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## Development of versatile allele-specific siRNAs able to silence all the dominant dynamin 2 mutations

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1 **Development of versatile allele-specific siRNAs able to silence all the dominant Dynamin**  
2 **2 mutations**

3

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19 **Running title:** Allele-specific silencing of dominant *DNM2* mutations

20

21

22 **Abstract**

23

24 Dominant centronuclear myopathy (CNM) is a rare form of congenital myopathy associated  
25 with a wide clinical spectrum, from severe neonatal to milder adult forms. There is no available  
26 treatment for this disease due to heterozygous mutations in the *DNM2* gene encoding Dynamamin  
27 2 (DNM2). Dominant *DNM2* mutations also cause rare forms of Charcot-Mare-Tooth disease  
28 and hereditary spastic paraplegia and deleterious DN M2 overexpression was noticed in several  
29 diseases. The proof of concept for therapy by allele-specific RNA interference devoted to  
30 silence the mutated mRNA without affecting the normal allele was previously achieved in a  
31 mouse model and patient-derived cells, both expressing the most frequent *DNM2* mutation in  
32 CNM. In order to have versatile siRNAs usable regardless of the mutation, we have developed  
33 allele-specific siRNAs against 2 non-pathogenic single nucleotide polymorphisms (SNP)  
34 frequently heterozygous in the population. In addition, allele-specific siRNA against the  
35 p.S619L DN M2 mutation, a mutation frequently associated with severe neonatal cases, were  
36 developed. The beneficial effects of these new siRNAs are reported for a panel of defects  
37 occurring in patient-derived cell lines. The development of these new molecules allows  
38 targeting the large majority of the patients harbouring *DNM2* mutations or overexpression by  
39 only a few siRNA.

40

41

## 42 **Introduction**

43

44 Autosomal dominant centronuclear myopathy (AD-CNM, MIM #160150) is a rare  
45 congenital myopathy associated with a wide clinical spectrum from severe-neonatal to mild-  
46 adult forms <sup>1</sup>. The classical late-childhood or adult-onset form exhibits delayed motor  
47 milestones and diffuse skeletal muscle weakness mainly involving facial and limb muscles  
48 whereas paediatric patients affected by the severe neonatal form usually have generalized  
49 weakness, hypotonia, and facial weakness <sup>2,3</sup>. AD-CNM results from mutations in the *DNM2*  
50 gene which encodes dynamin 2 (DNM2) <sup>4</sup>. In addition, *DNM2* mutations also cause rare forms  
51 of Charcot-Marie-Tooth disease (CMT) <sup>5</sup> and hereditary spastic paraplegia (HSP) <sup>6</sup> and a  
52 deleterious *DNM2* overexpression was reported in several cancers <sup>7-12</sup> and the X-linked  
53 recessive CNM <sup>13</sup>, highlighting a large *DNM2* involvement in human diseases. *DNM2* belongs  
54 to the superfamily of large GTPases <sup>14</sup> acting as a mechanochemical scaffolding molecule that  
55 oligomerizes and deforms biological membranes leading to the formation and release of  
56 vesicles from the plasma membrane and intracellular membrane compartments. Furthermore,  
57 several studies have highlighted the role of *DNM2* as a regulator of both actin and microtubule  
58 cytoskeletons <sup>15,16</sup>. More than 30 *DNM2* mutations have been reported in AD-CNM patients <sup>17</sup>  
59 and, when tested, the mutant protein is normally expressed <sup>4,18</sup>. Mutations are thought to be  
60 responsible for a gain of function and/or a dominant-negative effect through an increased  
61 GTPase activity and formation of abnormal stable *DNM2* oligomers <sup>19,20</sup>. In addition, absence  
62 of haploinsufficiency in AD-CNM is supported by data from patients and absence of phenotype  
63 developed by heterozygous knockout mice expressing 50% *Dnm2* <sup>13,21</sup>.

64 We recently developed a therapeutic approach for the *DNM2*-related AD-CNM by allele-  
65 specific RNA interference (AS-RNAi) devoted to specifically suppressing the expression of the  
66 mutated protein from the mutated allele without reducing expression from the wild-type allele

67 <sup>22</sup>. AS-RNAi was demonstrated as a powerful strategy in cells from patients and animal models  
68 of numerous dominant inherited diseases <sup>23</sup> and led to two clinical trials targeting a Keratin 6a  
69 mutation causing Pachyonychia congenita <sup>24</sup> and one mutation of the *KRAS* gene involved in  
70 pancreatic cancer <sup>25</sup>. By applying this strategy, we reported functional rescue in the Knock-In-  
71 *Dnm2*<sup>R465W/+</sup> mouse model of AD-CNM and patient-derived fibroblasts, both expressing the  
72 most frequent mutation encountered in patients (p.R465W found in around 30% of patients) <sup>22</sup>.  
73 Extending this strategy to the entire AD-CNM patient population requires either to develop  
74 personalized medicine through specific siRNA for each reported mutation or to develop an  
75 alternative approach allowing to target the distinct dominant *DNM2* mutations using a limited  
76 number of allele-specific siRNA. In particular cases of triplet repeat diseases such as  
77 Huntington's disease or spinocerebellar ataxia in which the sequence of the genetic mutations  
78 makes allele-specific silencing challenging, AS-siRNA have been developed against disease-  
79 associated single nucleotide polymorphisms (SNP) <sup>23,26-28</sup>. We took advantage of the presence  
80 in the *DNM2* sequence of two non-pathogenic SNPs frequently heterozygous in the general  
81 population to develop a similar strategy for the AD-CNM. Here, we report the identification of  
82 effective AS-siRNA against the two nucleotide versions of the two non-pathogenic *DNM2*  
83 SNPs which may be used to silence any mutation carried by the same mRNA. In addition, the  
84 first AS-siRNAs targeting a *DNM2* mutation associated with severe neonatal phenotype, i.e.  
85 the p.S619L mutation, have been developed. We also report the functional benefits of this new  
86 set of siRNAs on several defects identified in patient-derived cell lines. The development of  
87 these new AS-siRNA, in addition to the previous ones against the p.R465W mutation, provides  
88 a panoply of allele-specific molecules able to target the large majority of AD-CNM patients.  
89 Interestingly, siRNAs against the *DNM2* SNPs represent versatile molecules with larger  
90 potential applications to silence *DNM2* mutations in CMT and HSP and to reduce *DNM2*  
91 expression in a controlled manner in diseases associated with deleterious overexpression.



93 **Results**

94

95 **Identification of targetable SNPs and heterozygous cells**

96 We performed an *in-silico* analysis devoted to identifying DNMT2 SNPs exhibiting the  
97 highest frequency of heterozygosity in Human. We looked in Ensembl Genome Browser  
98 (<https://www.ensembl.org> - human genome assembly GRCh38.p13) for synonymous variants  
99 in the *DNMT2* open reading frame or variants in the 5'- and 3'-untranslated transcribed regions  
100 (UTR) exhibiting a frequency of the second most common allele between 0.1 and 0.5 in the  
101 general population. Using these criteria, we identified two SNPs (Figure 1A). The first SNP  
102 was a T/C variation (rs2229920, thereafter called SNP1) identified as a synonymous variant of  
103 the Alanine 713 (c.2139T>C, NM\_001005361.3) of the DNMT2 protein. Allele frequencies for  
104 SNP1 were determined at 0.688 for the allele T and 0.321 for the allele C on 250 390 counts  
105 (gnomAD exomes r2.1.1) resulting in a theoretical heterozygous (HTZ) frequency of 0.43  
106 (calculated as 2 x frequency of T allele x frequency of C allele). In agreement, PCR and Sanger  
107 sequencing in a cohort of 52 CNM patients identified 42.3% of SNP1 heterozygosity. The  
108 second SNP was an A/T variation (rs12461992, thereafter called SNP2) identified as a 3'-UTR  
109 variant located 268 nucleotides after the stop codon (\*268A>T, NM\_001005361.3). Allele  
110 frequencies for SNP2 were determined at 0.824 for the allele A and 0.176 for the allele T  
111 resulting in a theoretical HTZ frequency of 0.29 on 143 008 counts (gnomAD exomes r3.0) and  
112 23% of SNP2 heterozygosity was found in our cohort of patient. No clinical sign was associated  
113 with SNP1 and SNP2.

114 We next thought to identify cells harbouring the SNPs at HTZ state which were required to  
115 screen for allele-specific siRNA against the SNPs. RT-PCR products encompassing the SNPs  
116 were amplified from fibroblast cell lines from 2 healthy controls and 2 CNM patients. Sanger  
117 sequencing of these RT-PCR products led to identifying one healthy control cell line

118 heterozygous for the 2 SNPs, one CNM cell line (p.R522H DNMT2 mutation) harbouring the 2  
119 SNPs at HTZ state, and one CNM cell line (p.S619L DNMT2 mutation) harbouring only the  
120 SNP1 at HTZ state (Figure 1B). Through RT-PCR, cloning, and Sanger sequencing of a single  
121 allele, we determined that in the R522H cell line, the mutated mRNA also harboured the C  
122 version of SNP1 and T version of SNP2 and that in the S619L cell line, the mutated mRNA  
123 harboured the C version of SNP1 (Figure 1C). Altogether, we identified 2 SNPs targetable by  
124 AS-RNAi, one healthy control cell line to perform the siRNA screening, and 2 patient-derived  
125 cell lines for the functional evaluation of the identified AS-siRNA.

126

### 127 **Allele-specific siRNA against the C allele of SNP1**

128 For all the screening for allele-specific siRNA against SNP1 and SNP2 reported in this study,  
129 we used one healthy control cell line and looked for allele-specific siRNA leading to around  
130 50% expression of DNMT2 mRNA and protein due to specific silencing of the targeted allele.  
131 We first thought to identify AS-siRNA against the C version of the SNP1 (Figure 2 and Supp.  
132 Figure 1). Twelve 19-nucleotide siRNA carrying a single mismatch with the untargeted T  
133 version on the positions 5 to 17 (called si5 to si17) were assessed. At low concentration (30  
134 nM), si6 and si11 were not able to significantly reduce *DNMT2* mRNA expression compared to  
135 scramble siRNA-transfected cells (Figure 2A). In addition, si5, si13, and si14 led to an  
136 excessive reduction of *DNMT2* transcript relative to the expected 50% and 7 siRNAs (si7, si8,  
137 si9, si10, si12, si15, and si17) reduced the *DNMT2* expression in the expected range (Figure 2A).  
138 RT-PCR and BglI restriction enzyme digestion assay was used to discriminate the C (digested)  
139 from the T (undigested) alleles of SNP1 (Supp Figure 2A). The 7 siRNA reduced the C/T ratio  
140 compared to scramble siRNA (Figure 2B) with the lower ratio reached by si8 and si9.  
141 Quantification of each allele relative to *HPRT* mRNA showed that si8, si9, and si10 reduced  
142 expression of the allele C without affecting the T (Supp Figure 1A). At the same concentration,



143 si8, si9, and si10 also reduced expression of the DNMT2 protein around the expected 50%  
144 compared to scramble siRNA in western blot (Supp Figure 1B). The maintenance of allele-  
145 specificity at higher concentration (100nM) was then assessed for si8, si9, and si10. The 3  
146 siRNA reduced total *DNMT2* mRNA content, and close to the expected 50% decrease for si8 and  
147 si9 (Figure 2C). Allele-specificity of si8 and si9 against the targeted C allele was maintained as  
148 demonstrated by the C/T ratio reduction (Figure 2D) and confirmed by quantification of each  
149 allele relative to *HPRT* expression (Supp Figure 1C). Maintenance of efficacy and allele-  
150 specificity of si8 and si9 at higher concentration was also demonstrated on DNMT2 protein by  
151 western blot showing a significant decrease in DNMT2 protein content which did not exceed  
152 50% (Figure 2E). Altogether, these data validate si8 and si9 as the best allele-specific siRNA  
153 against the C version of the SNP1.

