Title: Molecular Characterisation of Rare Loss-of-Function *NPAS3* and *NPAS4* Variants Identified in Individuals with Neurodevelopmental Disorders

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Figure S1: Alignment of Neuronal bHLH-PAS factors

The N-terminal amino acid sequences of neuronal bHLH-PAS factors, NPAS3, NPAS4, SIM1 and SIM2, were aligned using the CLUSTAL O (1.2.4) multiple sequence alignment tool. Orange sequence indicates the bHLH domain, blue sequence indicates PAS A and green sequence indicates PAS B. Domain boundaries were determined based on alignments performed for the NPAS1/NPAS3 crystal structure [1]. Relevant loss of function variants previously identified for NPAS4[2], SIM1[3], SIM2 (Whitelaw, unpublished), in addition to all missense/nonsense variants characterised in this study are highlighted in red. Finally, the start location of the two frameshift mutations are also indicated.

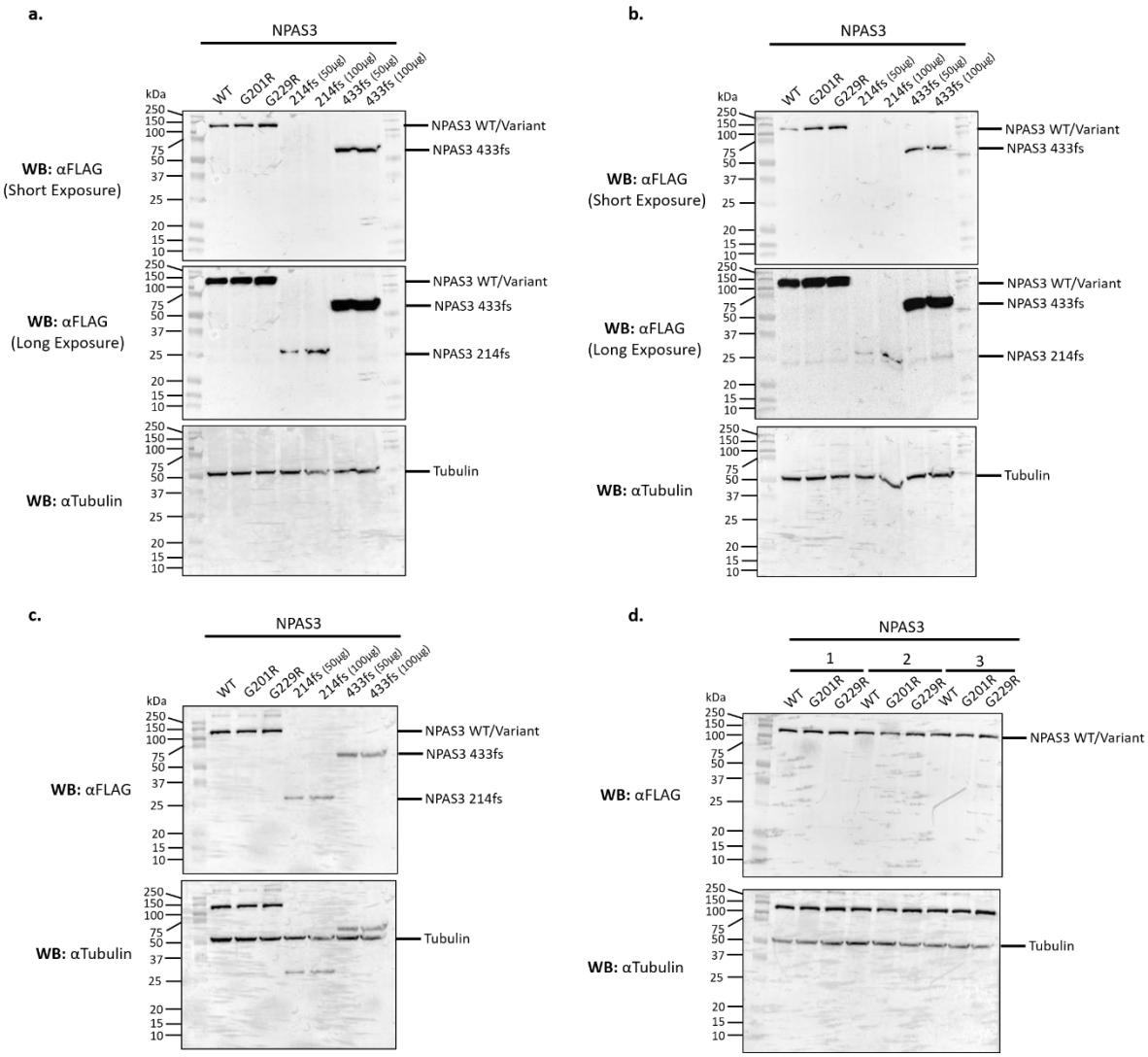

Figure S2: *NPAS3* Variant Expression – Full Blots and Replicates

a.-c. HEK293T cells were transiently transfected with *NPAS3* variants, and expression was detected by western blot. The results of 3 independent experiments are shown. **d.** Due to variability observed in NPAS3 WT expression across **a.-c.** three independent sets of lysates (1-3), were run simultaneously on a single blot. Of note, replicate 1 utilises the same lysates as **c.** and replicates 2 and 3 utilise new lysates. WT= wildtype; WB = western blot.

