

Supplemental Data

Biallelic Variants in *UBA5* Reveal that Disruption of the UFM1 Cascade Can Result in Early-Onset Encephalopathy

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Supplemental Note 1: Case reports

Case 1 (Family A, Individual II-1)

Individual 1 is a boy born to healthy non-consanguineous French parents. After a normal pregnancy, delivery was vaginal at 40 weeks of gestation. Growth characteristics at birth were normal: weight 3.48 kg, length 51cm, and occipitofrontal circumference (OFC) 36cm. Until 3 months of age, the child's development was considered normal. At age 3 months, he presented infantile spasms with a hypsarrhythmic EEG pattern, i.e. the West syndrome, associated with signs of failure to thrive. Seizures were refractory to polytherapy including vigabatrin, sodium valproate, clobazam, and corticosteroid. A ketogenic diet, started at age 20 months, initially decreased the frequency of seizures with an improved EEG pattern, but within a few months the seizures worsened, requiring further changes in treatment. On examination, at age 5 years, he had familial (non-dysmorphic) facial features, and still manifested significant failure to thrive despite gastric tube feeding. His length was then 91 cm (-4 SD), weight 10 kg (-4SD) and OFC 47cm (-3.5sd). He was minimally responsive to his environment, had no head control, no language skills, and could not sit up unaided. Axial hypotonia was associated with peripheral hypertonia. Visual pursuit was inconstantly present. Generalized tonic-clonic seizures occurred several times a day despite anticonvulsant polytherapy. Normal testing included plasma and cerebrospinal fluid (CSF) lactate, plasma and CSF amino acids, CSF glucose, neurotransmitters and interferon, urine organic acids, plasma and urine creatine and guanidinoacetate, plasma purines and pyrimidines, biotinidase, very long chain fatty acids, plasma phytanic and pristanic acid, plasma copper and ceruloplasmin. Screening for congenital disorders of glycosylation was done *via* plasmatic glycoprotein analyses. Sanger sequencing of *MECP2*, *STXBPI*, *CDKL5*, *FOXG1* and *ARX* showed no mutation. Brain CT-scan and MRI at the age of 3 months were normal. However,

subsequent brain MRIs at 15 months and 4.5 years showed a thin corpus callosum and hyperintensities in insula subcortical white matter (Figure 1B, a,f).

Case 2 (Family A, Individual II-2)

Individual 2 is brother to Individual 1. Pregnancy and delivery (vaginal, at 40 weeks of gestation) were uneventful. The growth characteristics at birth were normal: weight 3.3 kg, length 50cm, and OFC 35cm. Head control was acquired at age 2 months, but at age 4 months he had infantile spasms and showed signs of failure to thrive. Treatment with clobazam and corticotherapy, together with a ketogenic diet at age 5 months, initially resulted in a moderate reduction of seizures. Blinking reflexes and pupillary light reflexes were decreased but fundus examination and electroretinograms were normal. Corticotherapy was tapered down and stopped at age 7 months. Because of subsequent worsening of the EEG pattern at age 15 months, clobazam was discontinued and clonazepam was added. At age 27 months, he was presenting daily seizures with a prolonged postictal state. The ketogenic diet was stopped and sodium valproate was introduced. However, there was no improvement in seizure control. At age 4 years, he showed familial (non-dysmorphic) features, with severe developmental delay, with no head control, inability to sit up unaided, and no language skills. He presented axial hypotonia with peripheral hypertonia. Despite anticonvulsant polytherapy, generalized myoclonic seizures occurred several times a day. Visual pursuit was inconstantly present. Like his brother, he showed signs of failure to thrive, with length 89 cm (-2SD), weight 9.6kg (-3.5SD) and OFC 47cm (-2SD), leading to the insertion of a gastrostomy feeding tube. Extensive metabolic screening in blood, urine, and CSF was negative. Brain MRI at age 4 months was normal. However, a subsequent MRI at age 3 years showed widening of sylvian fissures (Figure 1B, b,g).

