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Supplemental Data

De Novo Variants in *MAPK8IP3* Cause Intellectual Disability with Variable Brain Anomalies

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Supplemental Tables

Table S1. Detailed clinical information of all individuals with causative *de novo* variants in *MAPK8IP3*

(Excel-file)

Table S2. *In silico* prediction of *de novo* missense variants and conservation of affected amino acids in *MAPK8IP3*

Chr16:g.	***De novo* variant**	**p.**	**REVEL** (Ioannidis *et al.*[1])	**CADD 1.4** (Kircher *et al.*[2])	**MutationTaster2** (Schwarz *et al.*[3])	**GERP** (Cooper *et al.*[4])	**Polyphen-2 v2.2.2r398** (Adzhubei *et al.*[5])	**SIFT** (Sim *et al.*[6])	**M-CAP** (Jagadeesh *et al.*[7])	**Conservation**[a] (up to indicated species)
1798706	c.1198G>A	p.Gly400Arg	0.560	32	DC	5.3	PD (1.000)	D (0.02)	PP (0.114)	high (zebrafish)
1810410	c.1331T>C	p.Leu444Pro	0.825	25.6	DC	5.47	PD (0.999)	D (0)	PP (0.799)	high (C. elegans)
1812389	c.1574G>A	p.Arg525Gln	0.521	28.2	DC	5.64	PD (0.998)	D (0)	PP (0.136)	high (C. elegans)
1812844	c.1732C>T	p.Arg578Cys	0.675	29.1	DC	4.42	PD (1.000)	D (0.02)	PP (0.284)	high (zebrafish)
1816769	c.2982C>G	p.His994Gln	0.379	23.3	DC	0.89	PD (0.998)	T (0.05)	PP (0.098)	high (fruitfly)
1817835	c.3436C>T	p.Arg1146Cys	0.472	25.3	DC	3.32	PD (1.000)	D (0)	PP (0.589)	high (C. elegans)

All genomic positions according to hg19, variant nomenclature corresponding to GenBank NM_015133.4.; D: deleterious; DC: disease causing; PD: probably damaging; PP: possibly pathogenic T: tolerated.

[a]Conservation was evaluated considering the following species: Homo sapiens, Pan troglodytes (chimp), Rattus norvegicus (rat), Mus musculus (mouse), Canis familiaris (dog), Ornithorhynchus anatinus (platypus), Gallus gallus (chicken), Xenopus tropicalis (frog), Tetraodon nigroviridis, Danio rerio (zebrafish), Drosophila melanogaster (fruitfly), Caenorhabditis elegans.

Supplemental Material and Methods

Exome Sequencing

Individuals 1 and 4. Parent-proband trio exome sequencing was performed using the IDT xGen Exome Research Panel v1.0. Bioinformatics filtering and data analysis were performed as previously described.[8] Candidate gene analysis and interpretation were performed as previously described.[9]

Individual 2. Whole exome analysis of the index individual was performed using the Nextera Exome capture on an Illumina HiSeq 4000. Bioinformatic analysis was carried out with an in-house developed pipeline relying on Burrows-Wheeler Alignment (BWA) and the Genome Analysis Toolkit (GATK). On target coverage was ≥ 10x in 96.0% with a median coverage of 97x.

Individuals 3, 6, 9, 10 and 12. Using genomic DNA from the proband and parents, the exonic regions and flanking splice junctions of the genome were captured using either the Clinical Research Exome kit (Agilent Technologies, Santa Clara, CA) for individual 3, individual 6 and individual 9 and the IDT xGen Exome Research Panel v1.0 for individual 10 and individual 12. Sequencing was done on an Illumina system with 100bp or greater paired-end reads. Reads were aligned to human genome build GRCh37/UCSC hg19, and analyzed for sequence variants using a custom-developed analysis tool. Additional sequencing technology and variant interpretation protocol has been previously described.[10]

Individuals 5 and 8. Agilent SureSelect All Exon V5 and V6 respectively were used for index and parents. Sequencing was performed using the Illumina HiSeq2500 and HiSeq400 respectively. Coverage on target was ≥ 10x for 98.78%, ≥ 20x for 97.31%, ≥ 30x for 94.84% and ≥ 10x for 96.76%, ≥ 20x for 95.57%, ≥ 30x for 93.25%, respectively. An analysis pipeline was used for both families as previously described.[11]

Individual 7. Trio exome sequencing was performed using the SureSelect All Human Version 6 (60 Mb) on a HiSeq 4000 platform. On target coverage was achieved ≥ 10x for 97.05% in the index individual. Analysis of the raw data was performed with the pipeline Varfeed (Limbus Medical Technologies) and variants were annotated and prioritized using the software Varvis (Limbus Medical Technologies).

