The American Journal of Human Genetics, Volume *94*

Supplemental Data

Mutations in *USP9X* Are Associated with X-Linked Intellectual Disability and Disrupt Neuronal Cell Migration and Growth

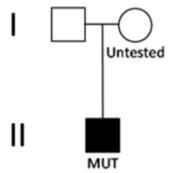
Claire C. Homan, Raman Kumar, Lam Son Nguyen, Eric Haan, F. Lucy Raymond, Fatima Abidi, Martine Raynaud, Charles Schwartz, Stephen A. Wood, Jozef Gecz, and Lachlan A. Jolly

Supplemental Reference

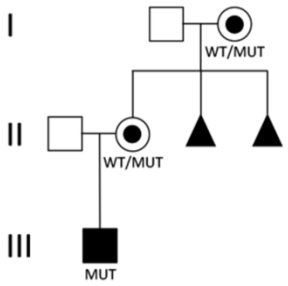
37. Kumar, R., Cheney, K.M., McKirdy, R., Neilsen, P.M., Schulz, R.B., Lee, J., Cohen, J., Booker, G.W., and Callen, D.F. (2008). CBFA2T3-ZNF652 corepressor complex regulates transcription of the E-box gene HEB. J. Biol. Chem. *283*, 19026–19038.

A

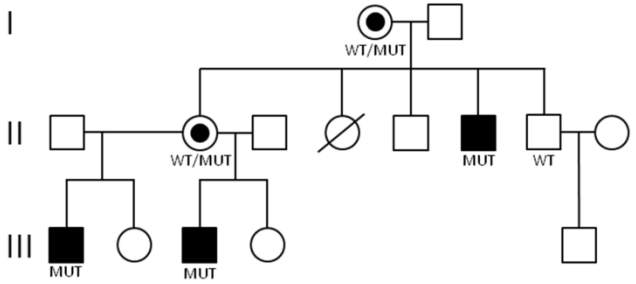
Family 1 c.6278T>A p.Leu2093His



Family 2 c.6469C>A p.Leu2157Ile



Family 3 c.7574delA p.Gln2525fs*18



B

	c. 6278 T>A p. Leu 2093 His	c. 6469 C>A p. Leu 2157 Ile	c. 7574 delA p. Gln 2525 fs*18
Homo sapiens USP9X	FAHNV**L**FNVSN	LSDHL**L**RAVLN	GSQYQ**Q**NNHVH
Homo sapiens USP9Y	FTHNV**L**FNVSN	LSDHL**L**RATLN	ASQYQ**Q**NNHVH
Mus musculus	FAHNV**L**FNVSN	LSDHL**L**RAVLN	GSQYQ**Q**NNHVH
Rattus norvegicus	FAHNV**L**FNVSN	LSDHL**L**RAVLN	GSQYQ**Q**P----
Danio Rerio	FAHNV**L**FAYPN	LSEHL**L**RAVLN	GSKFQ**Q**NNLPH
Xenopus tropicalis	FAHGV**L**FAYPN	LSDQL**L**RAVLN	GSQYQ**Q**NNHLH
Drosophila melanogaster	FANHA**L**LSPPS	LCEQV**L**ISVLR	SRQLF**G**AYTST
PolyPhen-2	Possibly Damaging (0.881)	Benign (0.164)	N/A
SIFT	Affect Function (0.03)	Tolerated (0.61)	N/A
iPTREE	Destabilizing	Destabilizing	N/A

C

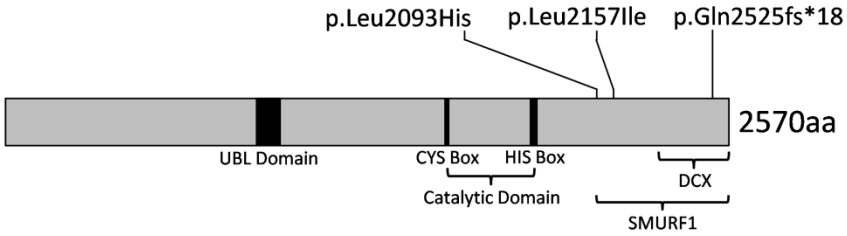
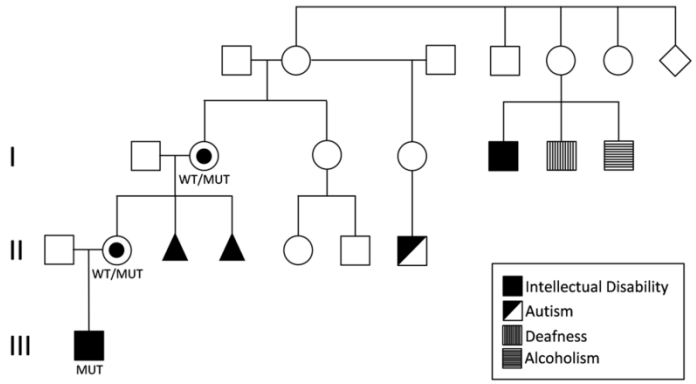


