Supp. Text 1: Clinical descriptions

Individual 1: Individual 1 was born to reportedly unrelated Hispanic parents and had a healthy older maternal half-brother. Her mother, maternal grandmother, and maternal great grandfather had strabismus (HP:0000486). At 27 years of age, her maternal aunt died of progressive cerebellar degeneration (HP:0001272) with ataxia (HP:0001251) and aphasia (HP:0002381), and her maternal first cousin once removed had gait abnormalities (HP:0001288).

Individual 1 was born full term by spontaneous vaginal delivery following an uncomplicated pregnancy with no exposures. She underwent phototherapy for elevated bilirubin (HP:0002904) and was discharged at 3 days of age. She did not open her eyes for her first week of life and, once she did, her eyelids were droopy and she could not look up.

By parental report, Individual 1 had hypotonia during her first year of life (HP:0008947) for which she received short-term physical therapy. Brain MRI at 20 months of age revealed thickening and irregularity of the posterior perisylvian cortex, with bilateral perisylvian polymicrogyria (HP:0032407). The lateral ventricles and caudate heads were asymmetrical (HP:0030047; HP:0002339). There was deficiency of the rostrum of the corpus callosum (HP:0002079). The brain parenchyma, olfactory sulcus and bulbs, cerebellum, and brainstem appeared normal (Supp. Table 3). The anterior commissure was present and slightly enlarged (HP:0030301). The oculomotor nerves appeared hypoplastic. The superior rectus and levator muscles were very small and the medial rectus muscles were somewhat small bilaterally (HP:0008049).

On examination at 2.5 years of age she had mild speech delay (HP:0000750) with otherwise normal social, fine motor, and gross motor development. She had a head

circumference of 50 cm (89th percentile, z=1.21), weight of 13.8 kg (67th percentile, z= 0.44), and height of 88 cm (18th percentile, z= -0.92).* She had mild hyperopia (HP:0000540), bilateral ptosis (HP:0007911), primary eye position in downgaze, and a preferred chin-up head position (HP:0031705). She had bilateral limitation of upgaze and mild bilateral limitation of downgaze. Horizontal eye movements were full. She had an intermittent exotropia (HP:0000577) of up to 40 prism diopters which became reduced and variable when she attempted to look up. She had abnormal conjugate eye movements (HP:0000549) with convergence on attempted upgaze and divergence and occasional convergence on attempted downgaze, consistent with aberrant innervation. She had slight asymmetry of her mouth, a hyperpigmented birthmark on her leg (HP:0000953), and a lordotic posture with inturning of her feet. Her exam was otherwise normal. Her presentation was felt to be consistent with mild malformation of cortical development (MCD) (HP:0032059) and congenital fibrosis of the extraocular muscles (CFEOM) (HP:0001491).

Individual 1 underwent multiple ophthalmological surgeries. At 2.5 years of age, she had bilateral 12 mm recessions of the inferior rectus muscles and 10 mm bilateral recessions of the lateral rectus muscles. At 5 years of age, she had 4 mm bilateral superior oblique tenotomies and 4 mm bilateral medial rectus muscle resections. The left superior oblique muscle was noted to have an anomalous insertion. Seven months later, she had recurrent exotropia (HP:0000577) and a new right hypertropia (HP:0025586) and underwent a 9 mm recession of the right superior rectus muscle and 4 mm advancement of the right medial rectus muscle. At 9 years of age, she had a residual exotropia (HP:0000577) of 20 prism diopters, with abnormal conjugate eye movements (HP:0000549) consisting of esotropia on attempted upgaze and exotropia on

attempted downgaze, and developed lower eyelid retraction (HP:0030802) with nocturnal lagophthalmos (HP:0030002) from these surgical procedures. At 10 years of age she underwent bilateral lower eyelid retraction repair with conjunctivoplasty. Following this surgery, she had a persistent exotropia (HP:0000577) of 25 prism diopters with satisfactory head position and ptosis (HP:0007911) managed with voluntary brow elevation.

At 13 years of age, her parents report that in addition to her CFEOM (HP:0001491), she struggles with school work (HP:0001328); she was held back one year and is now in the 6th grade.