154

#### 155 **Allele-specific siRNA against the T allele of the SNP1**

156 To identify AS-siRNA against the T version of SNP1, we assessed siRNA with mismatches  
157 at positions 8, 9, and 10, relative to the untargeted C allele in agreement with our previous  
158 screening. At low concentration (30 nM), si8 and si9 significantly reduced the *DNMT2*  
159 expression in the expected range (Figure 3A). The RT-PCR assay and BglII digestion used to  
160 distinguish the targeted T allele from the untargeted C allele showed that the 3 siRNA  
161 significantly reduced the T/C ratio compared to scramble siRNA (Figure 3B, Supp Figure 3A).  
162 Of note, an increase in the untargeted allele C was observed with si9 (Supp Figure 3A, right  
163 panel) suggestive of a genetic regulation promoting the expression of the untargeted allele. At  
164 this concentration, the DNMT2 protein content was not changed with these 3 siRNA (Supp  
165 Figure 3B). At higher concentration (100nM), total *DNMT2* mRNA amount was reduced for the  
166 3 siRNAs remaining close to the 50% threshold for si8 and si9 (Figure 3C), and allele-  
167 specificity of the 3 siRNAs against the targeted T allele was maintained as demonstrated by the

168 T/C ratio reduction (Figure 3D) and the quantification of each allele relative to *HPRT*  
169 expression (Supp Figure 3C). A significant increase in the untargeted allele was still observed  
170 (Supp Figure 3C, right panel) for the si9 and si10. At this concentration, western blot showed a  
171 significant decrease in DNMT2 protein content for the si8, si9, and si10 compared to scramble  
172 siRNA (Figure 3E). Altogether, these data validate si8 and si9 as allele-specific siRNAs against  
173 the T version of the SNP1.

174

### 175 **Allele-specific siRNA against the T allele of the SNP2**

176 To screen for AS-siRNA against the T version of SNP2, twelve siRNA carrying a mismatch  
177 with the untargeted A allele from positions 6 to 17 were assessed (Figure 4 and Supp Figure 4).  
178 Transfected at 30 nM for 48h, si11, si12, si13, si14, si15, si16, and si17 reduced *DNMT2* mRNA  
179 expression compared to scramble siRNA-transfected cells, and with the exception of si14 and  
180 si15 they all led to a reduction close to 50% (Figure 4A). RT-PCR and Psp5II restriction enzyme  
181 digestion assay was used to discriminate the T digested allele from the A undigested allele  
182 (Figure 4B, Supp Figure 1). Using this assay, we showed that the lower T/A ratio compared to  
183 scramble siRNA was reached by si11, si16, and si17 (Figure 4B). Quantification of each allele  
184 relative to *HPRT* mRNA showed that reduced T/A ratio resulted from a decrease in the  
185 expression of the T allele without reducing the A (Supp Figure 4A). For si17, a significant  
186 increase in the A allele was also noticed (Supp Figure 4A, right panel). At this low  
187 concentration, only si11 and si16 reduced expression of the DNMT2 protein around the expected  
188 50% when compared to scramble siRNA (Supp Figure 4B). We next assessed the maintenance  
189 of allele-specificity for si11, si16, and si17 at higher concentration (100nM). Semi-quantitative  
190 RT-PCR showed a significant reduction of around 50% of total *DNMT2* mRNA for each siRNA  
191 (Figure 4C). The 3 siRNA reduced the T/A ratio compared to scramble siRNA (Figure 4D)  
192 through a specific impact on the T allele for si11 and si16 (Supp Figure 4C). At this higher

193 concentration, DNMT2 protein content did not exceed 50% (Figure 4E). Altogether, these data  
194 validate si11 and si16 as efficient allele-specific siRNA against the T version of the SNP2.

195

#### 196 **Allele-specific siRNA against the A allele of SNP2**

197 Previous results on the T allele of SNP2 showed a large number of mismatch positions in  
198 the siRNAs efficient to develop AS-siRNA. Consequently, we started the screening for AS-  
199 siRNA against the A allele by introducing one mismatch in the central region of the siRNA  
200 (positions 9, 10, and 11) frequently showed with the highest specificity in previously reported  
201 studies (Figure 5 and Supp Figure 5) <sup>23</sup>. At low concentration (30 nM), si9, si10, and si11  
202 significantly reduced the DNMT2 expression in the expected 50% range (Figure 5A). The RT-  
203 PCR assay and Psp5II digestion used to distinguish the targeted A allele from the untargeted T  
204 allele showed that the 3 siRNA significantly reduced the A/T ratio compared to scramble siRNA  
205 through the highest impact on the A allele for si9 and si11 (Figure 5B and Supp Figure 5A). At  
206 this concentration, the DNMT2 protein content was significantly reduced with si10 and si11  
207 (Supp Figure 5B). At higher concentration (100nM), a significant reduction of total *DNMT2*  
208 mRNA was observed for the 3 siRNAs (Figure 5C) and Psp5II digestion of the RT-PCR  
209 products showed maintenance of allele-specificity (Figure 5D). Quantification of each allele  
210 relative to *HPRT* expression showed a reduction of the A allele using si9 and si11 and an  
211 upregulation of the untargeted allele T with the 3 siRNA (Supp Figure 5C). At this  
212 concentration, DNMT2 protein content, quantified by western blot, was reduced by si10 and si11  
213 (Figure 5E) but not by si9 probably due to the upregulation of the untargeted T allele (Supp  
214 Figure 5C). Altogether, these data validate si11 as the most efficient allele-specific siRNA  
215 against the A version of the SNP2.

216

#### 217 **Allele-specific siRNA against the p.S619L DNMT2 mutation**

218 In order to compare the functional benefit of siRNA directed against the SNPs or a mutation,  
219 we screened for AS-siRNA against the p.S619L (c.C1856T) (Figure 6 and Supp Figure 6). The  
220 S619L patient-derived fibroblasts were used to screen for 15 siRNA (si3 to si17) named  
221 depending on the position of the mismatch with the wild-type (WT) sequence of the *DNM2*  
222 mRNA. At low concentration (30 nM), si9 and si11 were not able to significantly reduce *DNM2*  
223 mRNA expression compared to scramble siRNA and si4, si5, si7 and si16 led to an excessive  
224 reduction of *DNM2* transcript relative to the 50% expected (Figure 6A). Nine siRNAs (si3, si6,  
225 si8, si10, si12, si13, si14, si15, and si17) significantly reduced the *DNM2* expression, and  
226 among them, the si6, si8, si10, si13, si15, si17 reduced mRNA amount in the expected range  
227 around 50% (Figure 6A). Given that the S619L mutation does not introduce or remove a  
228 restriction site relative to the WT sequence, we used the presence of the SNP1 at the  
229 heterozygous state in this patient cell line to quantify allele-specificity of the 9 siRNAs using  
230 BglI digestion of the SNP1 sequence (the C allele of SNP1 and the *DNM2* mutation carried by  
231 the same mRNA, Figure 1C and Supp Figure 7B). The 9 siRNA significantly reduced the C/T  
232 (i.e. the mutant/WT) ratio compared to scramble siRNA (Figure 6B) with the lower ratio  
233 reached by si10. Quantification of each allele relative to *HPRT* mRNA showed that the 9  
234 siRNAs reduced expression of the mutated allele with some of them inducing increased in the  
235 WT allele (Supp Figure 6A). We assessed the impact on the *DNM2* protein content of 4 siRNAs  
236 out of the 15 (si6, si8, si10, and si13) and showed a significant reduction which did not exceed  
237 50% for the 4 siRNAs (Supp Figure 6B). At higher concentration (100 nM), a significant  
238 reduction of total *DNM2* mRNA was observed for the 4 siRNA (Figure 6C) and allele-  
239 specificity was maintained (Figure 6D and Supp Figure 6C) with the maximum impact on the  
240 mutated mRNA reached with the si10. At this concentration, *DNM2* protein content, quantified  
241 by western blot, was significantly reduced by the 4 siRNAs and the decrease did not exceed

242 50% (Figure 6E). Altogether, these data validate 4 allele-specific siRNA (si6, si8, si10 and si13)  
243 against the S619L DNMT2 mutant allele.

244

### 245 **Rescue of clathrin-mediated endocytosis and cell surface by AS-siRNA**

246 DNMT2 is well recognized for its role in endocytosis and the defect of clathrin-mediated  
247 endocytosis (CME) was previously demonstrated in AD-CNM patient-derived fibroblasts<sup>18,29</sup>.  
248 We assessed CME through fluorescent transferrin uptake measurement in the healthy control  
249 and the 2 DNMT2-CNM fibroblast cell lines. Under basal conditions, CME was decreased in  
250 fibroblasts harbouring the R522H mutation (R522H-fibroblasts) and increased in fibroblasts  
251 harbouring the S619L mutation (S619L-fibroblasts) compared to control fibroblasts (Figure  
252 7A) and western blot showed similar expression of transferrin receptor between control and  
253 mutant cells (Supp Figure 8). Among the AS-siRNA identified by our *in vitro* screening, we  
254 used si8 against the C version of the SNP1 (SNP1-si8-C, thereafter called siSNP1), si11 against  
255 the T version of the SNP2 (SNP2-si11-T, thereafter called siSNP2), and si10 against the  
256 p.S619L mutation (S619L-si10, thereafter called siS619) for the functional evaluation (Supp  
257 Figure 7). The decrease in transferrin uptake noticed in R522H-fibroblasts was unchanged by  
258 siSNP1 and siSNP2 when transfected at 30 nM for 48 hours (Figure 7B). At 100 nM, the values  
259 of transferrin uptake were increased by siSNP1 and siSNP2 close to the control values for  
260 siSNP1 after 48 hours (Figure 7C). For the S619L-fibroblasts, the increase in transferrin was  
261 significantly reduced by siS619L and siSNP1 transfected at 30 nM and this is maintained at 100  
262 nM (Figure 7B and C).

263 By measuring cell surface for transferrin uptake quantification, a defect was evidenced with  
264 a significantly smaller size for the R522H-fibroblasts and bigger size for the S619L-fibroblasts  
265 compared to the control cell line (Figure 7D). The impact of transfection of the 3 AS-siRNA of  
266 interest was then evaluated on the cell surface changes in patient-derived cells (Figures 7E and

267 7F). Among the tested AS-siRNA, siSNP2 improved the cell size of the R522H-fibroblasts  
268 when transfected at 100 nM. For the S619L-fibroblasts, siSNP1 improved the cell size from 30  
269 nM and siS619L was able to restore cell size only when transfected at 100 nM. Overall, AS-  
270 siRNA directed against non-pathogenic SNPs were found to be as effective as AS-siRNA  
271 directly targeting the mutated nucleotide to restore CME and cell surface in patient-derived  
272 cells harbouring two distinct *DNM2* mutations.

273

#### 274 **Rescue of cell migration by AS-siRNA**

275 *DNM2* dysfunction through its overexpression is known to promote cell migration, invasion  
276 and metastasis in cancers <sup>12</sup>. Therefore, we looked for migration defects in the 2 fibroblast cell  
277 lines carrying the p.R522H and the p.S619L CNM mutations. We have tracked single cells  
278 during 24h and compared the motility behaviour in the 2 patient-derived cell lines and 2 healthy  
279 control cell lines. Both controls cells present similar motility behaviour (Figure 8A and Supp  
280 Figure 9A) but mutated fibroblasts presented cell motility defects including a reduced mean  
281 speed (Figure 8A), an increased duration of pause and a reduced speed when moving only for  
282 the S619L-fibroblasts (Supp Figure 9A).

