

1 **Severe Biallelic Loss-of-function Mutations in *Nicotinamide Mononucleotide***
2 ***Adenylyltransferase 2 (NMNAT2)* in Two Fetuses with Fetal Akinesia Deformation**
3 **Sequence**

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27 **Abstract**

28 The three nicotinamide mononucleotide adenylyltransferase (NMNAT) family members
29 synthesize the electron carrier nicotinamide adenine dinucleotide (NAD⁺) and are essential for
30 cellular metabolism. In mammalian axons, NMNAT activity appears to be required for axon
31 survival and is predominantly provided by NMNAT2. NMNAT2 has recently been shown to also
32 function as a chaperone to aid in the refolding of misfolded proteins. *Nmnat2* deficiency in
33 mice, or in its ortholog *dNmnat* in *Drosophila*, results in axon outgrowth and survival defects.
34 Peripheral nerve axons in NMNAT2-deficient mice fail to extend and innervate targets, and
35 skeletal muscle is severely underdeveloped. In addition, removing NMNAT2 from established
36 axons initiates axon death by Wallerian degeneration. We report here on two stillborn siblings
37 with fetal akinesia deformation sequence (FADS), severely reduced skeletal muscle mass and
38 hydrops fetalis. Clinical exome sequencing identified compound heterozygous *NMNAT2*
39 variant alleles in both cases. Both protein variants are incapable of supporting axon survival in
40 mouse primary neuron cultures when overexpressed. *In vitro* assays demonstrate altered
41 protein stability and/or defects in NAD⁺ synthesis and chaperone functions. Thus, both patient
42 *NMNAT2* alleles are null or severely hypo-morphic. These data indicate a previously unknown
43 role for *NMNAT2* in human neurological development and provide the first direct molecular
44 evidence to support the involvement of Wallerian degeneration in a human axonal disorder.

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50 INTRODUCTION

51 Fetal Akinesia Deformation Sequence (FADS) defines a broad range of disorders unified by
52 absent fetal movement resulting in secondary defects often leading to stillbirth or limited
53 postnatal survival^{1; 2}. These secondary features include edema, hydrops fetalis, craniofacial
54 anomalies including micrognathia, lung hypoplasia, rocker bottom feet, intrauterine growth
55 restriction, and decreased muscle mass³. Through previous experimental models of fetal
56 paralysis, the secondary findings have been shown to be primarily caused by a lack of fetal
57 movement^{1; 4}. FADS has both genetic and environmental causes that can affect any aspect of
58 the motor system including the central nervous system (CNS), peripheral nervous system
59 (PNS), neuromuscular junction (NMJ), and/or skeletal muscle. Although most cases of FADS
60 do not have a genetic diagnosis, multiple monogenic causes of FADS affecting PNS
61 innervation development have been identified to date including *RAPSN*, *DOK7*, *MUSK*⁵⁻⁷.

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63 Through whole exome sequencing and subsequent Sanger sequencing of a family with two
64 fetuses with FADS, we identified compound heterozygous mutations in a gene previously
65 unlinked to FADS, *nicotinamide mononucleotide adenylyltransferase 2 (NMNAT2)*. NMNAT
66 family members were first shown to play a role in axon degeneration with the discovery of the
67 slow *Wallerian Degeneration (Wld^S)* mutant mouse that showed delayed axon degeneration
68 post transection⁸. The *Wld^S* phenotype arose as the result of a spontaneous genomic
69 rearrangement generating a fusion protein of NMNAT1 and the N-terminus of UBE4B, an E4
70 type ubiquitin ligase^{9; 10}. Normally NMNAT1 is located only in the nucleus but the partial

71 axonal location of the fusion protein leads to a gain-of-function explaining the slow Wallerian
72 degeneration phenotype ¹¹.

73 There are three canonical NMNAT isoforms and each displays unique subcellular localization
74 and tissue specific expression. NMNAT1 is nuclear and broadly expressed, NMNAT2 is in the
75 cytoplasm and axoplasm and enriched in the brain. NMNAT3 is proposed to be localized to the
76 mitochondria and has lower expression in the brain ^{12; 13}. The functions of all three have been
77 studied in mice but until now only *NMNAT1* has been linked to human disease. *NMNAT1*
78 mutations cause Leber's Congenital Amaurosis 9 (LCA9) characterized by photoreceptor-
79 neuron degeneration resulting in congenital blindness ¹⁴⁻¹⁷. Two N-ethyl-N-nitrosourea
80 generated *Nmnat1* missense mouse mutants develop photoreceptor degeneration and closely
81 model the pathology observed in LCA9 ¹⁸. In contrast, *Nmnat3* homozygous null mice show no
82 nervous system phenotype and instead develop splenomegaly and hemolytic anemia ¹⁹.
83 Hikosaka, et. al. showed NMNAT3 is the predominant NAD producer in the cytoplasm of
84 mature erythrocytes and loss of *Nmnat3* resulted in defective glycolysis in these cells ¹⁹. To
85 date, no patients with mutations in *NMNAT3* have been identified. These data illuminate the
86 tissue specific requirements for NMNAT family members during development.

87 An essential role for NMNAT2 in axon growth and survival was established first by RNAi in
88 primary neuronal culture and subsequently in *NMNAT2-deficient* mice ²⁰⁻²². Acute removal of
89 NMNAT2 *in vitro* from established axons causes axon degeneration through the Wallerian
90 pathway, while its constitutive deletion in mice causes defects in PNS and CNS axon
91 outgrowth, and consequent underdevelopment of the skeletal muscle which lacks innervation
92 ^{21; 22}. Other features shared with FADS include craniofacial defects and perinatal lethality due
93 to a failure to inflate the lungs at birth ^{21; 22}. Furthermore, RNAi of the *Drosophila* ortholog

94 *dNMNAT* is also sufficient to trigger spontaneous degeneration of established axons and
95 genetic mutation causes growth and survival defects in axons and their presynaptic termini ²³⁻
96 ²⁵. Conversely, overexpression of NMNATs after peripheral nerve transection can delay
97 Wallerian degeneration and rescues all mouse NMNAT2 and *Drosophila* dNMNAT genetic
98 knockdown or deletion phenotypes mentioned above ¹³. Deletion of another Wallerian
99 pathway gene, *Sarm1*, also rescues axonal phenotypes in *NMNAT2-deficient* mice preventing
100 perinatal lethality and allowing survival into old age with no overt behavioral changes ^{26; 27}.
101 These data demonstrate NMNAT2 protects against an active Wallerian Degeneration pathway
102 mediated by SARM1. Recently, it has been discovered that NMNATs including NMNAT2 act
103 as chaperones for protein refolding as well as NAD-synthesizing enzymes ^{24; 28; 29}. *NMNAT2*
104 transcripts have been shown to be decreased in human neurodegenerative diseases and the
105 chaperone function of NMNAT2 has been shown to protect against neurodegeneration in a
106 variety of tauopathy models ^{29; 30}. While it remains controversial which function(s) of *NMNAT2*
107 are neuroprotective, we sought to investigate both functions in our patient variants of
108 *NMNAT2*. Interestingly, we found both functions are impaired. This finding and the striking
109 similarity to the homozygous null mouse phenotype strongly support a causative role for these
110 mutations.

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113 **MATERIALS AND METHODS**

114 **Subjects**

115 Initial exome analysis was performed as a clinical service (Ambry Genetics). Informed consent
116 to study the sequence data on a research basis was obtained according to Cincinnati

117 Children's Hospital Medical Center (CCHMC) institutional review board protocol # 2014-
118 3789. Following consent, residual DNA samples were obtained for Sanger sequencing
119 confirmation of exome sequencing analysis.

120

121 **Sanger Sequencing**

122 Sanger sequencing to confirm the results of whole exome sequencing was performed by PCR
123 amplification of exon 5 of *NMNAT2* with F primer 5'- gaggttcaggagcgatgaaa-3' and R primer 5'-
124 caggagaagagtgcacacca-3' using genomic DNA. Exon 9 of *NMNAT2* was PCR amplified from
125 genomic DNA with F primer 5'- gctcaaatgtgcttgctgaa-3' and R primer 5'- cagacatgggatggttgatg-
126 3'. Conservation and protein prediction scores for *NMNAT2* R232Q variant were generated by
127 SIFT, Polyphen, and MutationTaster algorithms. Schematic of NMNAT2 protein domains were
128 generated from existing literature and functional domains annotated by UniProt by homology.
129 The crystal structure of NMNAT1 (PDB ID: 1kku) in stereo ribbon view was generated by
130 PyMOL (v2.2.3)³¹.

131

132 **Histology**

133 Histology was performed on formalin-fixed, paraffin-embedded patient tissue collected at the
134 time of autopsy. Hematoxylin and eosin staining was performed according to standard
135 methods at the CCHMC Pathology Core Lab and analyzed by attending pathology physicians.

136

137 **Constructs**

138 The R232Q and Q135Pfs*44 *NMNAT2* mutations were introduced separately by QuikChangeII
139 site-directed mutagenesis (Stratagene) into the complete open reading frame of the canonical

140 307 amino acid human NMNAT2 isoform cloned into expression vector pCMV-Tag2
141 (Stratagene). The expressed NMNAT2 proteins have a Flag tag and short linker sequence (17
142 amino acids) at their N-terminus. The presence of the mutations and absence of other PCR
143 errors was confirmed by sequencing (Cogenics). pDsRed2-N1 (Clontech) was used for
144 expression of variant *Discosoma* red fluorescent protein (DsRed) to label micro-injected
145 neurons / neurites. pEGFP-C1 (Clontech) was used for expression of enhanced green
146 fluorescent protein (GFP) to act as a transfection control and reference for NMNAT2 turnover
147 in HEK 293T cells.