Individual 2: Individual 2 was born to unrelated Caucasian and Hispanic parents. His father had adult-onset unilateral ptosis (HP:0007687) and his maternal grandfather had esotropia (HP:0000565).

He was born full-term by vaginal delivery with a birth weight of 3.43 kg, and had mild neonatal jaundice (HP:0000952) and bilateral congenital ptosis (HP:0007911). He had decreased food intake (HP:0100738) and choking on solid foods (HP:0030842) early in life, but no aspiration pneumonia (HP:0011951) or diagnosed swallowing deficits (HP:0002015). A tongue release surgery was performed at 16 months of age to relieve his ankyloglossia (HP:0010296), which contributed to these issues.

Individual 2 underwent a series of ophthalmological exams between 4 and 15 months of age which reported a chin-up head position (HP:0031705) with bilateral ptosis (HP:007911), limited upgaze, full horizontal movements, and intermittent esotropia (HP:0000565). He had his first strabismus surgery at 16 months of age. On forced duction testing the left eye could not be elevated above the midline, while all other movements were free. He underwent a 5 mm left inferior rectus muscle recession along

with bilateral levator resections and Fasanella-Servat procedures for ptosis (HP:007911). At 27 months of age he underwent a 6 mm right medial rectus muscle recession with upward transposition by 1 tendon width, a 4 mm left medial rectus muscle recession with upward transposition by 1 tendon width, and bilateral frontalis slings.

On examination at two years and seven months of age, he had a head circumference of 48.9 cm (39^{th} percentile, z= -0.28), a height of 90.2 cm (28^{th} percentile, z= -0.57), and a weight of 12.587 kg (24^{th} percentile, z= -0.72).* On ophthalmological examination at three years of age, his development and behavior were normal to advanced. He had residual mild bilateral ptosis (HP:007911), greater on the right side, and a 20-degree chin-up head posture (HP:0031705). He could not elevate either eye and had mildly limited abduction (HP:0000634) and bilateral superior oblique overaction (HP:0025594). He had an exotropia (HP:0000577) of 12 prism diopters at near, with abnormal conjugate eye movements (HP:0000549) consisting of convergence on attempted upgaze. Both eyes were significantly intorted. He had left eyelid synkinesis triggered by specific gaze positioning or concentration, but not by movement of his eyes or jaw. His general and neurological exam were otherwise normal. His oculomotor phenotype was thought most consistent with CFEOM (HP:0001491).

Individual 2 underwent additional ophthalmological surgeries following the exam at 3 years of age. The frontalis slings were revised at 5 and again at 6 years of age, and at 6 years of age he again underwent strabismus surgery. The unoperated right inferior rectus muscle was noted to have a nasally displaced anomalous insertion. Both medial rectus muscles were resected and advanced 6.0 mm with downward displacement to the center of the original insertion. The superior and inferior rectus muscles were very tight.

Both inferior rectus muscles were recessed 5 mm (the left being a re-recession), with bilateral superior oblique tenotomies. The superior oblique muscles were felt to be normal.

Individual 2 underwent brain MRI at 13 months of age. He had slightly interdigitating frontal lobes consistent with a deficiency of the falx cerebri (HP:0010654) but no apparent MCD, with the caveat that the cortex could not be well assessed due to technical parameters and age-appropriate immaturity of myelination. There was slight asymmetry of the lateral ventricles (HP:0030047) and caudate heads (HP:0002339) and mild thinning of the posterior body and splenium of the corpus callosum (HP:0002079). Brain parenchyma, anterior commissure, optic nerves, olfactory sulci and olfactory bulbs, cerebellum, and brainstem appeared normal (Supp. Table 3). The superior rectus and medial rectus muscles appeared small bilaterally (HP:0008049), but imaging was not optimized for assessment of cranial nerves or extraocular muscles.