283 To assess the benefit of allele-specific siRNA, we performed the same experiment 48h after  
284 cell transfection with either scramble siRNA or allele-specific siRNA (Supp Figure 7). At the  
285 concentration of 30 nM, all measured parameters are impaired in mutant fibroblasts transfected  
286 with scramble siRNA compared with the control cell line transfected with a scramble. At this  
287 concentration, siSNP1 improved all impaired motility parameters in the R522H-fibroblasts (*i.e.*  
288 mean speed, number and duration of pause and speed in motion). The siSNP2 fully rescued all  
289 the parameters at the control values (Figure 8B and Supp Figure 9B). For the S619L fibroblasts,  
290 siSNP1 and siS619L were effective to rescue or nearly rescuing the motility parameters to the  
291 control values (Figure 8B and Supp Figure 9B). A second set of experiments was performed at

292 100 nM final concentration to evaluate the possibility of a full restoration of motility parameters  
293 for siSNP1 in the R522H cells. As shown in Figure 8C and Supp Figure 9C, 100 nM of siSNP1  
294 rescued all the motility parameters. Altogether these results highlight a decrease in cell motility  
295 associated with DNM2-CNM mutations which is restored using allele-specific siRNA.

296

### 297 **Rescue of cell adhesion by AS-siRNA**

298 The defects of both plasma membrane turnover due to endocytosis impairment and cell  
299 migration evidenced in the *DNM2*-mutated cell lines (Figures 7 and 8) may also suggest an  
300 impact of the *DNM2* mutations on cell adhesion. We next assessed the adhesion capacity of the  
301 2 CNM fibroblast cell lines by quantifying the number of adherent cells per  $\mu\text{m}^2$  1 hour after  
302 seeding on glass coverslips. A decrease of cell density around 60% was measured in the S619L-  
303 fibroblasts compared to control and no change occurred in the R522H-fibroblasts (Figure 8D).  
304 Consequently, we evaluated the impact of AS-siRNA transfected for 48 hours only in S619L-  
305 fibroblasts. At 30 nM, siS619L and siSNP1 were unable to revert the adhesion defect (Figure  
306 8E) whereas a partial rescue was achieved at 100 nM as intermediate values between patient-  
307 derived cells and healthy control cells values were measured (Figure 8F). Overall, these results  
308 showed that adhesion defect may be present in CNM patient-derived cells and that selected AS-  
309 siRNA against one SNP or the mutation similarly improve this phenotype.

310

311 **Discussion**

312

313 Efficient silencing of morbid genes by RNA interference (RNAi) has been at the origin of  
314 several clinical trials. Efficacy and specificity of RNAi were also used to specifically target the  
315 mutated allele in dominant inherited diseases as for the most frequent *DNM2* mutation causing  
316 CNM which is due to a single nucleotide change<sup>22</sup>. One bottleneck which may limit the  
317 development of therapy by AS-RNAi for the *DNM2*-linked CNM, and beyond for the other  
318 diseases linked to *DNM2* mutations, is the number of reported *DNM2* mutations and then the  
319 number of required AS-siRNA required to cover the entire patient population. Here, we  
320 addressed this key point for preclinical development of AS-RNAi therapy for AD-CNM by  
321 developing versatile AS-siRNA against two non-pathogenic SNPs displaying a wide spectrum  
322 of application for the *DNM2*-linked diseases.

323 Allele-specific siRNA against SNP have been first developed in Huntington's disease<sup>26-</sup>  
324<sup>28,30,31</sup> and Spinocerebellar ataxia<sup>27,32</sup> due to the nature of the mutation; i.e. a repeated sequence  
325 also carried by the normal allele in a shorter version which makes it difficult to develop siRNA  
326 targeting directly the disease-associated sequence. For these diseases, the development of  
327 siRNAs benefited from the presence of SNPs linked to the mutations. There is no disease-  
328 associated SNP in the *DNM2*-linked dominant centronuclear myopathy but the development of  
329 a similar strategy was made possible by the presence of two SNPs with a high frequency of  
330 heterozygosity (SNP1: 45% and SNP2: 30%). By this strategy, all the *DNM2* mutations can be  
331 silenced by choosing the siRNA against the version of the heterozygous SNP carried by the  
332 mutated mRNA. Consequently, we developed efficient AS-siRNA able to specifically silence  
333 each version of the 2 *DNM2* SNPs. Relative to their frequency of heterozygosity in the  
334 population, siRNA against only these 4 targets allows covering ~60% of the AD-CNM patients.  
335 By adding the previously developed siRNA against the most frequent p.R645W mutation (27%



336 of patients)<sup>17</sup> and the siRNA also developed in this study devoted to silence the most frequent  
337 p.S619L mutation associated with the severe neonatal form of the disease (11% of patients)<sup>17</sup>,  
338 ~75% of the AD-CNM population is covered.

339 One key step for the preclinical development of the AS-siRNA is the proof of principle for  
340 a therapeutic benefit in patient-derived cells using pertinent read-out related to the CNM  
341 pathomechanisms. Past studies have given contradictory results concerning the effect of CNM-  
342 associated *DNM2* mutations on clathrin-dependent endocytosis, some showing a decrease<sup>18,29</sup>,  
343 others an absence of effect<sup>33,34</sup> or even an increase<sup>35</sup>. Despite these discrepancies, highlighting  
344 potential cell type- and/or mutation-specific impacts, we evaluated this canonical role attributed  
345 to *DNM2*. As previously<sup>18</sup>, we show that clathrin-mediated endocytosis is impaired in patient  
346 fibroblasts and then may be used as a functional read-out for evaluating allele-specific siRNA.  
347 Interestingly, our results point towards distinct impacts relative to the *DNM2* mutation as the  
348 R522H reduces endocytosis whereas the S619L leads to increased endocytosis. We may  
349 hypothesize that these opposite impacts may participate in the diversity of the clinical spectrum  
350 associated with *DNM2* mutations. To identify additional readout, we took advantage of the  
351 growing number of studies showing the involvement of *DNM2* dysfunction in the invasive  
352 behaviour of cancer cells<sup>10,11,36-43</sup> to evaluate the migration of cells carrying CNM mutations.  
353 Indeed, *DNM2* dysfunction induced by both R522H and S619L mutations similarly impairs  
354 patient-derived cells' migratory properties. In contrast, only the S619L mutation associated  
355 with the severe neonatal CNM form also impedes cell adhesion. Like for clathrin-mediated  
356 endocytosis, the particular alteration of cell adhesion associated with the S619L mutation may  
357 start to highlight the cause of the clinical variability and especially the cause of the severe  
358 neonatal phenotype. Overall, our results represent a new argument in favour of impairment of  
359 the clathrin-mediated endocytosis by CNM-*DNM2* mutations and highlight two new potential  
360 pathomechanisms through alteration of cell migration and adhesion. Further assessment will be

361 required in a muscle context for a better characterization of the pathomechanisms in the DNM2-  
362 CNM as such migration and adhesion impairments may participate in the reduced muscle  
363 regeneration recently demonstrated in a mouse model of the disease <sup>44</sup>. In addition, this work  
364 allowed us to identify a panel of readouts for the functional evaluation of the AS-siRNA.

365 Functional evaluation was performed for the new AS-siRNA developed against the S619L  
366 mutation and the two SNP. By this study, we showed that, except for adhesion, which is only  
367 partially restored, endocytosis, cell size, and cell migration are rescued by siS619 and siSNP1  
368 in S619L-fibroblasts. In R522H-fibroblasts, endocytosis, size and migration have been  
369 similarly partially or fully restored by the AS-siRNA against the two SNPs. Beyond the efficacy  
370 obtained for each AS-siRNA, this study demonstrates that a similar benefit is reached using a  
371 siRNA targeting a mutation or a SNP. In addition, results obtained in the R522H-fibroblasts  
372 show that siRNA developed against SNP1 located in the coding region and SNP2 located in the  
373 3'-UTR region are similarly efficient. However, regardless of the siRNA used, some defects  
374 are difficult to fully correct as illustrated by adhesion impairment in the S619L cells. Such a  
375 highly integrated cell process may require a longer time to be restored. Nevertheless, the  
376 functional evaluation showed that all the allele-specific siRNA developed against the S619L  
377 mutation and the two SNPs are largely efficient to rescue the defects identified in two patient-  
378 derived cell lines without inducing cell toxicity even at 100nM.

379 Interestingly, the field of application of versatile siRNAs against SNPs goes far beyond the  
380 *DNM2*-linked CNM as these siRNAs may also be used for the other dominant diseases due to  
381 *DNM2* mutations including dominant forms of the Charcot-Marie-Tooth disease (CMT) and  
382 hereditary spastic paraplegia (HSP). Due to the respective frequency of heterozygosity in  
383 humans, the development of the versatile siRNA may allow allele-specific silencing therapy in  
384 ~60% (according to frequency of heterozygosity reported in gnomAD) of the CMT and HSP  
385 patient population. The field of application of the versatile siRNAs is even wider since it also

386 includes 60% of patients affected by diseases in which a deleterious overexpression of DNMT2  
387 has been reported such as several cancers <sup>12</sup> and the X-linked recessive CNM <sup>13</sup>. The allele-  
388 specific siRNA developed in this study against the SNP may be used in these pathological  
389 conditions to reduce DNMT2 expression in a controlled manner which avoids an excessive and  
390 potential toxic reduction below 50% by sparing one allele.

391 In conclusion, this study enriches the molecular toolbox with several novel allele-specific  
392 siRNA directed against the most frequent *DNMT2* mutation responsible for the severe neonatal  
393 dominant CNM and with versatile siRNA developed against two non-pathogenic SNPs able to  
394 target all the *DNMT2* mutations. These new molecular tools also enlarge the number of patients  
395 eligible for the therapy by allele-specific DNMT2 silencing by including diseases associated with  
396 either *DNMT2* mutations or deleterious overexpression. The current proof of concept in patient-  
397 derived cells represents an important step for the preclinical development of this therapy for  
398 AD-CNM patients.

399

400 **Methods**

401

402 **Cell cultures and transfection**

403 Healthy controls and CNM patient-derived fibroblast cell lines were obtained from the  
404 MyoLine platform for the immortalization of human cells (Institute of Myology, Paris, France)  
405 in accordance with European recommendations and French legislation. Cell lines were cultured  
406 at 37°C (5% CO<sub>2</sub>) in Dulbecco's modified Eagle's medium (DMEM, Life Technologies,  
407 France) containing 10% fetal calf serum (FCS) supplemented with Penicillin (100 Units/ml)  
408 and Streptomycin (100 µg/ml). Patient and control fibroblasts were immortalized using a  
409 lentiviral vector containing the sequence encoding the catalytic subunit of human telomerase  
410 (hTERT) as previously described<sup>45</sup>. For transfection, cells were grown to 70% confluency and  
411 transfected with siRNAs using RNAimax transfection reagent (Life Technologies, France)  
412 according to the manufacturer's protocol. The concentration of siRNAs for each experiment  
413 was indicated in corresponding figure legends. Allele-specific siRNA and scramble siRNA  
414 were purchased from Eurogentec (Belgium) and the sequences are available on request. Cells  
415 were used for functional evaluation or harvested for RNA and protein extraction 48h after  
416 transfection.