Figure S1. *USP9X* variants segregate with ID and result in changes to conserved regions of the protein C-terminus. A. Pedigrees of families identified in Tarpey *et al.,* 2009 with *USP9X* variants[3]. cDNA (NM_001039590.2) and protein (NP_001034679.2) annotation for individual variants are shown. Family 1: Missense variant found in one affected boy; Family 2: Missense variant found in one affected boy, his unaffected mother (displaying skewed X-inactivation; XI; data not shown) and grandmother (displaying skewed XI; data not shown). Affected male also has ~790kb deletion at 6q25.3 which includes *ARID1B*; Family 3: Single nucleotide deletion introducing a frame shift. Resulting cDNA truncates the protein sequence by 45 amino acids, and introduces 17 miscoded amino acids before a premature termination codon. Transcript not predicted to be subjected to NMD. Two generations of affected males recorded; variant found in affected half-brothers in generation III, their mother (XI status unknown), affected uncle and grandmother (XI status unknown). Two unaffected uncles also present in generation II, with one tested for the variant and found to be wild-type. Open symbols represent normal individuals, filled symbols represent affected individuals. Individual generations are numbered with Roman numerals (I, II and III). WT: wild-type; MUT: variant allele. B. Sequence alignment of variant amino acids across different species (using EBI CLUSTALW server), and *in silico* prediction (using PolyPhen2, SIFT and iPTREE algorithms) of the effect that the variant amino acid changes have on protein function and integrity. C. Linear scale cartoon of USP9X protein showing known structural features (Ubiquitin-like (UBL) and catalytic domains), binding sites of DCX and SMURF1, and the locations of variant amino acids. Note all variants cluster in the C-terminus.

Family 2 c.6469C>A p.Leu2157Ile



Family 3 c.7574delA p.Gln2525fs*18

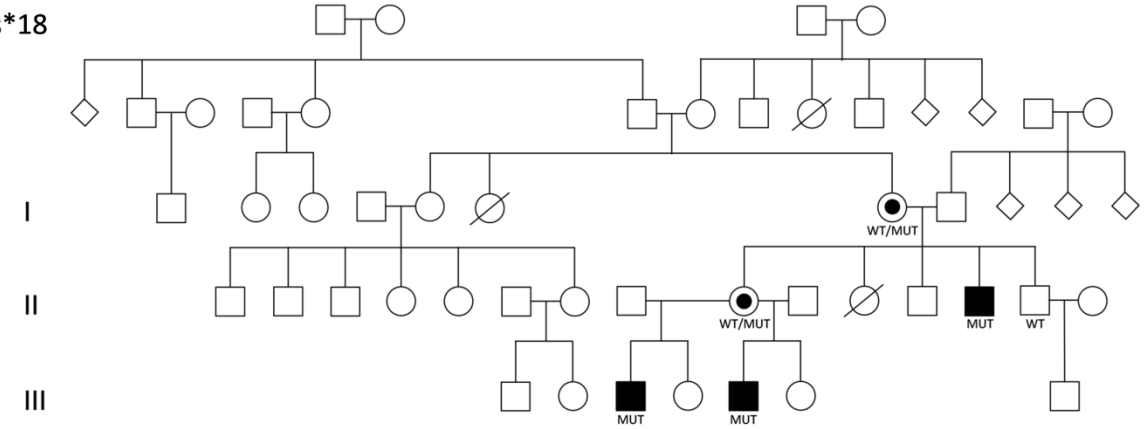


Figure S2. Extended pedigrees of families 2 and 3.