Case 3 (Family B, Individual II-2)

Individual 3 was a girl, the second child of healthy non-consanguineous American parents without a family history. Delivery was vaginal at 35 weeks of gestation after a pregnancy complicated by hyperemesis gravidarum. At birth, her weight was 2.8 kg and the OFC was 33 cm (4th centile); the perinatal evaluation was normal. The seizures, associated with feeding difficulties, began at the age of 2 months. She developed infantile spasms with hypsarrhythmia at the age of 5 months. These spasms proved refractory to drugs. A gastrostomy was placed at the age of 1 year. She acquired the ability to smile at age 13 months, but then lost it. Iterative brain MRI evidenced delayed myelination (Figure 1B, c,h). Normal results were found with assays of urine amino and organic acids, plasma amino acids, urine oligosaccharide and mucopolysaccharides, lysosomal enzymes, plasma VLCFA, and CDG screening. SNP-array and Sanger sequencing of *FOXG1* and of the genes responsible for neuronal ceroid lipofuscinose (*PPT1* and *TTP1*) also showed normal results.

The last physical examination at age 33 months revealed significant developmental delay: incapacity of sitting up unaided, failure to acquire language skills, and inability to smile. She still had seizures despite the ketogenic diet and the multiple seizure medications administered. A lack of visual pursuit associated with a vertical nystagmus was noted. Despite tube feeding, her height remained at the 14th centile, and her weight at the 3rd centile. She had microcephaly (OFC at the 4th centile) but no dysmorphic features. Despite intensive care, her medical condition deteriorated and she died at age 34 months.

Case 4 (Individual II-1, family C)

Individual 4 is a girl, the first-born child of non-consanguineous German parents. She was born by caesarean section at 38 weeks of gestation after a normal pregnancy. The growth characteristics at birth were: weight 3.2 kg (M), length 53 cm (M) and OFC 34.5 cm (M). She

presented with congenital strabismus, which improved spontaneously at age 2 years. The electroretinogram was normal. She underwent bilateral hip dislocation surgery at age 11 months. At age 2 years and 6 months, she started having hypnagogic leg jerks. At age 4 years, another kind of abnormal movement appeared, consisting of involuntary head deviation with arm and leg extension. These movements, lasting less than 10 seconds, occurred only during sleep with a frequency of two or three times a night. They were absent when the child was awake and were efficiently controlled with clobazam. Waking EEG monitoring results were normal but spikes and sharp waves were present on awakening. Brain MRI, performed at age 3 years, showed a mild cerebellar hypoplasia without brainstem anomalies, white matter hyperintensities periventricular region (Figure 1B, d,i). Physical examination at age 6 years showed significant developmental delay: incapacity to sit up without help, and failure to acquire language skills. Axial hypotonia was associated with peripheral hypertonia. She also had severe growth retardation: length 98cm (-4SD), weight 12kgs (-5 D) and microcephaly OFC: 47.5cm (-2.5SD), despite adequate caloric intake. She had no dysmorphic features.

Case 5 (Individual II-2, family D)

Individual 5 is a girl, the fifth child born to non-consanguineous parents originating from Kuwait. She was born at 32 weeks of gestation after a normal pregnancy. Her birth weight was 2.6 kg. She had congenital stridor and required treatment for hyperbilirubinemia. Developmental delay became obvious from the age of 6 months. She was able to maintain mutual gaze but has never been able to sit up unaided or to develop language skills. At age 2 years, her neurological condition suddenly worsened during an acute infectious disease that required intubation and ventilation. At that time, she developed a severe dystonia, which did not improve with baclofen, clonazepam, L-DOPA and valproic acid. Dystonic movements gradually disappeared but, at age 5 years, another acute neurological deterioration occurred,

including spastic/dystonic movements, stridor, and dysphagia. Neurological examination at age 6 years was significant for severe spasticity with dystonia, repetitive and prolonged episodes of opisthotonus, dysphagia and poor visual pursuit. The EEGs were difficult to interpret because of continuous muscle artifacts even when using sedative drugs. However, severe slowing down of the background activity was observed, with some epileptiform discharges over the central and frontal areas. Nevertheless, the movement disorders such as opisthotonus and limb spasms were not caused by epileptic seizures.

