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1 **A splice donor variant of *GAS8* induces structural disorganization of the**
2 **axoneme in sperm flagella and leads to nonsyndromic male infertility**

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25 **ABSTRACT**

26 Motile cilia and flagella are closely related organelles structured around a highly conserved
27 axoneme whose formation and maintenance involve proteins from hundreds of genes.
28 Defects in many of these genes have been described to induce primary ciliary dyskinesia (PCD)
29 mainly characterized by chronic respiratory infections, situs inversus and/or infertility. In men,
30 cilia/flagella-related infertility is usually caused by asthenozoospermia due to multiple
31 morphological abnormalities of the sperm flagella (MMAF). Here, we investigated a cohort of
32 196 infertile men displaying a typical MMAF phenotype without any other PCD symptoms.
33 Analysis of WES data identified a single case carrying a deleterious homozygous *GAS8* variant
34 altering a splice donor consensus site. This gene, also known as *DRC4*, encodes a subunit of
35 the Nexin-Dynein Regulatory Complex (N-DRC), and has been already associated to male
36 infertility and mild PCD. Confirming the deleterious effect of the candidate variant, *GAS8*
37 staining by immunofluorescence did not evidence any signal from the patient's spermatozoa
38 whereas a strong signal was present along the whole flagella length in control cells.
39 Concordant with its role in the N-DRC, transmission electron microscopy evidenced peripheral
40 microtubule doublets misalignments. We confirm here the importance of *GAS8* in the N-DRC
41 and observed that its absence induces a typical MMAF phenotype not necessarily
42 accompanied by other PCD symptoms.

43 **Keywords:** *GAS8*, *DRC4*, asthenozoospermia , MMAF, male infertility, PCD.

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50 INTRODUCTION

51 Disruption of spermatogenesis leads to a wide range of sperm defects including
52 asthenozoospermia which is defined by an alteration of sperm motility. One of the most
53 severe conditions of asthenozoospermia is due to Multiple Morphological Abnormalities of
54 the Sperm Flagella (MMAF), a phenotype characterized by a mosaic of sperm morphological
55 defects including absent, bent, short, irregular and coiled flagella (1). More than 40 MMAF-
56 associated genes have been so far related to human infertility, thus demonstrating the high
57 genetic heterogeneity of this phenotype (2). However about half of MMAF cases remain
58 unexplained (3).

59 In this study, we recruited and investigated by whole exome sequencing (WES), a patient
60 displaying a primary non-syndromic infertility due to MMAF. We identified and validated a
61 novel loss-of-function (LoF) variant in *GAS8* (OMIM: # 605178), a gene encoding a protein
62 localized in the axoneme of cilia and sperm flagella. Previous studies demonstrated that *GAS8*
63 LoF variants are responsible for mild symptoms of Primary Ciliary Dyskinesia (PCD, OMIM: #
64 244400), a genetic multisystemic disorder characterized by impaired ciliary function, leading to
65 a chronic sinopulmonary disease, referred to as Kartagener syndrome when associated to
66 situs inversus totalis (4). It has been reported that PCD patients carrying *GAS8* variants are
67 infertile but their phenotype was not fully explored and reported. Here, we demonstrated the
68 involvement of a novel pathogenic *GAS8* variant in a non-syndromic case of male infertility
69 due to MMAF.

70 MATERIAL AND METHODS

71 Participant and ethical approval statement

72 All samples, from 196 MMAF subjects were sent to Grenoble Alpes Hospital for genetic
73 analysis. This study respects all ethical guidelines (Declaration of Helsinki) and was approved
74 by local ethics committees and the French Data Protection Authority (DR-2016-392) and
75 samples collection (Fertithèque) was declared to the French Ministry of health (DC-2015-
76 2580). Informed consent was obtained from patients prior to sample collection and analyses.
77 Semen samples were prepared and analyzed as described previously (5)... The patient gave

78 his informed consent to donate and store spermatozoa not used for semen analysis in
79 GERMETHEQUE Biobank (BB-0033-00081).