417

418 **Total RNA extraction and cDNA analysis**

419 Total RNAs were isolated from cells using NucleoSpin RNA (Macherey-Nagel, France)  
420 according to the manufacturer's protocol. Cells were passed through a pipetting up-down  
421 several times for disruption in the lysis buffer. Total RNAs (500 ng) were submitted to reverse  
422 transcription using the Superscript III reverse transcriptase kit (Life Technologies, France)  
423 using oligo-dT primers in a final volume of 20 µl. Reverse transcription was performed at 50°C  
424 for 50 minutes, and a final step of 85°C for 5 minutes was added. To determine the allelic

425 version of each SNP present on the mutated allele, PCR encompassing SNPs and the mutation  
426 was performed and cloned using the pGEMT vector system (Promega) and about ten single  
427 clones were sequenced (Eurofins, France). The *DNM2* expression was quantified by semi-  
428 quantitative RT-PCR relative to the *HPRT* housekeeping gene expression. RT product (1  $\mu$ l)  
429 was submitted to PCR performed at 96°C for 3 minutes followed by 27 cycles including  
430 denaturation at 96°C for 25 seconds, annealing at 58°C for 25 seconds and polymerization at  
431 72°C for 40 seconds and a final step at 72°C for 5 minutes. The number of 27 PCR cycles has  
432 been selected to have the amplification in the exponential range for *DNM2* and *HPRT*. To  
433 quantify allele-specificity of the assessed siRNA, assays were developed for SNP1 and SNP2  
434 using restriction enzymes allowing discrimination between the 2 alleles after digestion of the  
435 RT-PCR products. PCR was designed to amplify regions of the *DNM2* transcript encompassing  
436 the SNPs. RT product (1  $\mu$ l) was submitted to SNP1 or SNP2 PCR performed at 96°C for 3  
437 minutes followed by 40 cycles including denaturation at 96°C for 25 seconds, annealing at 58°C  
438 for 25 seconds and polymerization at 72°C for 40 seconds and a final step at 72°C for 5 minutes.  
439 The number of cycles has been selected to be at the end of the exponential phase of  
440 amplification. Ten  $\mu$ l out of the 20  $\mu$ l PCR products were digested overnight at 37°C using 7  
441 units of BglII (New England Biolabs, France) for SNP1, and 15  $\mu$ l out of the 30  $\mu$ l PCR products  
442 were digested overnight at 37°C using 7 units of Psp5II (New England Biolabs, France) for  
443 SNP2. Image acquisition of the PCR products after agarose gel electrophoresis was performed  
444 using a Geni2 gel imaging system (Ozyme, France), and the associated signal was quantified  
445 using ImageJ Software (NIH; <http://rsbweb.nih.gov/ij>). All the PCR primers used in this study  
446 were from Eurogentec (Belgium) and sequences are available on request.

447

448 **Protein extraction and western blot**

449 Cell pellets were homogenized in lysis buffer containing 50 mM of Tris-HCl pH 7.5, 150 mM  
450 NaCl, 1% IGEPAL, 0.5% Deoxycholate Sodium and protease inhibitor cocktail 1% (Sigma-  
451 Aldrich, France) and kept on rotator for 20 minutes at 4°C. After cell lysates scraping, samples  
452 are lysed by sonication 2 times for 10 seconds at 30% of a maximum power of VCX 130 Vibra-  
453 cell ultrasonic processors (Sonics, USA). After centrifugation (12,000 g, 4°C, 20 minutes),  
454 protein concentration in the supernatant was determined with the BCA Protein Assay Kit  
455 (Thermo Scientific Pierce, France). Five micrograms of proteins were mixed with a loading  
456 buffer (50 mM Tris-HCl, SDS 2%, glycerol 10%,  $\beta$ -mercaptoethanol 1% and bromophenol  
457 blue) and denatured at 90°C for 5 minutes. Protein samples were separated on SDS-PAGE 10%  
458 pre-stained gels and transferred onto PVDF membranes (0.45  $\mu$ m pore size, Life Technologies,  
459 France) overnight at 100 mA at 4°C or nitrocellulose membranes (BioRad Turbo transfer  
460 System) by trans blot at 25V, 2.5 mA for 8 minutes. Membranes were blocked for 2 hours at  
461 room temperature in PBS containing non-fat dry milk 5% and Tween-20 0.1% and then exposed  
462 to rabbit polyclonal anti-Dynamin 2 antibody (ab3457 or ab65556, Abcam, France) or rabbit  
463 polyclonal anti-Transferrin receptor antibody (ab84036, Abcam, France) or rabbit polyclonal  
464 anti-GAPDH antibody (sc-25778 Santa Cruz, France) in PBS-Tween-20 0.1%, non-fat dry milk  
465 1% overnight at 4°C. Membranes were rinsed in PBS-Tween-20 0.1% and incubated 2 hours  
466 with horseradish peroxidase-conjugated secondary antibody (anti-rabbit from Jackson  
467 ImmunoResearch, United Kingdom) in PBS-Tween-20 0.1%. Chemiluminescence was  
468 detected using ECL detection Kit (Merck-Millipore, Germany) in a BioRad Chemidoc MP  
469 imaging system and signal quantification was performed using ImageJ software.

470

#### 471 **Transferrin uptake assay and cell surface**

472 Transfected cells were cultured in DMEM at 37°C for 45 minutes. Transferrin-AlexaFluor488  
473 (Life Technologies, France) was added at 20  $\mu$ g/ml at 37°C for 15 minutes. Cells were then

474 washed in DMEM pH 2 and PBS and fixed in paraformaldehyde 4% for 15 minutes. Images z-  
475 stacks were acquired using an Axio Observer Apotome.2 microscope (Zeiss, Germany) using a  
476 20x Plan Apochromat Zeiss objective. Individual cells were outlined manually on the sum  
477 projection of the confocal stacks to measure cell surface ( $\mu\text{m}^2$ ) and the transferrin uptake was  
478 calculated using the ImageJ software for each cell according to the formula: total corrected cell  
479 fluorescence (CTCF) = integrated density of the cell – (mean fluorescence of background x cell  
480 area).

481

### 482 **Adhesion assay**

483 Cells were harvested by trypsinization 48 hours after transfection or under basal conditions and  
484 35000 cells were seeded on a 12-millimetre diameter glass coverslip in 24-well-plates (3  
485 technical replicates for each transfection). After 1 hour incubation at 37°C in DMEM-10% FCS  
486 supplemented with Penicillin (100 Units/ml) and Streptomycin (100  $\mu\text{g}/\text{ml}$ ), the non-attached  
487 cells were removed by PBS washing and cells attached on coverslips were fixed in  
488 paraformaldehyde 4% for 15 minutes. Glass coverslips were mounted on slides with  
489 Vectashield medium (Vector Laboratories) containing DAPI to stain nuclei. Images were  
490 acquired using an Axio Observer Apotome.2 microscope (Zeiss, Germany) using a 2.5x Plan  
491 NeoFluar Zeiss objective and the number of attached cells per  $\mu\text{m}^2$  of coverslip were counted  
492 using ImageJ software through the counting of nuclei. Three independent experiments were  
493 done for each condition.

494

### 495 **Cell tracking and analysis**

496 Cells were seeded at low density on 24-wells plastic plates under basal conditions or 48 hours  
497 after transfection. Bright-field images were acquired at 10X magnification every 20 or 30  
498 minutes (for transfected cells and basal condition, respectively) for 65 hours using an inverted

499 video-microscope equipped with heat and CO<sub>2</sub> controlled chamber (Nikon Ti2, Oko-Lab)  
500 driven by NIS (Nikon). After reconstruction of a 24-hour movie, 50 to 70 cells per condition  
501 were randomly chosen and manually tracked by following the position of the nuclei using  
502 ImageJ software and MTrackJ plugin to determine mean and max speed<sup>46</sup>. Others motility  
503 parameters (mean speed in motion, number and duration of pause) were obtained using Skypad  
504 Microsoft Excel add-in that automatically analyzes particles in 2D trajectories<sup>47</sup>. A threshold of  
505 10 µm was set out to consider the cell in movement between two-time frames and a speed  
506 threshold of 0.1 µm/minute was set out to consider a relevant displacement.

507

### 508 **Data analysis and statistics**

509 Graphics and statistical analyses were performed with GraphPad Prism software versions 6 or  
510 9 (GraphPad Software, LaJolla, California, USA). Values were expressed as means ± SEM.  
511 The number of samples (n), represents the number of independent biological replicates, as  
512 indicated in the figure legends. Number of values analyzed, mean and SEM for all the graph  
513 presented in the figures are listed as supplementary table 1.

514 We used non-parametric statistical tests to analyze our data when the normality could not be  
515 assumed (Shapiro-Wilk test) or tested (n too small). In this case, statistical comparisons  
516 between groups were performed using unpaired two-tailed Mann–Whitney *U*-test for the  
517 siRNAs screening or using a Kruskal-Wallis test followed when significant by Dunn's for  
518 transferrin uptake, cell size and migration assays. Adhesion assay was analyzed using Anova  
519 followed by Bonferroni post-test or Welch's ANOVA to correct the inequality of variance  
520 followed Dunnett's as post-test. P values indicated for Post hoc tests are adjusted p value. P <  
521 0.05 were considered as statistically significant.

522

523



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530

531 **Author contributions**

532 DT and MB conceived and designed the experiments. SD, LM, BP, KM, DT, and MB  
533 performed the experiments. SD, LM, DT, and MB analyzed the data. DT and MB wrote the  
534 manuscript.

535

536 **Conflict of interest statement:** The authors have declared that no conflict of interest exists.

537

538 **Keywords:** allele-specific silencing therapy; dominant centronuclear myopathy; Dynamin 2;  
539 RNA interference, endocytosis, migration, adhesion.

540 Data Availability Statement:

541

542 **References**

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685

686

687 **List of Figure Captions**

688 **Figure 1. Identification of targetable SNPs and heterozygous cells.** **A.** Schematic  
689 representation of the *DNM2* mRNA showing the location of 2 CNM-mutations and 2 single  
690 nucleotide polymorphisms (SNP). For each SNP, the frequencies of the 2 alleles are indicated  
691 as well as the frequency of heterozygosity in the population. HTZ: heterozygous. **B.** Sequencing  
692 of the SNPs in healthy control and 2 patient-derived cell lines. **C.** Results of the genotyping of  
693 SNP1 and SNP2 on the wild-type (WT) and mutated *DNM2* mRNAs in the 2 patient-derived  
694 cell lines.

695

696 **Figure 2: Identification of allele-specific siRNA against the C version of SNP1.** **A.**  
697 Expression of the *DNM2* mRNA 48 hours after siRNA transfection at 30 nM. Agarose gel  
698 electrophoresis of *DNM2* and *HPRT* RT-PCR products and quantification of *DNM2* expression  
699 normalized to *HPRT* ( $n \geq 4$  per condition). **B.** BglI digestion profile on the *DNM2* PCR product  
700 including the SNP1 sequence and quantification of the C/T ratio ( $n \geq 4$  per condition) after  
701 transfection with siRNA at 30 nM. **C.** Expression of the *DNM2* mRNA 48 hours after siRNA  
702 transfection at 100 nM. Agarose gel electrophoresis of *DNM2* and *HPRT* RT-PCR products and  
703 quantification of *DNM2* expression normalized to *HPRT* ( $n \geq 4$  per condition). **D.** BglI digestion  
704 profile on the *DNM2* PCR product including the SNP1 sequence and quantification of the C/T  
705 ratio ( $n \geq 4$  per condition) after transfection with siRNA at 100 nM. **E.** *DNM2* western blot and  
706 quantification of the signal by densitometry after siRNA transfection at 100 nM concentration.  
707 GAPDH was used as a loading control ( $n \geq 4$ ). Data information: In scatter plots, the bars are  
708 mean values and error bars indicate SEM. \*\*\*\*P < 0.0001, \*\*\*P < 0.001, \*\*P < 0.01 and \*P <  
709 0.5 using a two-tailed Mann–Whitney *U*-test relative to the scramble siRNA (Sc).

