Actin type	Acetylation	Sequence	m/z	targeted $[M+2H]^{2+}$
beta	no	DDDIAALVVDNGSG**M**CK	1795.783	898.395
beta	$^{12}C/^{1}H$ acetate	**D**DDIAALVVDNGSG**M**CK	1837.793	919.400
beta	$^{13}C/^{2}H$ acetate	**D**DDIAALVVDNGSG**M**CK	1742.819	921.913
gamma	no	EEEIAALVIDNGSG**M**CK	1851.845	926.426
gamma	$^{12}C/^{1}H$ acetate	**E**EEIAALVIDNGSG**M**CK	1893.855	947.431
gamma	$^{13}C/^{2}H$ acetate	**E**EEIAALVIDNGSG**M**CK	1898.881	949.944

Supplementary Table S1: List of the 6 theoretical peptide sequences that were targeted by mass spectrometry. Meaning of bold amino acids: oxidation of **M** (+15.994 Da), carbamidomethyl of **C** (+57.021 Da), N-term acetylation for **E** or **D** (+42.010 Da for $^{12}C_2/^{1}H_3$ and +47.037 Da for $^{13}C_2/^{2}H_3$).

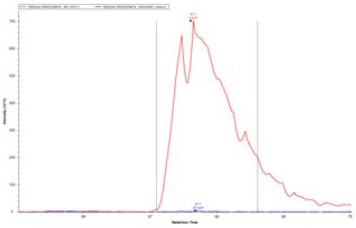
Label	Chromosome	Start	Stop	Size
ROH	3	45.476.694	54.264.197	8.788 MB
ROH	9	65.629.772	69.824.256	4.194 MB
ROH	5	129.486.905	132.169.532	2.683 MB
ROH	15	42.922.656	45.394.057	2.471 MB
ROH	1	142.541.502	144.931.626	2.39 MB
ROH	12	85.356.224	87.722.701	2.366 MB
ROH	5	44.442.578	46.404.402	1.962 MB

Supplementary Table S2: Identity by descent (IBD) regions found in proband 1.2 with SNP Array. Runs of Homozygosity (ROH) are contiguous regions of the genome where the individual is homozygous across all sites.

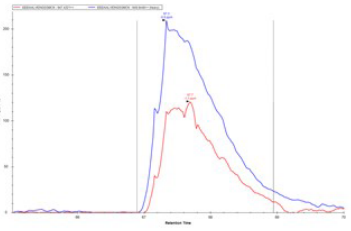
Supplementary Figure 1:

XIC for precursor ions
Targeted N-term peptide: Ac-EEEIAALVIDNGSGM$_{ox}$C$_{cm}$K