80 **WES analysis and variant verification**

81 WES and bioinformatics analyses were performed according to our previously described
82 protocol using the human genome assembly GRCh38 as a reference (6). The selected variant
83 was validated by Sanger sequencing using ABI 3130XL and SeqScape software (Applied
84 Biosystems; Foster City, CA). Primers are listed in Table S1.

85 **Transmission Electron Microscopy (TEM) analysis**

86 To better characterize the impact of the reported candidate variant on sperm ultrastructure,
87 sperm from a fertile control and from patient P0543 were subjected to transmission electron
88 microscopy as described previously (5).

89 **Immunofluorescence (IF) Analysis of Sperm Cells**

90 Immunostaining was carried out on sperm cells from fertile control subjects and from
91 individual P0543 . All the procedure is described in (5). Antibodies uses for these experiments
92 are listed in Table S2.

93 **RESULTS**

94 **WES identified a homozygous splicing variant in *GAS8***

95 Previous WES analyses of our MMAF cohort permitted to identify bi-allelic variants in a total
96 of 92 men (47%) in 22 confirmed MMAF-associated genes. In the remaining undiagnosed
97 individuals, we identified one subject (P0543) carrying a homozygous deleterious variants in
98 the intron 8 of *GAS8*, NM_001481.3:c.1011+2T>C (Fig. 1AB). This variant is very rare (MAF:
99 6.575×10^{-6} in gnomAD, v3.1.2).and was predicted to alter a consensus splice donor site (SPiP
100 score: 98.41% [91.47 % - 99.96 %]) (Fig. S1). Sanger sequencing confirmed the presence of this
101 variant (Fig. 1C). Finally, this variant was selected as a strong candidate among the list of the
102 other variants identified in P0543 to explain its infertility (Table S3).

103 **Patient phenotypic characterization**

104 Patient P0543, a 32 years old male, The subject consulted for infertility at the Reproductive
105 Biology laboratory-CECOS of Rouen University Hospital (France). This patient, originating from
106 West Africa, was born from related parents. He had fa brother showing primary infertility due
107 to severe asthenozoospermia who consulted to a different ART center and was described with
108 a MMAF phenotype (Fig. 2A). Physical examination of the patient was normal. Sperm analysis
109 evidenced a complete astheno-teratozoospermia with 100% of immotile and morphologically
110 abnormal spermatozoa, most of which (93%) displayed multiple morphological abnormalities
111 of the flagellum with 53% of absent, 24% of short, 16% of curled or bent and flagella (Fig. 2B).
112 Further clinical assessment of this patient suggested a condition of nonsyndromic infertility
113 without PCD-symptoms except for a medical past history of asthma and recurrent otitis during
114 childhood.

115 **TEM revealed N-DRC defects associated with abnormal flagellum assembly and elongation**

116 TEM analysis of patient's sperm evidenced severe and recurrent elongation defects associated
117 with fibrous sheath dysplasia and reduced numbers and abnormal arrangement of
118 mitochondria in the mid-piece (Fig. 2C). Moreover, most observed cross sections of flagella
119 showed a preserved "9+2" axonemal structure but also a frequent detachment or absence of
120 some peripheral microtubule doublets suggesting a possible disruption of the link given by the
121 N-DRC.

122 **IF experiments showed the loss of GAS8 in sperm flagella from patient carrying the**
123 **candidate variant**

124 GAS8 is localized in the axoneme of the flagella and is a component of the N-DRC (nexin-dynein
125 regulatory complex), which links adjacent peripheral doublet microtubules (DMTs). To
126 demonstrate the impact of the candidate *GAS8* variant on protein expression and localization,
127 we performed IF staining of sperm from controls and P0543 using an antibody raised against
128 the C-terminus domain of GAS8 (aa 379-469). In control spermatozoa, GAS8 and the axonemal
129 marker acetylated α -tubulin co-localize within the axoneme (Fig. 3). However, spermatozoa
130 from the patient showed no GAS8 signal in the flagellar axoneme indicating that this protein
131 was either absent or truncated (Fig. 3).