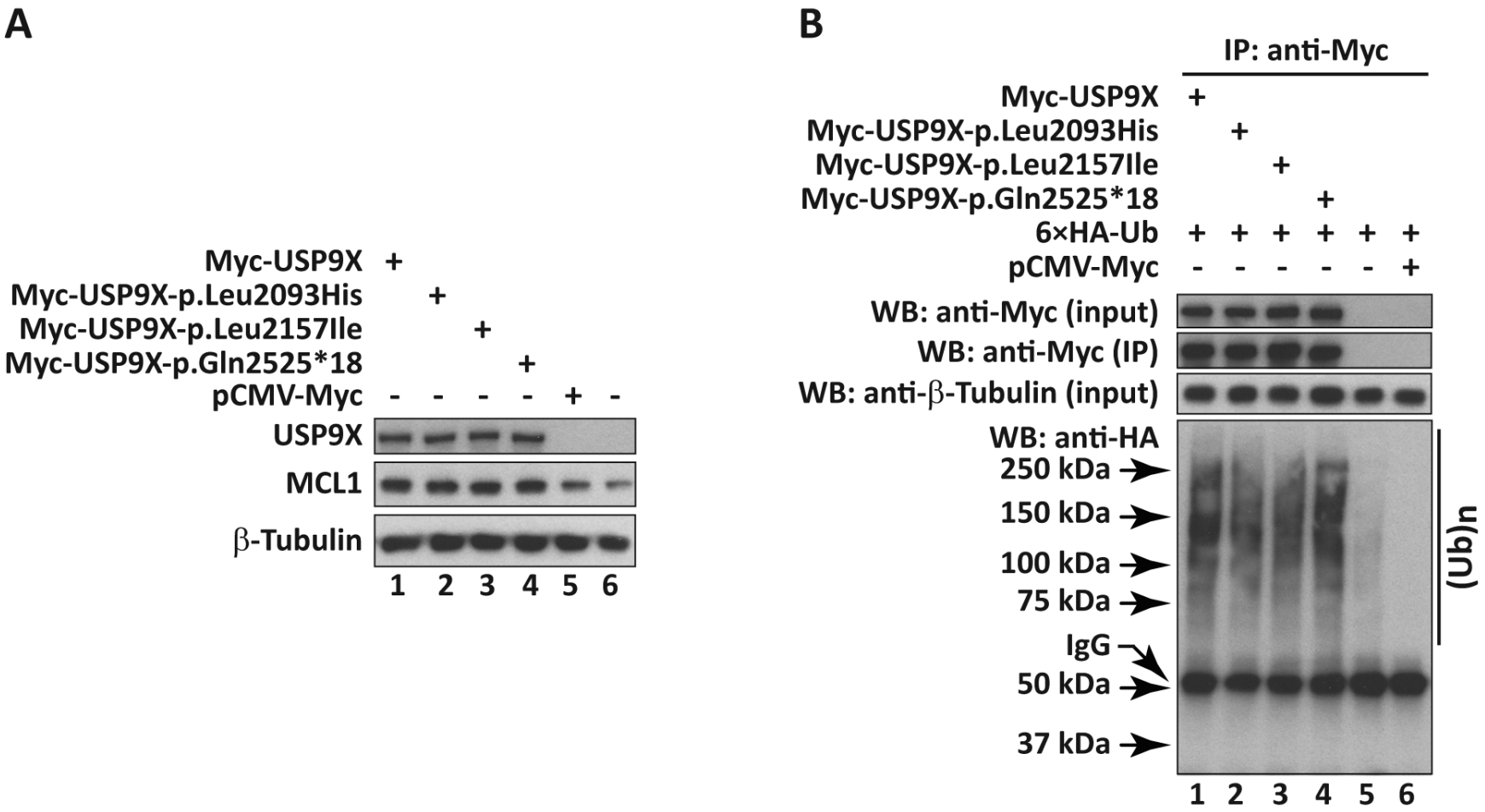

A
Myc-USP9X
Myc-USP9X-p.Leu2093His
Myc-USP9X-p.Leu2157Ile
Myc-USP9X-p.Gln2525*18
pCMV-Myc
USP9X
MCL1
β-Tubulin
1 2 3 4 5 6
B
IP: anti-Myc
Myc-USP9X
Myc-USP9X-p.Leu2093His
Myc-USP9X-p.Leu2157Ile
Myc-USP9X-p.Gln2525*18
6×HA-Ub
pCMV-Myc
WB: anti-Myc (input)
WB: anti-Myc (IP)
WB: anti-β-Tubulin (input)
WB: anti-HA
250 kDa
150 kDa
100 kDa
75 kDa
IgG
50 kDa
37 kDa
(Ub)n
1 2 3 4 5 6