When he was 5 years of age, his parents reported that he had difficulty with certain fine and gross motor tasks (HP:0002194; HP:0010862) and that he appeared clumsy. He had received an individualized educational program which included occupational therapy, and was later downgraded to an educational plan which provided visual accommodations. At his most recent examination at 11 years of age, his best-corrected visual acuity was 20/30 on the right and 20/40 on the left. He had a 5-10-degree chin-up head posture (HP:0031705) with no lower eyelid retraction. Ptosis (HP:0007911) was managed with brow elevation. He had satisfactory vertical alignment, limited supra- and infraduction bilaterally, abnormal conjugate eye movements (HP:0000549) with convergence on attempted upgaze, and an exotropia (HP:0000577) of 4 prism diopters at distance and approximately 12 prism diopters at near.

He is now in middle school and, by parental report, performing honors-level coursework. He continues to receive visual accommodations and to have some difficulty with balance (HP:0002172) and fine motor coordination (HP:0007010).

Individual 3: Individual 3 was born to French Canadian parents who were reported to have cognitive impairments (HP:0100543). She was adopted and additional family history and birth details are unavailable.

Ophthalmological exams early in life revealed severe bilateral congenital ptosis (HP:0007911), lack of vertical eye movements, limited horizontal eye movements (HP:0000634; HP:0000542), bilateral hyperopia (HP:0000540), bilateral accommodative esotropia (HP:0020046), and left-sided consecutive comitant exotropia (HP:0031718). She had axial hypotonia (HP:0009062) in her first year of life and global developmental delay (HP:0001263). She began developing words at 12-18 months, using 3-word sentences at 3-4 years of age, and reading words at 6 years of age. She was able to eat independently using her hands between 1-2 years of age. She could draw rudimentary shapes at 5 years of age and began using scissors at 6 years of age. She took her first steps at 2 years of age, but preferred to scoot or crawl until 3 years of age.

She has a history of constipation and gastrointestinal dysmotility (HP:0002579), and began to experience cyclic/episodic vomiting (HP:002572) at 4 years of age. These were recurrent episodes of intractable vomiting lasting 1-2 days, followed by nausea (HP:0002018) and inability to eat for 2-3 days (HP:0004396). She had multiple episodes per year with emergency room visits, some of which required hospital admission for rehydration. She also had shorter episodes in which vomiting could be stopped by Zofran and Maxeran. She required a gastrotomy tube to increase feeding after these episodes.

She had brain MRIs at 8 months of age and at 7 years of age that revealed bilateral perisylvian polymicrogyria (R>L) (HP:0032407), slight asymmetry of the lateral ventricles (HP:0030047) and caudate heads (HP:0002339), and thinning of the posterior body of the corpus callosum (HP:0002079) which was more pronounced than in Individual 2. The anterior commissure, optic nerves, olfactory sulci and olfactory bulbs, cerebellum and brainstem appeared normal. Imaging was not optimized for assessment of cranial nerves or extraocular muscles.

At 5 years of age, Individual 3 underwent strabismus surgeries. Her medial rectus muscles were recessed 3.5 mm bilaterally, and her inferior rectus muscles were recessed 7 mm bilaterally. She was left with a sizable exotropia (HP:0000577), and underwent a second surgery in which her medial rectus muscles were advanced 3.5 mm bilaterally.