132

133 **DISCUSSION**

134 Previous studies identified bi-allelic LoF variants in *GAS8* as genetic factors responsible for PCD
135 and severe primary male infertility due to asthenozoospermia, but few cases have been
136 reported and the reproductive phenotype has been seldom explored (7–9). Our study
137 identified a novel homozygous LoF variant in *GAS8* for a patient displaying primary infertility
138 due to MMAF without apparent PCD symptoms. *GAS8* is highly expressed in cilia of epithelial
139 cells of pulmonary bronchi and fallopian tubes, and also predominantly expressed in the adult
140 testes during the late stages of spermatogenesis concomitantly with the formation of flagella
141 (10). The sperm flagellum contains a structure known as the axoneme, which runs through its
142 entire length(11).

143 The axoneme comprises nine DMTs surrounding a central pair complex of two singlet
144 microtubules (9+2 configuration).. The N-DRC is a thin structure that bridges neighboring
145 DMTs and restricts sliding motions between DMTs. As a result, the N-DRC converts dynein-
146 generated sliding motion into flagellar bending motion (12). To date, biochemical and genetic
147 studies have revealed that the N-DRC is composed of 12 subunits (12,13). *GAS8* encodes the
148 DRC4 subunit of this complex. It was demonstrated that, during axonemal assembly, DRC4
149 travels within cilia and flagella as cargoes on intra-flagellar transport (IFT) particles (14). DRC4
150 was also described to interact with DRC1 (OMIM: # 615288) and *CCDC65* (OMIM: # 611088) subunits
151 to form the core structure of the N-DRC which serves as a scaffold for the assembly of the
152 functional subunits. Mutations in the main DRC subunit genes cause the disassembly of the N-
153 DRC, defects in ciliary movement and the abnormal assembly of several closely associated
154 structures (7,15,16). *CCDC65* pathogenic variants induce subtle ciliary beating defects and lead
155 to PCD, whereas DRC1 deficiency was reported to cause both PCD and male infertility due to
156 MMAF (16–18). *GAS8* deficiency leads to N-DRC disruption and cause PCD and
157 asthenozoospermia (7,8). Unlike *Gas8* knockout mice that display severe PCD phenotypes
158 including hydrocephalus and situs inversus, bi-allelic *GAS8* pathogenic variants in humans
159 have been associated to milder symptoms such as insufficient mucociliary clearance of the
160 airways which may result in a chronic destructive respiratory disorder (9). It is therefore
161 possible that our patient who does not present PCD symptoms is at an early stage of the

162 respiratory disease. Indeed, it was shown that the genetic background may play a role in
163 delaying the disease onset and progression in PCD-patients. In this issues, a recent study
164 performed a precise genotype-phenotype correlation analysis in a series of PCD patients
165 carrying mutations in *RSPH9*, a well-established causative PCD gene, reporting a wide
166 phenotypic variability even among patients bearing the same variant (19). In another study,
167 authors generated several *Drc1* mutant mouse strains and observed that the phenotypic
168 manifestations were strain-dependent (16). Overall, these data underscore the fact that
169 differences in genetic background may influence the penetrance of PCD-related mutations.

170 In conclusion, we herein identified a novel homozygous LoF *GAS8* variant in a MMAF patient.
171 We also propose the TEM analysis as a complementary approach in addition to WES for the
172 investigation of MMAF and particularly for the detection of N-DRC related-defects.

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178 **Author Contributions**

179 Z.E. K., N.T.M., Ch.C., Ca.C. and P.F.R. performed and analyzed the genetic data. A.L.B.
180 performed TEM analysis. G.M., Ca.C., A.M. performed the immunofluorescence assays. A.R.F.
181 and N.R. provided clinical samples and data. P.F.R and Z.E.K. designed the study, supervised
182 all molecular laboratory work. All authors have read and agreed to the published version of
183 the manuscript.

184 **Declaration of interests**

185 The authors declare no conflict of interest.

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239

240 **FIGURE LEGENDS**

241 **Figure 1. Identification of a rare homozygous splicing variant in *GAS8*.** **A)** *GAS8* is located at
242 chromosome (chr.) 16q24.3, g.90,019,629-90,044,975. **B)** *GAS8* (NM_001481.3) comprises 11 exons.
243 The novel *GAS8* variant, marked in blue, is located in intron 8 and affects the consensus GT sequence
244 of the splice donor site (c.1011+2T>C). Variants described in previous studies are marked in gray. **C)**
245 Sanger sequencing confirmed the presence of the candidate *GAS8* variant in P0543.