In addition, she was microcephalic (OFC -2.4 SD) and showed signs of failure to thrive (length -4.7 SD, weight -4.8 SD) despite adequate caloric intake by enteral tube feeding. At that time, a percutaneous endoscopic gastrostomy was placed because of swallowing difficulties and poor nutritional status. Normal testing included plasma lactate, urine lactate/creatinine ration, urine amino and organic acids, plasma amino acids, plasma biotinidase activity, CDG screening, lysosomal disorder screening, karyotype, and a search for mutations in *SLC19A3*, the gene encoding for the thiamine transporter. Fortuitously, plasma levels of thyroid hormones were increased in the blood because of the existence of familial dysalbuminemic hyperthyroxinemia.

Brain MRI performed at age 9 months showed mild brain atrophy with normal myelination. Subsequent brain MRI at age 5 years showed a severe cortical atrophy with a thin corpus callosum and a cerebellar atrophy without brainstem anomalies (Figure 1B, e,j).

Figure S1. Sequence Alignment of *UBA5*

p.Met57Val

HUMAN	RIEKMSSEVVDSNPYSRL M ALKRMGIVSDYEKIRTFA
ORANGUTANG	RIEKMSSEVVDSNPYSRL M ALKRMGIVSDYEKIRTFA
MOUSE	RIQEMSDEVLDSPYSRL M ALKRMGIVSDYKKIRTYA
CHICKEN	RIETMSPEVTDSNPYSRL M ALKRMGIVKDYEKIRFTT
ZEBRAFISH	KIEQMSAEVVDSNPYSRL M ALKRMGIVQDYEKIRSFA
C-ELEGANS	KIEKLSAEVVDSNPYSRL M ALQRMGIVNEYERIREKT

p.Gly168Glu

HUMAN	NITTVENFQHFMDRIS- N GGLEEGKPVDLVLSCVDNF
ORANGUTANG	NITTVENFQHFMDRIS- N GGLEEGKPVDLVLSCVDNF
MOUSE	NITTVEHFHFHFMNRIS- N GGLEEGQPVDLVLSCVDNF
CHICKEN	NITTLDNFEHFHFMNRIS- N GALEEGKPVDLVLSCVDNF
ZEBRAFISH	NITTMDFNFTHFMDRVRYH G GLEEGKPVDLILSCVDNF
C-ELEGANS	NITTMDFNFDTFVNRIR- K GSLTDG-KIDLVLSCVDNF

p.Val126Met

HUMAN	KTLKREGVCAASLPTTM G VVAGILVQNVLKFLNFGT
ORANGUTANG	KSLKREGVCAASLPTTM G VVAGILVQNVLKFLNFGT
MOUSE	KTLKREGVCAASLPTTM G VVAGILVQNVLKFLNFGT
CHICKEN	KTLKREGVCAASLPTTM G VVAGILVQNVLYFLLNFGT
ZEBRAFISH	KTLKRDGVCAASLPTTM G VVAGLLVQNVLYFLLGFGT
C-ELEGANS	RTLKRDGVCAASLPTTMA V VAGFLVMNVLYFLLNFGE