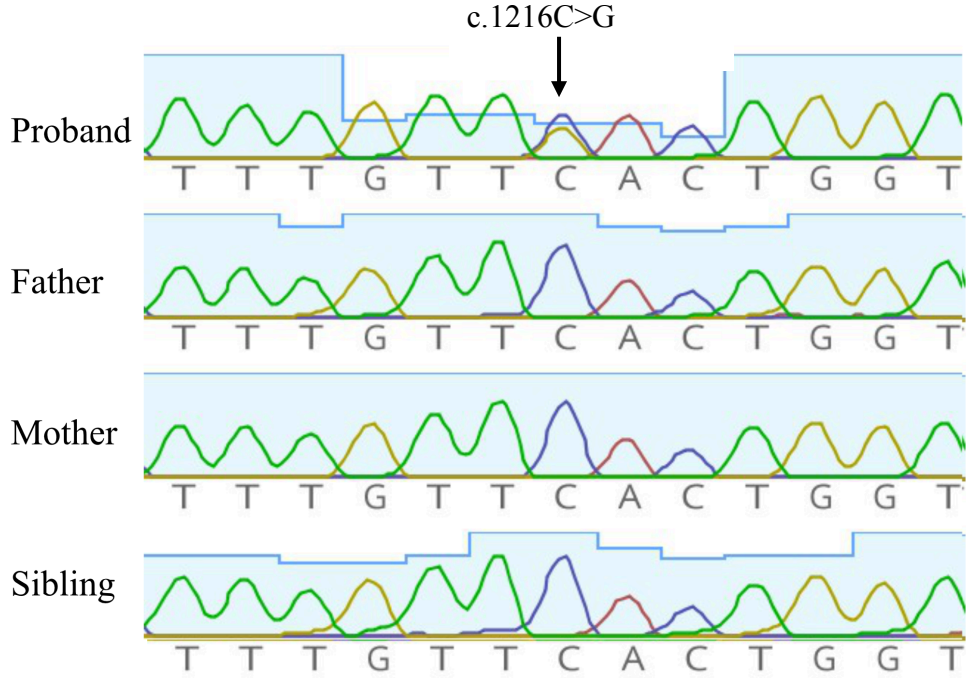
At 11 years of age, she follows an individualized educational program (HP:0001328; HP:0100543). She speaks in short, simple sentences with clear pronunciation. Her reading comprehension is at the 7- to 8-year-old level, and she can count to 100 and do some addition and subtraction. She has attention deficit hyperactivity disorder (HP:0007018) and oppositional defiant disorder (HP:0010865) with behavioral issues, impulsivity, and aggression. She has an awkward pencil grip and poor handwriting (HP:0007010). She is able to jump and run slowly but cannot turn quickly or ride a bike (HP:0007015). Her gastrotomy tube was removed and the cyclic vomiting (HP:0002572) has been treated with domperidone as well as metoclopramide and lorazepam as needed. The episodes have lessened considerably over the last few years, with only one episode in the last year. On examination at 10 years and 10 months of age, she had a head circumference of 50.7 cm (9^{th} percentile, z= -1.3, Nellhaus scale), a height of 146.3 cm (68.2 percentile, z= 0.44), and a weight of 31.5 kg (22^{nd} percentile,

z= -0.61). She has CFEOM (HP:0001491) and lower eyelid retraction (HP:0030802), likely a consequence of her inferior rectus surgery. She has thick ear helices (HP:0009894), a low anterior hairline resulting in a small forehead (HP:0000350), and deep-set eyes (HP:0000490). Her face is symmetrical with normal movements. Strength testing is normal except for hip flexion and extension, for which strength is 4/5 bilaterally. Reflexes are normal. Achilles tendons are tight (HP:0001771) and she walks on her toes (HP:0030051). Her gait is unsteady (HP:002317), and she lost her balance when using a tandem gait.

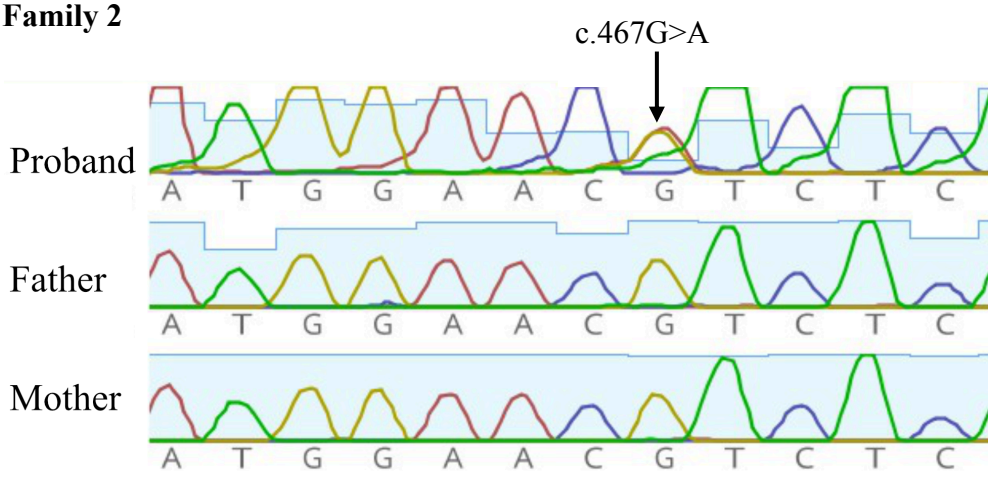
*Head circumference percentile and z-score for Individual 3 is based on the Nellhaus scale (Nellhaus, G. Head circumference from birth to 18 years. Practical composite international and interracial graphs. Pediatrics 1968; 41: 106-114). All other height, weight, and head circumference percentiles and z-scores are based on United States Centers for Disease Control and Prevention, National Center for Health Statistics. CDC growth charts: United States. http://www.cdc.gov/growthcharts/. May 30, 2000.

