Phenotypic and biochemical analysis of an international cohort of individuals with variants in *NAA10* and *NAA15*

Hanyin Cheng[1,#], Leah Gottlieb[2,3#], Elaine Marchi[4], Robert Kleyner[5], Puja Bhardwaj[5], Alan F. Rope[6,7], Sarah Rosenheck[5], Sébastien Moutton[8,9], Christophe Philippe [9,10], Wafaa Eyaid[11], Fowzan S. Alkuraya[12,13], Janet Toribio[14], Rafael Mena[15,16], Carlos E. Prada[17,18], Holly Stessman[19], Raphael Bernier[20], Marieke Wermuth[21], Birgit Kauffmann[21], Bettina Blaumeiser[22], R. Frank Kooy[23], Diana Baralle[24,25], Grazia M.S. Mancini[26], Simon J. Conway[27], Fan Xia[1,28], Zhao Chen[1,28], Linyan Meng[1,28], Ljubisa Mihajlovic[29], Ronen Marmorstein[2,3,30], Gholson J. Lyon[4,5*]

1. Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX 77030, USA.
2. Department of Chemistry, University of Pennsylvania, Philadelphia, PA 19104, USA
3. Abramson Family Cancer Research Institute, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA.
4. Department of Human Genetics New York State Institute for Basic Research in Developmental Disabilities (IBR), Staten Island, NY,10314, USA.
5. Stanley Institute for Cognitive Genomics, One Bungtown Road, Cold Spring Harbor Laboratory, NY, 11724, USA.
6. Kaiser Permanente Center for Health Research, Portland, OR 97227, USA
7. Genome Medical, South San Francisco, CA 94080, USA.

8. Reference Center for Developmental Anomalies, Department of Medical Genetics, Dijon University Hospital, Dijon, France.

9. INSERM U1231, LNC UMR1231 GAD, Burgundy University, F-21000, Dijon, France.

10. Laboratoire de génétique, Innovation en diagnostic génomique des maladies rares UF6254, Plate-forme de Biologie Hospitalo-Universitaire, CHU Dijon, Dijon, France.

11. King Abdulaziz Medical City, King Saud Bin AbdulAziz University- Health Science, King Abdullah International Medical Research Center, Riyadh, Saudi Arabia.

12. Department of Genetics, King Faisal Specialist Hospital and Research Center, Riyadh, Saudi Arabia.

13. Department of Anatomy and Cell Biology, College of Medicine, Alfaisal University, Riyadh, Saudi Arabia.

14. Division of Cardiology, CEDIMAT, Santo Domingo, Dominican Republic.

15. Neonatal Intensive Care Unit, Centro de Obstetricia y Ginecologia, Santo Domingo, Dominican Republic.

16. Division Of Neonatology, Cincinnati Children's Hospital Medical Center, Cincinnati, OH, 45229, USA.

17. Division of Human Genetics, Cincinnati Children's Hospital Medical Center, Cincinnati, OH, 45229, USA.

18. Department of Pediatrics, University of Cincinnati College of Medicine, Cincinnati, OH, USA.

19. Department of Pharmacology, Creighton University Medical School, Omaha, NE, USA.

20. Department of Psychiatry, University of Washington, Seattle, WA USA.

[21] Klinik für Kinder- und Jugendmedizin, Neuropädiatrie, Klinikum Links der Weser, Bremen, Germany.

[22] University and University Hospital of Antwerp, Antwerp, Belgium.

[23] Department of Medical Genetics, University of Antwerp, Antwerp 2000, Belgium.

[24] Human Development and Health, Faculty of Medicine, University of Southampton, UK.

[25] Wessex Clinical Genetics Service, Princess Anne Hospital, Southampton, UK.

[26] Department of Clinical Genetics, Erasmus MC University Medical Center, Rotterdam 3015 GD, the Netherlands.

[27] HB Wells Center for Pediatric Research, Indiana University School of Medicine, Indianapolis, IN 46202, USA.

[28] Baylor Genetics, Houston, TX, 77021, USA.

[29] Geneinfo (genetic counseling), Niš, Serbia.

[30] Department of Biochemistry and Biophysics, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA.

[#]These authors contributed equally.

*To whom correspondence should be addressed.

Gholson J. Lyon

Department of Human Genetics

New York State Institute for Basic Research in Developmental Disabilities (IBR)

Staten Island, New York 10314, USA

(Phone): 718-494-5388

(Fax) 718 494-1026

(email) gholsonjlyon@gmail.com

Abstract

N-alpha-acetylation is one of the most common co-translational protein modifications in humans and is essential for normal cell function. *NAA10* encodes for the enzyme NAA10, which is the catalytic subunit in the N-terminal acetyltransferase A (NatA) complex. The auxiliary and regulatory subunits of the NatA complex are NAA15 and HYPK, respectively. Through a genotype-first approach with exome sequencing, we identified and phenotypically characterized 30 individuals from 30 unrelated families with 17 different *de novo* or inherited, dominantly acting missense variants in *NAA10* or *NAA15*. Clinical features of affected individuals include variable levels of intellectual disability (ID), delayed speech and motor milestones, and autism spectrum disorder (ASD). Additionally, some subjects present with mild craniofacial dysmorphology, congenital cardiac anomalies and seizures. One of the individuals is an 11-year-old boy with a frameshift variant in exon 7 of *NAA10*, who presents most notably with microphthalmia, which confirms a prior finding with a single family with Lenz microphthalmia syndrome. Biochemical analyses of variants as part of the human NatA complex, as well as enzymatic analyses with and without the HYPK regulatory subunit, help to explain some of the phenotypic differences seen among the different variants.

Introduction

N-alpha-acetylation is a common co-translational protein modification that is essential for normal cell function in humans. *NAA10* encodes for the enzyme NAA10, which acts as the

catalytic subunit of NatA for the co-translational N-acetylation of proteins with Ser, Ala, Thr, Gly, and Val N-termini, in addition to other functions (1, 2). *NAA10*-related syndrome (3) is an X-linked condition with a broad spectrum of findings ranging from a severe phenotype in males with p.Ser37Pro in NAA10, originally described as Ogden syndrome (4), to the milder *NAA10*-related intellectual disability (ID) found with different variants in both males and females (5–12). There is also one previously reported family with Lenz microphthalmia syndrome (LMS) with a mutation in the intron 7 splice donor site (c.471+2T>A) of *NAA10* (13). Although postnatal developmental impairments and ID may be the presenting feature (and in some cases the only finding), many individuals exhibit additional differences including *in utero* instigated cardiovascular, growth, and dysmorphic findings, each of which vary in type and severity; therefore, this set of disorders has substantial phenotypic variability, and as such, should be referred to more broadly as *NAA10*-related syndrome (3). The auxiliary subunit of the NatA complex, NAA15, is responsible for the induction of conformational changes in to NAA10, including active site rearrangement for canonical NatA complex substrate-binding specificity (14). The Huntington Interacting Protein HYPK regulatory subunit inhibits intrinsic NatA activity and is proposed to modulate cognate NatA activity (15). We recently identified and phenotypically characterized 38 individuals from 33 unrelated families with 25 different *de novo* or inherited, dominantly acting likely gene disrupting (LGD) variants in *NAA15 (16)*. Clinical features of affected individuals with LGD variants in *NAA15* included variable levels of ID, delayed speech and motor milestones, and autism spectrum disorder (ASD). Additionally, mild craniofacial dysmorphology, congenital cardiac anomalies, and seizures are present in some subjects. In the present study, we have collected phenotypic information on individuals with missense variants in *NAA10* and *NAA15*, and we report herein 23 new individuals with *NAA10*-

related syndrome, while also reporting 7 different missense variants in *NAA15* that are likely associated with various neurodevelopmental phenotypes. To complement these findings and provide a survey of both the previously and newly reported variants, we have also conducted biochemical analyses of some of these variants, as part of the human NatA complex, as well as enzymatic studies with and without the HYPK regulatory protein (15, 17, 18). These functional results help to explain some of the wide-ranging phenotypic differences observed among the different variants

Results

NAA10 and *NAA15 variants*

We ascertained 22 individuals from 22 unrelated families with 9 different missense variants in *NAA10,* along with one other family with one male with a frameshift variant in *NAA10.* From these 23 families with variants in *NAA10,* 19 of these were *de novo,* 2 were maternally inherited and the remaining 2 had an unknown inheritance. None of the variants in *NAA10* were reported in public control databases such as gnomAD. **Figure 1** shows the location of these variants in *NAA10*. 7 individuals from 7 unrelated families with missense variants in *NAA15* were ascertained. In the 7 individuals with *NAA15* variants, 4 of these were *de novo,* one was maternally inherited, one was paternally inherited, and one had an unknown inheritance pattern. For *NAA15,* 5 of the variants were not present in public control databases such as gnomAD, whereas the c.1348A>G p.(Lys450Glu) and c.1424C>T p.(Ala475Val) variants were present only once and twice, respectively, in gnomAD. Phenotypic information as well as the variant inheritance are not available on these individuals in ExAC or gnomAD. Information regarding these *NAA10* or *NAA15* variants is given in **Table 1** and **Table S1**.

Clinical Features

Although each variant will be discussed below and in the Supplementary Information in greater detail, most individuals with variants involving *NAA10* or *NAA15* have variable degrees of neurodevelopmental disabilities, including impaired motor abilities (HP:0001270), ID (HP:0001249), impaired verbal abilities (HP:0000750) and ASD (HP:0000729) (**Table 1**, **Table S1**). The males with variants in *NAA10* are predictably much more severely affected, due to having only one X-chromosome, and some of these individuals died in infancy. Many subjects have impaired motor function, including fine motor difficulties, abnormality of movement, motor delay, and hypotonia. Various levels of ID are reported in almost all study subjects with available data, including mild, moderate or severe ID, and learning difficulties with or without behavioral issues (**Table 1**, **Table 2,** and **Table S1**). Most affected individuals have verbal issues including complete absence of speech, delayed language development, required use of sign language, or other speech difficulties. The common and less common features in the cohort are summarized in **Table 2**. Most subjects also present with either ASD and/or other behavioral challenges. A recognizable, regular pattern of dysmorphic facial features was not appreciated amongst the cases. That being said, commonly seen traits include thicker eyebrows and broad philtra (**Figure 2**). The birth weight was low (≤ 5th percentile) in a few individuals, and it was noted that some individuals failed to grow, so that their weights fell below the 5th percentile over time (**Table S1**).

Clinical presentation of individuals with variants in *NAA10*

One family presented with a boy (Individual 1) with a c.29A>G p.(Asp10Gly) variant who died in the first six months of life (**Figure 2**). This boy had agenesis and hypoplasia of the corpus callosum, ventriculomegaly, delayed motor development, muscular hypotonia, ptosis, hearing loss, hypertension, right-sided ventricular hypertrophy due to primary pulmonary hypertension, with eventual right-sided heart failure and death, and dysmorphic features (broad nose, broad forehead, long philtrum). This boy's facial features were remarkably similar to the boys with Ogden syndrome, which was originally named for two families with boys with the c.109T>C p.(Ser37Pro) variant (4, 19).

A 3-year-old girl (Individual 2) was discovered to have a c.32T>G p.(Leu11Arg) variant (see **Figure 2**). Hydrocephalus developed in part due to a large arachnoid cyst between the cerebellum and the occipital area, leading to increased intracranial pressure and requiring ventriculo-peritoneal drainage at the age of 2 months. Ventriculomegaly was already present at ultrasound in the 35th gestational week (GW); she was born in the 39th GW with a normal head circumference (HC) of 33 cm. After drainage, the brain MRI scan showed hypoplasia of the corpus callosum, hyperplasia of the choroid plexus and partial agenesis of the septum pellucidum. At the ages 2.7 and 3 years respectively, her head circumference measured at -1.4 SD and her height at -1.8 SD. She presented a mild pyramidal syndrome, but walked at around 3 years of age, has strabismus, and has no speech development at the age of 4 years. She experienced feedings difficulties. Renal ultrasound and cardiology screening, including ECG and echocardiography were normal.