148

149 **HEK 293T transfection and stability assays.** HEK 293T cells were cultured in DMEM with
150 4,500 mg/L glucose and 110 mg/L sodium pyruvate (PAA), supplemented with 2 mM glutamine
151 and 1% penicillin/streptomycin (both Invitrogen), and 10% fetal bovine serum. Cells were
152 plated in 24-well format to reach 50-60% confluence for transfection with Lipofectamine 2000
153 reagent (Invitrogen) according to the manufacturer's instructions. In standard turnover
154 experiments (Fig. 4A) 500 ng Flag-NMNAT2 expression construct (wild-type or mutant), 200
155 ng of an empty pCMV-Tag series vector, and 100 ng pEGFP were transfected per well. To
156 boost expression of the NMNAT2^{Q135Pfs*44} mutant 700 ng Flag-NMNAT2 expression construct
157 and 100 ng pEGFP-C1 were transfected per well (Fig. 4C). After treatment $\pm 10 \mu\text{M}$ emetine
158 hydrochloride (Sigma-Aldrich), cells from single wells were lysed directly in 100 μl 2x Laemmli
159 sample buffer and heated to 100°C for 5 mins. Equal amounts of extract (either 10 or 15 μl)
160 were resolved on 12% SDS polyacrylamide gels, transferred to Immobilon-P membrane
161 (Millipore) and probed with antibodies essentially as described previously²⁰. The following
162 primary antibodies were used: mouse monoclonal anti-FLAG M2 (1:2,000 Sigma-Aldrich

163 F3165), mouse monoclonal anti-GFP clones 7.1 and 13.1 (1:2,000, Sigma-Aldrich
164 11814460001) and rabbit polyclonal α -Tubulin (1:7,500, Thermo Fisher Scientific PA5-29444).
165 Appropriate HRP-conjugated secondary antibodies were used for band detection with
166 SuperSignal™ West Dura Extended Duration Substrate (Thermo Fisher Scientific) using an
167 Alliance chemiluminescence imaging system (UVITEC Cambridge). Relative band intensities
168 on captured digital images were determined (area under histogram peaks) using Fiji software
169 (<http://fiji.sc>)³².

170

171 **Microinjections and imaging.** The preparation of dissociated SCG neuron cultures from wild-
172 type P0-P2 pups, microinjections, Flag immunostaining and quantification of neurite survival
173 were all performed essentially as described previously^{20; 22}. Expression vectors and the
174 concentrations used in each specific injection experiment are described in the Figure 3 legend.
175 Fluorescence images were acquired with a Leica DFC365FX fluorescence monochrome
176 camera attached to a Leica DMI8 inverted fluorescence microscope (10x objective). Mean
177 intensities of Flag immunostaining and DsRed fluorescence signals in injected SCG neurons
178 were determined using Fiji software (<http://fiji.sc>) by thresholding (20, dark background)
179 followed by particle analysis (size >250 pixels for 1392x1040 images) to identify neurons with
180 signal intensity above background (the threshold value was subtracted from the mean intensity
181 values obtained)³².

182

183 **NMNAT recombinant protein expression and purification.**

184 For biochemistry assays (Fig. 5A-G), pET28c plasmid constructs were generated for
185 NMNAT2^{R232Q} and NMNAT2^{Q135Pfs*44} pET28c to produce recombinant proteins with an N-

186 terminal His tag and linker (MGSSHHHHHSSGLVPRGSH) for affinity purification that
187 matched a previously generated NMNAT2^{WT} construct³³. Expression was carried out in *E. coli*
188 BL21(D3) cells (Invitrogen) following 0.5 mM IPTG induction for 4 h at 25°C with subsequent
189 purification using TALON chromatography (Clontech) as described³⁴. The purified proteins
190 were desalted on PD-10 columns (GE Healthcare) in 50 mM HEPES/NaOH buffer, pH 7.5, 1
191 mM Tris(2-carboxyethyl)phosphine (TCEP), 20 % glycerol, and stored at -80 °C. Their amount
192 was measured by the Bio-Rad protein assay. Their purity was evaluated on SDS
193 polyacrylamide gels either after Coomassie staining or immunoblotting. Proteins were
194 transferred from gels to Immobilon-P membrane (Millipore) and probed with antibodies as
195 described²⁰. Monoclonal anti-NMNAT2 (1:1,000 Abcam AB5698) or anti-tetra His (0.1 µg/ml
196 Qiagen 34670) were used as primary antibodies, followed by appropriate HRP-conjugated
197 secondary antibodies. SuperSignal™ West Dura Extended Duration Substrate (Thermo Fisher
198 Scientific) was used for detection on an Alliance chemiluminescence imaging system (UVITEC
199 Cambridge).

200

201 **NMNAT enzymatic activity assay.**

202 Routine assays were done by a spectrophotometric coupled method as described, in 0.5 mL
203 mixtures containing 30 mM HEPES/NaOH buffer, pH 7.5, 0.5 mg/mL bovine serum albumin
204 (BSA Sigma-Aldrich A7906), 75 mM ethanol, 30 mM semicarbazide (Sigma-Aldrich S2201),
205 12.5 U/mL alcohol dehydrogenase (ADH Sigma-Aldrich A7011)³⁵. NMNAT2^{WT} was assayed at
206 25 mM MgCl₂ and 1 mM of both ATP and NMN (Fig. 5C). The mutant R232Q was assayed at
207 5 mM MgCl₂, 5 mM Mg-ATP, and 1 mM NMN (Fig. 5D). For Mg²⁺-dependence studies the
208 MgCl₂ was increased up to 100 mM. Temperature studies were carried out under various

209 treatments as indicated (Fig. 5E-G) using apo-enzyme solutions in buffer. With assays at 52
210 °C, the reaction mixture at the end of incubation was cooled down to 37 °C and then enzyme
211 was re-added to check for activity recovery, thus ruling out heat inactivation of the ancillary
212 enzyme ADH. The K_m and K_{cat} values were calculated at 37 °C as described using 0.5-5 mM
213 Mg-ATP and 0.05-1 mM NMN for the mutant R232Q, or 0.05-0.6 mM ATP and 0.01-0.15 mM
214 NMN for the wild type ³⁶. Due to the known instability of NMNAT2 preparations after thawing,
215 enzyme was always added as the last component to start the reaction, and control assays
216 were performed in parallel ³⁴. One Unit (U) of NMNAT activity refers to the amount of enzyme
217 that forms 1 μ mol/min of product at the indicated temperature.

218

219 **Gel filtration.** Gel filtration of pure NMNAT2^{R232Q} was carried out by FPLC with a Superose 12
220 HR 10/30 column (Amersham Pharmacia), equilibrated with 50 mM HEPES/NaOH buffer, pH
221 7.5, 0.15 M NaCl, 1 mM DTT. Bovine serum albumin, ovalbumin, and carbonic anhydrase
222 were used as the standards.

223

224 **In-cell luciferase refolding assay.**

225 HEK 293T cells were cultured in six-well plates and double transfected using jetPRIME
226 transfection reagent (VWR International, Radnor, PA, USA) with pCMV-luciferase, and one of
227 the following plasmids: pDsRed2 vector (control), pCMV-Hsp70, pCMV-Nmnat3, pCMV-
228 Nmnat2^{WT}, pCMV-NMNAT2^{R232Q}, and pCMV-NMNAT2^{Q135Pfs*44}. At 48 hrs after transfection,
229 protein synthesis was inhibited by adding 1 μ g/ml cycloheximide. Cells were subjected to heat
230 shock at 42 °C for 45 mins, and then recovered at 37 °C for 3 hours ³⁷. Cells were lysed in
231 lysis buffer containing 100 mM KCl, 20 mM HEPES, 5% glycerol, 0.1% Triton X-100, and 1mM

232 dithiothreitol. Luciferase activity was measured with the Luciferase Assay System (Promega,
233 Madison, WI, USA).

234

235 **Statistics.** Statistical testing of data was performed using Excel (Microsoft) or Prism
236 (GraphPad Software Inc., La Jolla, USA). The specific tests used are described in Figure
237 legends.

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241 **RESULTS**

242 **Clinical Summary of two fetuses with FADS**

243 **Fetus 1.** Fetus 1 was born to a 32yo Caucasian female evaluated for non-immune hydrops
244 fetalis identified at 21 weeks gestation by ultrasonography. Ultrasonography identified multiple
245 abnormalities including cystic hygroma, skin edema, ascites, and pleural effusion. Fetal MRI
246 confirmed these findings and revealed profound hydrocephalus and cystic hygroma (Fig.
247 1A,B). The fetus was motionless though there was a normal amount of amniotic fluid.
248 Amniocentesis showed a normal 46, XX karyotype and microarray analysis was negative for
249 aneuploidy. Whole genome chromosome SNP microarray analysis was normal as well. Alpha
250 fetoprotein was elevated and viral PCRs for toxoplasmosis, parvovirus, cytomegalovirus, and
251 HSV were all negative.