Figure S3. USP9X variants do not alter its deubiquitylating activity. HEK 293T cells were transfected with plasmids encoding Myc-tagged USP9X species or empty vector control using Lipofectamine 2000 as per manufacturer's instruction (Life Technologies). A. Over-expression of either USP9X or variant forms leads to stabilisation of the USP9X substrate MCL-1. Western blot analysis of MCL-1 (mouse anti-MCL-1, 1:500, BD Pharmingen), Myc-USP9X (mouse monoclonal anti-Myc, 9E10, 1:1000, Santa Cruz Biotechnology) and β-tubulin (loading control; rabbit anti-β tubulin, 1:15000, Abcam). Note MCL-1 levels are increased by USP9X and variant form over-expression. B. USP9X variants do not alter ubiquitylation status of interacting proteins. Cells were also transfected with 6×HA-Ubiquitin as indicated. USP9X species (and interacting proteins) were co-immunoprecipitated from lysates using anti-Myc antibody (see below) and analysed by western blot using anti-Myc, β-tubulin, and anti-HA (mouse anti-HA, 1:1000, Sigma) antibodies. HA blot indicates ubiquitylation status of USP9X interacting proteins. Note HA blots are comparable between wild-type and variant USP9X species. All methods were essentially as previously described[37]. The generation of Myc-tagged USP9X constructs was as follows: Briefly, coding regions of USP9X species (wild-type and variant) were excised from respective pRK5-USP9X plasmids and ligated in-frame into pCMV-Myc (Clonetech) producing N-terminal Myc-tags. Transfected cell lysates were prepared 24 hours post-transfection using lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Triton X-100, 50 mM NaF, 0.1 mM Na_3VO_4 and protease inhibitor cocktail (Roche)) and sonication before centrifugation at 16000×g. For immunoprecipitation, lysates were incubated with 800ng of mouse monoclonal anti-Myc antibody overnight at 4°C followed by the addition of Dynal protein G magnetic beads (Life Technologies). Beads were washed three times with lysis buffer and two times with low salt buffer (20mM Tris-HCl pH 7.5). Proteins were eluted with 1× protein-loading buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol and 0.001% bromophenol blue) for 5 min at 100°C. All samples were separated using SDS-PAGE, transferred to nitrocellulose membranes, and analyzed by western blotting using standard procedures.

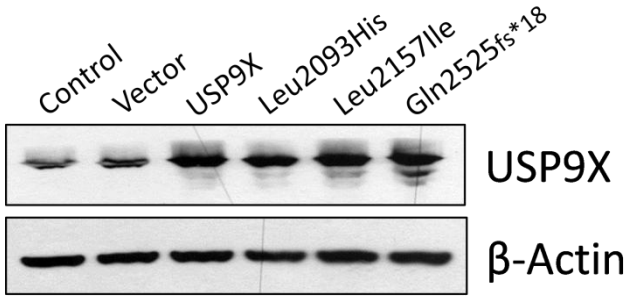


Figure S4. Uniform expression of USP9X and USP9X variants following transient transfection. Expression constructs encoding USP9X or variant forms were transfected into HEK 293T cells using lipofectamine 2000 reagent as per manufactures instructions (Invitrogen). Cell lysates were collected after 48 hours and subjected to SDS-PAGE and western blot analysis as previously described[23]. Generation of variant *USP9X* constructs was as follows: The C-terminal region of *USP9X* was isolated by PCR (F: GTACATGGGAGAAGTGTTTGAT, R: ACAACTAGAATGCAGTGAAAA) from the Prk5-*USP9X* construct, cloned into the pGEM-T vector (Promega) and used as a template for mutagenesis. Mutagenesis was carried out using the QuikChange Lightning multi site-directed mutagenesis kit (Stratagene) according to manufacturers protocol. *USP9X* variants were introduced using the following primers: c.6278T>A,p.L2093H, R:CGGAGAAGCGATTTG AAACATTAAAA**T**GGACGTTATGAGCAAACC; c.6469C>A,p.L2157I, R:GAGATTTAGTACTGCTCTTA**T**TAAGTGATCACTCAAGC; c.7574delA, p.Q2525fs*, R:GAGGGTTGTTCATG**/**GATGTGCTGCTGGGC. Sites introducing variants are in bold. Fragments containing the variants used for replacing the wild-type sequences in the Prk5-*USP9X* construct using *XbaI* and *BsaBI*. All constructs were confirmed by sequencing.