246 **Figure 2. A consanguineous family with an infertile man displaying primary infertility due to multiple**
247 **morphological abnormalities of the sperm flagella (MMAF).** **A)** Pedigree analysis. **B)** Morphological
248 analysis of sperm illustrating a typical MMAF phenotype. **C)** Ultrastructure of the spermatozoa from
249 P0543 compared to that of control sperm. Yellow head-arrows indicate abnormal DMT assemblage
250 and cohesion. CPC, central pair complex; FS, fibrous sheath; MS, mitochondrial sheath; ODF, outer
251 dense fibers.

252 **Figure 3. *GAS8* immunostaining of sperm cells from a fertile control subject and the infertile patient**
253 **carrying the candidate *GAS8* variant.** Sperm cells were stained with a polyclonal anti-*GAS8* (red) and
254 anti-acetylated α -tubulin (green) antibodies. DNA was counterstained with DAPI (4',6-diamidino-2-
255 phenylindole). *GAS8* signal was strongly detected in control sperm over the entire length of the
256 flagellum. However, the *GAS8* signal was not detected in the sperm flagella from P0543 who carries
257 the *GAS8* variant.

258 **SUPPLEMENTARY MATERIAL**

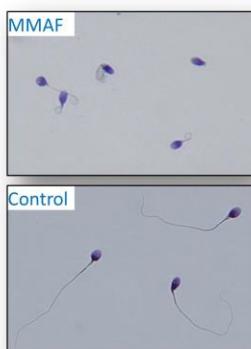
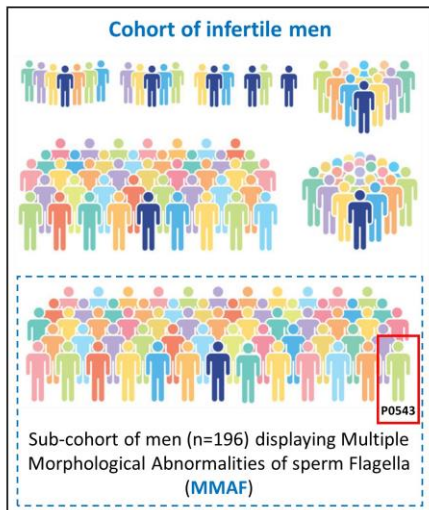
259 **Figure S1. Prediction of the impact of the identified GAS8 variant on mRNA splicing and translation.**

260 **Table S1. List of primers used for Sanger verification.**

261 **Table S3. List of antibodies used for IF experiments.**

262 **Table S3. List of variants identified in the reported case P0543 by WES analysis.**

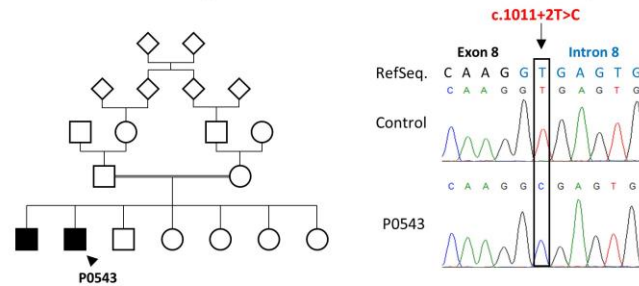
263



Whole Exome Sequencing (WES)