252 Fetal echocardiogram showed normal fetal cardiac anatomy, function, and rhythm
253 approximately 2 weeks prior to delivery. Fetal MRI showed a head circumference that
254 appeared to be within the normal range but showed evidence of severe dilation of the lateral

255 and third ventricles (Fig.1A). The pons, cerebellum, and spinal cord were thin. Both kidneys
256 were below the 5th percentile in size for gestational age. Contrary to the findings in *MNMAT2-*
257 *deficient* mice, there were no abnormalities in the bladder either grossly or microscopically ²¹.
258 Both extremities remained in position and muscle planes were markedly diminished during
259 fetal MRI. The fetus was delivered stillborn at approximately 27 weeks gestation.
260 Fetal autopsy was performed at Cincinnati Children's Hospital Medical Center. Gross
261 inspection identified multiple congenital anomalies including: hydrops fetalis, cystic hygroma,
262 bilateral hypoplastic lungs, hydrocephalus, hypoplastic cerebellum, severely reduced skeletal
263 muscle mass or absence, flexion contractures of all extremities, micrognathia, cleft palate, and
264 hydroptic placenta (Table 1). All tissues were extremely edematous with focal hemorrhage of
265 soft tissues. The musculature was very poorly developed and nearly absent in all extremities.
266 The lungs were hypoplastic. The placenta was grossly hydroptic with evidence of
267 chorioamnionitis and very friable, spongy, dark red tissue with no focal areas of discoloration
268 or evidence of infarcts. The umbilical cord had an eccentric insertion and the fetal membranes
269 were not discolored. The spinal cord was thin and poorly developed throughout most of its
270 length. A frozen section of the quadriceps muscle showed no recognizable skeletal muscle
271 tissue and appeared to be composed essentially of immature fat tissue. There were no
272 inflammatory cell infiltrates or evidence of degenerating or dysplastic skeletal muscle fibers.
273 The brain was poorly developed and collapsed when the calvaria was opened along the suture
274 lines. Evaluation of the brain was severely limited due to autolytic changes, however, sections
275 showed very immature neural glial tissue and germinal matrix. Only one slide showed a few
276 areas of recognizable cortex.
277

278 Given this constellation of phenotypes, the cause of death in fetus 1 was determined to be
279 hydrops fetalis with multiple congenital anomalies and fetal akinesia deformation sequence
280 (FADS). It is unlikely the phenotypes were due to a congenital myopathy based on evaluation
281 of the muscle biopsy taken at autopsy.

282

283 **Fetus 2.** Fetus 2 was a second stillborn fetus of the same parents with an intervening healthy
284 child (Fig.2A). The genetic analysis described below was used for prenatal counseling and
285 fetus 2 was the product of prenatal genetic diagnosis/*in vitro* fertilization (PGD/IVF) and was
286 intended to be a carrier for *NMNAT2* but developed severe hydrops at 16 weeks gestation as
287 determined by Ultrasound (US). Given the prognosis of fetus 1, fetus 2 was delivered at 23
288 weeks gestation by Cesarean section and submitted for autopsy. Fetus 2 was diagnosed with
289 hydrops fetalis with multiple fetal anomalies similar to fetus 1. Upon gross inspection, the fetus
290 displayed hydropic changes including diffuse body wall and soft tissue edema with prominent
291 nuchal fold (Fig. 1C). Markedly, there was apparent absence of skeletal muscle, especially that
292 of the shoulder, extremities, pelvic girdle and absence of the psoas muscles, bilaterally (Fig.
293 1C-E). Long bone formation appeared adequate in length but slender with reduced formation
294 of the femoral neck and trochanters. Sections of the limbs showed adequately formed long
295 bones with adequate bony trabecular marrow space with scant trilineage hematopoiesis. The
296 surrounding tissue was composed of mainly fibroadipose tissue in an edematous background
297 with very few skeletal muscle fibers (Fig. 1H, J). The nuclei of the skeletal muscle were plump
298 and evenly distributed at the edges of the pink proteinaceous myocyte fibers (Fig. 1I).The
299 carpal cartilages were fused. Both the upper and lower limbs showed an abnormal absence of

300 bundling of skeletal muscle fibers. The hands and feet had an unusual flattened appearance
301 with severe contractures but adequate ray and digit bony formation (Fig. 1F,G).

302 The heart was of normal weight with apparently normal myocardium, indicating the skeletal
303 muscle was affected but the cardiac muscle was spared (Fig. 1K). The lungs were hypoplastic
304 with normal lobar formation and no focal lesions or other abnormalities. No lesions were
305 identified in the kidneys or bladder. The brain showed no significant gyration as expected for
306 gestational age.

307 The placenta showed variable villous immaturity with irregular contours and occasional
308 trophoblastic inclusions. There was a vascular distribution of fetal thrombotic vasculopathy.

309 The cause of death for fetus 2 was likely related to placental insufficiency with placental
310 immaturity, hydrops fetalis, and high-grade fetal thrombotic vasculopathy. Fetus 2 thus
311 displayed many features of FADS including severely diminished muscle mass, pulmonary
312 hypoplasia, joint contractures, and micrognathia.

313

314 **Exome Sequencing and Filtering**

315 Genetic analysis was performed upon birth of fetus 1. Karyotyping and whole genome SNP
316 microarray were normal suggesting a monogenic disorder. Clinical whole exome sequencing of
317 the trio (Father, Mother, and Fetus 1) (Fig. 2A) was performed at Ambry Genetics. Mean
318 coverage was 77.0, 105.6, and 86.7 reads per base for the father, mother and fetus,
319 respectively. Filtering was performed by Ambry as detailed in Table S1. Briefly, multiple
320 inheritance models were tested resulting in 26 potential gene candidates (with 40 total coding
321 alterations) over all models. Manual review was performed for sequencing artifacts, known
322 polymorphisms, additional artifacts and benign alterations.

323 *NMNAT2* was identified as the remaining candidate in an autosomal recessive model with two
324 unique alterations suggesting a compound heterozygous inheritance pattern (Supp Table 1).
325 By co-segregation analysis, both parents were found to be heterozygous for one of the two
326 mutations identified in fetus 1. *NMNAT2* was deemed clinically novel as no patients have been
327 identified to date but there is significant phenotypic overlap between the patient's phenotype
328 with a mouse model of *NMNAT2* deficiency and the mutations are predicted to be damaging by
329 SIFT and Polyphen ^{21; 22}. When fetus 2 was diagnosed with such similar features, *NMNAT2*
330 was directly tested with Sanger sequencing and found to have the same compound
331 heterozygous variants.

332

333 ***NMNAT2* Variants**

334 The maternally inherited variant was a single duplication of a cytosine at position 403 in exon 5
335 resulting in a frameshift and premature stop after 44 amino acids in *NMNAT2* (c.403dupC,
336 p.Q135Pfs*44; confirmed by Sanger Sequencing; Fig. 2B,D). The paternally inherited variant
337 was a missense mutation in exon 9 (c.695G>A, p.R232Q; confirmed by Sanger Sequencing;
338 Fig. 2B) resulting in a coding change of arginine to glutamine at position 232 (R232Q).
339 Conservation alignment shows R232 is highly conserved to *D. melanogaster* and has a
340 PhastCons score of 1 (Fig. 2C). Multiple algorithms predict this to be damaging sequence
341 change (e.g., PolyPhen score of 0.97, SIFT 0.0 and is predicted to be “disease causing” by
342 MutationTaster. The family has one unaffected daughter who was identified to carry only one
343 of the *NMNAT2* mutations. This finding supports a recessive model in which both affected
344 alleles must be inherited in order to develop FADS.

345 Although the human NMNAT2 crystal structure has not been characterized, it is predicted that
346 the enzyme activity domains share the same structure folds as those of human NMNAT1 and
347 NMNAT3³⁸. The R232 equivalent residue is invariant in all three human NMNAT isoforms and
348 NMNAT homologs across distant phyla (Fig. 2C), suggesting its importance in protein function.
349 Given that the R232 residue is located within the conserved region, we examined the crystal
350 structure of human NMNAT1 to evaluate the potential structure-function consequences of the
351 R232Q mutation in NMNAT2³⁹ (Fig. 2E). The residue is located at the end of a β strand
352 connecting the substrate binding domains for NMN and ATP, suggesting it is part of a
353 conformational change upon binding the adenine group of ATP substrate or NAD/NaAD³⁹. It is
354 predicted that the significant change in the side chain from arginine to glutamine will alter the
355 electrostatic distribution of the substrate binding sites. In addition, R232 is at the bend between
356 the β strand and a helix, positioned at the surface of the protein that likely participates in the
357 interface of protein-protein interaction (Fig. 2E).

358

359 **Both the NMNAT2^{R323Q} and NMNAT2^{Q135Pfs*44} variants have reduced capacity to delay**
360 **Wallerian Degeneration**

361 Overexpression of mouse or human (Flag-tagged) NMNAT2 is sufficient to delay Wallerian
362 degeneration in cultured mouse superior cervical ganglion (SCG) neurons. This affords us a
363 ready assay to measure the capacity of a variant to support axon survival^{20; 40}. We therefore
364 assessed whether this property is affected by the NMNAT2^{R232Q} or NMNAT2^{Q135Pfs*44} variants
365 found in the affected patents. We introduced expression vectors for Flag-tagged wild-type or
366 variant NMNAT2 into SCG neurons by microinjection. We used a concentration for which the
367 resulting expression from the Flag-NMNAT2^{WT} construct preserves integrity of the majority

368 (~70%) of neurites of the injected neurons for at least 24 hours after transection. Under these
369 conditions we found little or no preservation of cut neurites from SCG neurons injected with
370 either Flag-NMNAT2^{R232Q} or Flag-NMNAT2^{Q135Pfs*44} expression vectors (Fig. 3A, B). This lack
371 of protection is comparable to the lack of protection seen after injection with empty vector or
372 eGFP expression vector^{20; 40}.

373

374 Importantly, even when using 2.5 times the vector concentration previously used in the
375 Wallerian degeneration assays, expression of the NMNAT2^{Q135Pfs*44} variant was barely
376 detectable above background by Flag immunostaining in injected neurons (Fig. 3C). In
377 contrast, robust expression of Flag-NMNAT2^{R232Q} was observed that closely matched that of
378 Flag-NMNAT2^{WT} (Fig. 3C). Therefore, if the truncated Flag-NMNAT2^{Q135Pfs*44} mutant retains
379 any functionality, its inability to protect transected neurites in this assay could simply reflect
380 very low levels of expression, whereas the failure of Flag-NMNAT2^{R232Q} to protect must
381 instead reflect either much more rapid loss of the mutant protein after injury relative to Flag-
382 NMNAT2^{WT}, or a substantial loss of function.