One of the males (Individual 3) was determined to have a c.215T>C p.(Ile72Thr) variant, and this male was previously reported (as Patient II-1) (11). His picture is shown in **Figure 2**. He had some coarse facial features with an abnormally shaped head with a small chin, high arch palate, and prominent musculature. More information can be found in **Supplementary Information**. Most notably, he had a history of cardiomyopathy, tachycardia, and a prolonged QT interval (493 msec was last reported QT interval, and it was never above 500 msec). He died suddenly at the age of 3 years in January 2018 due to cardiopulmonary issues, likely due to complications from the cardiomyopathy. It is uncertain however if there was an arrhythmia event that preceded this decompensation. An autopsy was not performed. The mother, who is a carrier of the variant, reported that she graduated college and was gainfully employed for 12 years thereafter. She had test-related anxiety during schooling and did receive math tutoring, but there is no evidence for ID or any learning disability and there was never any formal IQ testing.

We obtained clinical information on ten females (Individuals 4-14) with the c.247C>T p.(Arg83Cys) variant, which was *de novo* in all of them. Facial photographs are presented in **Figure 2** for those with consent to publish photographs. The clinical presentation of these girls is similar to what has been presented with other children with this variant (5).

We identified three individuals with *de novo* c.259G>T p.(Ala87Ser) variants. The first is a 14-year-old girl (Individual 15) (see **Figure 2**). She was born after an unremarkable pregnancy and a normal delivery. She is diagnosed with global developmental delay, epilepsy with tonic clonic seizures, attention deficit hyperactivity disorder (ADHD), ASD, fine motor delay, bilateral hearing impairment, absent speech, and optic nerve hypoplasia. In terms of facial

dysmorphology, she has a thin vermillion, microcephaly, malar flattening, and a long philtrum. She has normal cardiac function and a normal QTC interval. Despite being on medication, the seizures still occur daily.

The second individual is a 2.5-year-old female (Individual 16) also with a *de novo* heterozygous c.259G>T p.(Ala87Ser) variant. She is diagnosed with global developmental delay, hypotonia, relative microcephaly, mild dysmorphic features and has significantly elevated alkaline phosphatase levels. She is essentially non-verbal but can say “da-da”. At age 1, she could not crawl or sit up independently. At age 2, she could crawl, pull to stand, and cruise along furniture, but she is not able to stand or walk independently. A brain MRI showed hypoplasia of corpus callosum, and a mild myelination defect. The child has a happy disposition and readily smiles. She receives physical therapy and speech therapy.

The third recurrent variant is in a 14 year-old female (Individual 17) with a *de novo* heterozygous c.259G>T p.(Ala87Ser) variant. She has severe global developmental disorder. Her first symptoms occurred in the 5th week of life with muscle hypotonia and difficulty swallowing. At age 4 years, free walking with a dystonic broad-based-gait was reported. She was also diagnosed with urinary and fecal incontinence, has no expressive speech, and has severe scoliosis. She had a dystonic movement disorder with a degenerative course due to three dystonic crisis at the age of 8.5 years, 10 years and 12 years, respectively, which worsened with each crisis: at the age of 12 years she developed a myoclonus epilepsy with generalized sharp waves noted on the EEG, which was treated with valproic acid and topiramate. Her general condition worsened; her behavior included biting, hair pulling, and vomiting. After another three

months, she developed a life-threatening status dystonicus with rhabdomyolysis and fever, which included profuse sweating. Neuro MRI reported subcortical hyperintensities in the white matter and basal ganglia, some of them were reversible, and some left a defect/cyst. At age of 14 years, operative spinal fusion was performed without postoperative complications. She is in a good clinical status now, has no pain (medicated with gabapentin), no vomiting, and acceptable dystonic movements. She is now wheelchair bound.

We also report a 10-year-old girl (Individual 18) with a *de novo* c.311C>A p.(Ala104Asp) variant (see **Figure 2**). She was born full-term from a normal pregnancy. She is diagnosed with global developmental delay, growth hormone deficiency, sensory processing disorder, ADHD (combined type), mixed receptive and expressive language disorder, hypotonia, fine motor delay, short stature, anxiety, astigmatism, anisometropia conjunctivae and sleep disorder. Her anxiety manifests with public avoidance, skin picking, and stereotypies. Her clinical history was also significant for hip dysplasia and she wore a pelvic harness for the first six weeks of life. She has no history of seizures. Cognitive testing reports ID, although testing is complicated by her ADHD. Additional information is provided in the Supplementary Information.

We identified two individuals with a recurrent c.361C>G p.(Leu121Val) variant. The first is a 6-year-old girl (Individual 19) with a *de novo* c.361C>G p.(Leu121Val) variant (see **Figure 2**). Growth cessation occurred at week 37 gestational age and she was born at 39.4 weeks via an emergent C-section. Her birth weight was 2.5 kg. Her head circumference was under 20th percentile until she was 3 years old, and with growth is now is just under 50th percentile. She

was diagnosed with hip dysplasia at 9 months old and global developmental delay at 18 months old. She is non-verbal and has severe ID. She has mixed muscle tone. She did not start walking until she was 2 years of age. She was diagnosed with severe ASD at 3 years of age, lacks eye contact, becomes easily anxious, toe walks, and engages in self-stimulatory behavior. A Neuro MRI performed at 2.5 years was normal. There are no cardiovascular issues and no sign of seizures. She is happy but has very little patience, challenging behaviors, and struggles with sensory issues in new and loud environments. She has no feeding issues; however, she does not have the fine motor skills to feed herself. She cannot run (she skips instead) and cannot coordinate to use a bicycle. She has severe sleep issues. As an infant she had silent reflux which resolved. She has normal weight and stature for her age.

The second individual with the c.361C>G p.(Leu121Val) variant is a 14-year-old female (individual 20). She was first evaluated at 20 months for speech delay and possible hearing loss. She has significant receptive and expressive language delay, anxiety, avoidance behaviors, mild dystonia, fine and gross motor delays, and borderline non-verbal abilities with adaptive abilities in the mildly delayed range. She is diagnosed with mild ASD. At age 12 she was diagnosed with moderate to profound ID. At age 13 years she was identified as having an NAA10, c.361C>G p.(Leu121Val) mutation and a duplication on chromosome 7. She does not have feeding issues and eats independently. She attends school with the assistance of an aide. She receives support services for speech, hearing and physical therapies. Medication includes sertraline for anxiety. According to her mother the girl sweats profusely. The family history includes two older siblings with speech articulation issues. The mother is a college graduate who teaches school.

We report a 1.5-year-old female (Individual 21) with a *de novo* c.384T>G p.(Phe128Leu) variant. There is no pre- or perinatal history available. Her mother states early feeding problems were alleviated with the insertion of a gastric tube at two months of age. The child has gastric reflux and is successfully medicated with ranitidine. The mother states that the child's head growth such as head circumference and weight is less as compared to other girls the same age. The child has cortical vision impairment and does not make good eye contact. The child has delayed motor development, chorea, overriding toes, and a single palmar crease. Neuro MRI exam reported bilateral pyramidal characteristics with deviations in the basal ganglia. The mother states that the child can roll from side to side if she is lying on her back. Hand usage is limited but she actively moves her legs.

We report a 8-year-old girl (Individual 22) with a *de novo* c.440T>C p.(Met147Thr) variant (see **Figure 2**) who has developmental delay, coordination issues, sensory processing disorder, and self-stimulatory behaviors, but she is very social and there is no other evidence of ASD. Following intense speech therapy intervention, she now speaks a few words (~12). She can show testing in the near age-appropriate reading comprehension and has an above-age-level math ability when she can escape from her major sensory and behavioral impairments. Neuroimaging reports a thinning corpus callosum. She has microcephaly, stigmatism, cortical visual impairment, acne, body odor, adrenarche, and is a light sleeper (so no hypersomnolence). There are no cardiac issues.

An 11 year-old boy with a maternally inherited frameshift variant c.455_458delCTCA p.(Thr152fs) was identified (Individual 23, **Figure 3**). This variant is not present in gnomAD.

His clinical features include agenesis of corpus callosum, craniosynostosis, severe ID, severe global developmental delay, growth delay, generalized hypotonia, microcornea and microphthalmia, scoliosis, pectus excavatum, equinovarus, craniosynostosis (corrected by surgery), and an atrial septal defect, with some rotation of the heart in the chest. He is severely underweight, at 17 kg (<1st %tile), with height of 140cm (~25th %tile). He had hypospadias, which was surgically corrected, and also has syndactyly (webbing) on two digits (2nd and 3rd digits) on his feet with webbing mildly present on two digits (3rd and 4th fingers). Behaviorally, he can be stubborn and also aggressive at times toward his mother. There are no issues with chewing and swallowing, but he has a very low appetite and often refuses to eat. He has no speech, does not sleep well, and is not toilet-trained. He is immobile, as per parent report. The mother, who is a maternal carrier, finished high school and three years of college. She characterizes herself as an "average student", who did not finish college after the birth of her child. She subsequently had two daughters (ages 6 and 9), who have not yet been tested for inheritance of the variant, but appear to be developing normally. The six-year-old daughter had a ductus arteriosus and an atrial septal defect at birth, but both conditions were surgically corrected in the first few months of her life. The mother developed multiple sclerosis after the second childbirth but does not have any other health issues.

Clinical presentation of individuals with variants in *NAA15*

Individual 1 has a c.334G>A p.(Asp112Asn) variant, with an unknown inheritance pattern. This boy was 16.9-years old at his last assessment. He was born at 38 weeks gestation via an emergent C-section (due to breech position). Birth weight was 7.9 pounds. The mother's prenatal history is notable for placenta previa and first trimester mild bleeding but no other

concerns. The boy was diagnosed with Asperger's syndrome at 8 years of age, with notable attention problems. He is apparently very intelligent for his age and there is no other history of developmental or language delays. He walked at 12-14 months of age and began using 2-word sentences at 18 months of age. He was toilet-trained at approximately 2 years of age. He was diagnosed with generalized epilepsy and had his first seizure episode at 6 years of age. He has had multiple abnormal EEGs, and his seizures are now well-managed by medication. Neuro MRI results were normal. Height and weight have been somewhat below average but otherwise normal on the growth curves. He has a curved fifth finger on both hands. There is a history of mild fifth finger curving in his mother. He has mild constipation, and there are no other medical concerns.

Individual 2 has a *de novo* c.1014G>T p.(Lys338Asn) variant. The only available clinical information is that this individual has ASD.

Individual 3 is a 10-year-old female with a paternally inherited c.1348A>G p.(Lys450Glu) variant. She was born at 40 weeks via vaginal delivery with placental detachment. She has hypertelorism and a broad nasal bridge as well as behavioral problems including: selective mutism and pervasive developmental disorder (PDD).

Individual 4 was age 12-year-old at his last visit, and he has a *de novo* c.1413A>C p.(Glu471Asp) variant. The child was diagnosed with ADHD and there was concern of unclear speech. The patient had epileptic discharges over the left temporal region and occasionally the frontal central. MRI indicated mild, deep white matter, hyperintense signal changes.

Individual 5 is an 11-year-old female with a maternally inherited c.1424C>T p.(Ala475Val) variant. She was diagnosed with ASD (confirmed with ADOS, ADI, and clinical judgment using DSM-IV criteria). Her cognitive abilities fall into the extremely low range (Verbal IQ = 36). Her parents report that her adaptive abilities were impaired (Adaptive composite = 41). Abnormalities were first noted in her development at 12 months of age. She first used single words at 66 months and has not developed phrase speech.

