVLRLSPTV  
EPSSTVVSLEWVDVQPAIGTKVSDYILQHKKVDEYTDTDLYTGLHQCPKDPDQREQSGTYG  
ETKGR

**Red:** peptide against which antibody was raised (Wilson et al. 2010) and also used for depleting the antibody sera for anti-ASTN2 antibody

**Green:** endosomal/lysosomal sorting signals

**Blue/Black:** protein sequences encoded by alternating exons

**Yellow:** FN III domain

**Gray:** MACPF domain

**Linker sequence** in the EGFP tagged JDUP-EGFP construct

### **Figure S2. Endo/lysosomal signals in ASTN2 and JDUP protein sequences**

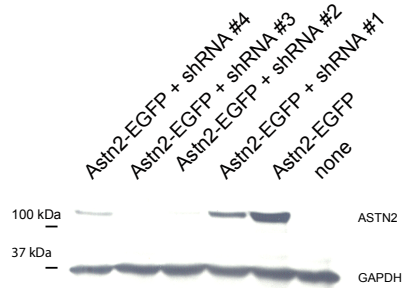
Protein sequences of the full length mouse ASTN2 and the truncated version (JDUP) modeled on the patient family CNV. Red box indicates the peptide against which the ASTN2 antibody was raised (1) and also used for depleting the antibody sera for use as a control in IPs (Fig. 4 and Fig. S6). Green boxes highlight endosomal and lysosomal sorting signals. Blue/black texts highlight protein sequences encoded by alternating exons. MACPF domain in gray, FNIII domain in yellow.