383

384 **NMNAT2^{Q135Pfs*44} variant produces an unstable protein whereas NMNAT2^{R232Q} variant is**
385 **slightly more stable than wild-type NMNAT2**

386 To investigate whether the stability of either variant Flag-NMNAT2 protein is altered relative to
387 Flag-NMNAT2^{WT}, we assessed their relative rates of turnover in transfected HEK 293T cells
388 after a protein synthesis block. Expression of the exogenous proteins was kept low to avoid
389 saturation of the degradation machinery. Levels of Flag-NMNAT2^{WT} and Flag-NMNAT2^{R232Q} at
390 the start of the protein synthesis block were comparable, whereas Flag-NMNAT2^{Q135Pfs*44}

391 levels were greatly reduced (Fig. 4A, B). Flag-NMNAT2^{Q135Pfs*44} migrates at the expected size
392 for the truncated protein but, intriguingly, Flag-NMNAT2^{R232Q} consistently migrates slightly
393 slower than Flag-NMNAT2^{WT} (Fig. 4A). To give a more representative comparison of turnover
394 rate of the Flag-NMNAT2^{Q135Pfs*44} mutant we increased its expression to better match starting
395 levels of the Flag-NMNAT2^{WT} and Flag-NMNAT2^{R232Q} (Fig. 4C). In broad agreement with
396 previous analyses, we saw almost complete loss of Flag-NMNAT2^{WT} within the 8 hour
397 timeframe of these assays with only ~25% remaining at 2 hours (Fig. 4A,D)^{20; 40}. In
398 comparison, Flag-NMNAT2^{Q135Pfs*44} was undetectable on blots even at 2 hours, even from the
399 highest starting levels (Fig 4C), whereas significantly more Flag-NMNAT2^{R232Q} was detectable
400 at both 2 and 4 hours (Fig. 4A,D). Notably however, Flag- NMNAT2^{R232Q} was also almost
401 completely lost by 8 hours (Fig. 4A,D).

402

403 These data indicate that Flag-NMNAT2^{Q135Pfs*44} is much less stable than Flag-NMNAT2^{WT}
404 whereas Flag-NMNAT2^{R232Q} is modestly more stable. The reduced stability of Q135Pfs*44
405 Flag-NMNAT2 could thus partly explain both its lower expression level in SCG neurons and
406 transfected HEK cells and its greatly reduced capacity to protect injured axons (above), even
407 in the unlikely event the severely truncated protein remains functional. In contrast, the slightly
408 increased stability of Flag- NMNAT2^{R232Q} instead suggests that its lack of axon-protective
409 capacity is likely due to a loss of one or more other functional properties.

410

411 **NMNAT2^{R232Q} variant displays impaired NAD synthase and chaperone functions**

412 Recombinant human NMNAT2^{WT}, NMNAT2^{R232Q} and NMNAT2^{Q135Pfs*44} were obtained as His-
413 tagged fusion proteins after bacterial expression and His-tag affinity chromatography.

414 Typically, this yielded ~2 mg of relatively pure recombinant protein per 0.5 L of bacterial culture
415 for both NMNAT2^{WT} and NMNAT2^{R232Q} (Fig. 5A and 5B). Notably, the purified NMNAT2^{R232Q}
416 was found to have NMNAT activity of just 0.51 ± 0.04 U/mg in these preparations compared to
417 11.2 ± 0.23 U/mg for purified NMNAT2^{WT} (Fig. 5C). In contrast, NMNAT2^{Q135Pfs*44} purifications
418 yielded 0.1 mg or less of protein per 0.5 L of bacterial culture with a ~22 kDa His-tagged
419 protein, corresponding in size to NMNAT2^{Q135Pfs*44}, seemingly a relatively minor component of
420 the preparations (Fig. 5A and 5B). This is consistent with low level expression of the truncated
421 protein in bacteria, as in mammalian cells. While the highly heterogeneous NMNAT2^{Q135Pfs*44}
422 preparations did have detectable NMNAT activity, size-exclusion and ion exchange
423 chromatography revealed that none of the activity was associated with the 22 kDa protein
424 species (not shown). Instead, the invariant presence of a ~34 kDa protein recognized by both
425 anti-His and anti-NMNAT2 antibodies (Fig. 5A and 5B), and thus likely to be His-tagged
426 NMNAT2^{WT}, probably accounts for any activity in these preparations. Although the origin of this
427 full-length protein remains unknown (correction of the frameshift mutation in the construct by
428 ribosomal frameshifting or transcriptional slippage in bacteria is one possibility), this analysis
429 suggests that the truncated NMNAT2^{Q135Pfs*44} protein is inactive as expected.

430

431 The purity of NMNAT2^{R232Q} preparations allowed us to perform further characterization that
432 was not possible for NMNAT2^{Q135Pfs*44}. NMNAT2^{R232Q} was eluted as a monomer following size-
433 exclusion chromatography and was stable at -80 °C for months but was progressively
434 inactivated after thawing, similar to wild type NMNAT2^{34; 41}. There was also a linear decline of
435 NMNAT activity for NMNAT2^{R232Q} at Mg²⁺ concentrations above 5 mM (Fig. 5D) so all
436 subsequent assays were performed at MgCl₂ concentrations only marginally exceeding the

437 ATP concentration (see Methods) thus avoiding the large excess of free Mg^{2+} ions in solution
438 usually employed for assaying NMNAT2^{WT} 35; 36. Further assays revealed a relative thermal
439 resistance and stability of NMNAT2^{R232Q} which showed a markedly higher residual activity, at
440 least relative to its lower baseline (Fig. 5C), than NMNAT2^{WT} after 1 hour incubations at
441 temperatures ranging from 25 °C to 47 °C (Fig. 5E) and, at ~40 min, the activity half-life of
442 NMNAT2^{R232Q} at 37 °C was more than twice that of NMNAT2^{WT} (Fig. 5F). Nevertheless, the
443 optimum temperature for activity was the same for NMNAT2^{WT} and NMNAT2^{R232Q} (Fig. 5G).
444 Crucially, however, the R232Q mutation had a profound negative effect on kinetic properties of
445 the enzyme: K_{cat} was found to be reduced by ~20-fold and the K_m values for NMN and ATP
446 were both increased ~10-fold (Table 2). These striking changes predict a ~200-fold reduced
447 catalytic efficiency (K_{cat}/K_m) of NMNAT2^{R232Q} compared to NMNAT2^{WT} (Table 2). In fact, the
448 loss of catalytic activity may even be greater *in vivo* where physiological concentrations of ATP
449 (~1 mM) and NMN (5 μ M) in brain predict a 500-fold or greater reduction compared to the wild
450 type enzyme 42.

451
452 Together, these data suggest that both NMNAT2^{R232Q} and NMNAT2^{Q135Pfs*44} have a substantial
453 loss of NMNAT activity. Although the R232Q mutation makes the enzyme slightly more
454 resistant to heat denaturation *in vitro*, any increased stability is likely to be completely negated
455 by the substantial detrimental effect it has on catalytic activity. In contrast, reduced
456 expression/stability and impaired catalytic activity (largely predicted from the absence of key C-
457 terminal motifs resulting from truncation) likely combine to severely impair the activity of
458 NMNAT2^{Q135Pfs*44}. The presence of only NMNAT2^{R232Q} and NMNAT2^{Q135Pfs*44} in neurons would

459 thus be predicted to be highly limiting for NMN consumption and NAD⁺ biosynthesis in axons,
460 thereby limiting survival.

461

462 To characterize the chaperone function, we used an in-cell luciferase refolding assay to
463 measure the ability of NMNAT isoforms to facilitate the refolding of unfolded luciferase after
464 heat shock^{43; 44} (Fig. 6). Chaperones may act as “holdases” to protect their client protein from
465 unfolding, or as “foldases” to assist the folding to the native state^{45; 46}. The in-cell luciferase-
466 refolding assay allows the measurement of the luciferase unfolding after heat shock (red bars),
467 as well as the luciferase refolding after recovery (green bars)^{43; 44}. We found that both
468 NMNAT2^{WT} and NMNAT2^{R232Q} greatly protected luciferase from unfolding during heat shock,
469 indicating strong “holdase” activity (Fig. 6, red bars). However, when “foldase” activity was
470 analyzed, we found a remarkable loss of foldase activity specifically in NMNAT2^{R232Q}
471 expressing cells, while NMNAT2^{WT} facilitated the refolding of luciferase after heat shock,
472 comparable to heat shock protein 70 (Hsp70) and NMNAT3 (Fig. 6, green bars). Compared to
473 NMNAT2^{WT} and NMNAT^{R232Q}, NMNAT2^{Q135Pfs*44} did not exhibit either “holdase” or “foldase”
474 activity, indicating a lack of stable or functional protein.

475

476 Collectively, these biochemical and cellular analyses revealed two functional consequence of
477 the NMNAT2 mutation p.R232Q: a significant loss of enzymatic activity and a complete loss of
478 chaperone foldase activity. Considering the essential neuronal maintenance function of
479 NMNAT2^{20; 47; 48}, disruption of both activities provides the molecular basis for compromised
480 embryonic metabolism and neuronal development, contributing to FADS.

481

482 **DISCUSSION**

483 The critical role of NMNAT2 in promoting axon survival in mice has been well established
484 using *in vitro* and *in vivo* models. Declining NMNAT2 levels have been associated with a
485 variety of neurodegenerative diseases, including Alzheimer's disease and other tauopathies ^{24;}
486 ^{25; 29; 47; 49}, but a direct role in human disease causation has not previously been demonstrated.
487 Here we report two related fetuses that are both compound heterozygous for severe loss-of-
488 function *NMNAT2* alleles. They both present with a FADS phenotype closely resembling that of
489 homozygous null mice. Thus, for the first time, we present strong evidence that *NMNAT2* loss
490 of function causes a human disorder.

491
492 We performed detailed molecular analyses of the *NMNAT2* variants to support our claims. We
493 first tested the ability of the variants to protect axons in a Wallerian Degeneration model.
494 Consistent with previous reports, the *NMNAT2*^{WT} construct was able to protect nearly 75% of
495 axons from Wallerian Degeneration. However, both *NMNAT2* variants were severely
496 compromised in their ability to delay degeneration with 10% or less of neurites remaining intact
497 24 hours post transection. These data argue both patient variants significantly impair *NMNAT2*
498 function in PNS axons.