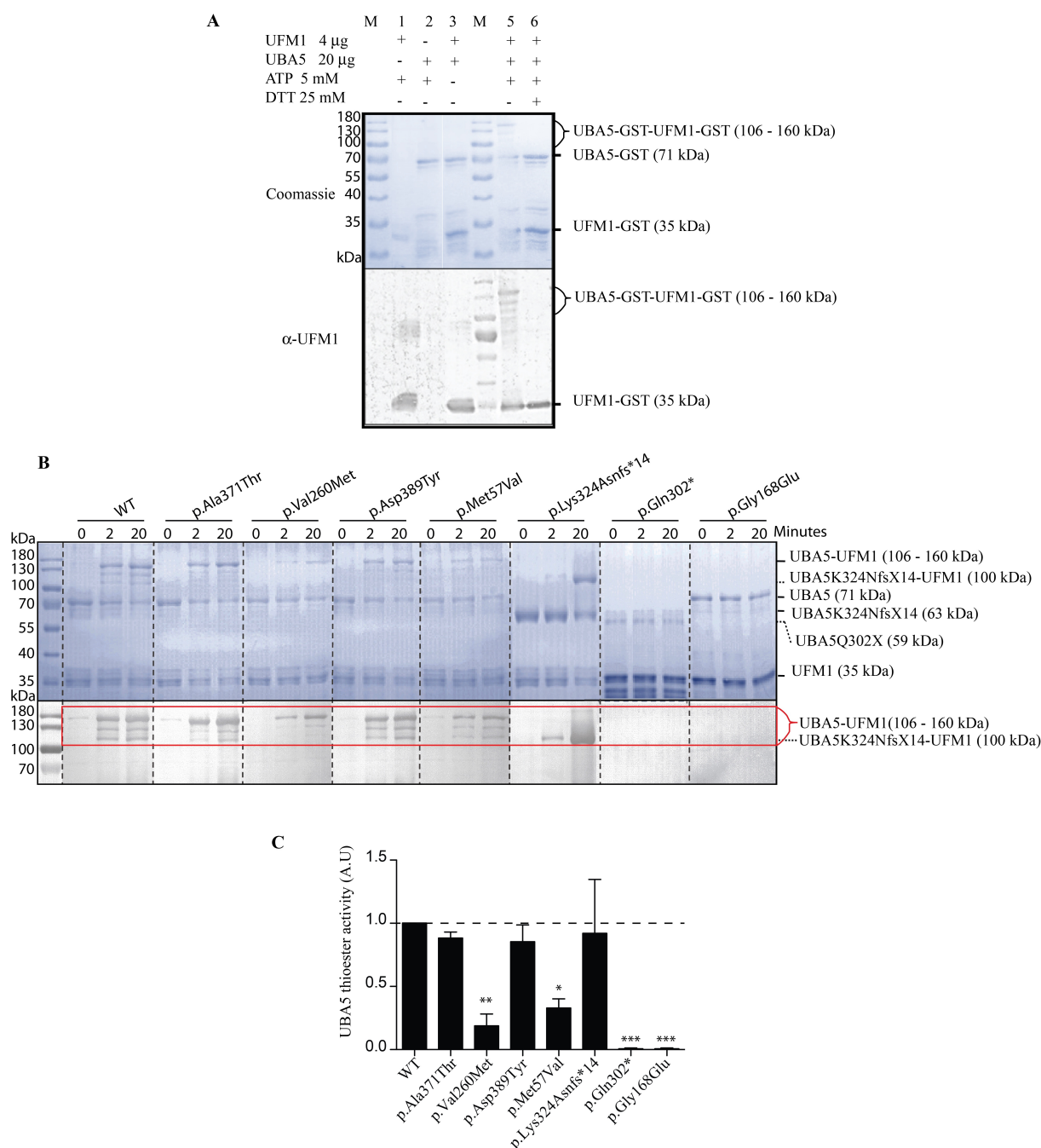
p.Ala371Thr

HUMAN	ELKNFSGVPVDPDLPEGITV A YTIPKKQEDSVTELTVED
ORANGUTANG	ELKNSSGPVDPDLPEGITV A YTIPKKQEDSVTEVTVED
MOUSE	ELKNSSGPVPTLPEGITV A YTVPKKTEDSASEVTVED
CHICKEN	ELKAASGPVDPDLPVGITV A YTIPNKEENLTAEETVAE
ZEBRAFISH	ELQDASGPIPDLPPEGITV A YTIPEKDGG--GETVEE
C-ELEGANS	AEQSS--LNAGTGLKF A YEPIKRDAQT--EL--SP

p.Asp389Tyr

HUMAN	ITVAYTIPKKQEDSVTELTVEDSGESLE D LMAMKMN
ORANGUTANG	ITVAYTIPKKQEDSVTEVTVEDSGESLE D LMAMKMN
MOUSE	ITVAYTVPKKTEDSASEVTVEDSGESLE D LMARMKMN
CHICKEN	ITVAYTIPNKEENLTAEETVAESEE S LE D LMAMKMN
ZEBRAFISH	ITVAYTIPEKDGG--GETVEETE S LE E LMAMKMN
C-ELEGANS	LKFAYEPIKRDAQT--EL--SPAQAATH D FMKSIKDK