710



711 **Figure 3: Identification of allele-specific siRNA against the T version of SNP1. A.**  
712 Expression of the *DNM2* mRNA 48 hours after siRNA transfection at 30 nM. Agarose gel  
713 electrophoresis of *DNM2* and *HPRT* RT-PCR products and quantification of *DNM2* expression  
714 normalized to *HPRT* ( $n \geq 4$  per condition). **B.** BglI digestion profile on the *DNM2* PCR product  
715 including the SNP1 sequence and quantification of the T/C ratio ( $n \geq 4$  per condition) after  
716 transfection with siRNA at 30 nM. **C.** Expression of the *DNM2* mRNA 48 hours after siRNA  
717 transfection at 100 nM. Agarose gel electrophoresis of *DNM2* and *HPRT* RT-PCR products and  
718 quantification of *DNM2* expression normalized to *HPRT* ( $n \geq 4$  per condition). **D.** BglI digestion  
719 profile on the *DNM2* PCR product including the SNP1 sequence and quantification of the T/C  
720 ratio ( $n \geq 4$  per condition) after transfection with siRNA at 100 nM. **E.** *DNM2* western blot and  
721 quantification of the signal by densitometry after siRNA transfection at 100 nM concentration.  
722 GAPDH was used as a loading control ( $n \geq 4$ ). Data information: In scatter plots, the bars are  
723 mean values and error bars indicate SEM. \* $P < 0.5$  using a two-tailed Mann–Whitney *U*-test  
724 relative to the scramble siRNA (Sc)

725

726 **Figure 4: Identification of allele-specific siRNA against the T version of SNP2. A.**  
727 Expression of the *DNM2* mRNA 48 hours after siRNA transfection at 30 nM. Agarose gel  
728 electrophoresis of *DNM2* and *HPRT* RT-PCR products and quantification of *DNM2* expression  
729 normalized to *HPRT* ( $n \geq 4$  per condition). **B.** Psp5II digestion profile on the *DNM2* PCR  
730 product including the SNP2 sequence and quantification of the T/A ratio ( $n \geq 4$  per condition)  
731 after transfection with siRNA at 30 nM. **C.** Expression of the *DNM2* mRNA 48 hours after  
732 siRNA transfection at 100 nM. Agarose gel electrophoresis of *DNM2* and *HPRT* RT-PCR  
733 products and quantification of *DNM2* expression normalized to *HPRT* ( $n \geq 4$  per condition). **D.**  
734 Psp5II digestion profile on the *DNM2* PCR product including the SNP2 sequence and  
735 quantification of the T/A ratio ( $n \geq 4$  per condition) after transfection with siRNA at 100 nM.

736 **E.** DNMT2 western blot and quantification of the signal by densitometry after siRNA  
737 transfection at 100 nM concentration. GAPDH was used as a loading control ( $n \geq 4$ ). Data  
738 information: In scatter plots, the bars are mean values and error bars indicate SEM. \*\*\*\* $P <$   
739 0.0001, \*\*\* $P <$  0.001, \*\* $P <$  0.01 and \* $P <$  0.5 using a two-tailed Mann–Whitney *U*-test relative  
740 to the scramble siRNA (Sc).

741

742 **Figure 5: Identification of allele-specific siRNA against the A version of SNP2. A.**

743 Expression of the *DNMT2* mRNA 48 hours after siRNA transfection at 30 nM. Agarose gel  
744 electrophoresis of *DNMT2* and *HPRT* RT-PCR products and quantification of *DNMT2* expression  
745 normalized to *HPRT* ( $n \geq 4$  per condition). **B.** Psp5II digestion profile on the *DNMT2* PCR  
746 product including the SNP2 sequence and quantification of the A/T ratio ( $n \geq 4$  per condition)  
747 after transfection with siRNA at 30 nM. **C.** Expression of the *DNMT2* mRNA 48 hours after  
748 siRNA transfection at 100 nM. Agarose gel electrophoresis of *DNMT2* and *HPRT* RT-PCR  
749 products and quantification of *DNMT2* expression normalized to *HPRT* ( $n \geq 4$  per condition). **D.**  
750 Psp5II digestion profile on the *DNMT2* PCR product including the SNP2 sequence and  
751 quantification of the A/T ratio ( $n \geq 4$  per condition) after transfection with siRNA at 100 nM.  
752 **E.** DNMT2 western blot and quantification of the signal by densitometry after siRNA  
753 transfection at 100 nM concentration. GAPDH was used as a loading control ( $n \geq 4$ ). Data  
754 information: In scatter plots, the bars are mean values and error bars indicate SEM. \*\* $P <$  0.01  
755 and \* $P <$  0.5 using a two-tailed Mann–Whitney *U*-test relative to the scramble siRNA (Sc).

756

757 **Figure 6: Identification of allele-specific siRNA against the S619L DNMT2 mutation. A.**

758 Expression of the *DNMT2* mRNA 48 hours after siRNA transfection at 30 nM. Agarose gel  
759 electrophoresis of *DNMT2* and *HPRT* RT-PCR products and quantification of *DNMT2* expression  
760 normalized to *HPRT* ( $n \geq 4$  per condition). **B.** BglI digestion profile on the DNMT2 PCR product

761 including the SNP1 sequence and quantification of the mutant/WT ratio ( $n \geq 4$  per condition)  
762 after transfection with siRNA at 30 nM. The mutant allele harbors the C version of SNP1. **C.**  
763 Expression of the *DNM2* mRNA 48 hours after siRNA transfection at 100 nM. Agarose gel  
764 electrophoresis of *DNM2* and *HPRT* RT-PCR products and quantification of *DNM2* expression  
765 normalized to *HPRT* ( $n \geq 4$  per condition). **D.** BglI digestion profile on the *DNM2* PCR product  
766 including the SNP1 sequence and quantification of the mutant/WT ratio ( $n \geq 4$  per condition)  
767 after transfection with siRNA at 100 nM. **E.** *DNM2* western blot and quantification of the signal  
768 by densitometry after siRNA transfection at 100 nM concentration. GAPDH was used as a  
769 loading control ( $n \geq 4$ ). Data information: In scatter plots, the bars are mean values and error  
770 bars indicate SEM. \*\*\* $P < 0.001$  and \* $P < 0.1$  using a two-tailed Mann–Whitney *U*-test relative  
771 to the scramble siRNA (Sc)

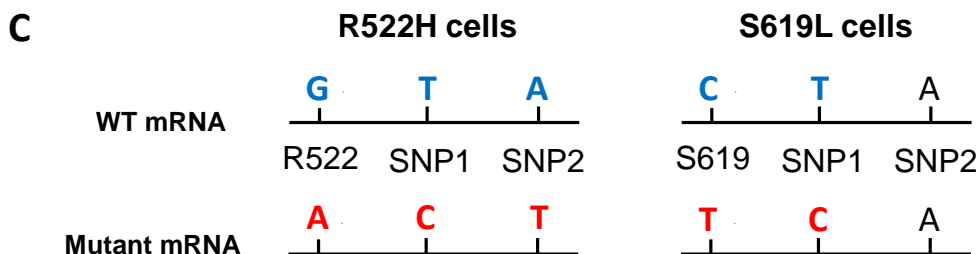
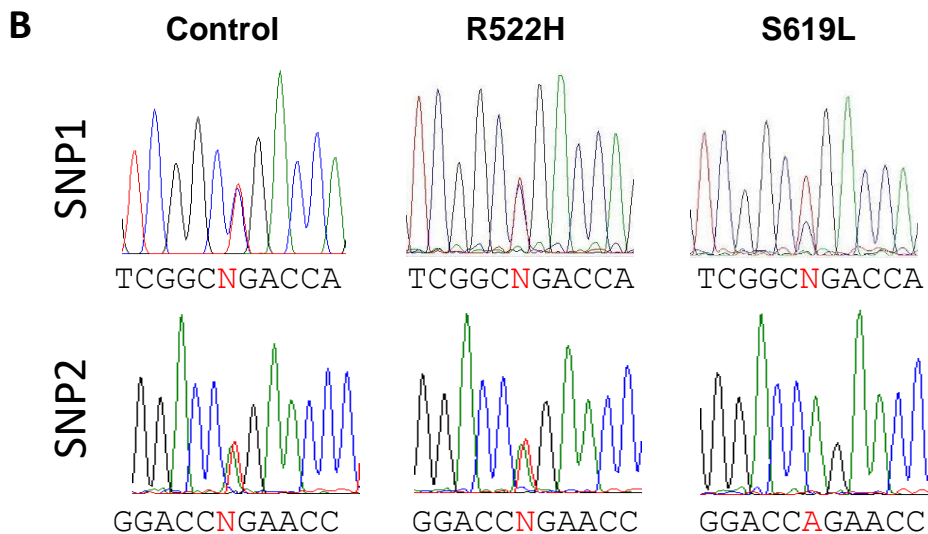
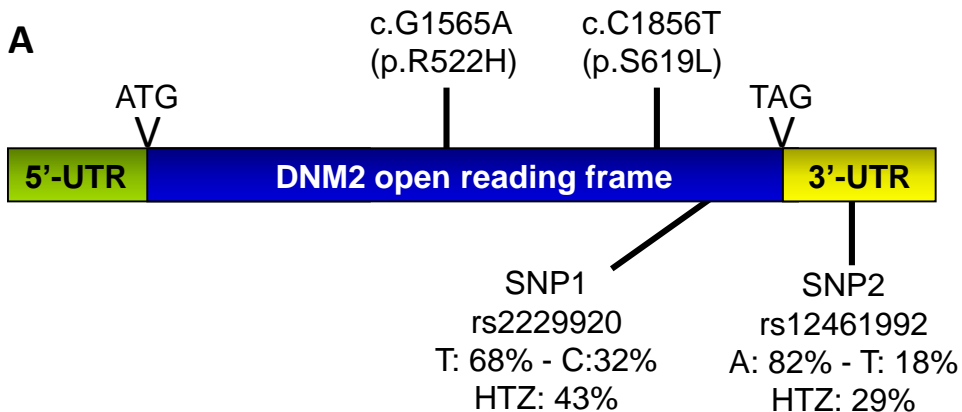
772

773 **Figure 7: Impact of AS-siRNA on transferrin uptake and cell surface of patient-derived**  
774 **fibroblasts.** **A.** Transferrin uptake under basal conditions ( $n = 500-700$  cells from 3 independent  
775 experiments). CTCF: Corrected total cell fluorescence. **B.** Transferrin uptake after 48 hours  
776 transfection with 30 nM scramble siRNA (sc) or allele-specific siRNA against the S619L  
777 mutation or the 2 SNPs ( $n = 200-300$  cells from 2 independent experiments). **C.** Transferrin  
778 uptake after 48 hours transfection with 100 nM scramble siRNA (sc) or allele-specific siRNA  
779 against the S619L mutation or the 2 SNPs ( $n = 200-300$  cells from 2 independent experiments).  
780 **D.** Cell surface ( $\mu\text{m}^2$ ) under basal conditions ( $n = 500-700$  cells from 3 independent  
781 experiments). **E.** Cell surface after 48 hours transfection with 30 nM scramble siRNA (sc) or  
782 allele-specific siRNA against the S619L mutation or the 2 SNPs ( $n = 200-300$  cells from 2  
783 independent experiments). **F.** Cell surface after 48 hours transfection with 100 nM scramble  
784 siRNA (sc) or allele-specific siRNA against the S619L mutation or the 2 SNPs ( $n = 200-300$   
785 cells from 2 independent experiments). Data information: bars represent mean values and error

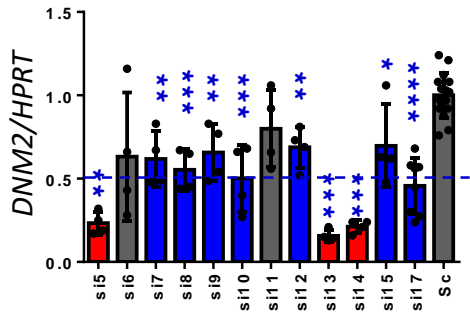
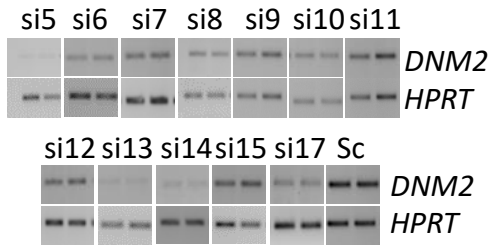
786 bars indicate SEM. Statistical analysis are performed using Kruskal-Wallis for A to F (\*\*\*\*  
787  $p < 0.0001$  in all histograms), followed by Dunn's test relative to control cells in A and D (\*\*\*\*  
788  $P < 0.0001$ , \*\*\*  $P < 0.001$  adjusted p-value), control cells transfected with scramble siRNA in B,  
789 C, E and F (\*\*\*\*  $P < 0.0001$  adjusted p-values), or the patient cell lines transfected with scramble  
790 siRNA in B, C, E and F ( $\dagger P < 0.05$ ,  $\dagger\dagger P < 0.01$ , and  $\dagger\dagger\dagger P < 0.0001$  adjusted p-values).