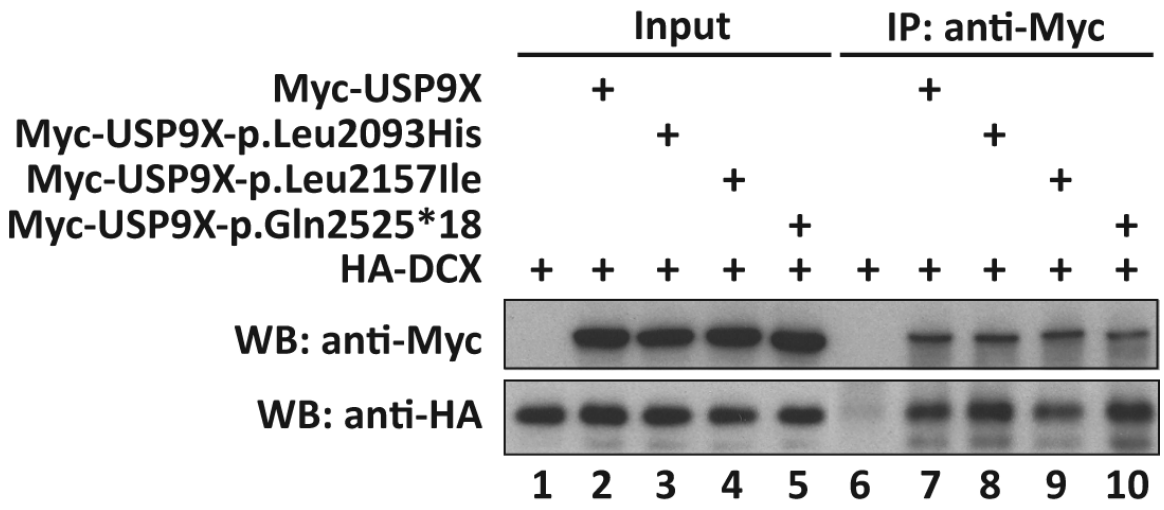


Figure S5. USP9X variants do not abolish physical interactions with Doublecortin in HEK 293T cells. Cells were transfected with expression plasmids encoding wild-type and variant USP9X species, with or without expression plasmids encoding HA- tagged Doublecortin as indicated. Cells were harvested 24hr post-transfection, lysates prepared and USP9X species and interacting proteins co-immunoprecipitated using mouse monoclonal anti-Myc antibody. Inputs and immunoprecipitates were analysed by western blotting. Note HA-DCX is present in both USP9X and variant immunoprecipitation samples. All methods are as described in Supplemental Figure 3. FLAG-tagged DCX expression plasmid was a kind gift of Prof. Orly Reiner, Weizmann Institute of Science Rehovot, Israel. The DCX coding sequence was excised and ligated into pCMV-HA (Clontech) for generating N-terminal HA-tagged DCX expression plasmid.