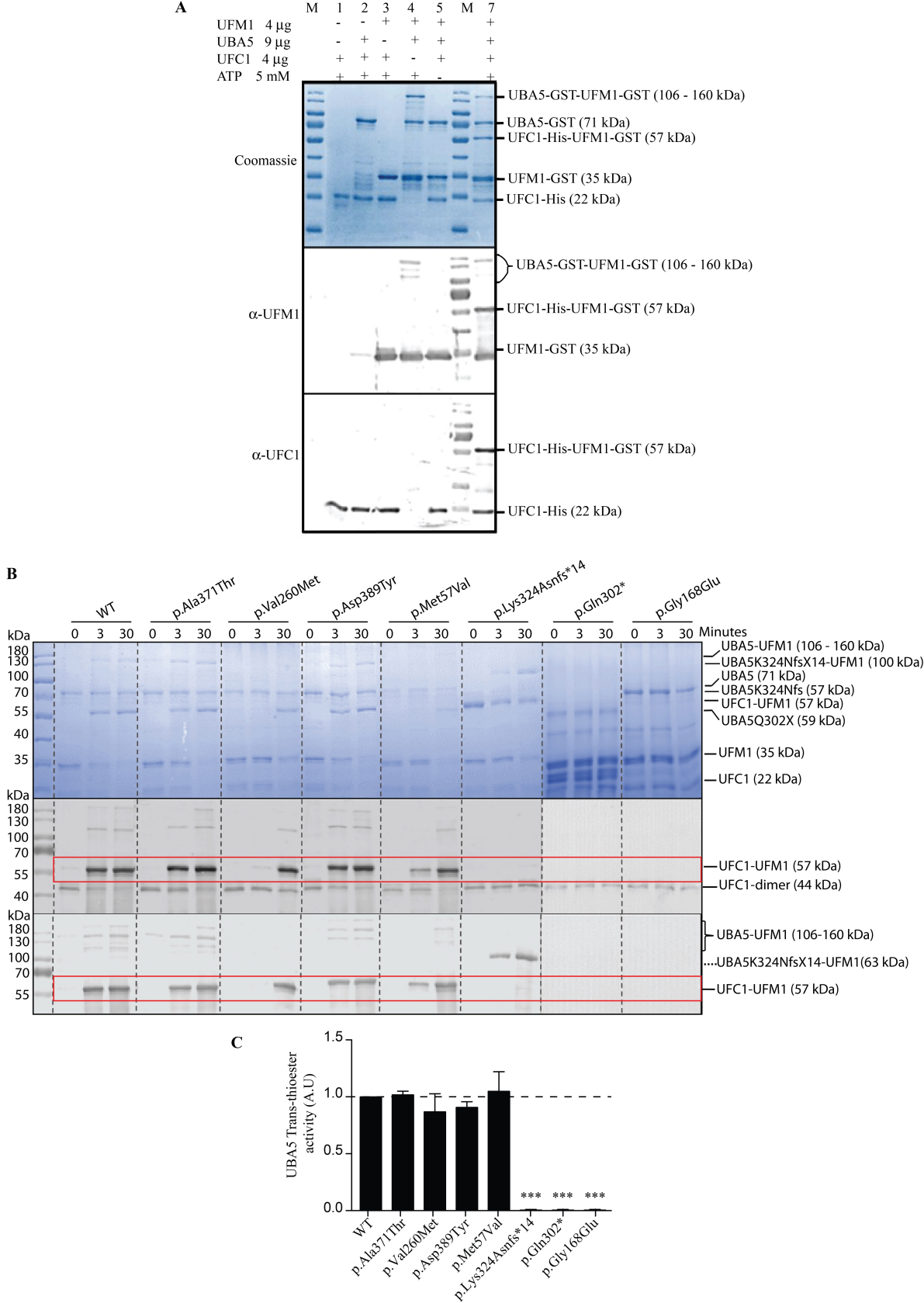
Figure S2: Biochemical Characterization of the Disease-Causing Mutations in UBA5 Protein using the Thioester Assay