791

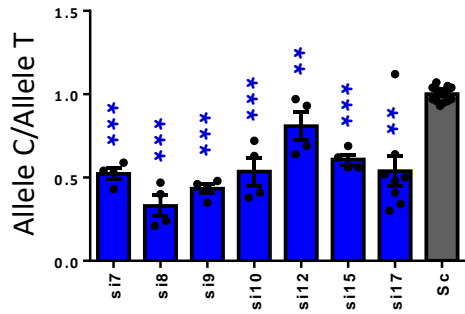
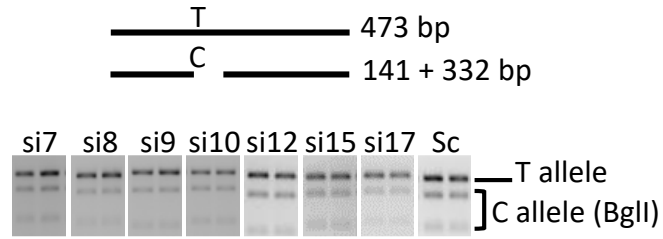
792 **Figure 8: Migration and adhesion assays in patient-derived cells.** **A.** Mean speed of CNM-  
793 and control-fibroblasts ( $\mu\text{m}/\text{minute}$ ) under basal conditions ( $n=50$  cells tracked). **B.** Mean speed  
794 of cells after 48 hours transfection with 30 nM scramble siRNA (sc) or allele-specific siRNA  
795 against the S619L mutation or the 2 SNPs ( $n= 50-70$  cells tracked). **C.** Mean speed of cells after  
796 48 hours transfection with 100 nM scramble siRNA (sc) or allele-specific siRNA against the  
797 SNP1 ( $n= 50-70$  cells tracked). **D.** Adhesion under basal conditions ( $n=8$  independent assays).  
798 **E.** Adhesion assay after 48 hours transfection with 30 nM scramble siRNA (sc) or allele-  
799 specific siRNA against the S619L mutation or the SNP1 ( $n=5$  or 6 independent transfections).  
800 **F.** Adhesion assay after 48 hours transfection with 100 nM scramble siRNA (sc) or allele-  
801 specific siRNA against the S619L mutation or SNP1 ( $n=6$  independent transfections). Data  
802 information: bars represent mean values and error bars indicate SEM. Statistical analysis are  
803 performed using Kruskal-Wallis for migration assay A to C (\*\*\*\*  $p < 0.0001$  in all histograms),  
804 followed by Dunn's test relative to control cells 1 (\*\*\*  $P < 0.001$ ) or control cells 2 ( $\phi\phi P < 0.01$ )  
805 in A, or to control cells transfected with scramble siRNA in B and C (\*\*\*\*  $P < 0.0001$ ), or to  
806 patient cell lines transfected with scramble siRNA in B and C ( $\dagger\dagger\dagger\dagger P < 0.0001$ ). Anova tests  
807 were performed for adhesion assay in A (\*\*  $P < 0.01$ ), B and C (\*\*\*\* $P < 0.0001$ ) followed by  
808 post-tests relative to control cells in A (\*\* $P < 0.01$ ), to control cells transfected with scramble  
809 siRNA (\*\*\*\*  $P < 0.0001$ , \*\*\*  $P < 0.001$  \*\*  $P < 0.01$ ) and to patient cell lines transfected with  
810 scramble siRNA ( $\dagger\dagger p < 0.01$ ,  $\dagger\dagger p < 0.05$ ) in B and C



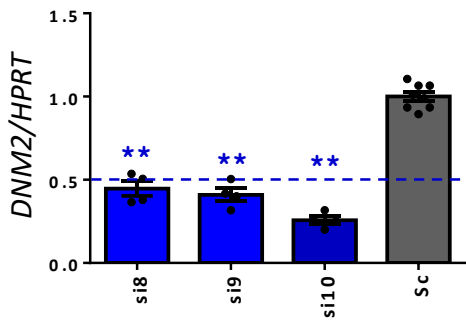
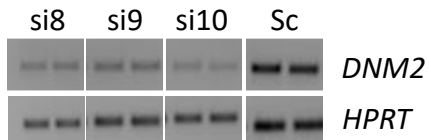
**A** *DNM2* mRNA (siRNA at 30 nM)



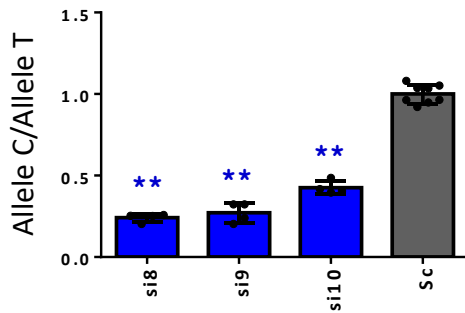
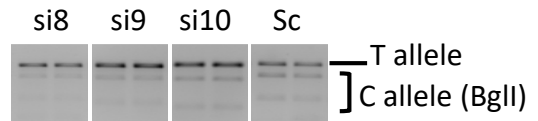
**B** *Bgl*I digestion (siRNA at 30 nM)



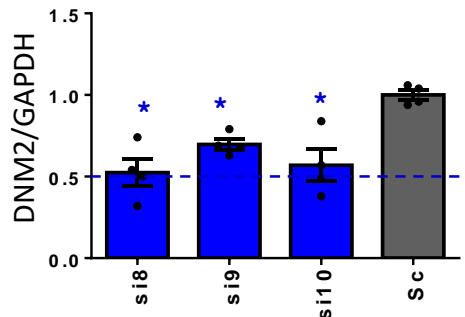
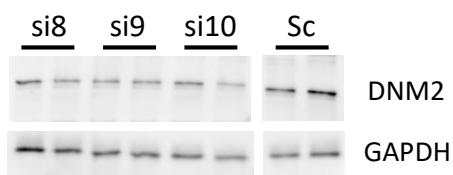
**C** *DNM2* mRNA (siRNA at 100 nM)

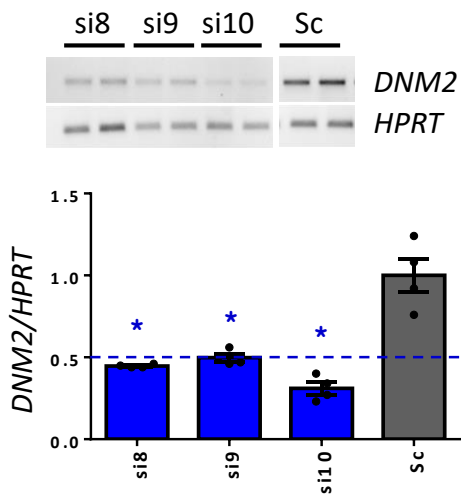
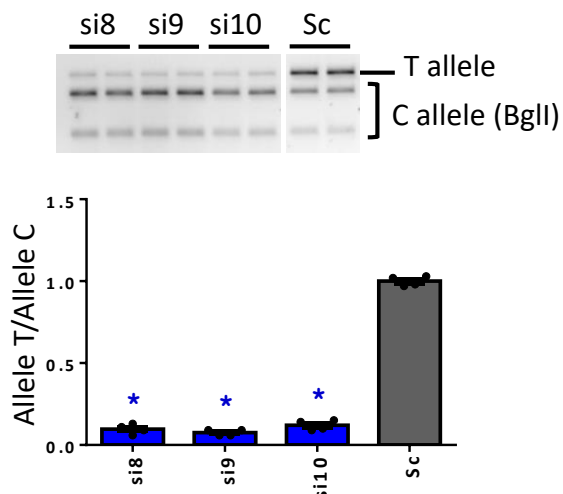
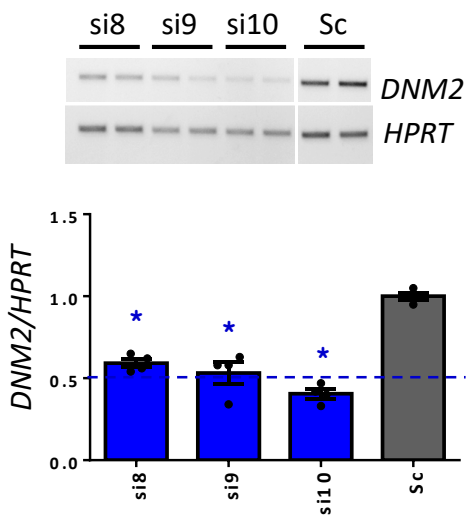
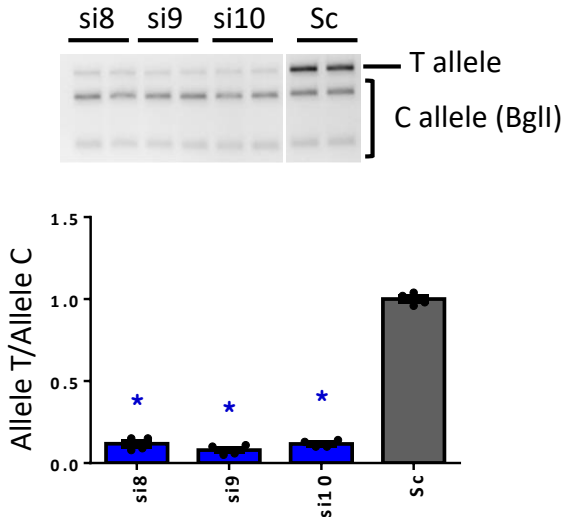


**D** *Bgl*I digestion (siRNA at 100 nM)

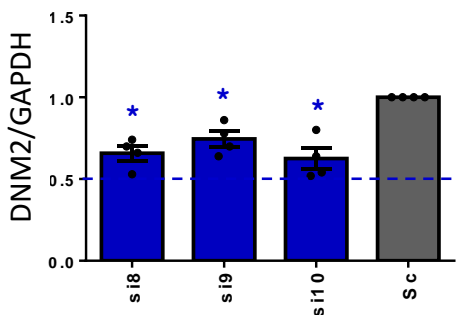
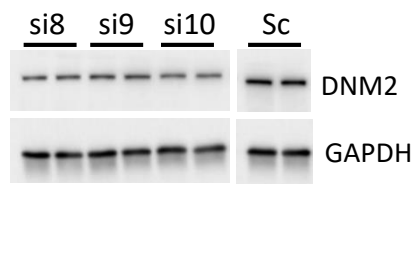