499
500 We also performed *in vitro* experiments to specifically query enzymatic and chaperone
501 functions for *NMNAT2*. We most conclusively demonstrated severe loss-of-function of the
502 *NMNAT2*^{R232Q} variant. The strong conservation of the R232 residue across evolutionarily
503 distant *NMNAT* homologues suggested that the R232 residue is functionally relevant. The
504 invariable R residue is at the end of a β strand connecting the substrate binding domains for

505 NMN and ATP, therefore we predicted the R232Q mutation likely affects substrates and NAD⁺
506 binding. Indeed, we found the R232Q substitution substantially reduces affinity for both
507 substrates and largely abolishes NMNAT activity of the variant protein. Furthermore, R232
508 forms the bend between the β strand and a helix and is positioned at the surface of the protein
509 that likely participates in the interface of protein-protein interactions. The reduced refolding
510 activity of NMNAT2^{R232Q} is also thus consistent with reduced protein-protein interaction(s) with
511 the cellular refolding machinery as a result of the missense mutation. Interestingly,
512 NMNAT2^{R232Q} also consistently shows retarded migration during electrophoresis. While the
513 R232Q missense mutation could influence migration by altering the charge and/or structural
514 rigidity of the protein, the possibility that it might be the result of altered posttranslational
515 modification also needs to be considered, especially in the context of the loss-of-function and
516 increased stability of this variant.

517

518 The relative instability of the NMNAT2^{Q135Pfs*} variant protein largely precluded the same degree
519 of functional assessment. However, this instability is probably sufficient on its own to explain
520 the observed loss-of-function in our assays. Nevertheless, the fact that the frameshift mutation
521 results in a truncated protein lacking its entire C-terminal half, including many residues that are
522 critical for ATP binding, makes it extremely likely that NMNAT2^{Q135Pfs*44} will also be defective
523 for NMNAT activity and chaperone function. Interestingly, because we expressed
524 NMNAT2^{Q135Pfs*44} from an intronless construct in our assays, its relative instability likely reflects
525 an increased susceptibility of the truncated peptide to direct proteolytic cleavage. However, it
526 remains possible that nonsense mediated decay of the aberrant mRNA could also further limit
527 expression the FADS cases.

528

529 Importantly, the FADS phenotype seen in the human patients shows broad overlap with that of
530 *MNMAT2-deficient* mice, in particular the severely reduced skeletal muscle mass and akinesia,
531 which are both likely due to failed peripheral innervation^{21; 22}. However, the human cases and
532 the mouse model also show a number of notable differences. First, viability is further reduced
533 in humans; fetal patient demise occurs at around 27 weeks in gestation whereas *MNMAT2-*
534 *deficient* mice die perinatally. Second, at least one patient developed hydrocephalus in which
535 the cortex was essentially spared. We do note *Nmnat2* shows strong expression in the CNS
536 and PNS and therefore it is possible that *Nmnat2* deficiency is related to the hydrocephalus⁵⁰.
537 Third, the bladder is consistently distended in the mouse model but we did not identify defects
538 in the bladder of either patient. Crucially, these differences could all be related to significantly
539 longer gestation and longer axons in humans than mice which likely allow for more severe
540 neurodegeneration in humans *in utero* and an increased likelihood of fetal demise⁵¹. In
541 addition, some symptoms specific to the human cases, including cystic hygroma, ascites, and
542 edema are likely to be a consequence of the fetal demise *in utero*. Interestingly, mice
543 nullizygous for other FADS-associated genes, such as *Dok7* and *Musk*^{52; 53}, have a phenotype
544 remarkably similar to *MNMAT2-deficient* mice, providing additional support for a direct link
545 between the *NMNA2* loss-of-function alleles in these cases and their FADS presentation.

546

547 There is strong evidence in the literature from several independent groups suggesting that
548 NMNAT2 enzymatic activity is the key activity for preventing activation of Wallerian-like axon
549 degeneration and that enzyme dead / chaperone competent mutants broadly fail to protect
550 axons¹³. At the moment it is not known whether NMNAT2 chaperone function also contributes

551 to axon protection and the finding that blocking the Wallerian degeneration pathway by
552 removal of SARM1 “fully” rescues axon defects and survival of mice lacking NMNAT2
553 suggests that chaperone activity is dispensable for survival or overt health in mice, at least in
554 the context of a relatively non-stressful home cage environment ^{26; 27}. However, as we found
555 the R232Q variant affects both chaperone and NAD synthase functions of NMNAT2, we
556 cannot definitively exclude a critical requirement for the chaperone function in human
557 development.

558

559 We conclude that the compound heterozygous variant *NMNAT2* alleles in the FADS cases
560 described here encode proteins whose enzymatic and chaperone functions are both either
561 directly or indirectly impaired and are a likely underlying cause of the disorder. As in *NMNAT2*-
562 *deficient* mice, we propose that defects in PNS axon outgrowth and/or survival primarily lead to
563 decreased innervation of the skeletal muscle in the fetuses resulting in severely reduced
564 skeletal muscle mass. We argue *NMNAT2* may be added to the growing list of genes involved
565 in developing or maintaining PNS innervation that have been linked to FADS or FADS-
566 associated symptoms such as *DOK7*, *MUSK*, *RAPSN*, *ADCY6*, *GPR126*, *ECEL1*, *GLDN*, and
567 *PIEZO2* ¹. *NMNAT2* mutations should be investigated in other cases with fetal hydrops, fetal
568 akinesia, and widespread skeletal muscle deficiency.

569 It will also be important to determine whether more modest *NMNAT2* loss-of-function alleles
570 are associated with other disorders. Interestingly, in the accompanying paper [Huppke et al;],
571 another set of patients has been identified that are homozygous for a temperature-sensitive,
572 partial loss-of-function *NMNAT2* allele who develop a childhood-onset peripheral neuropathy
573 phenotype. This raises the possibility of an *NMNAT2* allelic series with mutations that have a

574 less severe effect on NMNAT2 function leading to childhood or later-onset neuropathies, rather
575 than prenatal lethality, and/or preclinical phenotypes that predispose to adult-onset disorders.

576

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579 **Supplemental Data**

580 Two tables with more information on variants identified in whole exome sequencing.

581

582 **The authors declare no competing interests**

583

584

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595 **Figure 1. Gross phenotype and histology of affected fetuses.** (A,B) Fetal MRI of Fetus II-1

596 in which hydrocephalus is noted by asterisk and cystic hygroma by arrowhead. (C) Dorsal view

597 of fetus II-3 with notable edema and lack of skeletal muscle in the extremities. (D) Fetus II-3
598 displays malrotation of the gut with the appendix in the upper left quadrant. (E) Absence of the
599 psoas muscles bilaterally is noted by the asterisks. (F,G) Fetus II-3 displays flattened hands
600 with contractures of the elbow (F), and nearly complete absence of skeletal muscle of the leg
601 (G). (H) Histology of the right radius and ulna shows reduced skeletal muscle fiber packing
602 near the bone. (I) Histology of the interosseous muscles of the right hand show sparsely
603 spaced muscle fibers with plump nuclei. (J) Histology of the hip joint shows fibrofatty tissue
604 replacement of the musculature of the hip (K) Histology of the left ventricle shows normal
605 architecture of the myocardium.

606

607

608 **Figure 2. Whole exome sequencing identifies compound heterozygous mutations in**
609 ***NMNAT2***. (A) Family pedigree. Stillborn infants are depicted as filled triangles with slashes
610 (A). (B) Sanger sequencing of *NMNAT2*. (C) Conservation of arginine at aa232 in *NMNAT2*
611 homologues across distant phyla. (D) Diagram of functional domains of *NMNAT2* with patient
612 variant positions in red. (E) 3D structure model of *NMNAT1*. The conserved β strands and α
613 helices important for enzymatic function are marked in yellow and cyan, respectively. The
614 disordered region in *NMNAT* that contains the nuclear localization sequence is indicated by a
615 dashed line. The R232 equivalent residue is labeled in red.

616

617 **Figure 3. *NMNAT2*^{R232Q} and *NMNAT2*^{Q135Pfs*44} both have reduced capacity to maintain**
618 **neurite survival.** (A) Representative images (of n = 4 independent experiments) of cut
619 neurites of SCG neurons co-injected with expression vectors for Flag-*NMNAT2*^{WT}, or Flag-

620 NMNAT2^{R232Q}, or NMNAT2^{Q135Pfs*44} (10 ng/μl) and DsRed (pDsRed2, 40 ng/μl). Neurites were
621 cut 48 hours after injection when DsRed expression allows clear visualization of the distal
622 neurites of the injected neurons. Images show transected neurites, just distal to the lesion,
623 immediately after (0h) and 24 hours after cut. The lesion site is located the bottom edge of
624 each field. Brightness and contrast have been adjusted for optimal visualization of neurites. (B)
625 Quantification of neurite survival at 24 hours after cut for experiments described in panel A.
626 The number of intact neurites with continuous DsRed fluorescence at 24 hours is shown as a
627 percentage of intact neurites at 0h. Individual values and means ± SEM are plotted (individual
628 values represent the average of two fields per separate culture). n.s. = not significant (p >
629 0.05), *** p < 0.001, one-way ANOVA with Tukey's multiple comparisons test. (C) Relative
630 expression level of Flag-NMNAT2 variants in injected SCG neuron cell bodies. Representative
631 fluorescent images of SCG neurons 24 hours after co-injection with expression vectors for
632 Flag-NMNAT2^{WT}, Flag-NMNAT2^{R232Q} or Flag-NMNAT2^{Q135Pfs*44} and DsRed (each at 25 ng/μl).
633 DsRed identifies injected neurons, Flag immunostaining shows expression of the Flag-
634 NMNAT2 proteins, and DAPI labels nuclei. Relative intensities (± SEM) of Flag
635 immunostaining and DsRed signal are shown after transformation to the mean of levels in
636 neurons injected with the Flag-NMNAT2^{WT} construct. The data for WT, R232Q and
637 Q135Pfs*44 were calculated from 47, 62 and 40 injected neurons (DsRed positive) of which
638 87.2%, 81.3% and 22.5% were Flag-positive respectively.