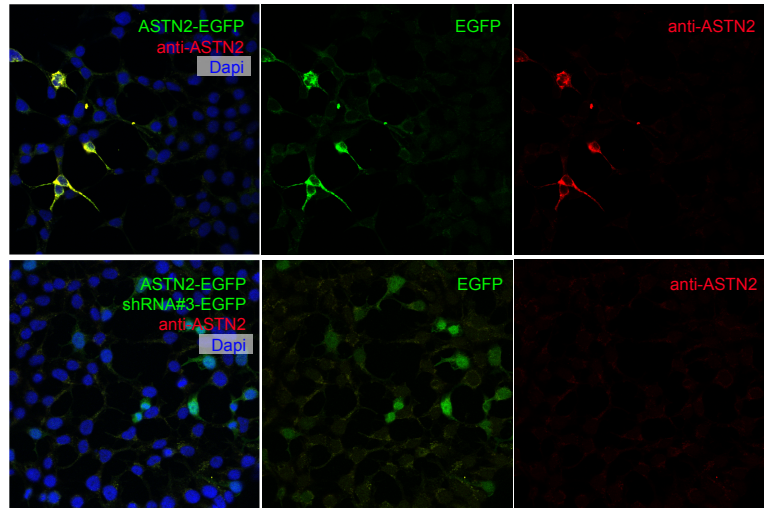
Fig. S3

HEK cells

**a**

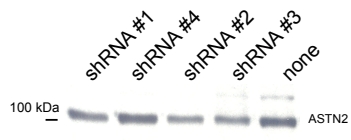


**b**

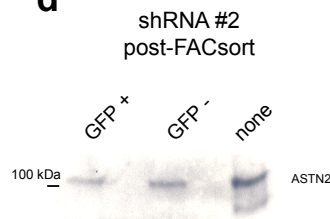


neurons

**c**

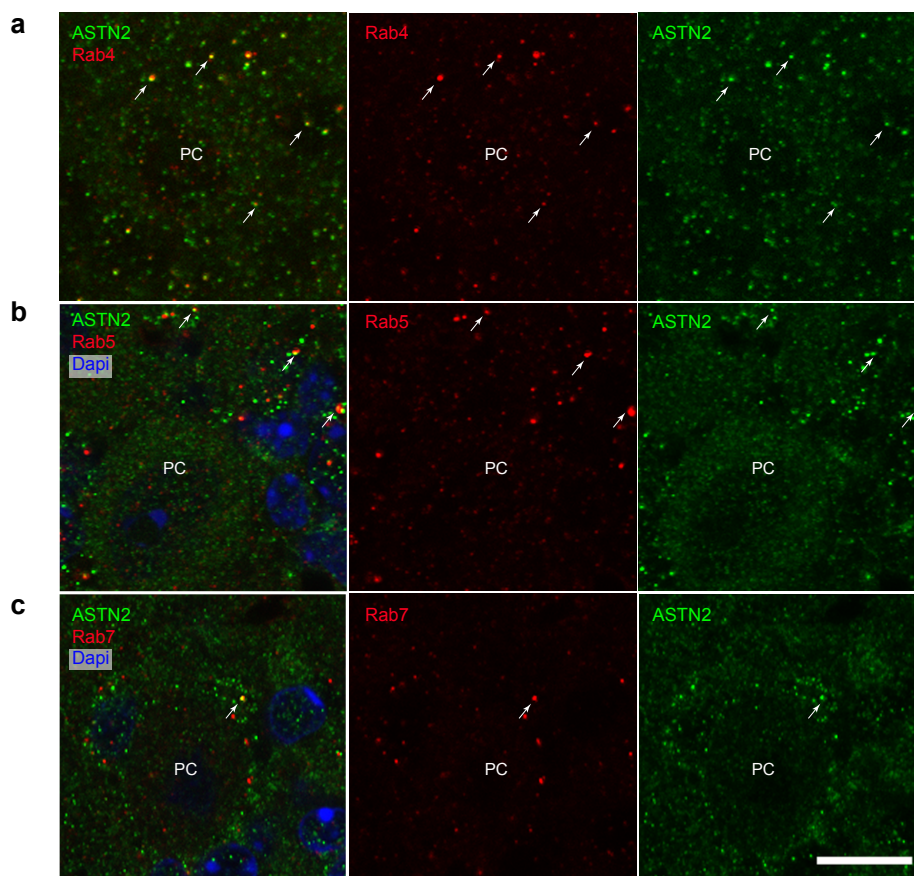


**d**



**Figure S3. shRNA-mediated knockdown of ASTN2 and antibody specificity**

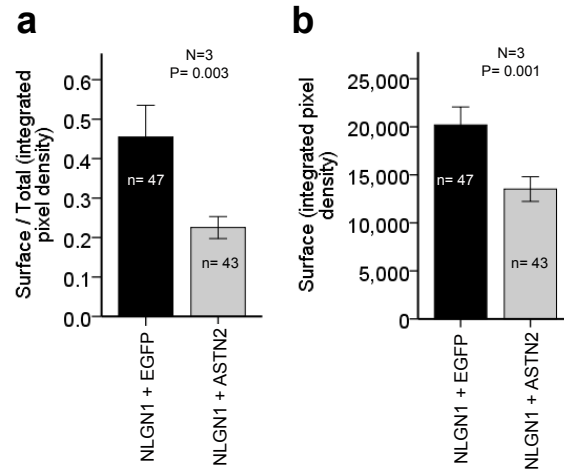
**(a, b)** Knockdown of ASTN2-EGFP by four different shRNA constructs in HEK 293T cells by Western blot (a) and immunohistochemistry (b) using a rabbit antibody against ASTN2. GAPDH (at 37 kDa) was used as loading control in a. In b, top panel shows ASTN2-EGFP (green, cytoplasmic/membrane) and detected by anti-ASTN2 (red), bottom panel shows EGFP expressed from the shRNA construct (green, nuclear and cytoplasmic) but no trace of ASTN2-EGFP labeling with the ASTN2 antibody (red channel) in the presence of shRNA#3. Dapi marks nuclei. **(c, d)** Western blots showing ASTN2 protein expression in cerebellar granule cells transfected with the same shRNA constructs as in a. (c) shows Western blot on non-sorted mixed transfected cells and (d) shows lysates from FACsorted GFP-positive (expressing the shRNA construct) and GFP-negative populations.



**Figure S4. ASTN2 co-localization with endosomal markers**

Immunohistochemistry of ASTN2 (green) with markers for recycling (Rab4, **a**), early (Rab5, **b**), and late (Rab7, **c**) endosomes in red in the postnatal cerebellum. Each section (sagittal) shows a PC soma and its immediate surroundings. Arrows point to examples of co-labeled puncta. Nuclei are marked by Dapi (blue) in b and c. PC, Purkinje cell, Scale bar: 10  $\mu$ m.