**E** *DNM2* protein (siRNA at 100 nM)

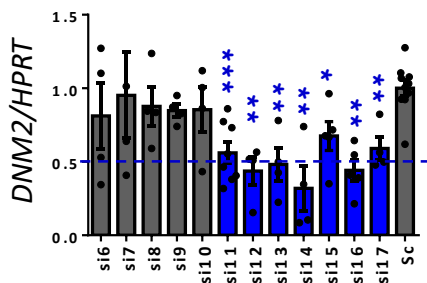
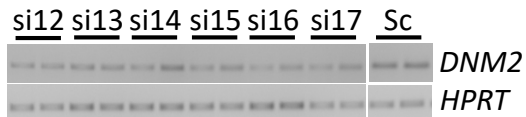
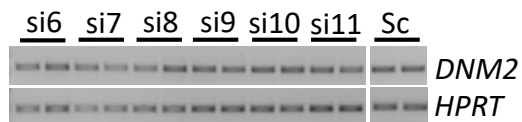


**A** *DNM2* mRNA (siRNA at 30 nM)**B** BglI digestion (siRNA at 30 nM)**C** *DNM2* mRNA (siRNA at 100 nM)**D** BglI digestion (siRNA at 100 nM)

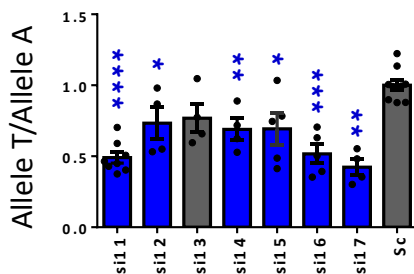
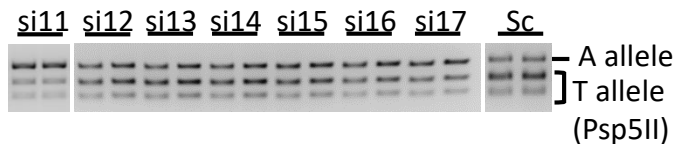
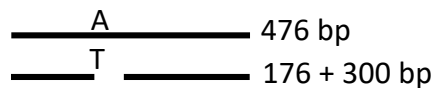
## DNM2 protein (siRNA at 100 nM)

**E**

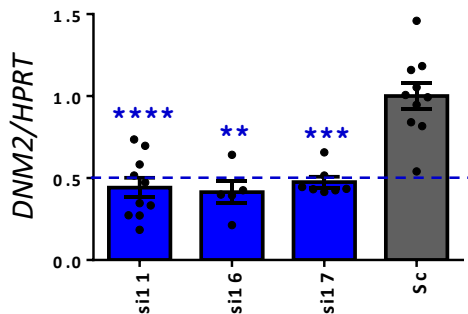
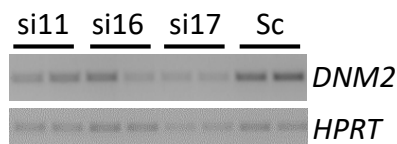
**A** *DNM2* mRNA (siRNA at 30 nM)



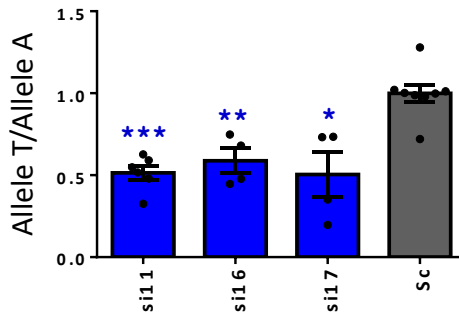
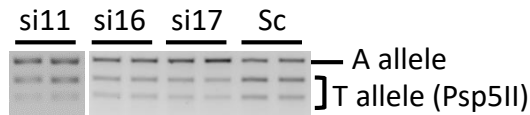
**B** Psp5II digestion (siRNA at 30 nM)



**C** *DNM2* mRNA (siRNA at 100 nM)

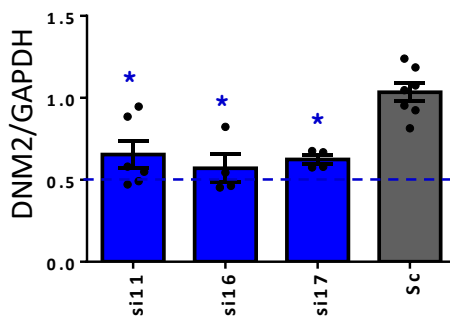
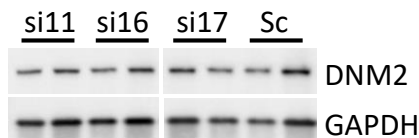


**D** Psp5II digestion (siRNA at 100 nM)

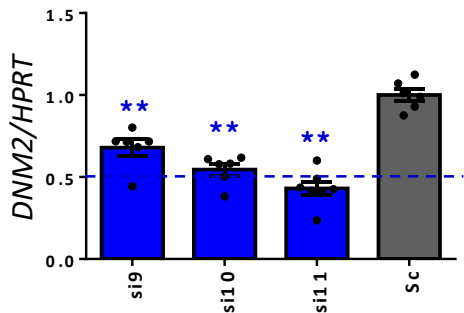
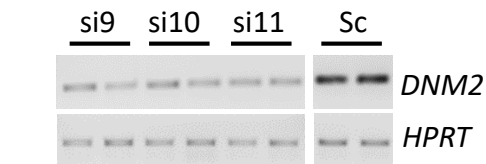
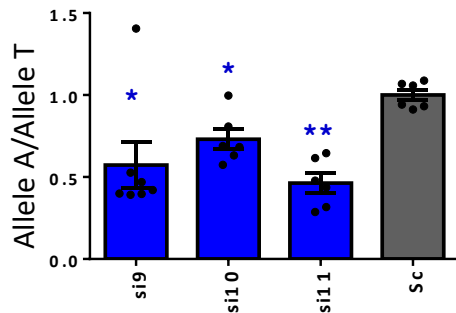
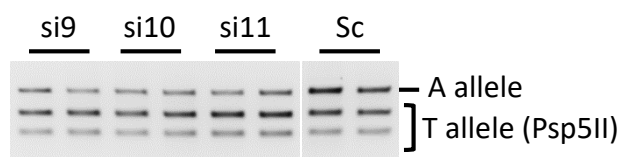
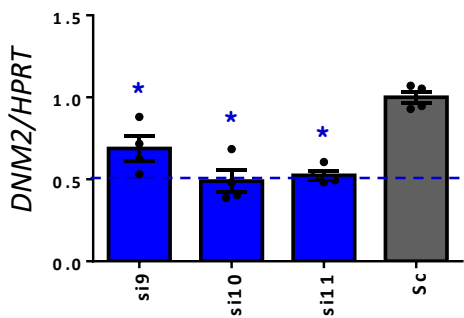
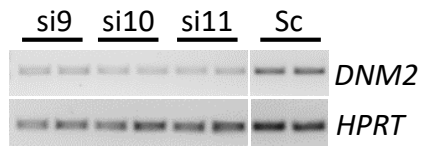
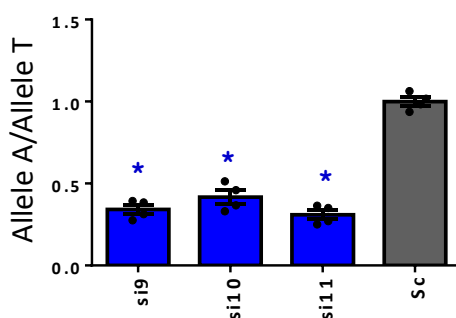
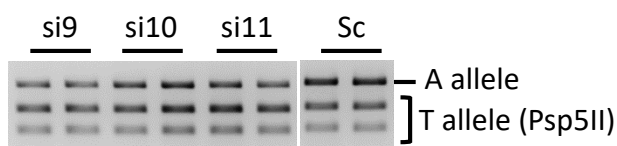


**DNM2** protein (siRNA at 100 nM)

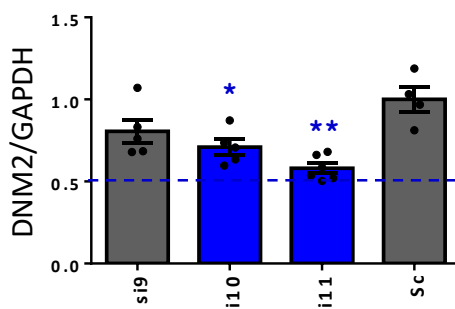
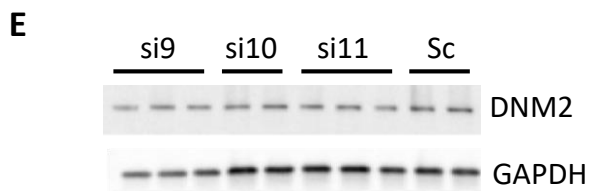
**E**



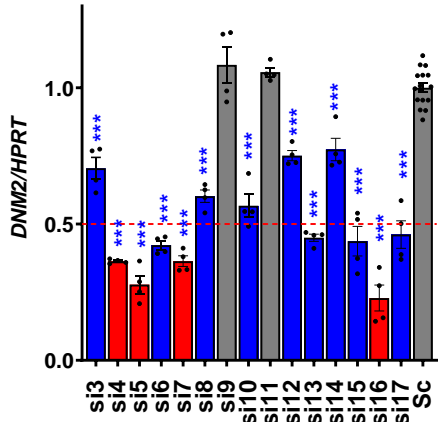
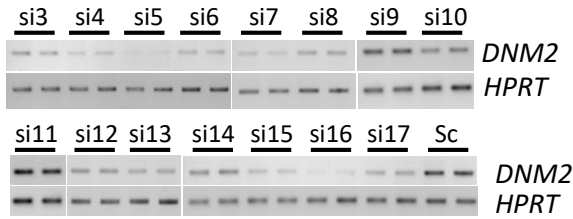


**A** *DNM2* mRNA (siRNA at 30 nM)**B** Psp5II digestion (siRNA at 30 nM)**C** *DNM2* mRNA (siRNA at 100 nM)**D** Psp5II digestion (siRNA at 100 nM)

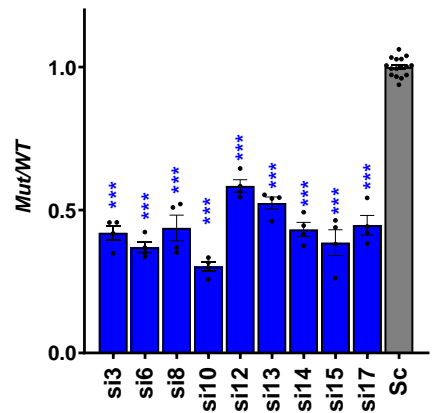
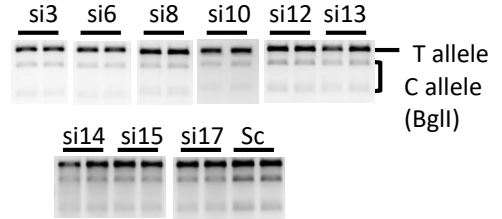
## DNM2 protein (siRNA at 100 nM)

**E**

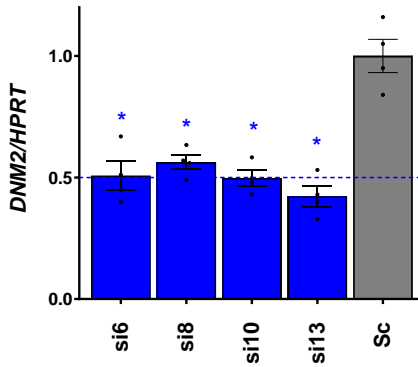
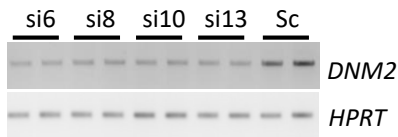
**A** *DNM2* mRNA (siRNA at 30 nM)