Individual 11. Exome sequencing and variant calling using a parent-offspring trio approach was performed as described previously.[12] Briefly, the Agilent SureSelectXT Human All Exon v5 library prep kit was used (Agilent Technologies, Santa Clara, CA, USA). Sequencing was performed on an Illumina HiSeq 4000 instrument (Illumina, San Diego, CA, USA) with 101 bp paired-end reads at a median coverage of ×75 at the BGI Europe facilities (BGI, Kopenhagen, Denmark). Using Burrows-Wheeler Alignment (BWA) version 0.5.9-r16.14, sequence reads were aligned to the hg19 reference genome. Variants were subsequently called by the Genome Analysis Toolkit (GATK) unified genotyper, version 3.2-2. Annotation was performed using a custom built diagnostic annotation pipeline (DG.2.8).

Individual 13. Whole exome sequencing of index and parents was performed using the Agilent SureSelect Clinical research Exome v.2 on an Illumina NextSeq500 platform. An analysis pipeline was used as previously described.[13] Coverage on target for the index was ≥ 10x for 98.6% with a mean coverage of 200x.

C. elegans culture and strains

Worm culture and manipulation essentially followed previously described methods.[14–16] Briefly, culture media was modified NGM (referred to as NGM-LOB).[17] Prior studies defined the culture plate types “streak plates”, “locomotion plates”, “24-well plates”, and “96-well solid media culture plates”.[18–20] “96-well thrashing plates” were made similar to “96-well solid media culture plates”,[19] except the media volume per well was 305 µl, and the wells were not seeded with bacteria. “5 ml unseeded plates” were made by dispensing 5 mls of modified NGM into standard 60 mm petri plates,

allowing them to set overnight at room temperature lid-side-up, and storing unseeded at 4° until needed. Wild type was strain N2. Other *C. elegans* strains used in this study include KG2338 unc-16(ce483),[21] KG2430 ceIs56 [unc-129::CTNS-1-RFP, unc-129::nlp-21-Venus],[22] KG4192 unc-16(ce483); ceIs56,[23] and the 6 unc-16 mutants and their wild type revertant counterparts listed in Figure 3A in the main text.

Generation of unc-16 mutants and wild type revertants in C. elegans

With the exception of unc-16(ce856) and its mutationally-rescued counterpart, all mutations were generated using oligonucleotide-templated repair after producing targeted double strand breaks using the CRISPR-Cas9 system. All edits were targeted within 31 bp of the cut site. In addition to the targeted mutation, we introduced a silent mutation in the repair template that altered a restriction site, allowing us to screen for conversion by PCR followed by restriction digest. The oligo template contained ~50 bases of homology on each side of the mutated sequences. The oligo was ordered from Sigma at the 0.2 µmole scale with PAGE purification. The polarity of the oligonucleotide template was sense when the edit was left of the cut site and anti-sense when the edit was right of the cut site, as suggested by published guidelines.[24] Adult worms were injected as described.[25] Except where indicated (see below), the injected animals were N2 (wild type). F1 progeny containing the Co-CRISPR or co-transformation marker were allowed to lay eggs for 1-2 days before testing the F1's for the targeted edit using PCR and restriction digest. We chose homozygous lines not expressing the Co-CRISPR or co-transformation markers for analysis. The sequences around the edits were confirmed by PCR and sequencing.

To generate the unc-16(ce851) and unc-16(ce852) mutations, we used the oligonucleotide-templated co-conversion strategy ("Co-CRISPR"),[26] using dpy-10(cn64) as a co-conversion marker. We cloned the 20 bp Cas9 target sequence into the pJP118 gRNA expression cassette as previously described for cloning targeting sequences into pRB1017.[26] pJP118 is a modified version of the published pRB1017 plasmid.[26] It contains a modified sgRNA (F+E), with an extended Cas9 binding structure and removes a potential Pol III terminator by an A-U base pair flip. The injection mixture was pDD162 (Cas9

plasmid; 50 ng/µl),[27] pJA58 (dpy-10 gRNA plasmid; 25 ng/µl),[26] the experimental gRNA plasmid at 25 ng/µl, and the dpy-10(cn64) and experimental oligonucleotide repair templates (500 nM and 2400 nM, respectively). We injected ~60 wild type animals with this mixture and cloned 192 F1 rollers for each conversion. For unc-16(ce852), 6% of F1 rollers showed a restriction pattern consistent with the targeted edit, all of which were heterozygous. One of these lines was chosen and screened for homozygosity in the following generation. One homozygous line was chosen, and the sequence around the edit was confirmed by PCR and sequencing. For unc-16(ce851), 33% of F1 rollers showed a restriction pattern consistent with the targeted edit. One line was homozygous, indicating that edits occurred in both germlines.