<u>Supplemental Figure 1: Sanger sequencing of *TUBA1A* variants.</u> All variants annotated with NM_006009.4.

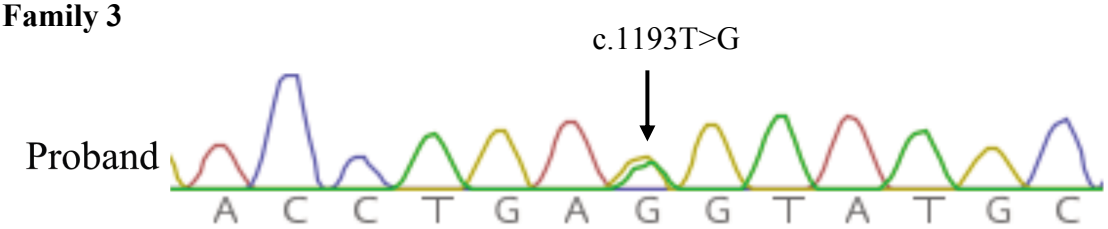
Family 1



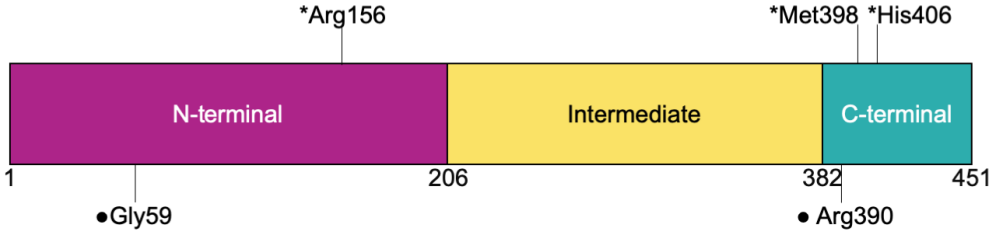
Family 2



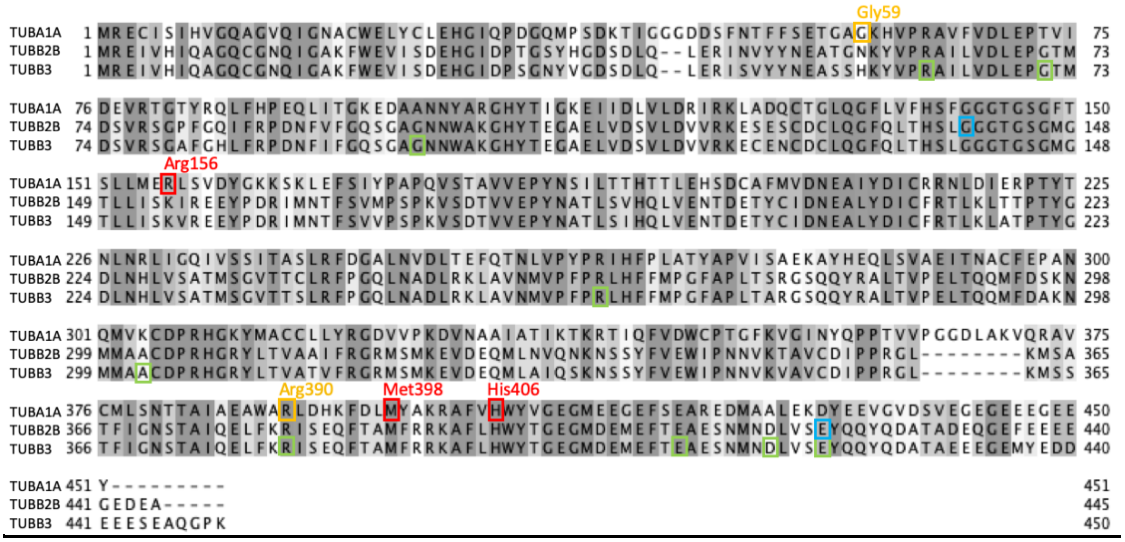
Family 3



Supplemental Figure 2: 2D structural mapping of *TUBA1A*-encoded residues associated with CFEOM or putative CFEOM. Residues associated with CFEOM or putative CFEOM are mapped to their respective protein domains. Key: *Residues reported for the first time in this work (Arg156, Met398, and His406). ●-Previously reported residues associated with putative CN3 phenotypes (Romaniello et al., 2017)



Supplemental Figure 3: Tubulin multiple sequence alignment. Multi-sequence alignment was performed for amino acid sequences of TUBA1A (NP_006000.2), TUBB2B (NP_821080.1), and TUBB3 (NP_006077.2). The level of conservation among residues in the alignment is indicated by the level of shading; dark gray shading indicates full conservation of the residue among all 3 tubulins, while white shading indicates lack of conservation among the 3 tubulins. TUBA1A-CFEOM residues reported here are boxed in red. TUBA1A residues reported in the literature in association with putative CFEOM are boxed in orange (Romaniello et al., 2017). Residue numbers are designated above boxed TUBA1A residues. TUBB2B-CFEOM associated residues are boxed in blue. TUBB3-CFEOM associated residues are boxed in green.



Supplemental Table 1: Sanger sequencing primer sequences

Family	F Sanger sequencing primer (5'>3')	R Sanger sequencing primer (5'>3')	Product size (bp)
1	GACCAAGCGTACCATCCAGT	AAATGGACAGCTTGGGTCTG	522
2	TCGCAAGCTGGTATGTTTCTT	GCGCCGGGTAAATAGAGAAC	476
3	CTCGCCTGGACCACAAGT	CCTAGGATATGTATAAACCACAGG CA	507