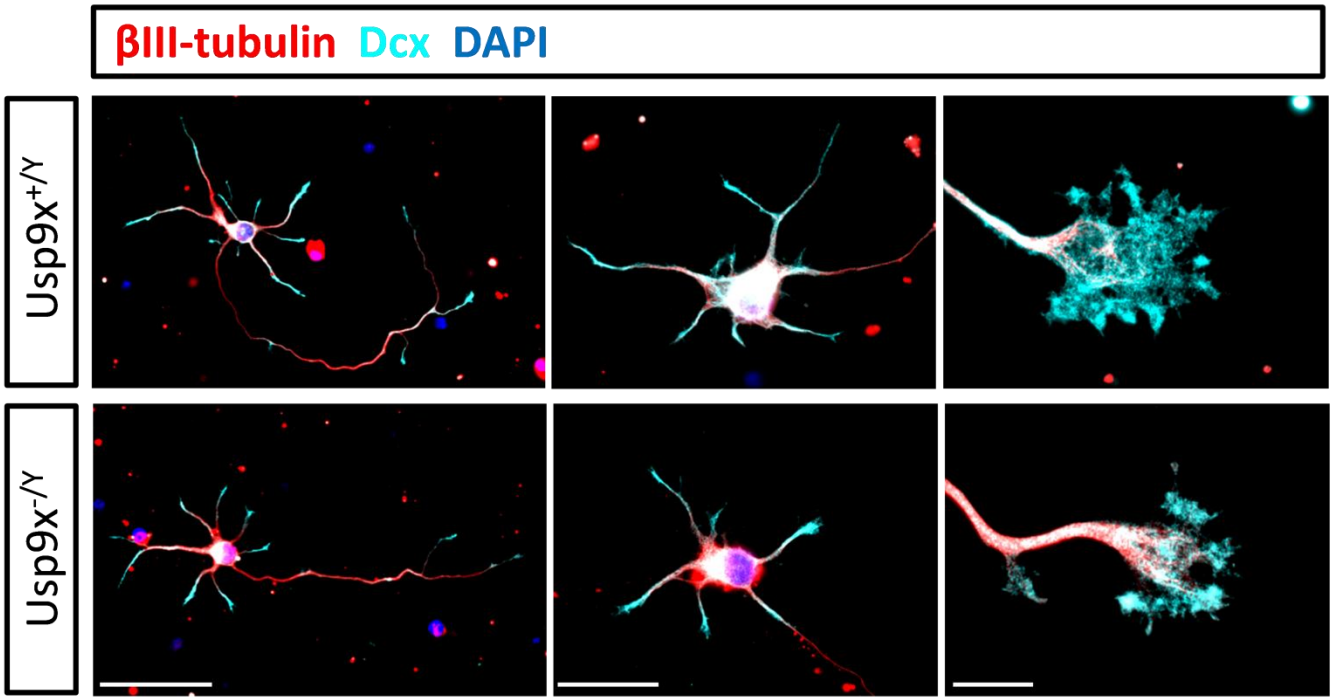


Figure S6. Loss of Usp9x does not alter localisation of Doublecortin in hippocampal neurons. Wild-type ($Usp9x^{+/Y}$) or knockout ($Usp9x^{-/Y}$) hippocampal neuronal cultures were generated as described previously (Figure 1). Immunofluorescent staining of Doublecortin (Dcx; Cyan; Guinea Pig anti-Dcx, 1:2000, Merck Millipore), and βIII-tubulin (red). Cell nuclei were stained with DAPI (Blue). Left panels: whole neurons, scale bar=100µm; Middle panels: neuronal cell bodies, scale bar=50µm; Right panels: axonal growth cone, scale bar=20µm.