To generate the mutationally-rescued (reverse-engineered) counterparts of unc-16(ce851) and unc-16(ce852), as well as the ce853, ce854, ce857 unc-16 alleles and their reverse-engineered counterparts, we used the high efficiency Co-CRISPR independent method described by Prior et al.[28] This method calls for injecting sgRNA-Cas9 complexes along with a myo-2::RFP co-transformation marker to select for progeny that incorporated the injection components. The complexes are made by combining the Alt-R® S.p. HiFi Cas9 Nuclease 3NLS (IDT) with a synthetic sgRNA with an optimized 80mer Synthego Scaffold (Sythego). The injection mixture was 300 mM KCl, 20 mM HEPES, pH 7.4, KG#381 (myo-2::RFP), 2.0 µM of the experimental Oligo repair template, 5 µM of the sgRNA, and 5 µM of the Cas9 enzyme. The injection mixture was incubated 30 min – 4 hr at 23° before injecting. Depending on the distance between the edit and the cut site, which ranged from 4 – 31 bp, we injected 60 – 100 animals and cloned 48 – 192 myo-2::RFP – positive F1's. An average of 35% of lines showed a restriction pattern consistent with the targeted edit (range 2.6% - 57%). 25% - 80% of positive F1's were homozygous, indicating that the conversions occurred in both germlines.

For unc-16(ce856), the targeted mutation site was 45 bp from the nearest cut site. We therefore generated this mutation in two steps. In the first step we deleted 108 bp between two cut sites that flanked the targeted edit site by co-injecting 2 sgRNAs with an oligonucleotide template homologous to the flanking regions to be joined, but that also inserted a 4 bp restriction site at the join. The restriction site insertion allowed us to use PCR and restriction digest to test whether the locus came

together by non-homologous end joining, which is prone to errors, or by homologous repair. It also allowed us to improve the protospacer for one of the cut sites that we would use in the second step. For the second step, we use GenePlus Economy (Genscript) to synthesize the 108 bp region to be re-inserted, including the targeted amino acid change, plus 35 – 37 bp homology arms. We amplified the DNA using eight 50 µl Q5 PCR reactions and concentrated the products to 818 ng/µl in 9 µl using the MinElute PCR purification kit (Qiagen). For both the first and second step, the injection mixtures were as described above for the high efficiency Co-CRISPR independent method. The second step injection mixture included 81 ng/µl of the PCR product. The first step used N2 (wild type as the host) and the second step used the strain containing the 108 bp deletion made in the first step. To mutationally rescue this strain back to the wild type sequence, we repeated these steps starting with the mutant strain, deleting the 108 bp region and replacing it with the wild type region.

Genetic crossing of C. elegans unc-16 mutations

For assaying lysosomes in axons, each mutation and its mutationally-rescued counterpart were crossed with the genomically integrated transgene ceIs56, which also expresses the ttx-3::RFP co-transformation marker. To cross the mutants with ceIs56, we crossed ceIs56 males with the mutant strain and, after 3d at 20°, cloned 5 ttx-3::RFP-positive L4-stage progeny to streak plates, grew them 4d at room temperature, and then cloned 14 ttx-3::RFP (red) putative homozygotes to streaks. After growing 4d at room temperature we tested all of these lines for homozygosity of the mutant using PCR and restriction digest. Homozygosity of ceIs56 was confirmed by checking for 100% ttx-3::RFP-positive animals in the chosen lines, final genotype: unc-16(ce____); ceIs56.

To cross the mutationally-rescued alleles with ceIs56, we crossed them through the original mutant background since the mutationally-rescued alleles could not be distinguished from wild type alleles by PCR and restriction digest. To do this, we made unc-16(ce____); ceIs56 males by heat shock and crossed them with hermaphrodites from the rescued strain. After 3d at 20°, we cloned 5 ttx-3::RFP-positive L4-stage progeny to streak plates, grew them 4d at room temperature, and then cloned 14 ttx-3::RFP (red) putative homozygotes to streaks. After growing 4d at room temperature we tested

all of these lines for homozygosity of the mutationally-rescued allele using PCR and sequencing (for ce863) or PCR and restriction digest (all other mutationally-rescued alleles). Homozygosity of ceIs56 was confirmed by checking for 100% ttx-3::RFP-positive animals in the chosen lines.

Quantitative fluorescence imaging of axonal lysosomes in C. elegans

Strain growth: Young adult progeny that had not previously been starved were grown for imaging as described.[23] ~55 young adults were selected and transferred to an unseeded plate immediately prior to mounting as described below.