Individual 6 was age 6.2 years old at her last assessment, and she has a *de novo* c.1450T>C p.(Cys484Arg) variant. At her initial presentation, she was 2 years and 8 months. At that age, she weighed 11 kg., and was 80 cm. tall, with a head circumference of 43 cm. She had global development delay which improved. Developmentally, she sat at 10-11 months and walked at 2 years. Cognitively, she developed normally. She has facial dysmorphisms, abnormal dentition, broad philtrum, high palate, hypertelorism, microcephaly, persistent open anterior fontanelle, and tented upper lip vermillion (**Figure 4**). She is short for her age. She also had *café-au-lait* macules on each leg of the lower limbs and hypermobility of the digits. She also has a *de novo* c.1837C>T, p.(Arg613Cys) variant in *TCF12*, a gene previously associated with an autosomal dominant non-syndromic coronal craniosynostosis (20).

Individual 7 was 8.6-year-old at the last known visit, with a *de novo* c.2441T>C p.(Leu814Pro) variant. She has global development delay, language delay and fine motor difficulties. Developmentally, she walked at 26 months and spoke her first words at 18 months. Cognitively, she has mild to moderate ID. WISC-testing reported scores of: reasoning 67, visuospatial 57, speed 60. Vineland II scores were communication: 30, daily life 67, socialization

82, and motor skills 60. Since 6-months-old she has had six febrile seizures, and since 6.5 years of age, she has had nonfebrile seizures (absence), which were treated with ethosuximide. She has a prominent forehead and nasal tip, triangular nose, and mild retrognathism (**Figure 4**).

Biochemical Analysis

Thus far, mutant NatA *in vitro* acetyltransferase activity has been measured with either *E. coli*-expressed recombinant hNAA10 protein solubilized using a maltose binding protein (MBP)-tag (or other affinity tag) as a monomer (4–6, 8, 21) or also in the context of an immunoprecipitated NatA complex (11, 12, 21). Furthermore, studies have been conducted using an *S. cerevisiae* model system (22, 23). Protein stability of some variants has been measured in HeLa cells with cycloheximide-chase experiments (5, 8, 12). Herein, we have expressed and purified recombinant NatA complex from insect Sf9 cells, with this complex being comprised of human NAA10 and NAA15 (15, 18). We introduced and tested many of the published and novel variants, which are shown on the recent crystal structure in **Figure 5**. The acetyltransferase activities of these variants, in the presence and absence of the regulatory binding partner, HYPK, are shown in **Figure 6**, along with the thermal stability of the NatA complex with and without these various variants (associated *p*-values in **Tables S2 and S3,** respectively).

Functional Characterization of Human NatA Missense Variants of NAA10.

The following mutants (D10G, L11R, S37P, Y43S, I72T, R83C, A104D, and M147T) were soluble and purified to homogeneity. A few of the variants (A87S, L121V, and F128L) were only characterized clinically at a much later date and were thus not assessed biochemically

for this study. We found that, except for R83C and M147T, alterations in the catalytic subunit caused a significant decrease in enzymatic activity (**Figure 6A and Table S2**). Particularly, the activity of residues that make important contributions to the integrity of the fold of the catalytic subunit resulted in the largest decrease (D10G, S37P, and A104D). These protein changes likely alter numerous interactions including the loss of hydrogen bond interactions and the perturbation of hydrophobic pockets (**Figure 5**). Similarly, L11R, Y43S, and I72T likely promote changes in the hydrophobic surfaces that they occupy, but they do not promote larger-scale structural changes that would impede N_t-acetyltransferase activity. For example, while bulky, L11R can occupy other rotameric positions, allowing the accommodation of its long aliphatic chain so that it may find a favorable orientation. M147T, on the other hand, does not appear to have any effect on NatA catalytic activity. By contrast, R83C appears to enhance NatA activity. It may promote the formation of a disulfide bond with NAA15-C322, which could allow for improved positioning of acetyl-CoA for catalysis. Interestingly, previous studies with monomeric human NAA10 have demonstrated that $NAA10_{R83C}$ has a diminished catalytic capacity (9), which is consistent with our findings indicating that in the absence of NAA15, $NAA10_{R83C}$ would no longer benefit from the catalytic stabilization provided by $NAA10_{R83C}$-$NAA15_{C322}$ disulfide bond formation.

We were surprised to find that when we supplemented the NatA mutant reactions with HYPK and re-evaluated their enzymatic activities, HYPK-mediated inhibition of wild-type NatA activity was potentiated, decreased or nullified depending on the specific variant (**Figure 6B** and **Table S2**). M147T, which had no effect in the heterodimeric state, significantly decreased NatA activity upon addition of HYPK. D10G, Y43S, I72T, and A104D also appeared to sensitize NatA to HYPK-mediated inhibition while L11R, S37P, and R83C did not have a significant

effect. In WT, the NAA15 α26-44 helical stalk serves as the primary point of tight-binding for the NAA15-HYPK interaction. It is possible that L11R and S37P are stabilized in the HYPK-bound complex relative to other mutants due to packing-induced stabilization between the NAA15 helical stalk and either NAA10 α1 (L11R) or NAA10 α2 (S37P) helices. In contrast, HYPK binding nullified the enhancing effects of R83C due to the HYPK-induced conformational changes in the acetyl-CoA binding pocket.

Next, we compared the thermostability of heterodimeric NatA complexes with mutant heterodimeric complexes in the absence and presence of its cofactor, acetyl-CoA, using differential scanning fluorimetry (DSF) (**Figure 6C and Table S3**). We found that all of the missense changes, except I72T, R83C, and M147T, were destabilized in the absence of co-factor when compared to WT. By contrast, all changes, except I72T, were destabilized in the presence of acetyl-CoA with respect to WT. These results indicate that the missense changes are overall destabilizing for the heterodimeric complex, where changes that destabilize the GNAT fold – such as S37P, D10G, L11R, Y43S, and A104D – reduce the stability of the complex in the absence as well as the presence of acetyl-CoA. On the other hand, I72T may play a minor role in stabilizing the apo-state of the mutant complex where the smaller side chain promotes the compaction of the hydrophobic pocket between the NAA10 β4-strand and α3 helix. The stabilization of the R83C mutant complex likely is a result of the formation of a disulfide bond with NAA15-C322. By contrast, the R83C complex is likely no longer able to fully benefit from the acetyl-CoA stabilizing effects due to the loss of electrostatic interaction between the positively charged Arg83 side chain and the negatively charged phosphodiester acetyl-CoA backbone. Similarly, M147T likely diminishes the packing between the α4 helix and β7-strand, diminishing the stabilizing hydrophobic packing effects of acetyl-CoA binding.

Functional Characterization of Human NatA Missense Variants of NAA15.

Except for K450E, all the variants tested (D112N, K338N, A475V, C484R, and L814P) were soluble and were purified to homogeneity. One variant (E471D) was only characterized clinically at a much later date and was thus not assessed biochemically for this study. We found that K450E appeared to break the NatA heterodimeric complex, causing $NAA15_{K450E}$ to elute throughout the S200 column rather than as a single peak (**Figure 7**). Since K450 makes important interactions with the structural inositol hexaphosphate (IP_6) molecule, we reasoned that K450E would result in the loss of a hydrogen bond as well as introduce a charge-charge repulsion between the mutant residue and the negatively IP_6 phosphate moieties. Consistent with this possibility, we found that we could rescue the NAA10-NAA15 association and enzymatic activity when the complex was prepared with 15 μM IP_6 , as documented in (24), suggesting that the K_D for the IP_6-NatA complex interaction may be decreased in the K450E variant (**Figure 7** and **Table S4**). Consistent with the overall destabilization of the complex, due to an enduring charge-charge repulsion and the loss of a hydrogen bond interaction, we observed that the additional IP_6 did not improve the melting temperature of the $NAA15_{K450E}$ mutant complex (**Figure 7** and **Table S5**).

To understand how the other NAA15 missense changes impacted NatA complex activity and thermostability, we characterized the overall effect of these variants on NatA activity – in the absence and presence of HYPK – and performed DSF assays in the absence and presence of acetyl-CoA. We found that D112N and K338N both did not significantly impact N_t-acetyltransferase activity (**Figure 6A** and **6B, Tables S2** and **S3)**, likely due to the role and position of D112 as a surface residue and K338N as a residue that is distantly located from the

NAA10-NAA15 interface (**Figure 5B**). However, D112N and K338N did appear to stabilize the complex with respect to WT, in the absence of acetyl-CoA, which was also the case for all of the NAA15 missense changes (**Figure 6C**). K338N may serve to form new hydrogen bond interactions, which may stabilize the complex. A475V and C484R had reduced N_t-acetyltransferase activity in the absence of HYPK, and, interestingly, L814P had increased N_t-acetyltransferase activity in the presence of HYPK, though it maintained its ability to interact with HYPK (**Figure 6A, 6B and S1**). As a heterodimeric complex, C484R results in a significant reduction in NatA activity due the inherent steric bulk of the Arg side chain. In the case of A475V, the Val side chain, while larger than the wild-type Ala residue, likely contributes just enough to a small shift in the NAA15 α26 helix packing against the NAA10 α1 and α2 helices, shifting the helices so that the complex catalyzes activity less efficiently. These mutant-induced steric shifts, however, are nullified upon HYPK-binding, likely due to the stabilizing effect of $HYPK_{UBA}$ (C-terminal) binding to the NAA15 metazoan-specific C-terminus (**Figure 6A, 6B**).

A475V, C484R, and L814P were found to all likely impact the stability of the NAA10-NAA15 interface through the stalk of helices (α26-45), depending on whether or not acetyl-CoA is bound to the mutant complex (**Figure 6C**). Although A475V and C484R are located in the same area of the NAA15 helical stalk, these missense changes have different effects on the thermostability of the NatA complex (**Figure 6C**). This difference likely arises from the positioning and identity of the variants: they are on opposite ends of the same α26 helix and are steric opposites. In the case of C484R, the bulky Arg variant located near the NAA10 α1/α2 helices potentially pushes away NAA15 α26, resulting in diminished NAA10-NAA15 contact area. A475V likely minimally perturbs NAA15 α26 so that it may pack more closely with the NAA10 α1/α2 helices. A larger residue in this position, however, would likely lead to a similar

effect that is observed with C484R. In contrast, L814P has no effect on NatA complex activity unless HYPK is bound (**Figure 6A,6B**), likely due to the steric restraints generated by a Pro substitution, reducing HYPK binding integrity and, therefore, the inhibitory capacity of the interaction. Unsurprisingly the L814P mutant reduces the thermostability of the complex, regardless of the binding of acetyl-CoA (**Figure 6C**). This data is consistent with a model where the L814P variant results in a dramatic reduction in the dynamics of the NAA15 α26-45 helical stalk which is necessary for the clamp-like binding of HYPK binding as well its intrinsic NatA inhibitory activity.