639

640 **Figure 4. Relative stabilities and activities of NMNAT2^{R232Q} and NMNAT2^{Q135Pfs*44} in HEK**
641 **293T cells.** (A) Representative immunoblots (of n = 3) of extracts of HEK 293T cells co-
642 transfected with expression vectors for Flag-NMNAT2^{WT}, Flag-NMNAT2^{R232Q} or Flag-

643 NMNAT2^{Q135Pfs*44} and eGFP at the indicated times after addition of 10 μ M emetine. Emetine
644 was added 24 h after transfection. Extract from non-transfected cells is also shown (NT). Blots
645 were probed with Flag, eGFP and α -Tubulin antibodies. To avoid saturation of the protein
646 degradation machinery that might artificially slow rates of turnover, expression of the Flag-
647 NMNAT2 proteins was kept relatively low by including empty vector as part of the transfection
648 mix (see Materials and Methods). Co-transfected eGFP or endogenous α -Tubulin (present in
649 transfected and non-transfected cells) are both relatively stable proteins and were respectively
650 used as a reference for Flag-NMNAT2 protein turnover (to control for transfection efficiency)
651 and for loading. Arrows indicate the positions of bands corresponding to Flag-NMNAT2^{WT}
652 (black, ~34 kDa), Flag-NMNAT2^{R232Q} (red, ~37 kDa), and Flag-NMNAT2^{Q135Pfs*44} (green, ~22
653 kDa). An asterisk indicates the position of a non-specific band. (B) Relative steady-state Flag-
654 NMNAT2 protein band intensities (0h, just before emetine addition) after normalization to co-
655 transfected eGFP for blots described in panel A. Individual values (n = 4-5) and means \pm SEM
656 are plotted. n.s. = not significant (p > 0.05), ** p < 0.01, one-way ANOVA with Tukey's multiple
657 comparisons test (only comparisons to Flag-NMNAT2^{WT}). (C) Representative immunoblots (of n
658 = 4) of extracts of HEK 293T cells, as in panel A, but transfected with a higher concentration of
659 Flag- NMNAT2^{Q135Pfs*44} expression vector and with increased loading per lane to maximize
660 Flag band intensity at the 0h time point so that its level at 0h is similar to that of Flag-
661 NMNAT2^{WT} in panel A. This allows for a more accurate comparison of turnover rates. (D)
662 Relative turnover rates of Flag-NMNAT2 proteins after emetine addition. Flag-NMNAT2 band
663 intensities on blots described in panel A (Flag-NMNAT2^{WT} and Flag-NMNAT2^{R232Q}) and panel
664 C (Flag-NMNAT2^{Q135Pfs*44}) were normalized to co-transfected eGFP and intensities at each
665 time point after emetine addition were calculated as a proportion of the intensity of the 0h,

666 untreated band. Means \pm SEM (n = 4) are plotted. n.s. = not significant ($p > 0.05$), ** $p < 0.01$
667 and *** $p < 0.001$, two-way ANOVA with Sidak's multiple comparisons test for effects between
668 variants. One-phase decay curves were fitted to the data sets for Flag-NMNAT2^{WT} and Flag-
669 NMNAT2^{R232Q} using non-linear regression. The R² value and half-life ($t_{1/2}$) are reported. No
670 intensity values could be obtained for Flag-NMNAT2^{Q135Pfs*44} at any timepoint assessed after
671 emetine addition precluding curve fitting and statistical analysis.

672

673

674 **Figure 5. Bacterial expression and *in vitro* characterization of the activity of**
675 **recombinant NMNAT2^{R232Q} and NMNAT2^{Q135Pfs*44}.** (A) Coomassie blue stained 12 % SDS
676 polyacrylamide gel loaded with similar amounts (~3 μ g) of NMNAT2^{WT} and each indicated
677 recombinant NMNAT2 variant arising from His-tag affinity chromatography. (B) Immunoblots of
678 ~0.3 μ g of the same protein samples as in A probed with anti-His and anti-NMNAT2 antibodies
679 as indicated. As in HEK cells, bacterially-expressed NMNAT2^{R232Q} migrates slower than
680 NMNAT2^{WT} and NMNAT2^{Q135Pfs*44}, which lacks the epitope recognized by the NMNAT2
681 antibody (raised against the C-terminus of the full-length protein), is expressed at a low level.
682 The NMNAT2^{Q135Pfs*44} preparation contains a ~34 kDa protein recognized by both anti-His and
683 anti-NMNAT2 antibodies that is likely to be His-tagged NMNAT2^{WT} (red boxes). (C) NMNAT
684 specific activity of His-tag purified preparations measured at 37 °C with saturating
685 concentrations of substrates. The less pure NMNAT2^{Q135Pfs*44} preparation is omitted despite
686 some activity found since it was not associated with the His-tagged 22 kDa truncated protein
687 arising from the frame shift mutation (see text). (D) Magnesium-dependent rates of NMNAT
688 activity referred to 1 mM MgCl₂ (arbitrary 100 % value). (E) Enzyme stability after 1 hour

689 treatment at different temperatures. Treated enzyme solutions were then assayed at 37 °C.
690 Relative rates are expressed as percentages of the untreated enzyme kept at 4 °C (100 % not
691 shown). (f) Enzyme stability at 37 °C as function of time. Rates are relative to time zero. (g)
692 Optimum temperature after heating of whole assay mixtures at the indicated temperatures.
693 Relative rates are expressed as percentages of the maximum observed (42 °C for both
694 enzymes). All data presented are the mean \pm SEM from n = 3 independent measures. T test p
695 values vs corresponding WT are marked by (*) p < 0.015 or by (**) p < 0.005 (Two Sample t
696 Test, unequal variances).

697

698

699 **Figure 6. NMNAT2^{R232Q} and NMNAT2^{Q135Pfs*44} have reduced chaperone activities.** HEK

700 293T cells were co-transfected with luciferase and one of the following plasmids: DsRed2
701 vector (control), Hsp70, NMNAT3, NMNAT2^{WT}, NMNAT2^{R232Q}, and Nmnat2^{Q135Pfs*44}. At 48 hrs
702 after transfection, protein synthesis was inhibited, and cells were subjected to heat shock at 42
703 °C for 45 mins, and then recovered at 37 °C for 3 hours. Quantification of luciferase activity
704 measured without heat shock (blue bars), after heat shock (red bars), and after recovery
705 (green bars). Luciferase activity in each group was normalized to no heat shock (set to 1). All
706 data were presented as mean \pm SD, n=4. Statistical significance was established by two-way
707 ANOVA post hoc Tukey's multiple comparison test. ***P<0.001, ****P<0.0001, NS: not
708 significant.

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859 **Table 1.** Clinical Features of fetuses II-1 and II-3.

Measurement	II-1	II-3	Reference (24 weeks)
Weight	810 g	282.2 g	586 ± 74g
Crown to rump length	23 cm	23.5 cm	21 ± 1.4 cm
Head circumference	25.5 cm	17.4 cm	21.8 ± 1.4 cm
Inner canthal distance	2c m	1.9 cm	1.5 ± 0.17 cm
Outer canthal distance	5c m	3.4 cm	4.21 ± 0.41 cm
Foot length	4.5 cm	3 cm	4.4 ± 0.2 cm
Lung weight	3.6 g	1.7 g	15.8 ± 5.3g
Heart weight	3.4 g	0.9 g	4.4 ± 0.9g
Brain weight	100 g	45 g	81.7 ± 14.8g
Placenta weight	475 g	152.4 g	225 ± 69g

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Placenta area	18x17 cm	N/A	
Fetal hydrops	+	+	
Cystic hygroma	+	+	
Flexion contractures	+	+	
Lung hypoplasia	+	+	
Hydrocephalus	+	Unknown	
Hypoplastic cerebellum	+	Unknown	
Muscle atrophy	+	+	
Micrognathia	+	+	
Cleft palate	+	+	
Hydropic placenta	+	+	
Gut malrotation	+	+	

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871 **Table 2.** Kinetic parameters of human NMNAT2 WT and R232Q.

Enzyme	Substrate	K_m (μM)	K_{cat} (s^{-1})	K_{cat}/K_m ($\text{s}^{-1}\text{M}^{-1}$)
NMNAT2 ^{WT}	ATP	159.6 \pm 28.6	6.84 \pm 3.21	0.429 * 10 ⁵
	NMN	22.3 \pm 8.13	6.84 \pm 3.21	3.070 * 10 ⁵
NMNAT2 ^{R232Q}	ATP	1820.9 \pm 121.1	0.31 \pm 0.03	0.002 * 10 ⁵
	NMN	178.5 \pm 10.8	0.31 \pm 0.03	0.017 * 10 ⁵

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873 Data represent Mean \pm SEM of 3 independent experiments.