Agarose pad slide production: Clean glass slides were produced as described.[23]

Mounting animals on agarose pad slides: We applied a 30 µl drop of 30 mg/ml BDM (2, 3-Butanedione monoxime; Sigma B0753) in M9 buffer onto a 24 X 30 mm coverslip. We then transferred the prepicked animals in one pick-full to the drop on the coverslip and incubated them for 10 min, placing the coverslip on a 1.5 cm square pad of folded paper towel tissue under a Petri plate lid. After the incubation, we removed ~19 µl of the solution using a P20 microinjection tip (Eppendorf 5242 956.003), leaving the worms behind in the remaining anesthetic, and inverted the coverslip onto a ~18-19 mm diameter 4% agarose pad that had been dried without its protective coverslip for the final 4 min of the incubation. We marked animal positions on the slide using a sharpie and imaged animals over the next 35 min.

Image acquisition and processing: We viewed animals using a Nikon Eclipse Ti-E inverted microscope equipped with a Nikon CFI Apo TIRF 100X/ 1.49 N.A. objective, a Nikon motorized high resolution z-drive, and a motorized filter turret containing GFP, YFP, and Texas Red filter cubes (Semrock). Our illumination source was a SOLA Light Engine LED source (Lumencor). We acquired images with an ORCA Flash 4.0 16-bit camera (Hamamatsu, Bridgewater, NJ) controlled by Metamorph v. 7.7. We controlled exposure times by using Metamorph to turn the LEDs on and off rather than using a shutter. We only collected images from animals with their dorsal surfaces facing the objective and used the “center quad” (center quadrant) mode of the camera. Z-series interval sizes (0.312 µm) and plane numbers (16) were the same for all strains. Exposure times were identical for different strains

in each experiment and chosen to collect at sub-saturating levels. Before imaging each strain, we measured the light power of the peak emission wavelength at the objective plane using an XR2100 power meter (Lumen Dynamics) and an XP750 objective plane light sensor (Lumen Dynamics) with the stage position set at a standard distance (z-position) from the objective. We then adjusted the percent power of the SOLA Light Engine to produce the targeted mW power for the experiment. We used AutoDeblur Gold CWF (Media Cybernetics) to deconvolve the image stacks using the Adaptive PSF blind method and 10 iterations at the low noise setting. After deconvolving, we used Metamorph to make maximum intensity projections of each image stack.

Quantifying Images: We used Metamorph 7.7 for all analysis and quantification. To quantify puncta per micron, we set a minimum pixel intensity threshold after viewing a series of images collected from unc-16(ce483) mutant dorsal axons. We then used the Threshold plug-in of Metamorph to highlight all pixels in the region that exceeded the threshold and counted the pixel clusters that exceeded this value, irrespective of the number of pixels in the cluster. We used the same threshold value in all strains throughout the experiment.

Producing Representative Images: After quantifying an image set we produced representative images for display by saving 8-bit versions of an image that was close to mean +/- standard error for the set. All representative images were scaled identically.

C. elegans locomotion assays

To grow animals for the assay, we transferred 13 L2 stage larvae to each of 4 Locomotion Plates and cultured the plates at 20° for 5 2/3 days to produce F1 young adults that had never been starved. The day before the assay, we adjusted the assay area temperature to 22.5 – 23.5° and set the following items in this area: one “96-well thrashing assay plate” (see “*C. elegans* Culture and Strains”), three “5 ml unseeded plate” (see “*C. elegans* Culture and Strains”), a 2L bottle of M9 with a 10 ml bottle top dispenser (set on 6 mls), a 100 ml bottle of M9, a count-down timer set on 3:00 min, a tally counter, and a 2.5 ml Combi-tip attached to a repeat pipetter and set on 75 µl. We made a “25 µl glass pipet

tip" by scoring and breaking off ~4 cm from the end of a Pasteur pipet, inserting a plastic gel-loading tip into the broken end, and securing the junction with Para film.

Prior to and during the assay, room thermostats were adjusted to keep the assay area temperature at 22.5° to 23.5°, as monitored by a CheckTemp digital thermometer kept near the stereoscope stage. To start the assay, one of the source plates was transferred from the 20° incubator to the assay area. A 2.5 ml Combi-tip on a repeat pipetter was used to fill the first 12 wells in row A of the 96-well Thrashing Plate with 75 µl of M9. The 5 ml Unseeded Plate was then filled with 12 mls of M9 using the bottle top dispenser. 12 young adult animals were transferred from the source plate into the 12 mls of M9 in the unseeded plate. We then used a P20 set on 25 ul and the 25 ul glass pipet tip to transfer those animals one at a time, in a full 25 µl of M9 to the first 12 wells of the assay plate. We then immediately focused on the animal in the first well, started a count-down timer, and used a tally counter to count swimming cycles for 3 min. We then recorded the counter value on the datasheet, reset the timer and tally counter, focused on the animal in the next well, started the timer, and counted for 3 min, etc. until we had assayed all 12 animals. We then repeated this for 5 more sets of 12 animals for 72 animals total, using a fresh 5 ml unseeded plate for every 24 animals assayed.

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