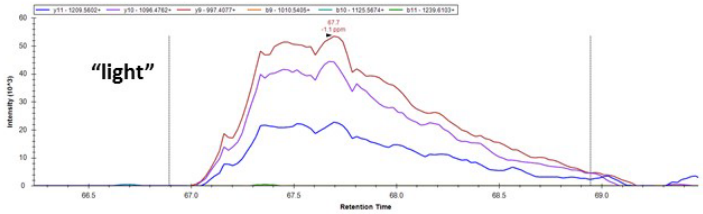
Gamma actin
Wild-type

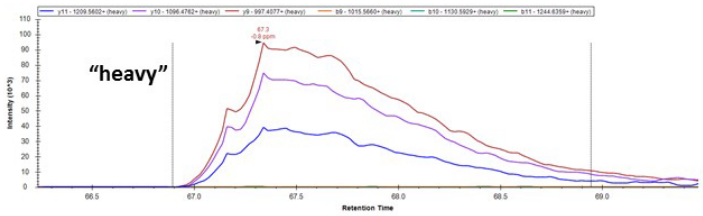


Gamma actin
NAA80 deficient

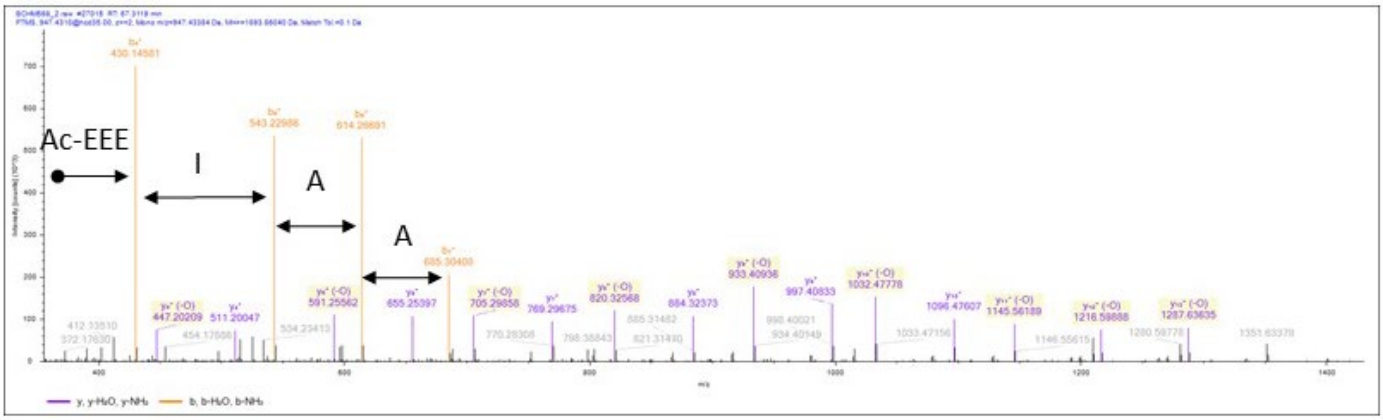


XIC for daughter ions from Nat6 deficient cells
Targeted N-term peptide: Ac-EEEIAALVIDNGSGM$_{ox}$C$_{cm}$K