Discussion

The amino-acid changes D10G, S37P and A104D in NAA10 were shown to have very low NatA acetyltransferase activity in the absence of HYPK (**Figure 6**). The variants L11R, Y43S, and I72T also have activity that is lower than wild-type in the absence of HYPK, but not nearly as low as the D10G, S37P and A104D changes. The overall activity of WT NatA is substantially decreased in the presence of HYPK (15). However, in the presence of HYPK, the variants that are significantly less active than WT are: D10G, Y43S, I72T, A104D, and M147T, although the overall activity of the WT enzyme is itself only at ~10% of the activity seen without HYPK (see y-axis) and thus the signal to noise ratio is also lessened. The results in the absence of HYPK are more consistent with the observation that males with the D10G variant (reported herein) and S37P variants (4) are very severely affected (and have very similar facial features), whereas males with the Y43S variant can live to adulthood (8). Furthermore, boys with the I72T variant are also not as dysmorphic (see **Figure 2**) and the two brothers in one family were 5.5 and 8.5 years old, respectively, at the time of that report (11). It is notable that the individual in

the second family with the I72T variant (**Individual 3** in **Table 1**) died suddenly in January 2018 due to cardiopulmonary issues, likely due to complications from the cardiomyopathy. It is uncertain, however, if there was an arrhythmia event that preceded this decompensation, and an autopsy was not performed. The difference in severity between the S37P and the I72T variant is further demonstrated in **Figure 8, Table 3** and **Table S6)**, in which the acetyltransferase activity, in addition to the thermostability (**Figure 6**), of the S37P variant is considerably lower than the I72T variant. To date, we are not aware of any known males with A104D or L11R variants, but the prediction based on these functional studies is that the A104D variant would be more severe than the L11R variant, although both variants would have a major impact on the quality of life. It is interesting to note that the M147T variant in NAA10 only has decreased NatA acetyltransferase activity in the context of the NatA/HYPK complex (**Figure 6B** vs. **6A)**. In conjunction with the thermostability data showing only decreased stability in the presence of acetyl-CoA (**Figure 6C**), this suggests that the reduction in NatA/HYPK activity arises from a diminished ability to bind to the acetyl-CoA cofactor. HYPK has been demonstrated to improve the acetyl-CoA K_M of wild-type NatA through a shift in the acetyl-CoA binding pocket (15). Due to the inherent difference in size between Met and Thr, mutation of Met147 to Thr would reduce the effect of HYPK binding, thus impacting the overall activity and stability of NatA in the presence of HYPK and acetyl-CoA, respectively. This further demonstrates the utility and superiority of conducting these *in vitro* assays with NatA and NatA/HYPK as opposed to with Naa10 alone.

The phenotype of a female heterozygous for an *NAA10* disease-contributory variant can range from asymptomatic to having some of the same clinical findings as affected males, including a range of ID and cardiac findings, depending on specific variants and presumed

favorable vs. non-favorable X-chromosome inactivation (XCI). Skewed X-chromosome inactivation in B cells in female carriers of different *NAA10* disease-associated variants has been observed, but the direction of skewing was not demonstrated in many cases, making it very difficult to speculate about the impact of X-chromosome skewing on the phenotypic expression in those individuals. In addition, it is possible that X-chromosome skewing can occur in different ratios in different tissues, particularly if expression of the protein from the mutated X-chromosome confers either a growth advantage or disadvantage in specific tissues. Although one group did report a significant correlation ($r(s) = 0.51$, $p = 0.035$) between XCI values for blood and/or spleen and brain tissue (25), the sample size was small and there was substantial scatter in their data. It is certainly the case that some female carriers of these variants are much less affected (if at all) with cognitive disabilities. The carrier mothers in the Ogden syndrome family with c.109T>C p.(Ser37Pro) were never diagnosed with ID or any other serious medical condition. It was previously shown that their X-chromosome inactivation was nearly completely skewed toward the wild type allele in blood (21), and it is possible that this might have been true in other tissues of their bodies as well. Although it was reported that the carrier mother with c.128A>C p.(Tyr43Ser) might have had a “learning disability” or “learning problems” when compared to her siblings (8), the mother reports that she finished her formal education and was gainfully employed, and there is no documented evidence for ID or learning disability, and there was never any formal IQ testing. The maternal carrier in Family 3 with c.215T>C p.(Ile72Thr) completed college and was gainfully employed. She did have test-related anxiety during schooling and did receive math tutoring, but she did not have any formal IQ testing, nor was she diagnosed with an intellectual or learning disability. One major problem in the field is that many female carriers have not had systematic IQ testing, so a quantitatively reliable way to compare

and contrast them does not currently exist. Considering this, we recommend that future studies should include systematic IQ testing of female carriers.

It is surprising that the R83C variant has increased (not decreased) NatA acetyltransferase activity in the absence of HYPK, and it does not exhibit any significant difference in NatA acetyltransferase activity in the presence of HYPK (**Figure 6A, 6B**). This variant also has only slightly decreased thermal stability compared to the WT NatA complex, and it is not nearly as destabilized as some of the other NAA10 variants (D10G, L11R, S37P) (see **Figure 6C**). These results differ from the decreased activity that was reported when testing acetyltransferase activity of monomeric NAA10 with peptides starting with EEEI-, DDDI-, and SESS- (5). There is only one reported male hemizygous for the R83C variant who died in the first week of life with supraventricular tachycardia, pulmonary hypertrophy, mild ventricular hypertrophy, and diffuse small cortical kidney cysts. Brain imaging was not reported, so it is unknown whether he had any hydrocephaly (5). The R83C variant may promote the formation of a disulfide bond with NAA15 C322. In the absence of acetyl-CoA, this change can promote a minor stabilization of the NatA mutant complex. However, the binding of acetyl-CoA to the mutant NatA complex does not impart the same level of stabilization as in WT due to the loss of the electrostatic interaction with the acetyl-CoA phosphodiester backbone in addition to the restricted conformation. Overall, when considering the activities of the monomeric NAA10 versus dimeric NatA complex, the monomeric mutant NAA10 is less active for S37P and R83C. However, in the case of the heterodimeric complex NatA, S37P exhibits reduced activity while R83C exhibits an enhanced activity (likely afforded by the disulfide bond formation with NAA15). This suggests that the phenotype of individuals with the R83C may manifest through a different mechanism of action than the other missense variants, which seem to be entirely hypomorphic, at least as related to

canonical acetyltransferase catalytic activity. There is a wide spectrum among individuals with *NAA10*-related disorder with regard to number of findings and severity of phenotype. Males with *NAA10*-related syndrome may have symptoms at different ages; those with arrhythmias or other serious cardiac conditions may be embryonic lethal, while others are apparent at birth with cardiac concerns, hypotonia and dysmorphic features, and others come to attention later with relatively nonspecific developmental/intellectual and growth impairments. The current results reveal that the various variants have different effects on the overall NatA acetyltransferase activity and complex stability, which can also certainly have a major effect on disease outcome.

Prior studies report that NAA10 I72T is destabilized, while binding to NAA15 most likely is intact (11). That study concluded that the NatA activity of NAA10 I72T appeared normal while its monomeric activity was decreased (11). Our current results contradict this and suggest that the NatA activity of NAA10 I72T is decreased, in the presence and absence of HYPK, but that the stability of the NatA complex was unaffected. This result seems to be more consistent with the phenotypic presentation of the probands with NAA10 I72T variants.

When a variant occurs *de novo* in an affected proband, this is itself a piece of evidence in favor that the variant is likely contributing to the phenotype of interest (as a PS2 in the ACMG criteria) (26), so the *de novo* variants reported herein are classified as Pathogenic, based on the following ACMG criteria: 1) PS2 De novo (both maternity and paternity confirmed) in a patient with the disease and no family history; 2) PS3 Well-established *in vitro* functional studies supportive of a damaging effect on the gene or gene product, 3) PM1 Located in a mutational hot spot and/or critical and well-established functional domain (e.g., active site of an enzyme) without benign variation, 4) PM2 Absent from controls (or at extremely low frequency if recessive) in Exome Sequencing Project, 1000 Genomes Project, or Exome Aggregation

Consortium, and 5) PP2 Missense variant in a gene that has a low rate of benign missense variation (Z=2.41 for *NAA10* and Z=3.81 for *NAA15* in gnomAD v.2.1.1) and in which missense variants are a common mechanism of disease (26).

For those variants presented herein that are inherited from a parent and are thus not *de novo*, additional individuals with the exact same variants should be identified in the future to provide further evidence for their contribution to the phenotypes of interest. For now, we classify these variants as Likely Pathogenic, based on the same ACMG criteria, but excluding the PS2 *de novo* criteria (26). Although all of the variants in *NAA15* were shown to affect either NatA activity or complex stability, the variant D112N has an unknown inheritance pattern, K450E has paternal inheritance, and A475V has maternal inheritance. We note that two of the inherited variants in *NAA15*, K450E and A475V, were also found to be present at a very low frequency in gnomAD (once for K450E and twice for A475V), so the above PM2 criteria cannot be used for those two variants, given that *NAA15* is autosomal, whereas very low-frequency variants in *NAA10* would be eligible, as those are X-linked recessive (26). As such, these two parentally-inherited variants in *NAA15*, K450E and A475V, only meet the ACMG criteria of PS3, PM1, and PP2, which nonetheless still classifies them as Likely Pathogenic, based on "1 Strong (PS1–PS4) AND 1–2 moderate (PM1–PM6)" (26). There is clearly variable expressivity, however, as the father with the K450E variant was never diagnosed formally with any ID or ASD, although there was some question of whether he may or may not be mildly affected. There is no information available for the mother with the A475V variant. This issue of variable expressivity was also found with truncating variants in *NAA15* (16), so it is not surprising to find this also for missense variants; however, we fully acknowledge that the support for pathogenicity is somewhat weak for these two inherited variants. Additional families will need to be identified with these exact

same variants in order to help prove or disprove their disease contribution, andfuture studies of patient-derived cell lines could also help to further prove the pathogenicity of these variants.

The boy (Individual 23) with the maternally inherited frameshift variant c.455_458delCTCA p.(Thr152Argfs*6) in *NAA10* has many phenotypic features that are shared with the previously reported family with Lenz microphthalmia syndrome with a mutation in the intron 7 splice donor site (c.471+2T>A) of *NAA10* (13). This most notably includes the microphthalmia, severe ID, scoliosis, and syndactyly. This variant is not present in gnomAD, and there are only two other truncating variants present in gnomAD in the canonical NAA10 transcript, namely p.Ser228Ter and p.Ser233Ter, with the former being present only one time in a heterozygous female and the latter variant being present three times (one time in a heterozygous female and two times in hemizygous males). The canonical transcript of *NAA10* encodes a protein that is only 235 amino acids in length, so these two truncating variants in gnomAD occur at the very C-terminus of the protein, thus likely having no functional effect, whereas p.(Thr152Argfs*6) removes the C-terminus of NAA10, but leaves the acetyltransferase domain intact (**Figure 1**). It was previously shown that the mutation in the intron 7 splice donor site (c.471+2T>A) generated a very small amount of truncated NAA10 protein, which could have had residual NatA activity, although it was also shown that this protein likely aggregates in the cytoplasm (13). Although some of the individuals with missense changes in NAA10 have milder eye or visual anomalies, including astigmatism, cortical visual impairment, hyperopia, and/or myopia, none of them have microcornea or microphthalmia. Even if any truncated NAA10 protein has NatA activity, the overall level of this activity should be much less due to its much lower expression level (13). The expression level for the frameshift variant c.455_458delCTCA p.(Thr152Argfs*6) is presumably also substantially less, likely due to

nonsense-mediated decay of the messenger RNA, although there are currently no patient-derived cells from this proband available to test this hypothesis.

From a mechanistic perspective, it is worth noting that our identification of a frameshift variant in the C-terminal region of NAA10 could be explained instead by loss of activity of some function associated with the C-terminal region. Although the serine residue at position 209 (Ser209) in NAA10 was shown to be a phosphorylation site of IKK-β, leading to the destabilization of NAA10 and its proteasome-mediated degradation (27), the absence of this IKK-β phosphorylation site in any truncated protein would be predicted to increase, not decrease, the protein levels of the truncated protein. Also, it has been reported that the C-terminus of NAA10 is required for interaction with TSC2, an inhibitor of the mTOR (mammalian target of rapamycin) pathway (28). After forming a complex with TSC2, NAA10 acetylates TSC2, both stabilizing TSC2 and increasing its cellular concentration (28); therefore, NAA10 may play a role in the regulation of the mTOR pathway, which could be disrupted with loss of the C-terminal region.