(A) **The thioester formation assay** was performed as described¹. In the presence of ATP three bands containing UFM1-GST / UBA5-GST conjugates (range 106-160 kDa) were visible. They were excised and verified using MALDI/TOF mass spectrometry. The addition of DTT in the sample buffer led to the disappearance of these bands, confirming a thioester bond between the proteins. (B) **Time-dependent UFM1 activation assay**. UBA5-GST fusion proteins, each carrying one of the mutations of the affected families, were analyzed in a time-

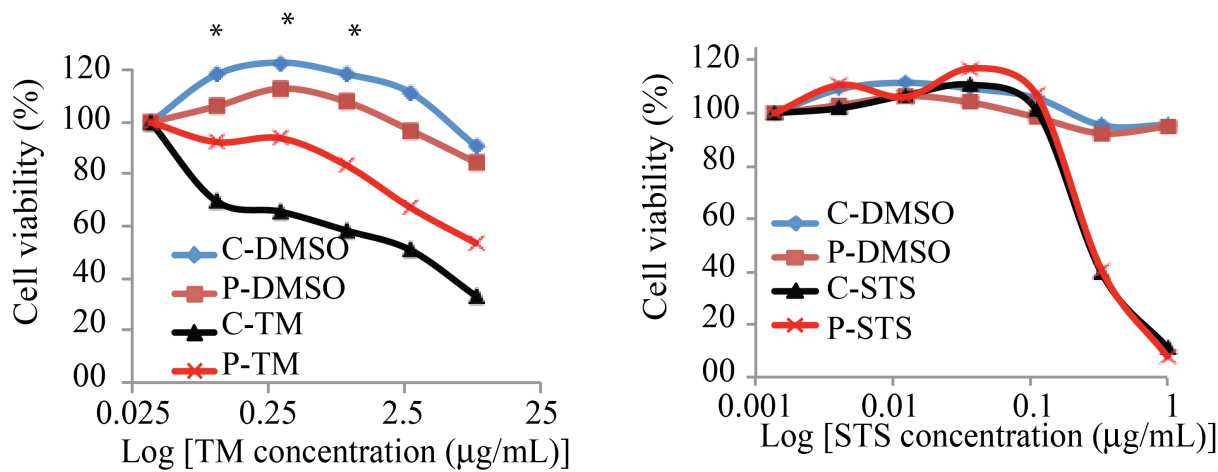
dependent (0, 2 and 20 min) *in vitro* thioester formation assay. Sample reactions were subjected to SDS-PAGE and analyzed by coomassie staining (top) and immunoblotting using anti-UFM1 (1:2000) (bottom). Singular UFM1-GST and UBA5-GST proteins and the formed intermediates are indicated on the right. Three bands appeared (range 106-130 kDa) as a result of the thioester formation, representing UBA5-UFM1 intermediates highlighted by the red box. While the assay with wildtype (WT) UBA5 protein resulted in one band during the first seconds (130 kDa, time 0) and three strong bands (range 106-130 kDa) after 2 minutes (no increase after 20 min), the p.Val260Met and the p.Met57Val proteins displayed a highly significant reduction of UFM1-UBA5 conjugates ($p < 0.001$) after 2 and 20 min. The p.Ala371Thr and the p.Asp389Tyr protein did not display significant differences in the UFM1 activation compared to the wildtype protein. The frameshift mutation p.Lys324Asnfs*14 showed a delayed activity ($p < 0.001$, t-test), but was comparable to the wildtype after 20 min. The p.Gln302* and p.Gly168Glu proteins had no detectable thioester activity. (C) The thioester formation are observed after 20 minutes of incubation. Three independent experiments were used for quantification and statistical analyzes using One way ANOVA, Bonferroni's Multiple Comparaison test, * $p < 0,05$, ** $p < 0,01$, *** $p < 0,001$.