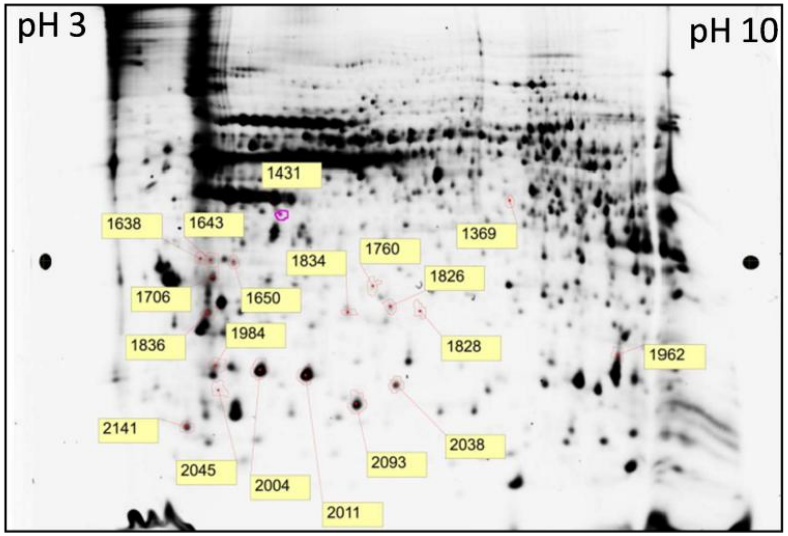


Figure S7. Representative DIGE gel. Cortical neurons were isolated from 4 wild-type (*Usp9x*$^{+/Y}$) and 4 knockout (*Usp9x*$^{-/Y}$) E18.5 embryos and cultured for 5 days *in vitro* before lysates prepared. 150µg of each lysate was provided to Adelaide Proteomics Centre (Adelaide, South Australia) for processing. Briefly, lysates were labelled with CyDyes (GE Healthcare), either Cy3 or Cy5. An internal pooled standard was labelled with Cy2. Samples were applied to 24cm 3-10 non-linear immobilised pH gradient (IPG) strips (Biorad) by anodic cup-loading. Isoelectric focussing (IEF) was performed on an IPGphorII (GE Healthcare). Proteins were focussed for 27000 Volt-hours at 8000V. Following IEF, SDS-PAGE in the second dimension was carried out using 12.5% 2DGel DALT NF precast poly-acrylamide gels (Gel Company). Electrophoretic separation was performed using an Ettan Dalt12 unit (GE Healthcare). Gels were scanned using the Ettan DIGE Imager (GE Healthcare). Image analysis was undertaken using the DeCyder 2D software (V7, GE Healthcare). Each gel image was processed separately in the Differential In-gel Analysis (DIA) module of DeCyder prior to export to the Biological Variation Analysis (BVA) Module. Spots remaining after DIA module filtering were manually inspected for resolution quality. Spot-matching and comparative analysis was conducted using BVA. Individual gel images were sorted into wild-type and knockout groups and subjected to t-test; $p<0.05$ were considered significant. A representative warped gel is shown. Numbers identify highest ranking differential spots found between knockout and wild-type cultures.

Protein	Gene	Fold Change	Ub	Functions
Voltage-dependent anion-selective channel protein 2	*Vdac2*	-1.46	Yes	Negative regulator of apoptosis; Increased expression correlated with refractory epilepy.
Actin related protein 2/3 complex subunit 2	*Arpc2*	-1.46	Yes	Regulates actin dynamics. Involved in neuronal migration and axon guidance.
Guanine nucleotide-binding protein subunit β2-like 1	*Gnb2l1*	-1.46	Yes	Adaptor protein (receptor for activated protein kinase C 1; aka Rack1) ; Regulates Ca^{2+} signalling; Regulates NMDA receptor excitation; Regulates acetylcholinesterase.
60S acidic ribosomal protein P0	*Rplp0*	-1.46	Yes	Ribosomal Protein
Guanine nucleotide-binding protein G(o) subunit α	*Gnao1*	-1.45	Yes	Influences G protein-mediated signalling and calcium channel regulation. Deletion in mice causes motor control and behaviour defects, altered pain perception and hyperactvity
GTP-binding nuclear protein Ran	*Ran*	-1.45	Yes	Regulates formation of the nuclear microtubule network; Regulates nuclear transport..
Cofilin-1	*Cfl1*	-1.37	Yes	Actin-depolymerizing activity; Rho signalling pathway - neurite growth; Reelin signalling pathway - neuronal migration. Brain specific deletion in mice results in ataxia and seizures.
Heterogeneous nuclear riboprotein K	*Hnrnpk*	-1.37	Yes	Gene expression, including chromatin remodeling, transcription, splicing, translation, and stability. Regulates mRNA trafficking in neurons. Involved in cell migration and dendritic spine morphology.
Tubulin α1A Chain	*Tuba1a*	-1.35	Yes	OMIM: 611603. Mutations cause Lisencephaly 3. Clinical features also include sever ID, seizures, thin corpus callosum, abnormal hippocampus. Involved microtubule formation and neuronal migration.
Tubulin βIIB Chain	*Tubb2b*	-1.35	Yes	OMIM: 614039. Mutations cause complex cortical dysplasia, with microcephaly, polymicrogyria, agenesis of corpus collusom, severe ID and seizures. Involved microtubule formation and neuronal migration.
Creatine kinase B-type	*Ckb*	-1.35	Yes	Energy metabolism. Additional roles in K and Cl transport in neurons.
Proteasome subunit α type-6	*Psma6*	-1.34	Yes	Subunit of the 20S Proteasome complex.
Keratin, type I cytoskeletal 14	*Krt14*	-1.33	Yes	Intermediate filament protein. Skin development.
Keratin, type II cytoskeletal 5	*Krt5*	-1.33	Yes	Cytoskeletal filament protein. Skin development.
Keratin, type II cytoskeletal 8	*Krt8*	-1.33	Yes	Cytoskeletal filament protein. Skin development.
Thioredoxin	*Txn1*	-1.33	Yes	Oxidoreductase enzyme , antioxidant with neuro-protective activities.
L-lactate dehydrogenase A chain	*Ldha*	-1.26	Yes	Subunit of lactate dehydrogenase. Energy metabolism.
Phosphoserine aminotransferase	*Psat1*	-1.26	Yes	OMIM: 610992. Clinical features include intractable seizures, acquired microcephaly, hypertonia, and psychomotor retardation. Enzyme involved in L-Serine synthesis.
Heterogeneous nuclear riboproteins A2/B1	*Hnrnpa2b1*	-1.26	Yes	Mutations cause inherited degeneration affecting muscle, brain, motor neuron and bone. RNA binding protein involved in packaging nascent mRNA, alternative splicing, trafficking and translation.
Glyceraldehyde-3-phosphate dehydrogenase	*Gapdh*	-1.23	Yes	Glycolytic enzyme. Energy metabolism
Stathmin	*Stmn1*	-1.23	Yes	Regulates microtubule polymerisation, neuronal migration and axon growth. In Mice, genetic deletion caused decreased fear conditioned memory.
Peptidyl-prolyl cis-trans isomerase A	*Ppia*	-1.21	Yes	Antioxidant and neuroprotective activities.
Dihydropyrimidinase-related Protein 3	*Dpysl3*	-1.17	Yes	Aka Crmp4. Variants associate with ALS. Microtubule associated protein. Regulates axonal growth and neuronal polarity.
Tubulin βIII Chain	*Tubb3*	-1.16	Yes	OMIM: 614039. Mutations cause complex cortical dysplasia, associated with ID, strabismus, axial hypotonia, and spasticity. Microtubule subunit involved in neuronal migration and neurite growth.
Tubulin βIIC Chain	*Tubb4b*	-1.16	Yes	Microtubule subunit.
Dihydropyrimidinase-related Protein 2	*Dpysl2*	-1.16	Yes	Aka Crmp2. Binds Tubulins. Mediator of growth cone collapse as well as modify axon number, length, and neuronal polarity.
Growth Factor receptor-bound protein 2	*Grb2*	1.8	Yes	Adaptor protein. Regulates receptor signalling and actin cytoskeletal reorganisation.
Peroxiredoxin 6	*Prdx6*	1.8	Yes	Lysosomal phospholipase, Hydrolyse the fatty acyl or alkyl bonds of membrane phospholipids; Reactive oxidative stress pathway control in astrocytes.