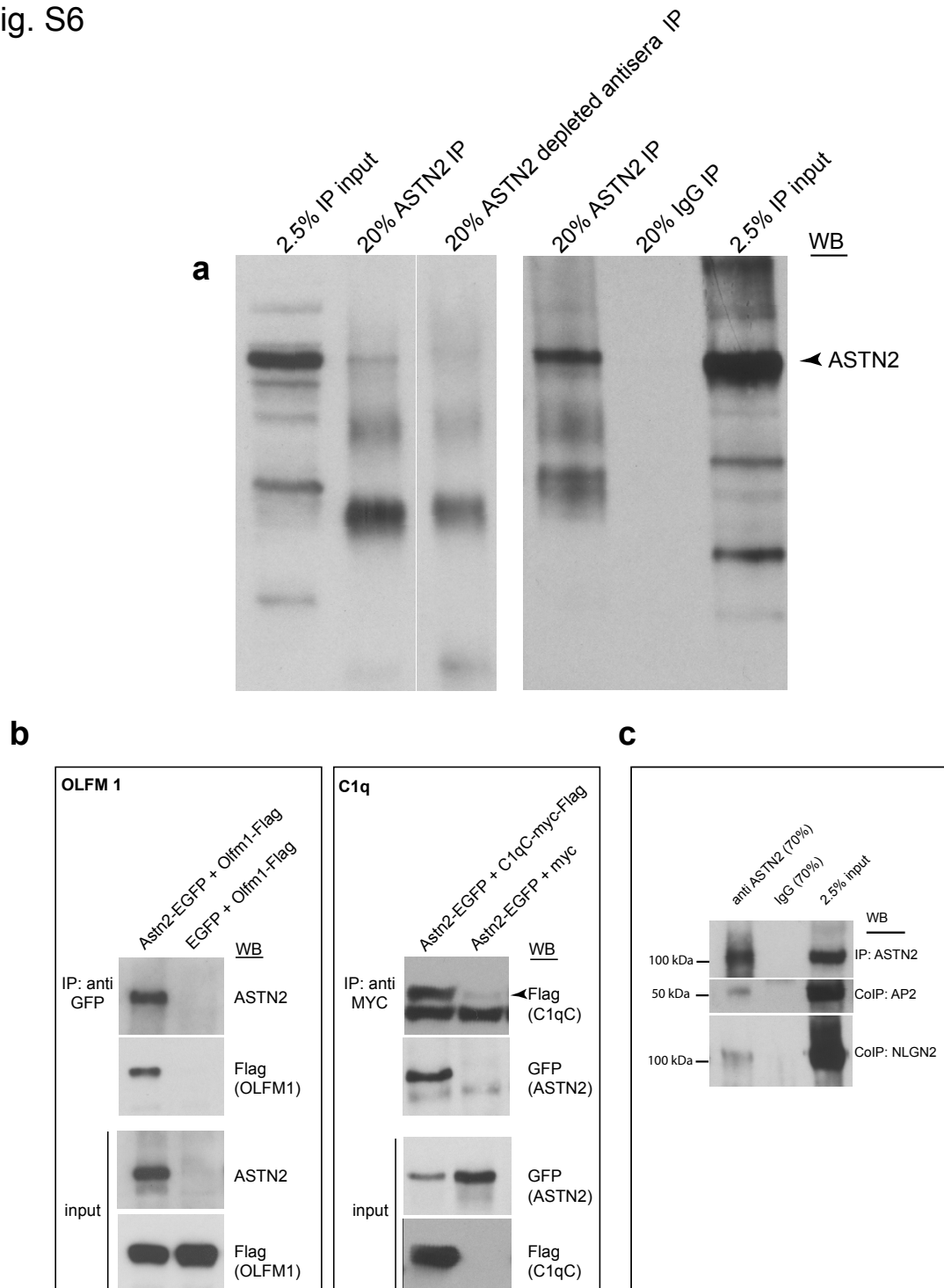
Fig. S5



**Figure S5. Quantification of surface labeling of NLGN1 in cerebellar granule cells**

Image quantifications of surface labeling of NLGN1-HA-YFP co-expressed with either EGFP or ASTN2-EGFP in GCs. Graphs show the integrated pixel density of surface labeling as an index of total labeling (corrected for background, **a**), as well as surface labeling alone (**b**). Bars show mean  $\pm$  1 SEM from three independent experiments. The number of cells analysed per condition are stated on each bar. P-values were obtained for comparison of surface labeling by ANCOVA in **a**, taking into account total labeling, and by ANOVA in **b**.