It was previously shown by quantitative real-time PCR analysis in the fibroblasts of three affected male individuals with LMS that the level of *STRA6* expression was significantly reduced, in comparison to controls, which suggested that these individuals may have retinol uptake deficiencies (13). Several studies had shown that cellular uptake of vitamin A/retinol from its RBP4-bound form is dependent on the membrane receptor protein, STRA6 (29, 30), and it was then demonstrated that these same three individuals (VI-9, VI-10, and VI-11) in the LMS family had deficiencies in retinol uptake (13). These results suggested that *NAA10* may play a role in the retinoic acid signaling pathway and in normal eye development.

In conclusion, we have presented phenotypic information on some new variants in *NAA10* and *NAA15*, including one frameshift variant in *NAA10* associated with microphthalmia, along with additional phenotypic information on some previously published *NAA10* variants. Biochemical analyses have suggested some possible explanations for the phenotypic differences among these variants, although it remains an open question why reduced expression and/or truncation of NAA10 can result in microphthalmia, whereas missense changes have not been shown to be associated with microphthalmia.

*Note. While this manuscript was under review, three families, including 15 affected individuals with syndromic X-linked microphthalmia, were reported in which hemizygous *NAA10* polyadenylation signal (PAS) variants segregated with the disease and were absent from gnomAD. Quantitative PCR and RNAseq showed ~50% reduction of *NAA10* mRNA levels and also abnormal 3′ UTRs in affected individuals (31).

Materials and Methods

Clinical features methodology

Phenotypic information was collected through clinicians and/or directly from families with missense or frameshift variants in *NAA10*, and from families with missense variants in *NAA15*. New families were identified via a world-wide collaboration between multiple institutions, via social media, or through GeneMatcher, a web-based tool for connecting researchers with an interest in the same gene (32). All families had been referred for the investigation of idiopathic developmental delay and ID. Several individuals were identified through the Deciphering Developmental Disorders (DDD) study. The study was performed in

accordance with protocols approved by the institutional review boards of participating institutions. Phenotypic information was obtained from clinical records with varying amounts of available data, ranging from a list of the key clinical features to detailed history and examination findings. Written informed consent was obtained for publication of photographs, and these photographs were reviewed by one medical geneticist (A.R.) to assign dysmorphic features in a systematic manner.

Detailed clinical summaries for each subject are provided in Supplemental File 1. Both pertinent positive and negative features were noted, with phenotypes classified as "pertinent negatives" only if the provided clinical information explicitly stated that a phenotype was denied by the parents, was noted to be absent during physical examinations, or not reported during diagnostic procedures. Clinical features were only reported as pertinent negatives if this was explicitly mentioned in the records, otherwise, these were classified as "unknown" or "not available". The relative prevalence of each phenotype was calculated by dividing the number of individuals positive for the phenotype by the sample size. Percentiles for head circumference, weight and height were calculated using CDC growth charts.

Variant identification and Bioinformatics Methodology:

Variants were identified using exome sequencing primarily through clinical diagnostic testing. The sequencing kits and technology varied based on the different companies involved, and the variants of interest were highlighted in the clinical diagnostic test reports.

Construction of E. coli Expression Vectors

A pET-m41 vector engineered to contain an N-terminally MBP-tagged HYPK was kindly donated to us by Thomas Arnesen (University of Bergen).

Construction of Baculoviruses

A plasmid containing the wild-type (WT) hNatA was generated using a pFastBac dual vector engineered with a C-terminal truncation construct coding for the human $NAA10_{1-160}$ and a non-cleavable 6xHis-tagged full-length NAA15 (866 residues). Individual mutations were introduced using the Stratagene QuikChange protocol: $NAA10_{D10G}$ (GGT), $NAA10_{L11R}$ (CGC), $NAA10_{S37P}$ (CCC), $NAA10_{I72T}$ (ACC), $NAA10_{R83C}$ (TGC), $NAA10_{A104D}$ (GAC), $NAA10_{M147T}$ (ACT), $NAA15_{D112N}$ (AAT), $NAA15_{K338N}$ (AAT), $NAA15_{K450E}$ (GAA), $NAA15_{A475V}$ (GTA), $NAA15_{C484R}$ (AGG), and $NAA15_{L814P}$ (CCC). Mutagenesis of the WT plasmid was performed by BioBasic to generate the $NAA10_{Y43S}$ mutation (TCC). For each of these constructs, a bacmid was generated by transposition into DH10 bac competent E. coli cells using the bac-to-bac system (Invitrogen). *Spodoptera frugiperda* (Sf9) cells cultured in SFM II medium were transfected with the bacmid using cellfectin reagent (Invitrogen). The resulting baculovirus was amplified until reaching a high titer.

Expression and Purification of wild-type and mutant 6xHis-tagged NatA constructs

Sf9 cells were grown to a density of $1x10^6$ cells/ml and infected using the amplified WT $NAA10_{1-160}$/NAA15 baculovirus to an MOI (multiplicity of infection) of ~1-2. The cells were grown at 27°C and harvested 48 hours post-infection. All subsequent purification steps were carried out at 4°C. Cells were isolated by centrifugation and lysed in lysis buffer containing 25 mM Tris, pH 8.0, 500 mM NaCl, 10 mM Imidazole, 10 mM β-ME, 10 µg/ml PMSF, DNase, and

complete, EDTA-free protease inhibitor tablet (Roche). The lysate was clarified by centrifugation and incubated with nickel resin (Thermo Scientific) for 1 hr. before washing the resin with ~125 CV of lysis buffer and then eluted with 10 CV of elution buffer (25 mM Tris, pH 8.0, 500 mM NaCl, 200 mM Imidazole, 10 mM β-ME) by batch elution. Eluted protein was diluted to a final salt concentration of 200 mM NaCl and loaded onto a 5 mL HiTrap SP ion-exchange column (GE Healthcare). The protein was eluted in the same buffer with a salt gradient (200mM-1 M NaCl) over the course of 20 CV. Peak fractions were pooled, concentrated to a volume of 500 µl (100 kDa concentrator), and loaded onto and run on a Superdex 200 Increase 10/300 GL gel filtration column in sizing buffer containing 25 mM HEPES, pH 7.0, 200 mM NaCl, and 1 mM TCEP. Peak fractions were pooled, concentrated to ~1-2 mg/mL, as measured by UV_{280-}, and flash-frozen for storage at -80°C until use. Mutant NatA proteins were prepared as described for the WT NatA with the following exception. Lysis and all purification steps of $NAA10_{1-160}$/$NAA15_{K450E}$ were performed in the presence and absence of 15 µM inositol hexaphosphate (IP_6) (Sigma) and analyzed by radioactive acetyltransferase assay (described below).

Expression and Purification of MBP-tagged HYPK

MBP-tagged HYPK was expressed in Rosetta (DE3) pLysS competent E. coli cells. Cells were grown in LB-media at 37°C to OD_{600-} 0.6-0.7 prior to inducing protein expression with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 18°C for ~16 hrs. All subsequent purification steps were carried out at 4°C. Cells were isolated by centrifugation and lysed in lysis buffer containing 25 mM Tris, pH 8.0, 150 mM NaCl, 10 mM β-mercaptoethanol (β-ME), 10 µg/ml phenylmethanesulfonyl fluoride (PMSF), and DNase (Invitrogen). The lysate was clarified

by centrifugation and incubated with amylose agarose resin (New England Biolabs) for 1 hr. before washing the resin with ≥80 column volumes (CV) of lysis buffer and then eluted with 10 CV of lysis buffer supplemented with 20 mM maltose by batch elution. Eluted full-length and truncated MBP-HYPK constructs were loaded onto a 5 mL HiTrap Q ion-exchange column (GE Healthcare). The protein was eluted in the same buffer with a salt gradient (150 mM – 1 M NaCl) over the course of 20 CV. Peak fractions were pooled and dialyzed overnight into buffer containing 25 mM HEPES, pH 7.0, 200 mM NaCl, and 1 mM Tris(2-carboxyethyl)-phosphine hydrochloride (TCEP). Dialyzed protein was concentrated to ~20-40 mg/mL by UV_{280} (Nanodrop 2000; Thermo Fisher Scientific), and flash-frozen for storage at -80°C until use.

Acetyltransferase Assays

Human NatA acetyltransferase assays, in the presence or absence of HYPK, were carried out in 100 mM HEPES, pH 8.0, 50 mM NaCl 2 mg/ml BSA, where reactions were incubated with 10 nM of 6xHis-tagged human NatA alone (WT or mutant) or mixed with 5 μM MBP-HYPK in a 30 μl reaction volume containing 50 μM each of substrate peptide and [^{14}C]acetyl-CoA (4 mCi/mmol; PerkinElmer Life Sciences) for 12 min at 25°C. The substrate peptide used in the assay corresponds to the first 19 residues of human H4 (Genscript), which was selected because it did not generate a substrate inhibition kinetic profile. $NAA10_{1-160}/NAA15_{K450E}$ purified in the absence (-IP_6) and presence of 15 μM (+IP_6) was utilized in the assay without additional IP_6. In the case of the latter sample, this resulted in a final concentration of ~1.5 μM IP_6 after reaction setup.

To determine steady-state catalytic parameters of WT, $NAA10_{1-160/S37P}$/NAA15, and $NAA10_{1-160/I72T}$/NAA15, a saturating concentration of radiolabeled [^{14}C]-acetyl-CoA (100 μM) was incubated at seven different concentrations of the H4 substrate peptide (ranging from 3.9–250 μM). Additionally, the acetyl-CoA K_m values of WT and $NAA10_{1-160/I72T}$/NAA15 were determined by titration of the acetyl-CoA at eight different concentrations (ranging from 0.73 to 94 μM) in the presence of 350 μM substrate peptide. GraphPad Prism, version 5.01, was used for all data fitting to the Michaelis–Menten equation.

Thermal Stability Assays

Frozen aliquots of WT and mutant NatA proteins (in the absence of HYPK) were thawed and diluted in size exclusion buffer (25 mM HEPES, pH 7.0, 200 mM NaCl, 1 mM TCEP) to a final concentration of ~0.94 μM (~0.13 mg mL^{-1}). 16 μL was added to the selected wells of a MicroAmp Optical 384 well plate (Applied Biosystems) to a final concentration of ~0.75 μM (~0.1 mg mL^{-1}). Sypro Orange (5000x stock, ThermoFisher Scientific) was diluted 1:500 using size exclusion buffer, and 4 μL of that diluted stock was added to each well. The plate was spun down and heated from 20 to 95 °C using a qPCR (ABI 7900 RealTime PCR) with a 2% ramp rate. Fluorescent readings were recorded every 2 min. Following signal normalization, melting curves were generated from these data and analyzed in GraphPad using a Boltzmann sigmoidal analysis where analysis was restricted to boundaries that included the transition of interest. The T_m reported corresponds to the inflection point of the sigmoidal curve, which represents the temperature at which half of the population of the protein is unfolded. The T_m reported corresponds to the inflection point of the sigmoidal curve, which represents the temperature at

which half of the population of the protein is unfolded. The resulting T_m was plotted as a scatter plot of each individual sample using GraphPad Prism. $NAA10_{1-160}$/$NAA15_{K450E}$ purified in the absence (-IP_6) and presence of 15 μM (+IP_6) was utilized in the assay with additional IP_6 to ensure a final concentration of 15 μM (+IP_6) after sample setup.