Identification of a splice donor variant of *GAS8* in a MMAF patient

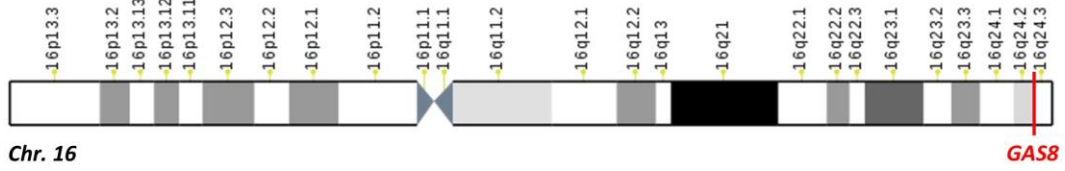
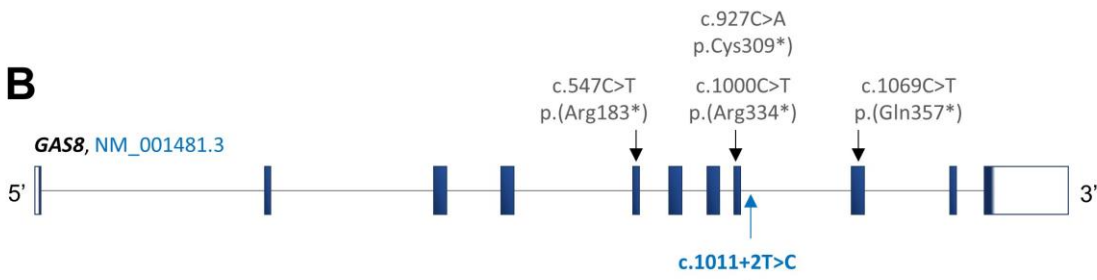
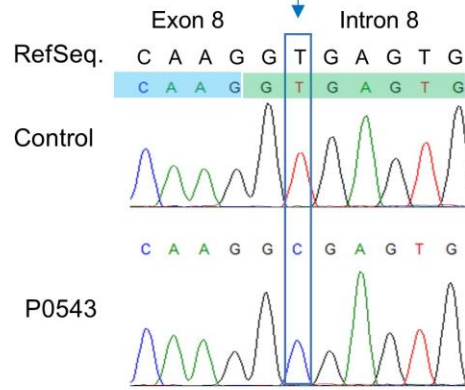