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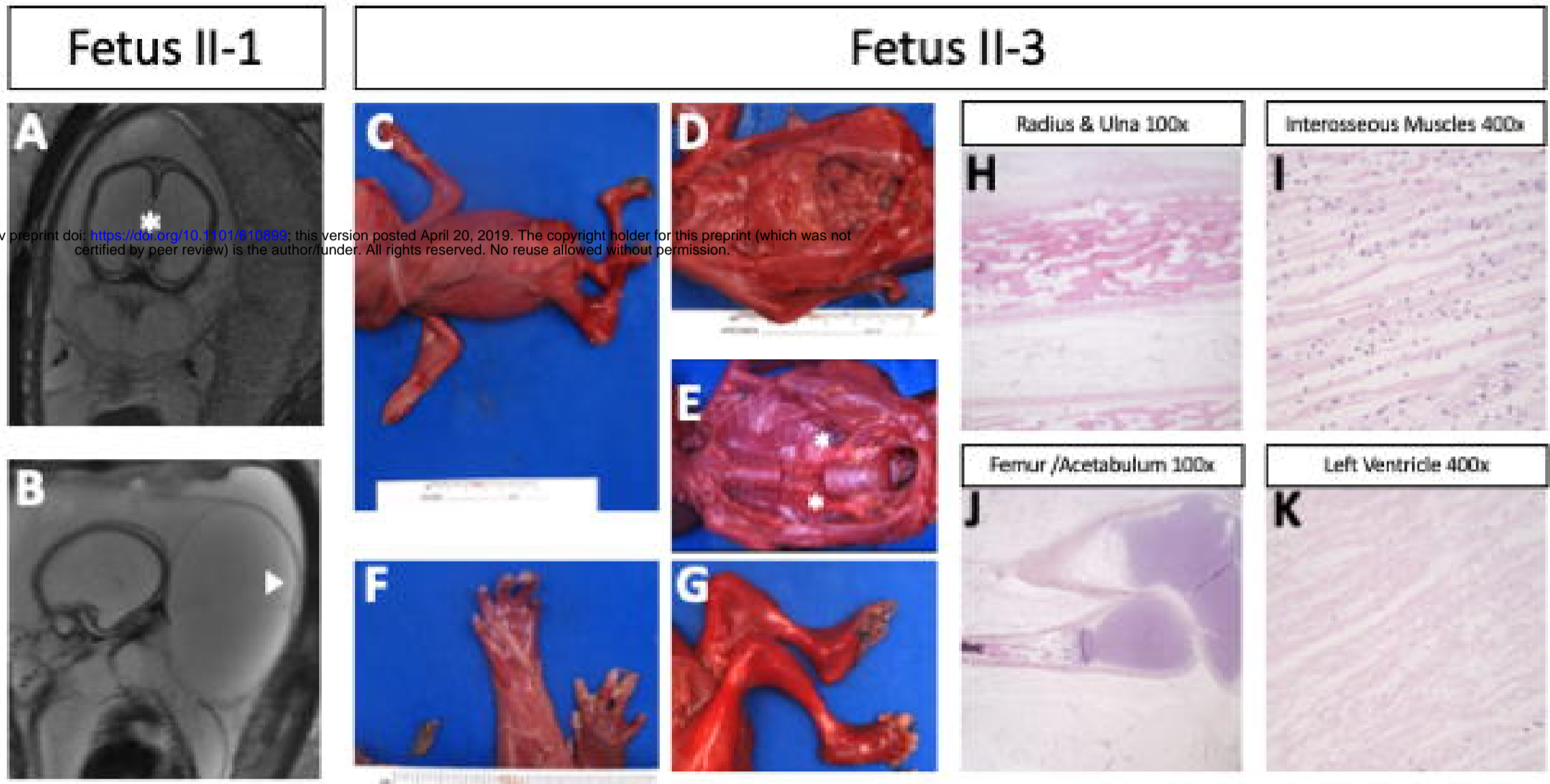


Figure 1

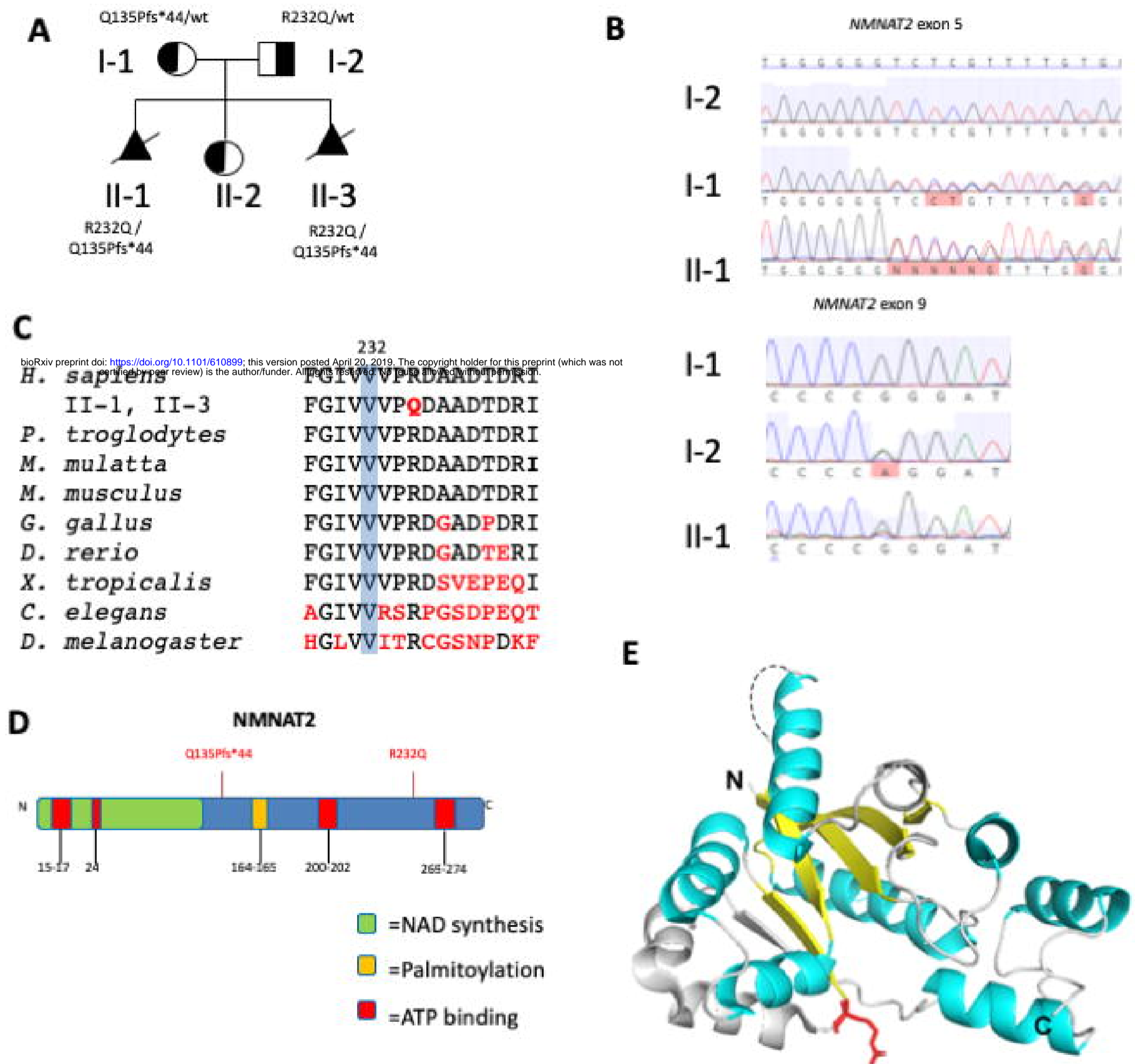
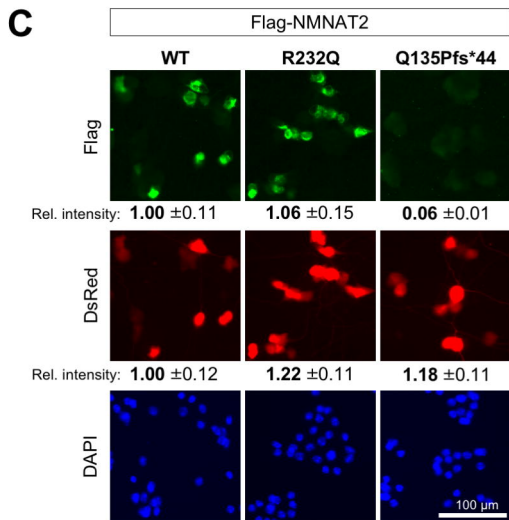
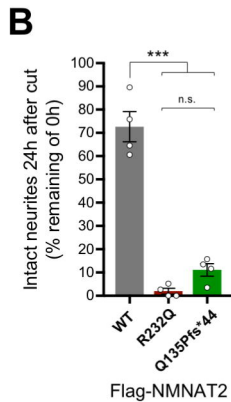
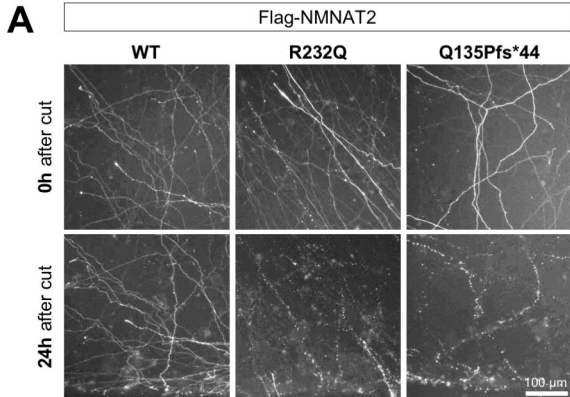
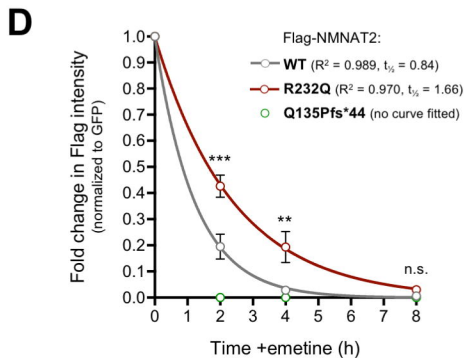
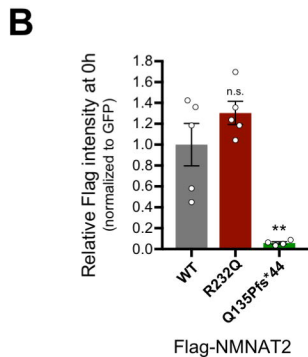
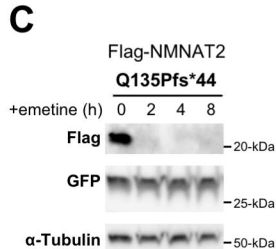