Pull-Down Assays

6xHis-tagged NatA (WT and L814P mutant) as well as MBP-tagged HYPK proteins were prepared as described above. Free MBP was prepared as described for MBP-HYPK above. The pull-down experiment represented in **Figure 1S** was conducted at 4°C where 2 μM prey (MBP-HYPK protein or free MBP) with 6 μM bait (6xHis-tagged hNatA WT or L814P mutant) were incubated together in sizing buffer 30 min. Proteins were then subjected to pull-down by incubation with amylose agarose resin (70μL slurry, New England BioLabs) for 30 min. Resin was washed with 80 CV of sizing buffer before elution of bound proteins by boiling resin in SDS gel-loading buffer. Results of the pull-down assay was analyzed though visual inspection of the input and pull-down samples using 15% SDS-PAGE stained with Coomassie Brilliant Blue G-250.

Acknowledgements

G.J.L. was supported by funds from the Stanley Institute for Cognitive Genomics at Cold Spring Harbor Laboratory, and he is currently supported by funding from the New York State Institute for Basic Research in Developmental Disabilities (*IBR*), Staten Island, NY, USA. The DDD study presents independent research commissioned by the Health Innovation Challenge Fund [grant #HICF-1009-003], a parallel funding partnership between the Wellcome Trust and the

Department of Health, and the Wellcome Trust Sanger Institute [grant #WT098051]. NIH grants R01 GM060293 and R35 GM118090 awarded to R.M. and T32 GM071339 grant awarded to L.G. also supported this work. The authors would like to thank the families who participated in this study. Ezzat El Ezzad, PhD. and Ms. Maryellen Carfaro of the Graphic Resource and Media Service at New York State Institute for Basic Research in Developmental Disabilities provided photographic guidance in formatting family images for this article. The authors would like to thank the Genome Aggregation Database (gnomAD) and the groups that provided exome and genome variant data to this resource. A full list of contributing groups can be found at http://gnomad.broadinstitute.org/about.

Conflict of Interest Statement

G.J.L serves on advisory boards for Seven Bridges Genomics, Inc., Phosphorus, Inc., and Fabric Genomics, Inc.

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Figure 1. *NAA10* mutations identified in this study. (A) Schematic representation of the genomic structure of human *NAA10*. Solid rectangles indicate exons, and the horizontal bars represent introns. (B) Exonic localization of *NAA10* mutations identified in this study and those previously reported elsewhere. Novel mutations identified in this study are marked in bold. * indicated published mutations in which new information is provided in this study. (C) The functional domains of human NAA10 protein.

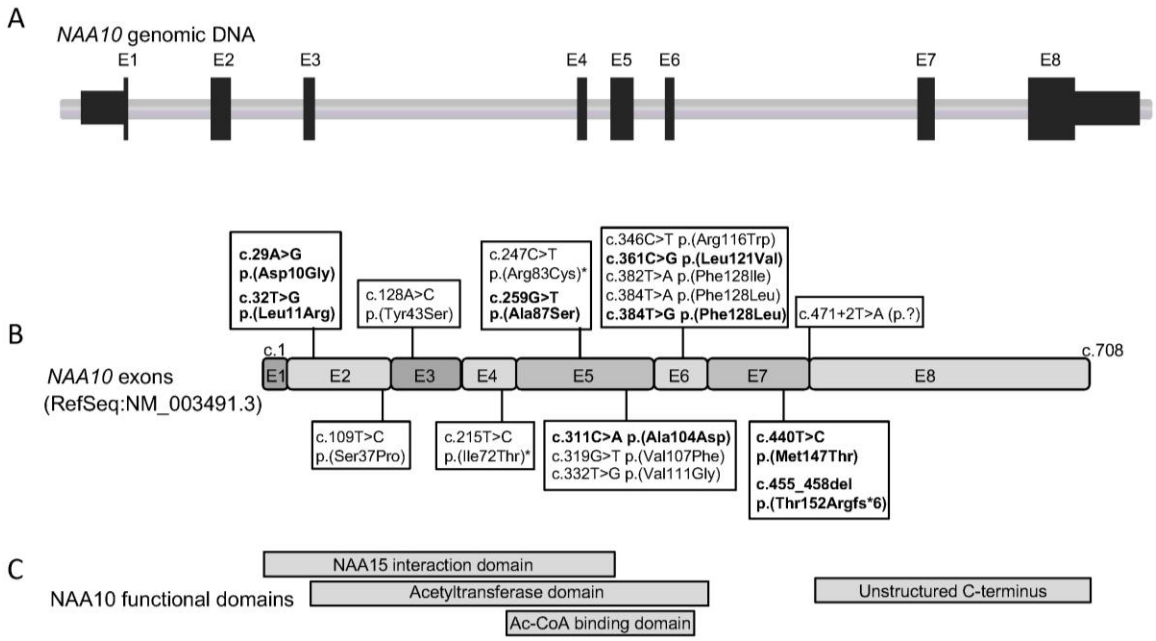

Figure 2. Facial morphology of individuals with *de novo* or inherited *NAA10* missense variants. A recognizable, regular pattern of dysmorphologic facial features was not discerned amongst the cases, other than perhaps thicker eyebrows and broad philtra. Individual 1: top picture at age 1 month, bottom picture at age 3.3 months. The child died at age 4 months due to heart failure. **Individual 3**: top picture at age 3 years old. Bottom picture at age 3 years, 11 months. This child died a few months later due to cardiopulmonary issues. **Individual 4**: at age 7 years old. **Individual 5**: top picture, age 10, bottom picture age 12. **Individual 6**: at age 11 in both pictures. **Individual 7**: top picture at age 4, bottom picture at age 32. **Individual 8**: at age 8.5 years and 10.3 years, **Individual 10:** at age 7. **Individual 15:** top picture age 7, bottom picture, age 14. **Individual 17**: at age 14 both pictures. **Individual 16:** at age 2 and 3, respectively. **Individual 19:** at age 5.7 years old. **Individual 22:** at age 8 years old.

Figure 3. An individual with *NAA10* c.455_458delCTCA variant. At 11-years of age, presenting with microcornea, microphthalmos, pectus excavatum, clubfeet, syndactyly (on 2nd and 3rd digits of feet) and also mildly so on the 3rd and 4th digits of his fingers.

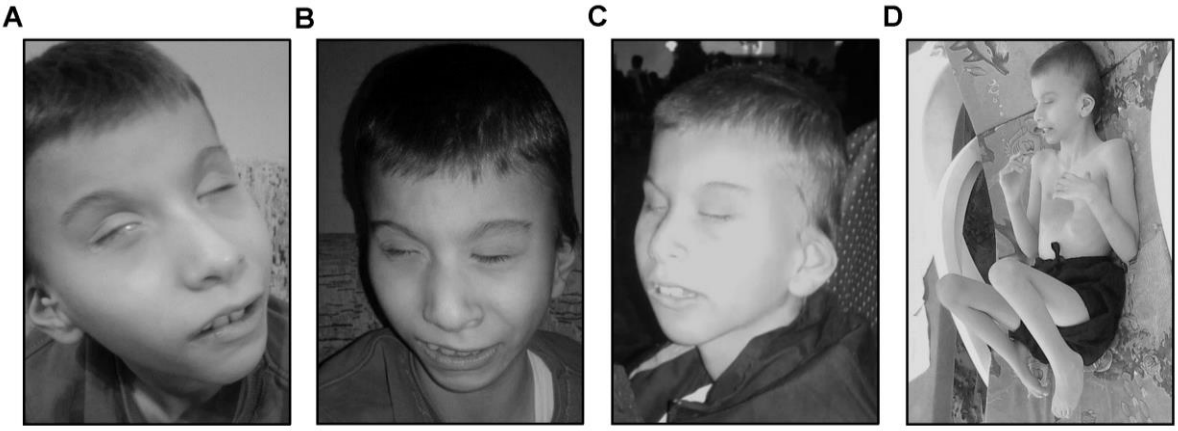

Figure 4. Mild facial dysmorphology and feet images of two individuals with *de novo NAA15* missense variants. **Individual 6:** photos were at 1.75 years and at 6.25 years, respectively. Abnormality of the dentition, broad philtrum, high palate, hypertelorism, microcephaly, persistent open anterior fontanelle and tented upper lip vermilion noted. Feet appear normal. **Individual 7:** at the age 8.6 years, with prominent forehead, prominent nasal tip, triangular nose, mild retrognathism and mild hypermetropia. Feet malposition was treated with one year physiotherapy.

Figure 5. Annotated Structure of Human NatA/HYPK.

A) Missense variants (yellow; stick format) found in NAA10 (purple) and B) NAA15 (dark teal) separately annotated on crystal structure of HYPK (light orange)-bound NatA (PDB: 6C95) in cartoon with bound IP_6(brown; stick format).

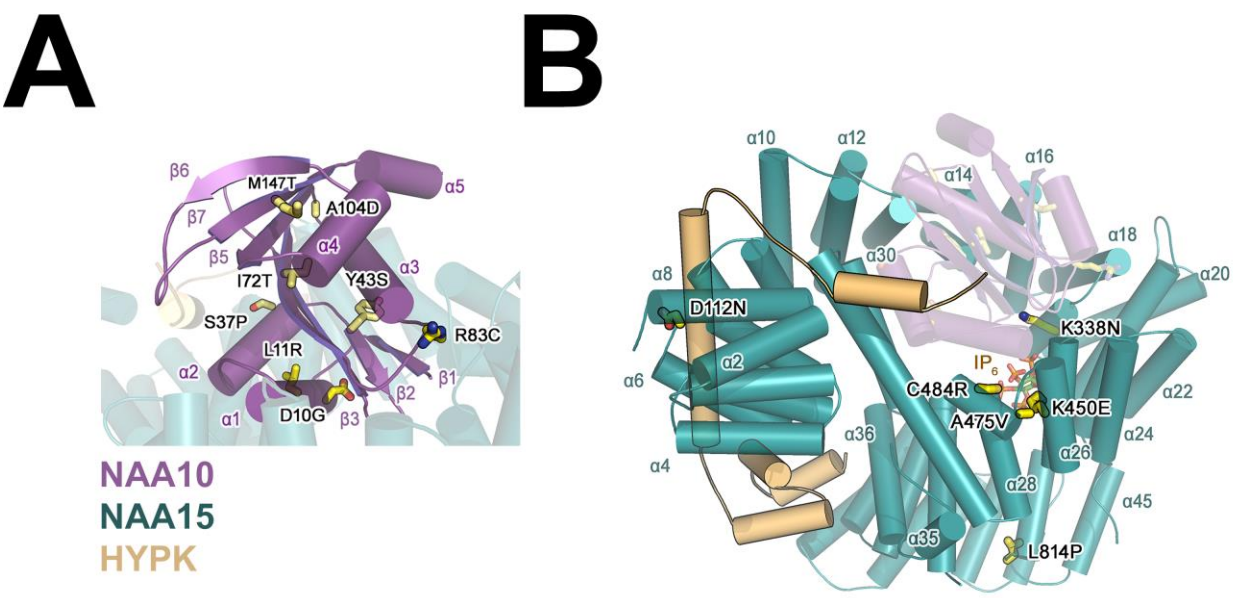

Figure 6. Comparison of NatA Complex Missense variants.