Fig. S6

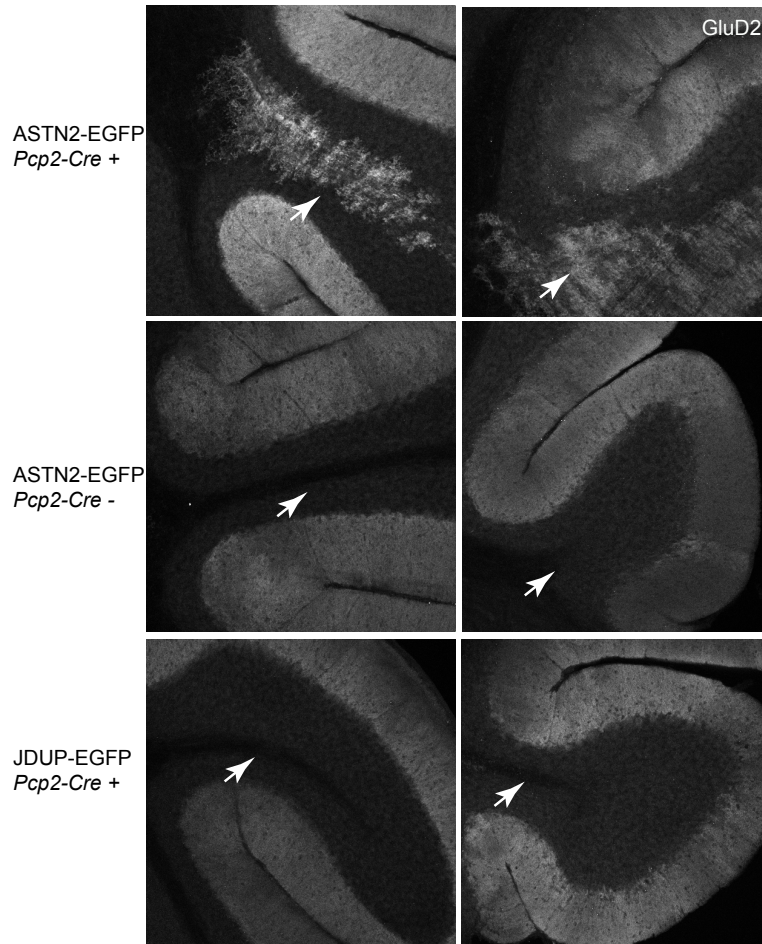


**Figure S6. Immunoprecipitation of ASTN2 and protein interactors**

(a) Representative Western blots of IPs with anti-ASTN2, rabbit IgG control (right panel), or depleted ASTN2 anti-sera (left panel) from the juvenile cerebellum used for mass spec analysis.

Blots show 20% of total IP volumes in relation to 2.5% inputs. As the IPs processed for mass spec were directly eluted in 8M Urea from beads and were not analysed by Western blot, examples of IPs carried out with the same conditions are shown. **(b)** Co-IP of OLFM1-Flag with ASTN2-EGFP and ASTN2-EGFP with C1qc-Flag-myc in HEK293T cells. **(c)** Co-IP of AP2 and NLGN2 with ASTN2 in lysates from the juvenile cerebellum.

Fig. S7



**Figure S7. Ectopic Purkinje cells upon conditional expression of ASTN2-EGFP in the cerebellum**

**(a)** Sagittal sections of the cerebellum showing ectopic PCs in the IGL and the WM (highlighted by arrows) of Lobules X (right panels) and I (left panels) marked by GluD2 expression, in *PCP2-Cre*<sup>+</sup> mice injected with pfU-cASTN2-EGFP (top), but not in pfU-cJDUP-EGFP (bottom) injected mice or in *PCP2-Cre*<sup>-/-</sup> mice injected with pfU-cASTN2-EGFP (middle).

**Additional data table: Dataset S1 (separate file)**

Dataset S1. Clinical features of the family with a paternally inherited *ASTN2* CNV

**Additional data table: Dataset S2 (separate file)**

Dataset S2. *ASTN2* interacting proteins identified by IP plus LC-MS/MS.

## References

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