Table S1. List of proteins found deregulated in *Usp9x* knockout neurons.

Gene ontology term	Gene count	*P* -value	Benjamini
GTPase activity	5	7.5×10^{-05}	5.5×10^{-03}
Cytoskeletal part	9	8.7×10^{-05}	7.7×10^{-03}
Structural molecule activity	7	1.2×10^{-04}	4.3×10^{-03}
Cytoskeleton	9	1.1×10^{-03}	4.8×10^{-02}
Protein polymerization	3	2.1×10^{-03}	4.4×10^{-01}
GTP binding	5	3.5×10^{-03}	8.2×10^{-02}
Guanyl nucleotide binding	5	3.8×10^{-03}	6.8×10^{-02}
Guanyl ribonucleotide binding	5	3.8×10^{-03}	6.8×10^{-02}
Microtubule-based process	4	5.2×10^{-03}	5.2×10^{-01}
Keratin filament	3	7.2×10^{-03}	1.9×10^{-01}
Cellular macromolecular complex subunit organization	4	7.9×10^{-03}	5.2×10^{-01}
Intracellular non-membrane-bounded organelle	10	9.2×10^{-03}	1.9×10^{-01}
Non-membrane-bounded organelle	10	9.2×10^{-03}	1.9×10^{-01}
Microtubule	4	1.2×10^{-02}	1.9×10^{-01}
Regulation of cytoskeleton organization	3	1.2×10^{-02}	5.7×10^{-01}
Microtubule-based movement	3	1.3×10^{-02}	5.0×10^{-01}
Mitochondrion	8	1.3×10^{-02}	1.7×10^{-01}
Cellular protein complex assembly	3	1.4×10^{-02}	4.8×10^{-01}
Cytosol	5	2.2×10^{-02}	2.5×10^{-01}
Macromolecular complex subunit organization	4	2.3×10^{-02}	6.1×10^{-01}
Cell projection	5	2.6×10^{-02}	2.5×10^{-01}
Regulation of organelle organization	3	2.8×10^{-02}	6.2×10^{-01}
Intermediate filament	3	3.0×10^{-02}	2.6×10^{-01}
Intermediate filament cytoskeleton	3	3.2×10^{-02}	2.5×10^{-01}
Nucleotide binding	9	3.3×10^{-02}	3.9×10^{-01}
Positive regulation of cytoskeleton organization	2	4.3×10^{-02}	7.4×10^{-01}

Table S2. **Summary of gene ontology terms identified following DAVID annotation tool analysis of the list of proteins found deregulated in *Usp9x*$^{-/Y}$ neurons**. p<0.05 by students t-test.