Supplemental Table 2: Summary of total numbers of genes and variants identified by ES/GS after filtering

Family 1

	De novo	Autosomal recessive--homozygous	Autosomal recessive--compound heterozygous
Variant Count	2	0	10
Gene Count	2	0	4

Family 2

	De novo	Autosomal recessive--homozygous	Autosomal recessive--compound heterozygous	X-linked recessive
Variant Count	1	0	8	2
Gene Count	1	0	4	2

Family 3

	Heterozygous	Homozygous	Putative compound heterozygous*	X-linked heterozygous
Variant count	164	2	12	4
Gene count	164	2	6	4

*Genes with a burden of >1 hit with at least 2 variants meeting allele frequency and quality score cutoffs. Note this is a singleton exome, so phase is unknown. These variants were not counted in the heterozygous variant number.

Supplemental Table 3: Clinical features assessed but not present in the cohort.

	Individual 1	Individual 2	Individual 3
Abnormal pupillary function (HP:0007686)	N	N	N
Epilepsy (HP:0001250)	N	N	N
Anosmia (HP:0000458)	N	N	N
Facial palsy (HP:0010628)	N	N	N
Trigeminal anesthesia (HP:0031912)	N	N	N
Hearing impairment (HP:0000365)	N	N	N
Poor suck (HP:0002033)	N	N	N
Retrognathia (HP:0000278)	N	N	N
Micrognathia (HP:0000347)	N	N	N
Abnormal palate morphology (HP:0000174)	N	N	N
Spasticity (HP:0001257)	N	N	N
Decreased muscle mass (HP:0003199)	N	N	N
Peripheral neuropathy (HP:0009830)	N	N	N
Brain MRI findings			
Abnormal cerebellum morphology (HP:0001317)	N	N	N
Abnormality of the internal capsule (HP:0012502)	N	N	N
Underdevelopment of the olfactory bulb (HP:0040326)	N	N	N
Aplasia/hypoplasia of the optic nerve (HP:0008058)	N	N	N
Abnormal eye morphology (HP:0012372)	N	N	N
Key: Y-yes, N-no, NA-not ascertainable/ unknown			

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