Bar graph representing relative effect of a missense mutant compared to the heterodimeric wild-type (WT) complex (-HYPK) on A) NatA complex activity and B) NatA/HYPK complex activity. In both cases, activity was normalized with respect to WT NatA (-HYPK). Assays were performed in triplicate; error bars S.D. Significance was calculated relative to WT using Sidak's multiple comparisons test. C) Differential scanning fluorimetry evaluation of WT and mutant NatA variants (-HYPK). Assays performed in absence (black squares) and presence of acetyl-CoA (grey circles). Assays performed in triplicate with each replicate presented in a staggered scatter plot. Significance was calculated with respect to the complex assayed in the absence of acetyl-CoA (black line) or in the presence of acetyl-CoA-bound (grey dotted line) using Sidak's multiple comparisons test. **** $p \leq 0.00001$; *** $p \leq 0.0001$; ** $p \leq 0.001$; * $p \leq 0.05$; ns $p > 0.05$

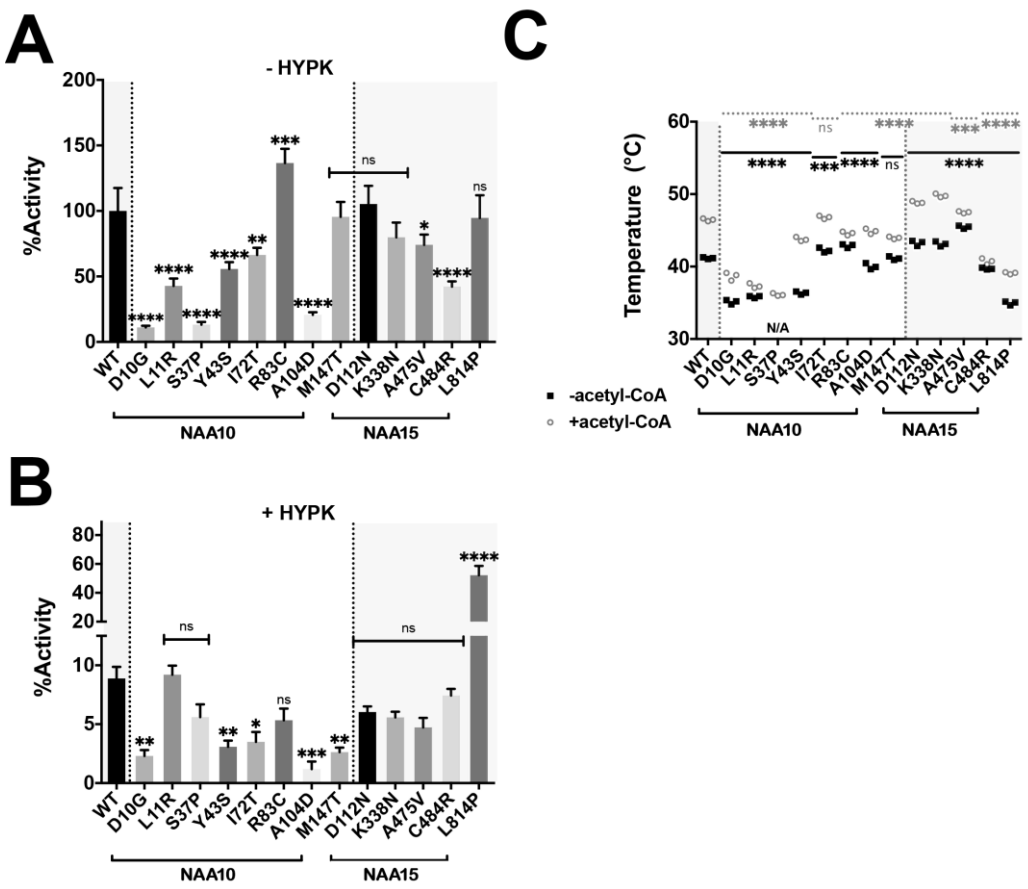

Figure 7. IP_6 Partially Rescues $NAA15_{K450E}$ Complex Function.

A) S200 gel filtration chromatogram comparing WT (solid black line) and HIS-$NAA10_{1-160}$/$NAA15_{K450E}$ (K450E) mutant prepared without (solid grey line) and with 15 μM IP_6 (dotted grey line).

B) Corresponding SDS-PAGE gel to the WT S200 elution profile for A.

C) Corresponding SDS-PAGE gel to the K450E complex –IP_6 elution profile for A.

D) Corresponding SDS-PAGE gel to the K450E complex +IP_6 elution profile for A.

E) Bar graph representing relative effect of K450E (grey) variant with respect to the heterodimeric WT (black) complex on NatA complex activity, when prepared without (Apo) or with IP_6 (+IP_6), as well NatA/HYPK complex (+IP_6/HYPK) activity.

Activity was normalized with respect to WT NatA (Apo). Assays were performed in triplicate; error bars S.D. Significance was calculated relative to WT using Sidak's multiple comparisons test.

F) Differential scanning fluorimetry (DSF) evaluation of WT and K450E. K450E was evaluated in the absence (-IP_6) and presence of IP_6 (+IP_6). Both WT and K450E DSF assays were performed in absence (black squares) and presence of acetyl-CoA (grey circles). Assays were performed in triplicate with each replicate presented in a staggered scatter plot. Significance was calculated either with respect to the WT in the absence of acetyl-CoA (black line) or in the presence of acetyl-CoA (grey dotted line) using Sidak's multiple comparisons test.

**** $p \leq 0.00001$; *** $p \leq 0.0001$; ** $p \leq 0.001$; * $p \leq 0.05$; ns $p > 0.05$

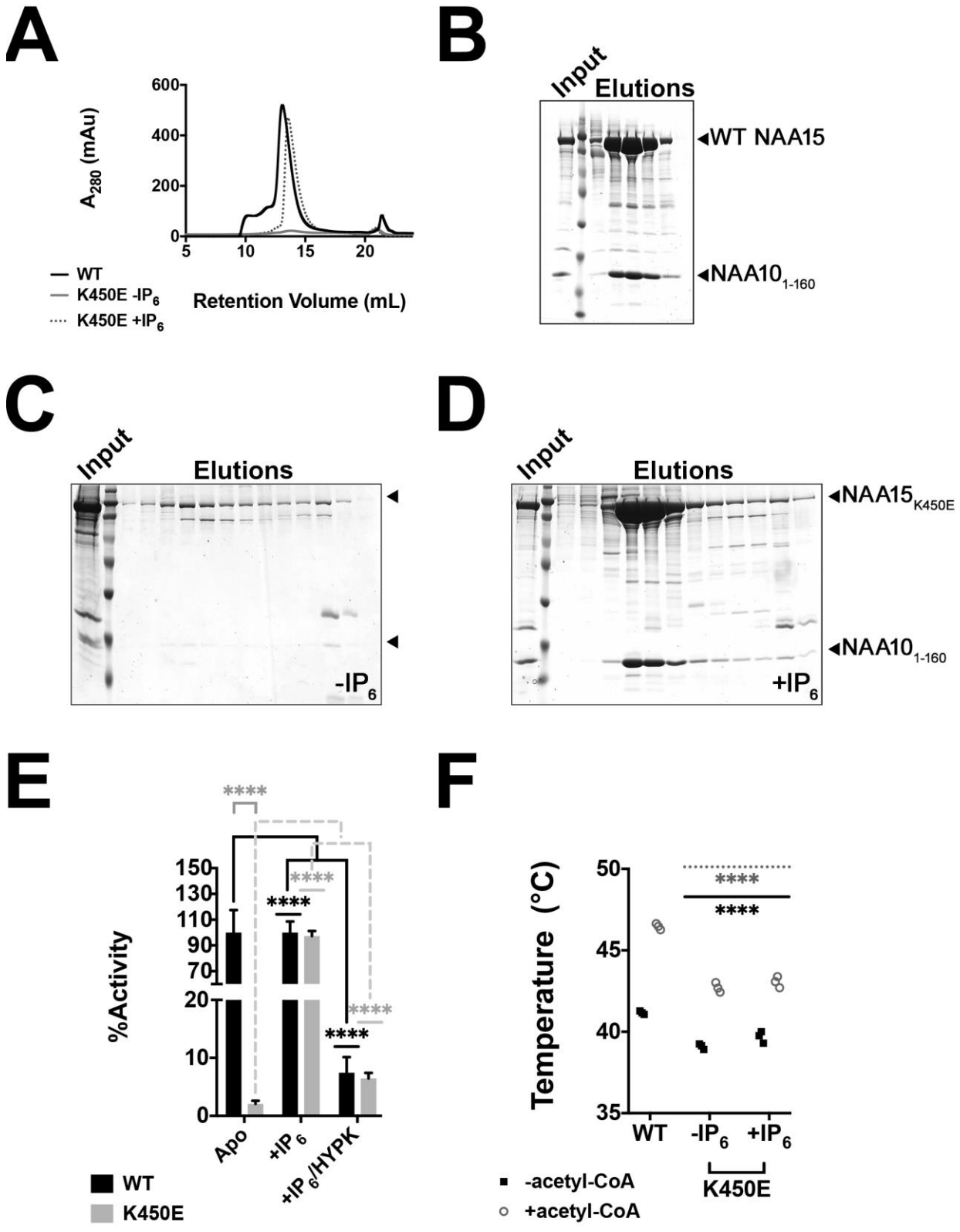

Figure 8. NAA10 Mutant Enzyme Kinetics. Michaelis-Menten kinetics of WT and catalytic mutant NatA complexes with respect to

A) H4

B) acetyl-CoA

Assays performed in triplicate; error bars correspond to the S.D. for each point.

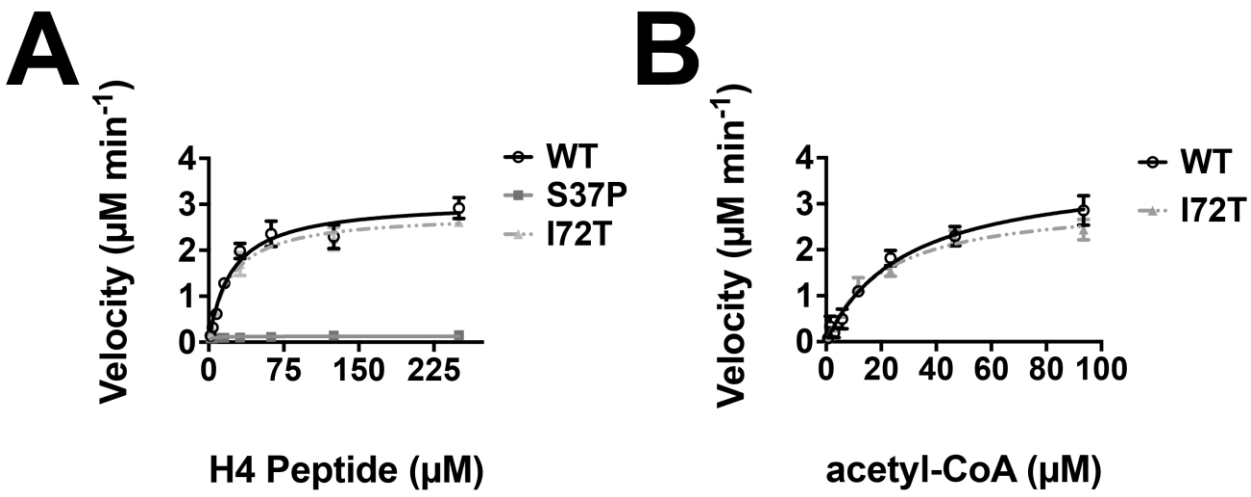

Table 1. Summary of clinical features and molecular findings in individuals with NAA10 variants in this study.