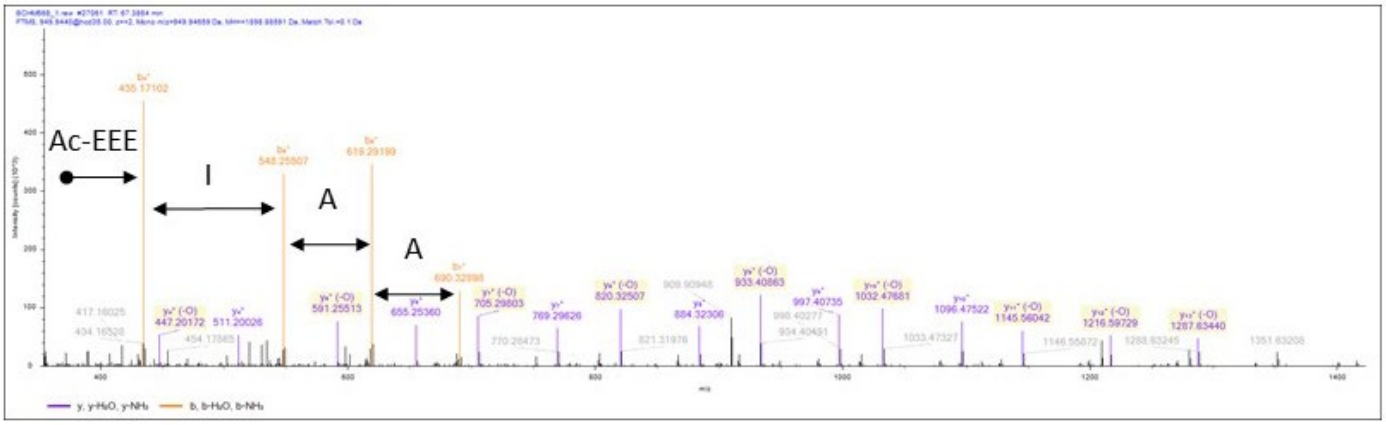




MS/MS spectra for $^{12}C_2/^{1}H_3$ "light" peptide

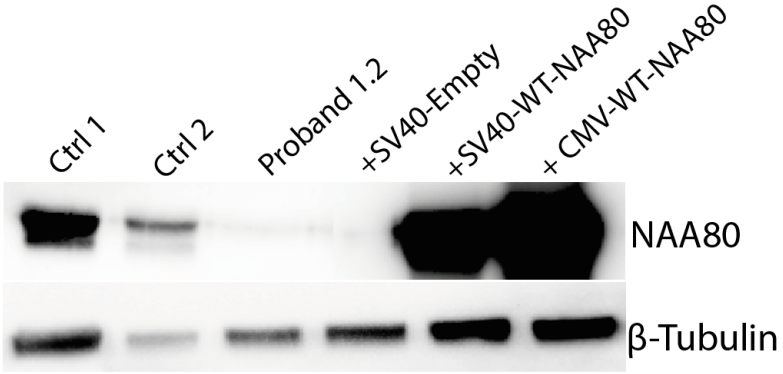


MS/MS spectra for $^{13}C_2/^{2}H_3$ "heavy" peptide



Supplementary Figure 1: Identification and quantification of the N-terminal acetylated peptide from gamma actin by targeted mass spectrometry. A PRM MS/MS method was designed to measure the two acetylated forms (light or heavy) of the processed N-term peptide taking oxidation of Met (M_{ox}) and carbamidomethylation of Cys (C_m) as fixed modifications. *Left upper panel*: XIC of the two precursors ions (light in red m/z= 947.432, heavy in blue m/z= 949.948) for a control and the NAA80 deficient individual. *Right upper panel*: example from a NAA80 deficient individual of the XIC of all daughter ions taken into account for quantification by their area under the curve (AUC). *Lower panel*: MS/MS spectra of the light and heavy +2 precursors ions, the b-ions series shows the incorporation of a heavy acetylated group at the N-term side of the peptide with a mass difference of 5.027 Da from their light counterpart. (-O) denotes neutral loss of H_4COS from the side chain of Cys.

Supplementary Figure 2.



Western blot showing NAA80 expression in healthy controls (Ctrl 1, Ctrl 2) and proband 1.2 with and without (SV40-Empty) vectors expressing NAA80 WT cDNA using either a CMV or SV40 promoter.

Supplementary Table S3:

Gene	Considerations regarding pathogenicity	Chromosome	Start	Stop	Genotype	cDNA	Protein	PhyloP and PhastCons
PRR14L	The PRR14L variant is not present in affected proband 1.4. Phenylalanine and Tyrosine have similar properties and this change is thus not expected to impact the protein structure significantly.	22	32084196	3.2E+07	A/T	c.6125T>A	p.F2042Y	PhyloP: 3.958, PhastCons: 1
PLXNB1	Segregates with affected individuals (proband 1.2 and 1.4). Affected residue is not conserved. Serine and glycine have similar properties and this change is thus not expected to impact the protein structure significantly. The variant is located in a region of low conservation in the protein. Serine to glycine has been observed in other species.	3	48460754	4.8E+07	C|C	c.2731A>G	p.S911G	PhyloP: -0.153, PhastCons: 0.
NAA80	Segregates with affected individuals (proband 1.2 and 1.4). Very	3	50334572	5E+07	G|G	c.323T>C	p.L108P	PhyloP: 4.572, PhastCons: 1

	conserved residue, amino-acid change predicted to result in altered protein confirmation (loss of proline).							
HDAC6	The HDAC6 variant is also present in one of the healthy male siblings (proband 1.1).	X	48663916	4.9E+07	C|	c.383G>C	p.C128S	PhyloP: 1.82, PhastCons: 0.998

Supplementary Table S3: Genetic variants in proband 1.2 found with Whole Exome Sequencing that remain after filtering.

Supplementary Table S4

Phenotypic Feautre	NAA80	ACTB	ACTG1	ACTA1	ACTA2	ACTG2	ACTC1
High-frequency sensorineural hearing impairment	50	0	35	0	0	0	0
Prominence of the zygomatic bone	25	1.515152	1.282051	0	0	0	0
Congenital ptosis	25	5.30303	8.333333	0	0	0	0
High-frequency hearing impairment	14.28571	0	1.848539	0	0	0	0
Snoring	14.28571	0	0	0	0	0	0
Peg Shaped maxillary lateral incisors	11.1111	1.31	0	0	0	0	0
Infantile axial hypotonia	7.69	0	0	0.3042156	0	0	0
Widely-spaced maxillary central incisors	7.14	0.67	0	0	0	0	0
Progressive sensorineural hearing impairment	2.325581	0.422833	1.471985	0	0	0	0
Brain imaging abnormality	1.5625	0.260417	0	0	0	0	0
Low posterior hairline	1.470588	0.039872	0	0	0	0	0
Prominent metopic ridge	1.25	0.113636	0	0	0	0	0
Sleep Apnea	1.162791	0	0	0	0	0	0
Tapered finger	1.07526	0.017618	0.053419	0	0	0	0
Everted lower lip vermilion	1.02	0.030921	0	0	0	0	0
Self-injurious behavior	0.99	0	0	0	0	0	0
Microretrognathia	0.980392	0.089127	0	0	0	0	0
Eclabion	0.9259259	0.028058	0	0	0	0	0
Synophrys	0.892857	0.040584	0	0	0	0	0
Downturned corners of mouth	0.862069	0.026123	0	0	0	0	0
Highly arched eyebrow	0.7462687	0.135685	0.0333	0	0	0	0
Failure to thrive in infancy	0.6493506	0.059032	0	0	0	0	0
Muscular hypotonia of the Trunk	0.5813953	0	0	1.0290698	0	0	0

Supplementary Table S4: Phenotypic features with their corresponding occurrence ratio in individuals with NAA80 genetic variants or actin mutations (ACTB, ACTG1, ACTA1, ACTA2, ACTG2, ACTC1).