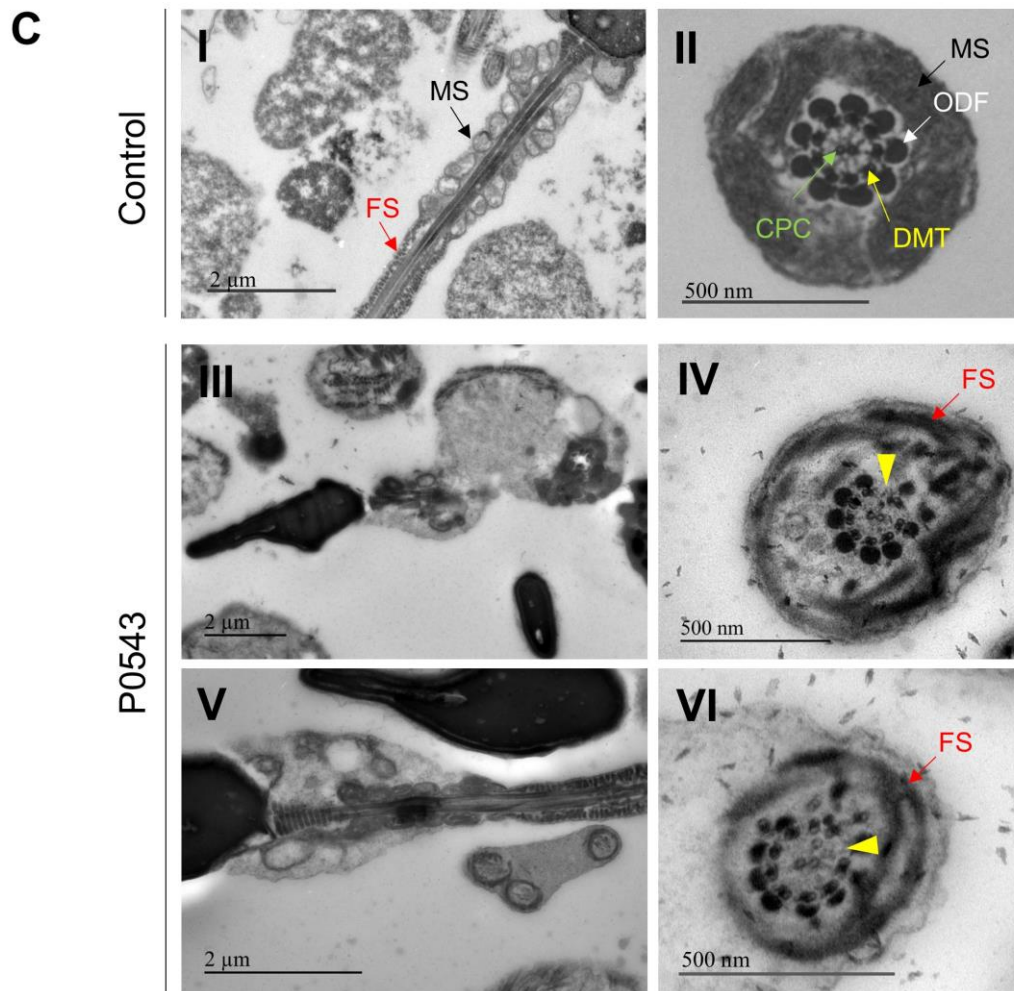
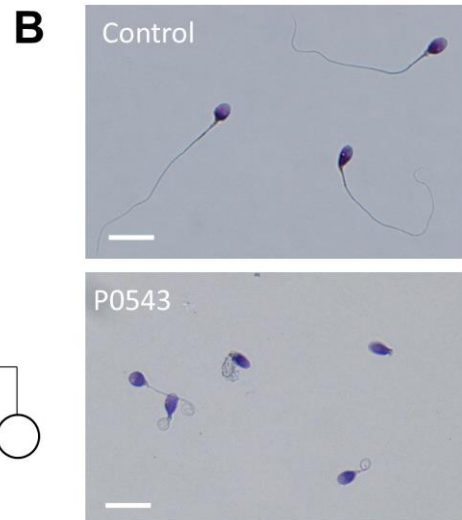
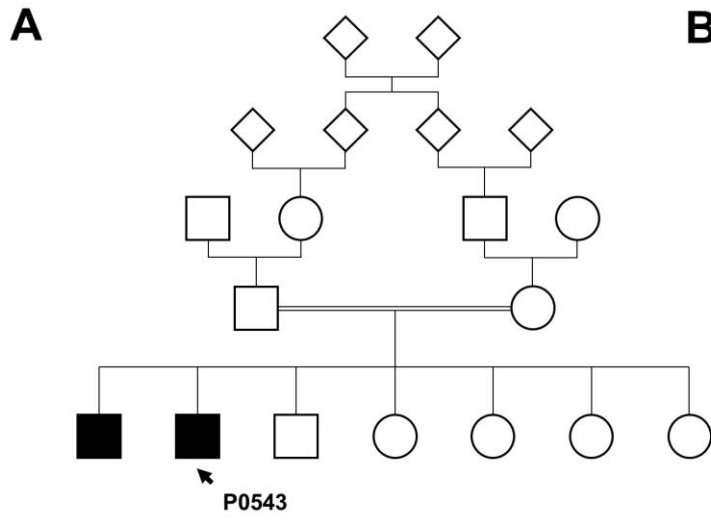
***GAS8* variant induces structural disorganization of the axoneme in flagella**