ID	Age/ Sex	Variant & Inheritance	Feeding	MRI	Neuro-developmental	Cardiac	Eye	Others
1	4 m/M	c.29A>G p.(Asp10Gly) *de novo*	difficulty swallowing, at risk for aspiration	MRI: agenesis of corpus callosum, Hypoplastic corpus callosum, Ventriculomegaly	hypotonia	ECG: right ventricular hypertrophy due to primary pulmonary Hypertension, with eventual right-sided heart failure and death	N/A	dysmorphic facial features, barrel chest, bilateral profound sensorineural hearing loss
2	3 y/F	c.32T>G p.(Leu11Arg) Unknown inheritance	no difficulties	MRI: congenital hydrocephalus, ventriculomegaly, hypoplasia of the corpus callosum, hyperplasia of the choroid plexus and partial agenesis of the septum pellucidum	delayed motor development, walked at around age 3y, speech delay	N/A	strabismus	N/A
3	4.2 y/M	c.215T>C p.(Ile72Thr) Maternal inheritance	gastrostomy tube care	MRI: medulloblastoma	intellectual disability, ataxia, mild motor delay, marked speech delay	ECG: enlarged heart muscle, tachycardia, prolonged QT interval, died due to cardio-pulmonary issues	strabismus,	dysmorphic facial features, posterior fossa syndrome, loss of height between T5 and T6
4	10 y/F	c.247C>T p.(Arg83Cys) *de novo*	functional bulbar palsy, esophagus and gut dysmotility syndrome, complete dysphagia	MRI: Normal	global developmental delay, severe intellectual disabilities, poor fine motor skills, apraxia	None	cortical/ cerebral visual impairment, bilateral astigmatism, alternating divergent squint	behavioral issues (autistic traits, severe sensor processing issues, anxiety), severe sleep disorder, short statue, bilateral talipes, reflux and constipation issues, kyphosis, hypertrichosis, mixed muscle tone
5	13 y/F	c.247C>T	PEG feeding	MRI: generalized	severe global	ECG:	moderate	absence seizures,

		p.(Arg83Cys) *de novo*		lack of white matter bulk with thinning of the corpus callosum and prominent CSF spaces	development delay, severe motor delay, poor fine motor skills, very limited speech	prolonged QT interval, bicuspid valve	astigmatism, borderline electro-diagnostics	pectus excavatum, premature precocious puberty, remains incontinent
6	11.5y/F	c.247C>T p.(Arg83Cys) *de novo*	PEG feedings, GI dysmotility, cyclical vomiting, food intolerance, allergic to most antibiotics	MRI: Small cyst	global developmental delay, poor fine motor skills, intellectual disability, non-verbal, but can read and type in full sentence	N/A	cortical visual impairment	micrognathia, moderate to severe hearing impairment; precocious puberty, body odor. autistic traits
7	34 y/F	c.247C>T p.(Arg83Cys) *de novo*	excessive vomiting, gastro-esophageal reflux, coeliac disease	N/A	global developmental delay, motor delay, severe mental retardation, no understandable speech	ECG: normal	N/A	facial dysmorphism, epilepsy, increased muscle tone and contracture, sleep disturbance
8	13 y/F	c.247C>T p.(Arg83Cys) *de novo*	gut dysmotility, food intolerance, frequent vomiting, severe allergies	N/A (not performed)	global developmental delay, motor delay, learning disability, limited speech	ECG: long QT syndrome	astigmatism, hyperopia	toe-walking, high anxiety, stereotypical behaviors, leg discrepancy, frequent nose bleeds, pica, noise sensitivity, cutis marmorata
9	15 y/F	c.247C>T p.(Arg83Cys) *de novo*	N/A	N/A	global developmental delay, unable to manage tasks independently such as dressing, toileting and feeding, non-verbal	Tetralogy of Fallot surgically corrected at age 4 months	N/A	ASD, challenging behaviors including pulling hair and a trichobezoar-laparotomy required; no sense of danger
10	7.5 y/F	c.247C>T p.(Arg83Cys) *de novo*	Feeding difficulties	Ventriculomegaly and intracranial hemorrhage at birth. VP shunt inserted at age one month.	severe global development delay, significant motor delay, hypotonia, cannot sit up alone, severe cognitive impairment, non-	None	central vision impairment; stigmatism and farsighted	Partial epilepsy, growth delay microcephaly, hydrocephalus status post shunt surgery

					verbal			
11	1.2 y/F	c.247C>T p.(Arg83Cys) *de novo*	feeding difficulties, fed with thick juices or puree	MRI: Normal	global development delay, motor delay	ECG: secundum ASD with left to right shunt, mild valvular pulmonary stenosis, dilated right atrium and ventricle, good LV/RV function	N/A	facial dysmorphism, bilateral minimal hearing loss
12	6 y/F	c.247C>T p.(Arg83Cys) *de novo*	failure to thrive; manages finger soft baby food	N/A	global developmental delay, motor delay, attending special education class, non-verbal	ECG: long QT syndrome	N/A	facial dysmorphism, seizures, extra rib, extra vertebrae, possible anterior beaking L1 vertebrae, behavioral issues
13	2.5 y/F	c.247C>T p.(Arg83Cys) *de novo*	Feeding difficulties; gastric reflux disease; esophagitis.	N/A	Global developmental delay, mild hypotonia, speech delay partially nonverbal intellectual disability		astigmatism	Growth delay; sensory processing disorder; avoidance disorder; facial dysmorphism;
14	7 y/F	c.247 C>T p.(Arg83Cys) *de novo*	feeding difficulties; food allergies: soy	MRI: Subtle abnormalities in white matter of both the occipital and temporal lobes. There was a decrease in the central white mass volume and a thinning of the corpus callosum.	Microcephaly; developmental delay: mild hypotonia; mild cerebral palsy; nonverbal Autism Spectrum Disorder intellectual disability		Astigmatism; myopia corrected with eyeglasses	Growth delay; sensory processing disorder; avoidance disorder; sensory seeking behaviors; microcephaly, facial dysmorphism
15	15 y/F	c.259G>T p.(Ala87Ser) *de novo*	feeding difficulty in infancy, abnormality of the gastrointestinal tract	MRI: normal	severe global developmental delay, gross motor development delay, severe intellectual disability, non-	ECG: normal cardiovascular examination: normal	optic nerve hypoplasia	facial dysmorphism; epilepsy, tonic clonic seizures; bilateral sensorineural hearing impairment; ADHD, autistic behavior, unpredictable

					verbal, generalized hypotonia, spastic paraplegia			behavior under stress
16	2.5 y/F	c.259G>T p.(Ala87Ser) *de novo*	No trouble swallowing, some feeding issues due to coordination	MRI: hypoplasia of corpus callosum, and mild myelination defect.	global developmental delay, hypotonia, non-verbal	N/A	None	mildly dysmorphic; relatively short stature, significantly elevated alkaline phosphatase
17	14 y/F	c.259G>T p.(Ala87Ser) *de novo*	swallowing difficulties eats mashed food only	MRI: subcortical hyper intensities in the white matter and basal ganglia. periventricular and pallidum (cysts)	global developmental delay, severe cognitive disability, dystonic movement disorder, non-verbal	ECG: normal	None	growth delay, myoclonic epilepsy, degenerative course due to 3 dystonic crisis, life-threating dystonic status, severe scoliosis, mixed muscle tone, wheelchair- bound
18	10 y/F	c.311C>A p(.Ala104Asp) *de novo*	chewing and swallowing difficulties	MRI: normal	global developmental delay, hypotonia, fine motor delay, intellectual disability, mixed receptive and expressive language disorder, apraxia	ECG: normal	astigmatism anisometropia conjunctivae and EOM noted as normal on examination	ADHD (combined type), stereotypies, anxiety, Sensory processing disorder, growth hormone deficiency, relatively short stature, hip dysplasia, hearing impairment, sleep disorder
19	6 y/F	c.361C>G p.(Leu121Val) *de novo*	gut issues which have been better with supplements, silent reflux as an infant	MRI: normal	global developmental delay, severe intellectual disability, motor delay and poor coordination, non-verbal	ECG: normal	N/A	facial dysmorphism, toe-walking, severe autism, self-stimulatory behavior, mixed muscle tone, hip dysplasia, severe sleep issues
20	14 y/F	c.361c>G p.(Leu 121 Val) Unknown inheritance	No feeding issues		delayed motor development, mild hypotonia, speech delay partially nonverbal intellectual disability, Autism Spectrum Disorder; avoidance		optokinetic nystagmus	Growth delay; sensory processing disorder; anxiety; avoidance disorder;

					disorder;			
21	1.3 y/F	c.384T>G p.(Phe128Leu) *de novo*	nasogastric tube feedings as infant; gastric reflux	MRI: bilateral pyramidal characteristics with deviations in the basal ganglia	delayed motor development	ECG: normal QT interval: normal cardiac exam:	cortical vision impairment	microcephaly, relatively short stature, poor eye contact, chorea; overriding toes; single crease hand line
22	8 y/F	c.440T>C p.(Met147Thr) *de novo*	Unable to drink from a bottle due to coordination issue, learned to chew through ABA therapy	MRI: thinning of corpus callosum	overall developmental delay, moderate intellectual impairment with advanced math skills, coordination issues, limited speech	ECG: normal	stigmatism, cortical visual impairment	sensory processing disorder, self-stimulating behaviors, but very social and no other evidence of autism, microcephaly, acne, body odor, adrenarche, light sleeper
23	11 y/M	c.455_458del p.(Thr152Argfs*6) Maternal inheritance	low appetite, no chewing or swallowing issues	MRI: agenesis of corpus callosum, craniosynostosis	global developmental delay, severe intellectual disability, generalized hypotonia, wheelchair-bound	ECG: defect in the atrial septum	microcornea, microphthalmia	growth delay

Table 2. Summary of the relative prevalence of each phenotype in 23 individuals with *NAA10* variants.

Phenotype	Number of individuals with phenotype	Number of individuals with relevant data	Percentage
Brain Structure and Function			
Global developmental delay	23	23	100%
Non-verbal or limited speech	21	21	100%
ASD, ADHD and other Behavioral issues	22	22	100%
Abnormal MRI	14	18	77%
Seizures	9	13	69%
Motor impairments			
Feeding difficulties	23	23	100%
Motor delay and related abnormalities	23	23	100%
Muscle tone issues	17	17	100%
Cardiovascular			
Long QT syndrome	7	19	37%
Heart failure and death	2	23	4%
Atrial septum defect	3	18	17%
Pulmonary hypertension	1	17	6%
Tetralogy of Fallot	1	17	6%
Other			
Facial dysmorphism	23	18	78.20%
Eye abnormalities	15	17	88.20%
Skeletal or connective tissues disorders	9	15	60%
Growth delay*	13	23	56.50%
Sleep disorder	7	16	43.70%
Hearing impairment	8	19	42.10%

* growth delay is indicated in individuals whose height and weight is below 3 percentile at last visit

Table 3. Naa10 Mutant Kinetic Parameters.

Mutant	Substrate	k_{cat} (min^{-1})	K_M (μM)	k_{cat}/K_M (μM min^{-1})
WT	H4	310 ± 13.3	23 ± 3.3	13 ± 0.2
S37P		13 ± 0.9 ****	1.9 ± 0.8 ****	6.6 ± 0.4 ****
I72T		280 ± 8.8 (ns)	22 ± 2.3 (ns)	13 ± 0.1 (ns)
WT	acetyl CoA	380 ± 24	29 ± 4.5	13 ± 0.2
I72T		310 ± 14 *	21 ± 2.5 (ns)	15 ± 0.1 ***

Michaelis-Menten parameters, corresponding to Figure 8A and B, of WT catalytic mutant NatA complexes with respect to H4 or acetyl CoA. Error bars correspond to the standard deviation for each point. n=3

Abbreviations

Attention deficit hyperactivity disorder (ADHD)

Autism spectrum disorder (ASD)

Huntington Interacting Protein (HYPK)

intellectual disability (ID)

Lenz microphthalmia syndrome (LMS)

N-terminal acetyltransferase A (NatA)