Figure S3: Biochemical Characterization of the Disease-Causing Mutations in UBA5 Protein using the Trans-Thioester Assay



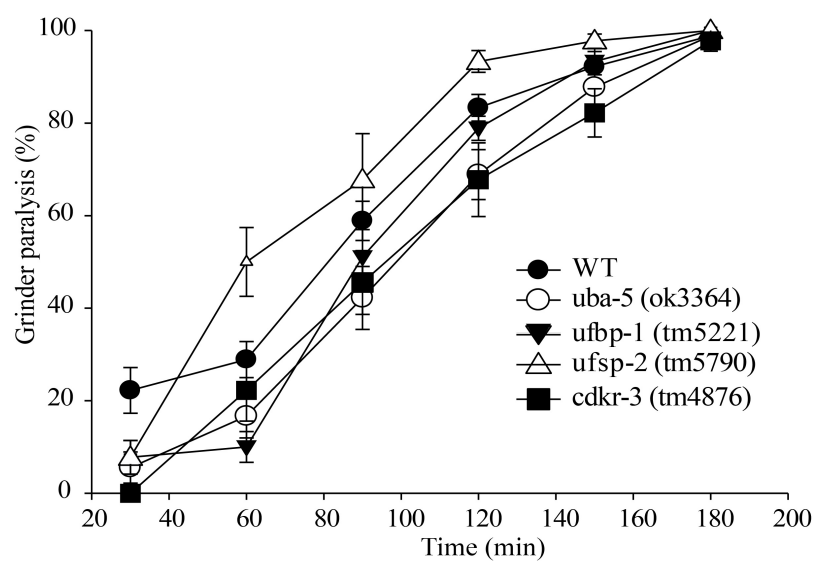
(A) The trans-thioester assay was performed as described¹. In the presence of ATP, UFM1, UBA5 and UFC1, a band at 57 kDa containing UFM1-GST and UBA5-GST was observed additionally to the UFM1-GST / UBA5-GST conjugates at 106-160 kDa, therefore demonstrating trans-thioester activity of UBA5. **(B) Time-dependent UFM1 conjugation assay.** The UBA5-GST fusion proteins were analyzed in a time-dependent (0, 3 and 30 min) *in vitro* trans-thioester formation assay with UFC1. Sample reactions were subjected to SDS-PAGE and analyzed by coomassie staining (top) and immunoblotting using anti-UFM1 (1:2000) (middle), anti-UFC1 (1:10000) (bottom). Bands corresponding to singular UFM-GST, UBA5-GST and UFC-His as well as the intermediates are indicated on the right. Wildtype UBA5 was able to conjugate UFM1 to UFC1 within the first three minutes and the amount of conjugates did not increase significantly after 30 min. The p.Asp389Tyr, the p.Val260Met and the p.Met57Val proteins displayed a significantly reduced density of UFM1/UFC1 conjugate bands after 3 min ($p < 0.05$), while no differences could be observed after 30 min. The conjugation activity of p.Asp389Tyr was not discernable from the wildtype protein. No conjugation activity was observed for the p.Gln302*, p.Gly168Glu and the p.Lys324Asnfs*14 proteins. **(C)** The trans-thioester formation are observed after 30 minutes of incubation. Three independent experiments were used for quantification and statistical analyzes using One way ANOVA, Bonferroni's Multiple Comparaison test, * $p < 0,05$, ** $p < 0,01$, *** $p < 0,001$.

Figure S4: UBA5 Mutant Fibroblasts Are Resistant to Tunicamycin



Cell viability assay after treatment with tunicamycin (TM, left) and staurosporine (STS, right). At day 3, cell viability was determined using Prestobblue Cell Viability reagent. DMSO was used as control in both TM and STS treatment. Abbreviations are as follow: C, wild type fibroblasts; P, *UBA5* mutant fibroblasts. Statistical analyzes: student t-test, $p < 0.05$.

Figure S5: Neurological Characterization of *C. elegans* Strains Deleted for Genes Involved in Ufmylation



Levamisole exposure motility assay (0.4 mM levamisole, 3 h) revealed no significant difference using ANOVA test in the forward movement ability between the UFM-1 cascade deletion mutants and the WT.