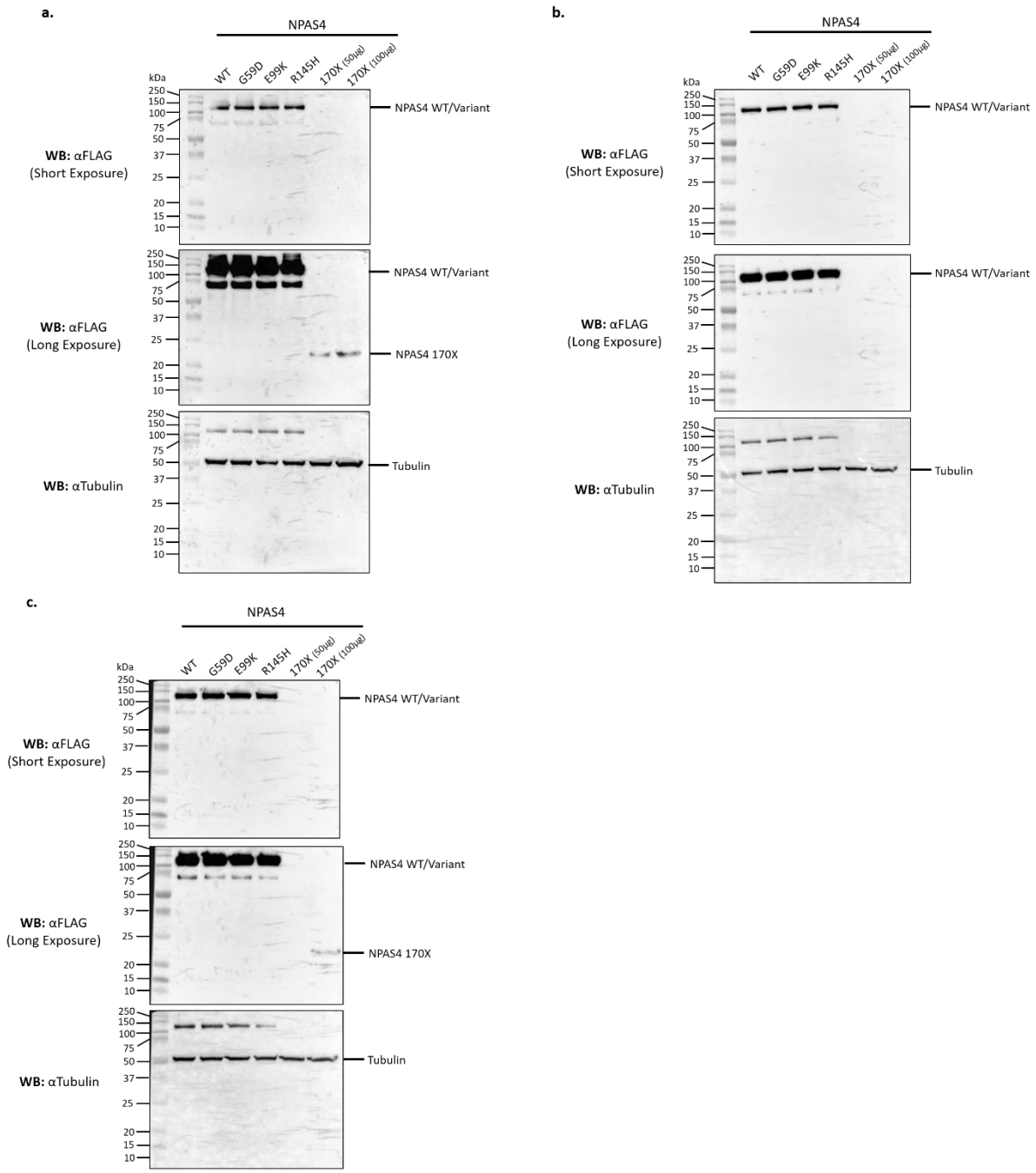

Figure S3: *NPAS4* Variant Expression – Full Blots and Replicates

a.-c. HEK293T cells were transiently transfected with *NPAS4* variants, and expression was detected by western blot. The results of 3 independent experiments are shown. WT= wildtype; WB = western blot.

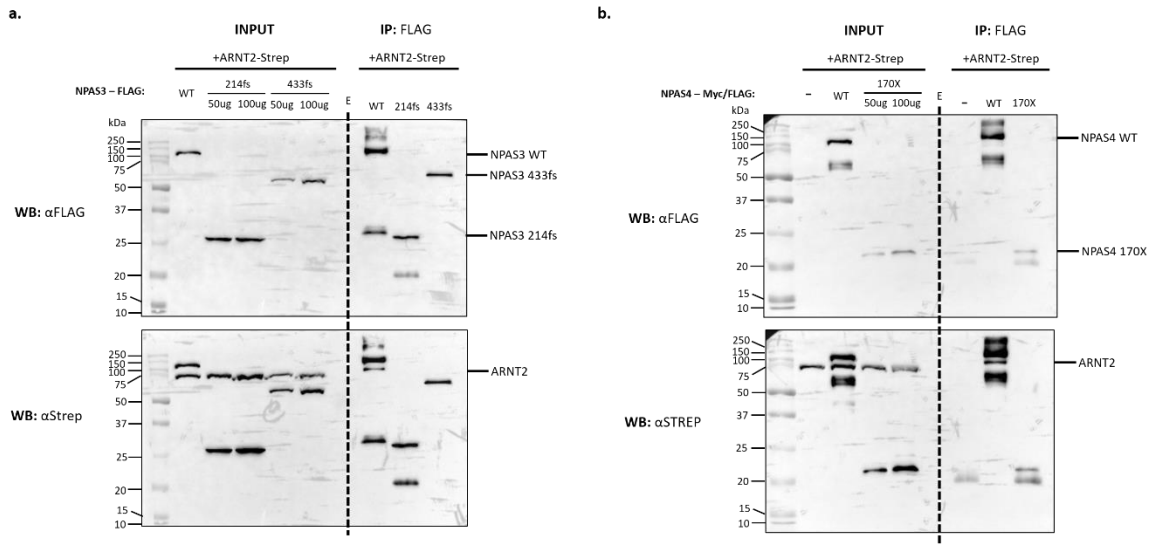

Figure S4: *NPAS3* and *NPAS4* Variant Co-immunoprecipitation of ARNT2 – Full Blots

a.-b. HEK293T cells were transiently transfected with a variant of either NPAS3-FLAG or NPAS4-Myc/FLAG and ARNT2-Strep. 500µg of whole cell lysate was then subjected to FLAG immunoprecipitation. Unless otherwise stated the input sample included 50µg of whole cell lysate. WT= wildtype; WB = western blot; IP = immunoprecipitation; E = lane left intentionally empty.