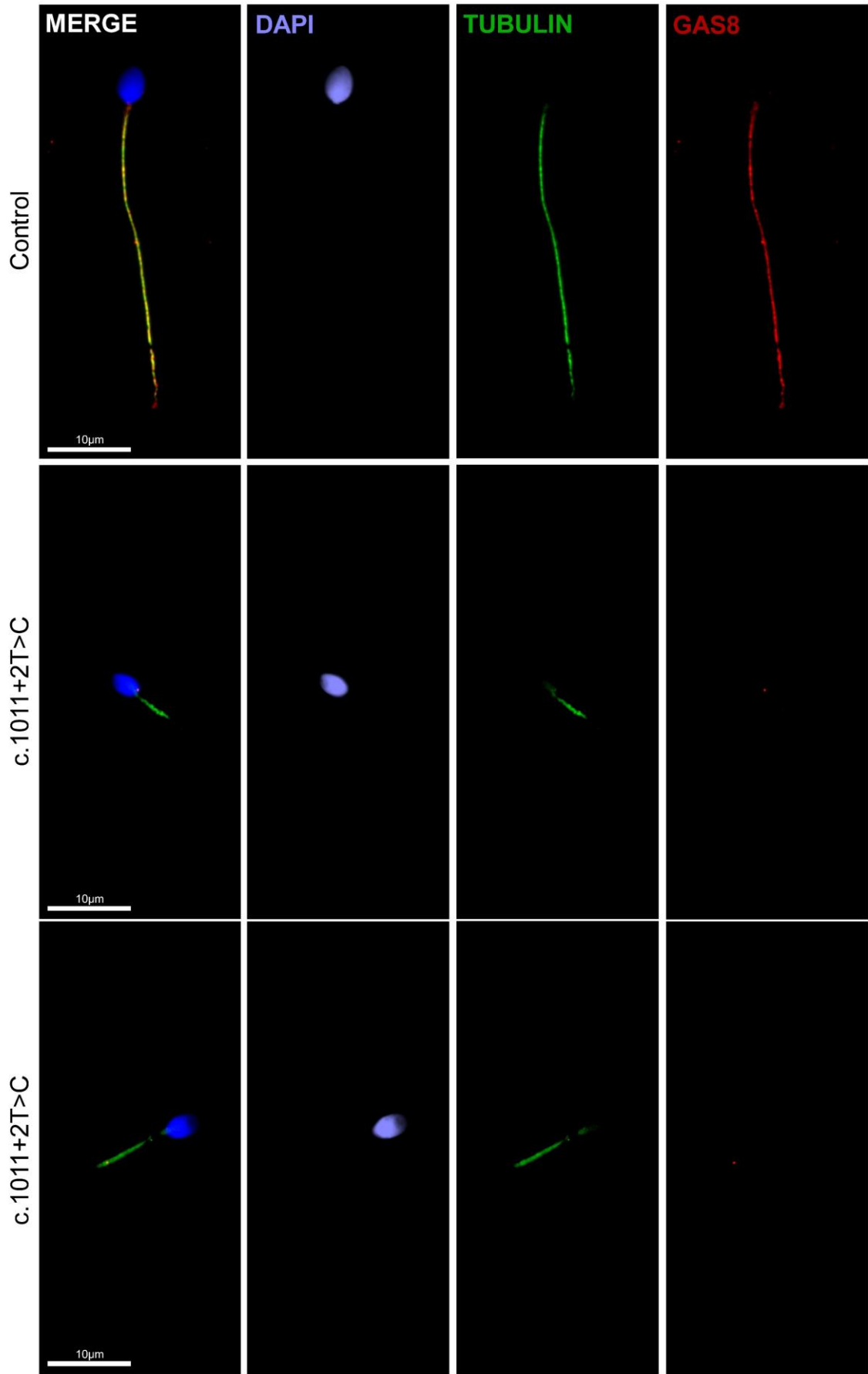


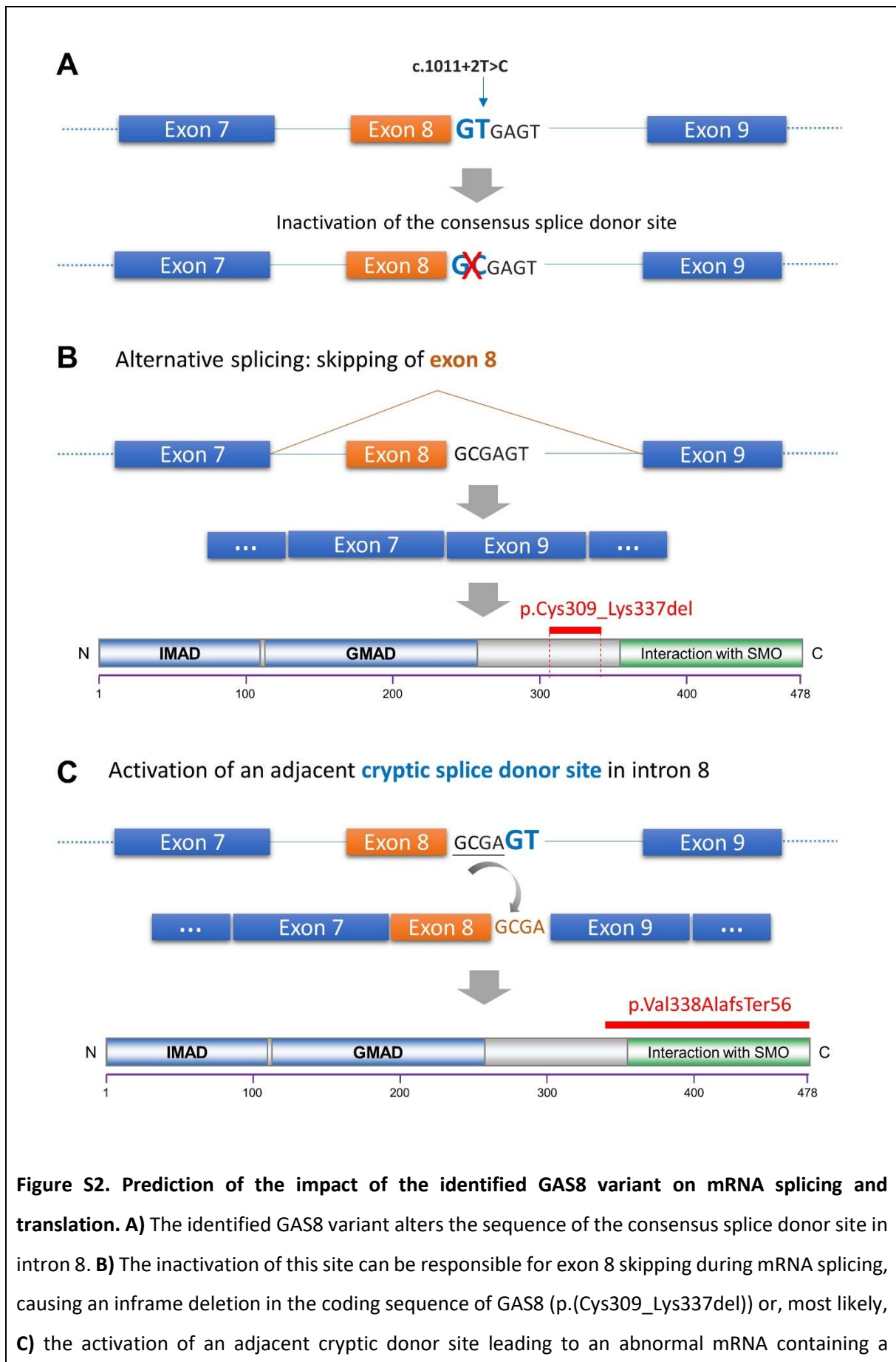
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A**B****C**







frameshift insertion of 4 bp between exons 8 and 9 that is responsible for the production of a truncated protein (p.(Val338AlafsTer56)).

269

270 **Table S1. Primers used for Sanger verification.**

271

Gene Name	Primer Sequence (5'-3')	Tm (°C)	Product size (bp)
GAS8_ Ex8-F	TGGAACCAGGTCTTTCTGGC	59.89	460
GAS8_Int8-R	AACAGGCCACAGGTTTTTGC	59.82	

272

273

274 **Table S2. List of antibodies used for IF experiments.**

275

Antibodies	species	supplier	Reference	Dilution
Primary antibodies				
Anti-GAS8 (polyclonal)	Rabbit	Sigma-Aldrich	HPA041311	1:100
Anti-acetylated α -tubulin (monoclonal)	Mouse	Abcam	AB61601	1:400
Secondary antibodies				
Anti-mouse IgG (conjugated)	Goat	Jackson ImmunoResearch	Dylight-549	1:1000

Anti-rabbit IgG (conjugated)	Goat	Jackson ImmunoResearch	Dylight-488	1:1000
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