Figure 2





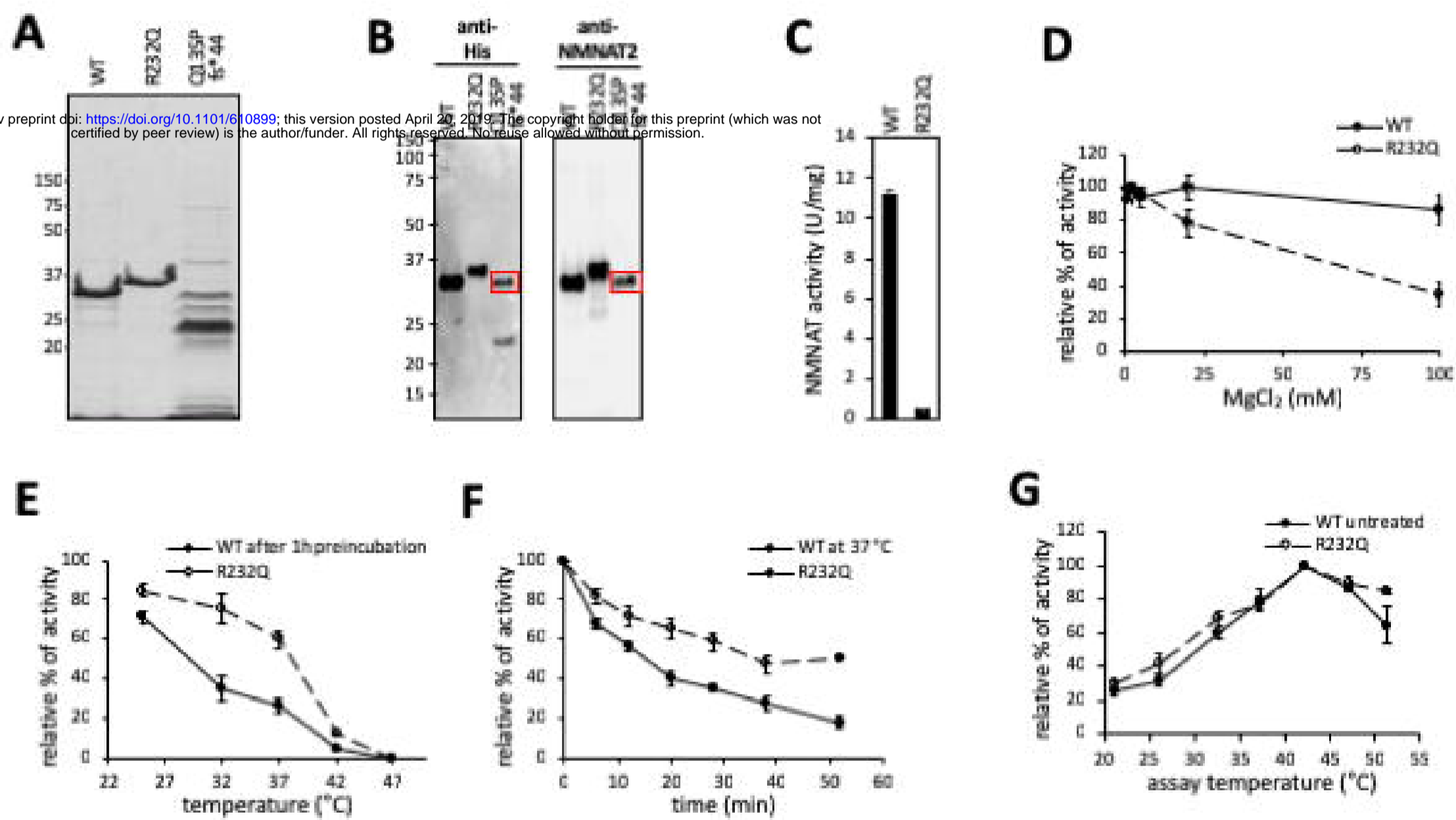


Figure 5

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In vivo luciferase folding assay

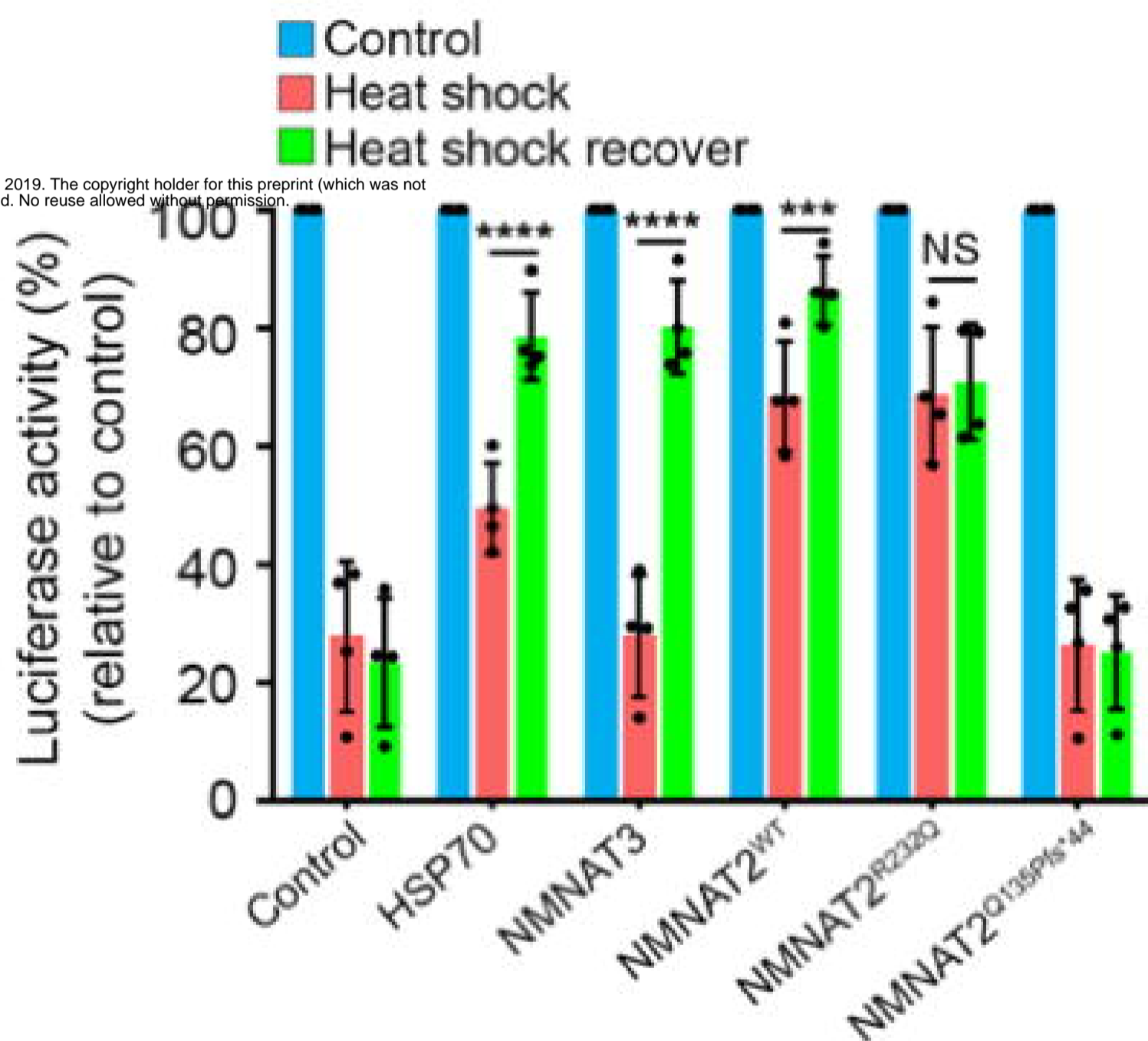
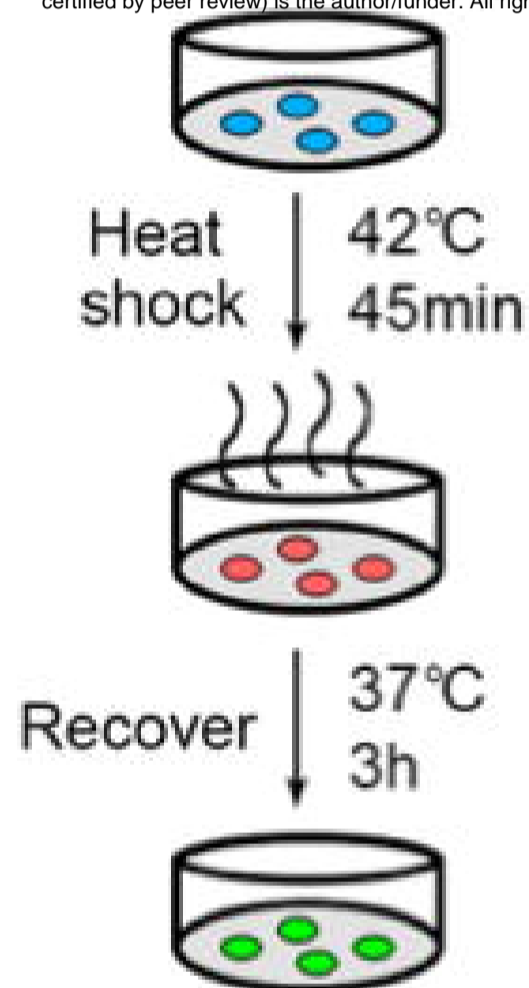


Figure 6