Table S1: Patient Information for *NPAS3* Variants

Gene	Age [Years] (Sex)	AA Change (Nt Change)	Zygosity	Reads (Var/ Ref†)	Patient Phenotype at Time of Sequencing	Other Mutations/Notes
*NPAS3**	4.3 (M)	p.G201R (c.601G>A)	Het	22/44	Swallowing issues in infancy, motor delays, difficulty walking, frequent falls, ophthalmoplegia, diffuse hypotonia, hyporeflexia, decreased muscle strength and overweight.	A heterozygous c.9847C>T (p.R3283X) variant and a heterozygous c.1250T>C (p.L417P) variant in the *RYR1* gene (refseq: NM_000540.3) were detected in this individual. Mutations in *RYR1* are the cause of multiminicore disease with external ophthalmoplegia (OMIM:255320). Family history is positive for a sister with ophthalmoplegia and the paternal grandmother with walking difficulties.
	0.048 (M)	p.G201R (c.601G>A)	Het	62/143	Congenital heart disease which includes transposition of the great arteries, coarctation of the aorta, and ventricular septal defect (VSD). Additionally, a history of intrauterine growth restriction and extremely low weight.	In a more recent assessment at an age of 3.75 years the individual did not have any developmental issues.
	2.3 (F)	p.G201R (c.601G>A)	Het	58/144	Delayed motor milestones, hypotonia, hypertonia/spasticity, seizure disorder, abnormal movements and microcephaly.	A homozygous c.79T>C (p.Y27H) variant in the *GAMT* gene (refseq: NM_000156.6) was detected in this individual. Homozygous mutations in *GAMT* are known to cause Cerebral Creatine Deficiency Syndrome 2 (OMIM: 612736).
	1.4 (F)	p.G229R (c.685G>A)	Het	25/54	Microcephaly, hypotonic cerebral palsy, profound intellectual disability, esotropia, delayed visual maturation, small optic nerves, nystagmus, mild distal lower extremity hyperreflexia, global developmental delay, and dysmorphic features.	A *de novo* novel heterozygous c.997delG (p.A333fs) variant in the *FOXG1* gene (refseq: NM_005249.5). Mutations in *FOXG1* can cause Congenital Rett Syndrome (OMIM: 613454). Family history is significant for both parents with delays and father with cardiomyopathy.
	2.1 (F)	p.G229R (c.685G>A)	Het	30/70	Intrauterine growth restriction, delayed motor milestones, delayed speech, hypotonia, dysmorphic features, hyperextensibility, short stature, microcephaly, eye anomalies (esotropia), bicuspid aortic valve and partial fusion of aortic leaflets, hemangioma over glabella, congenital hypothyroidism and stereotyped flapping movements of her arms.	A *de novo* heterozygous c.410_411del (p.137fs) variant in the *H3F3B* gene was detected in this individual. Defects in *H3F3B* are not currently associated with a disease in humans.
	16.3 (M)	p.L214fs (c.641dupT)	Het	49/78	Common variable immune deficiency, Evans syndrome, growth hormone deficiency, demyelination findings consistent with multiple sclerosis, eczema, hepatosplenomegaly, speech delay, intellectual disability, short stature, dysmorphic features, failure to thrive, complex dental caries resulting in the removal of all teeth, viral myocarditis, cardiomyopathy, eneuresis, neutropenia, thrombocytopenia, and intermittent headaches.	

* *NPAS3* refseq: NM_001164749.2 †Variant Reads/Reference Sequence Reads

Table S2: Patient Information for *NPAS4* Variants

Gene	Age [Years] (Sex)	AA Change (Nt Change)	Zygosity	Reads (Var/ Ref†)	Patient Phenotype at Time of Sequencing	Other Mutations/Notes
*NPAS4**	1.3 (M)	p.G59D (c.176G>A)	Het	16/30	Profound speech and motor delay, congenital hydrocephalus and congenital brain malformation (Chiari I malformation, absent corpus callosum and possible aqueductal stenosis), oculomotor apraxia, mixed tone abnormalities in the muscle, and poor weight gain.	In a more recent assessment at an age of 3.5 years, was non-verbal and had persistent, profound motor delays and an absent septum pellucidum.
	10.7 (M)	p.E99K (c.295G>A)	Het	21/49	Intrauterine growth restriction with motor delay, speech delay, intellectual disability, hypotonia, seizure, short stature, failure to thrive, and possible developmental regression.	A *de novo* heterozygous c.239T>C (p.I80T) variant in the *GNB1* gene (ref seq: NM_002074.5). Defects in *GNB1* are known to cause intellectual disability (OMIM: 616973).
	23.1 (F)	p.R145H (c.434G>A)	Het	33/63	Seizures, intellectual disability, hypotonia, mild dysmorphisms, and sensorineural hearing loss.	
	2.5 (F)	p.R170X (c.508C>T)	Het	24/51	Speech delay, hypotonia, dysmorphic features (hypertelorism, fetal finger pads, flattened nasal bridge and broad mouth), and hyperextensibility.	Family history indicates an older brother with delayed speech, hypotonia, and hyperextensibility; he does not carry the variant. The mother also has hyperextensible elbows and fingers.

* *NPAS4* refseq: NM_17886 †Variant Reads/Reference Sequence Reads

References

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