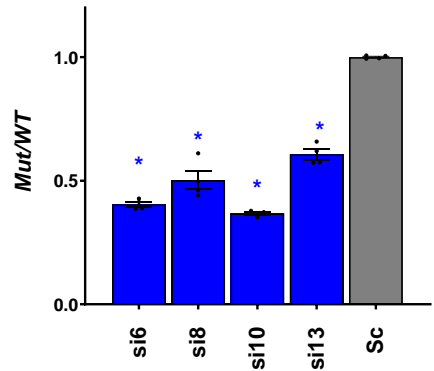
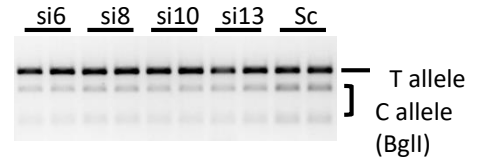
**B** *Bgl*I digestion (siRNA at 30 nM)



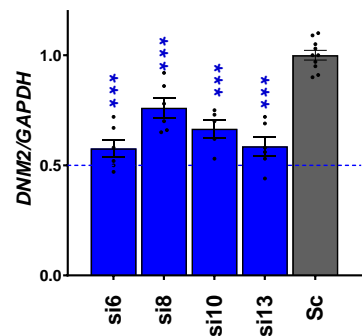
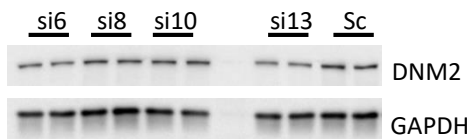
**C** *DNM2* mRNA (siRNA at 100 nM)

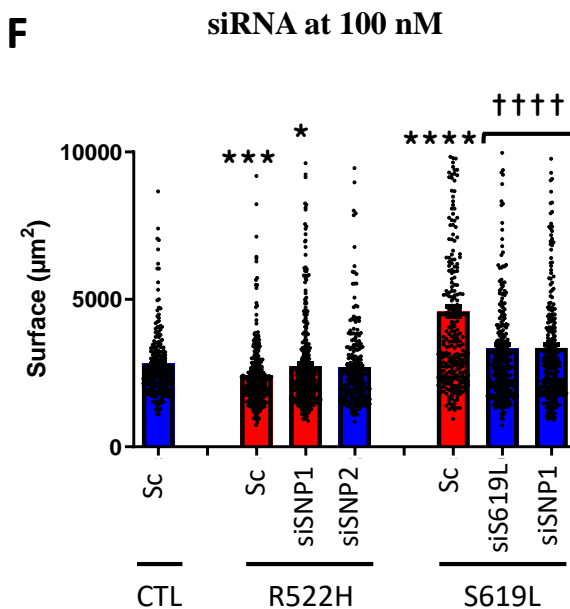
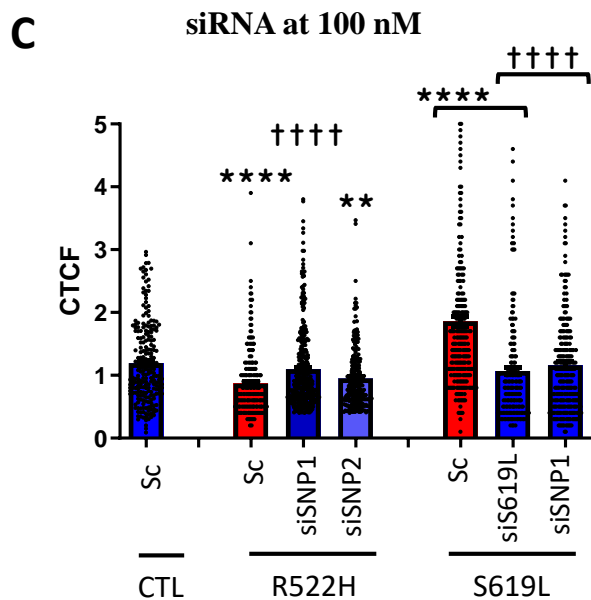
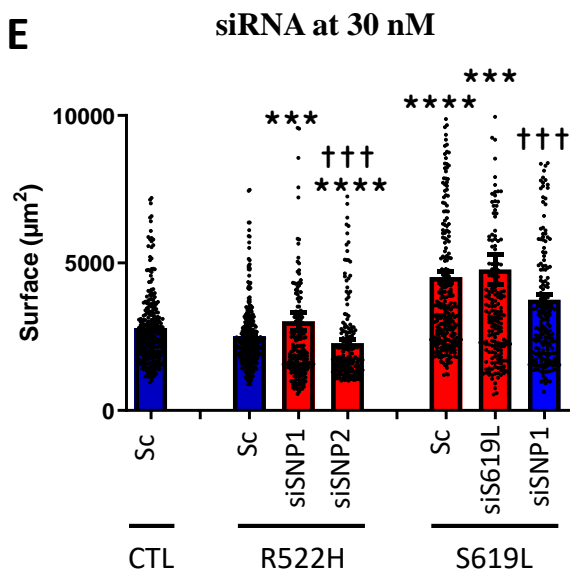
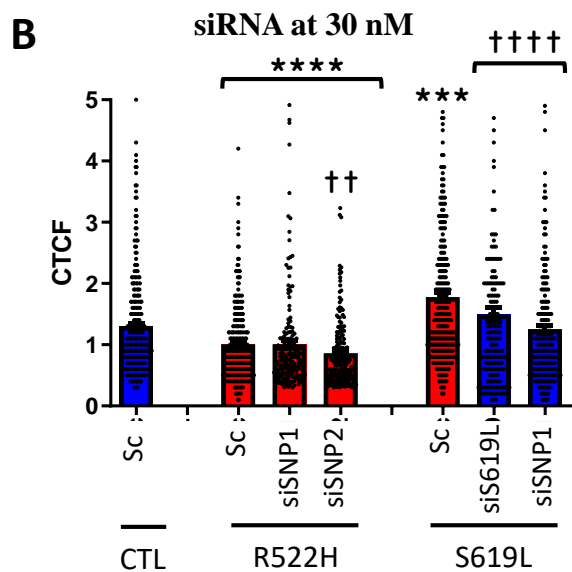
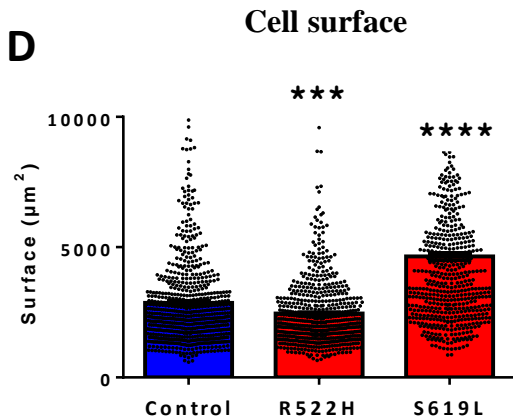
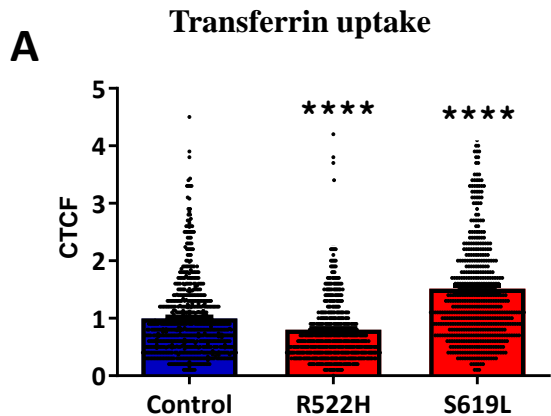


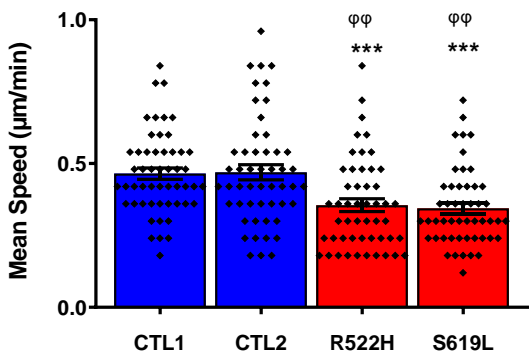
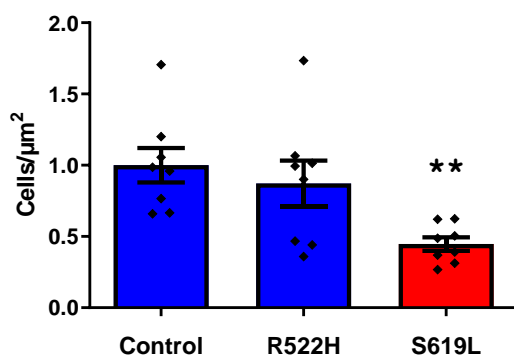
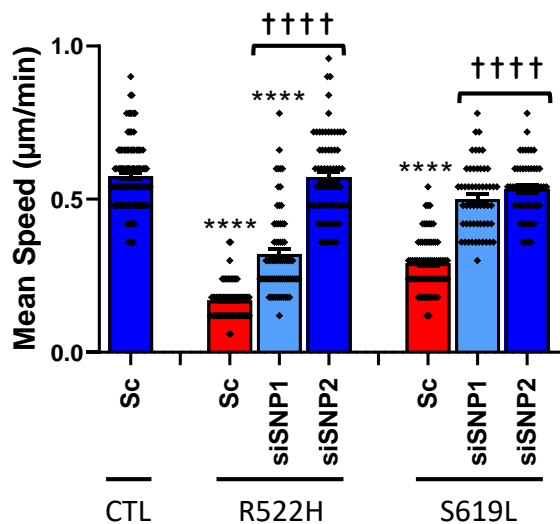
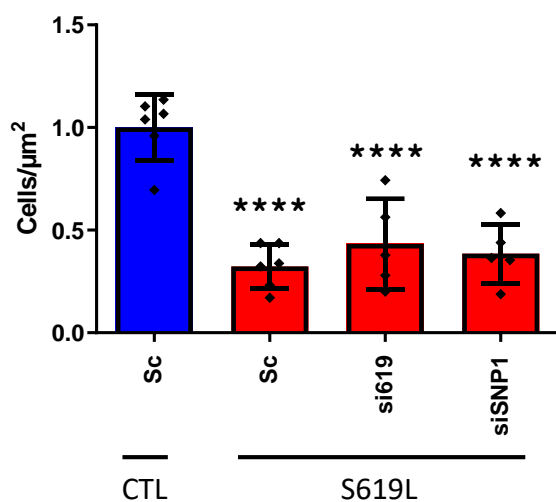
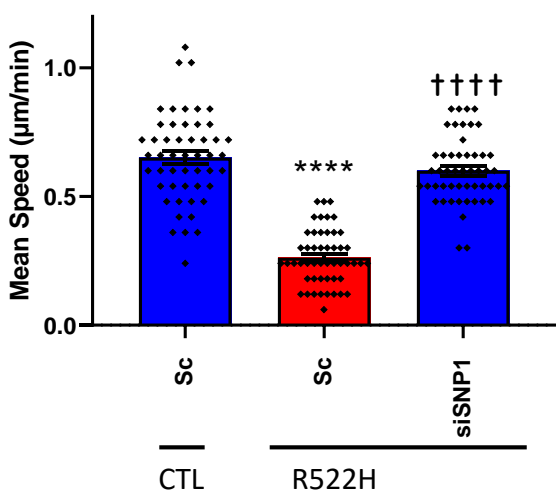
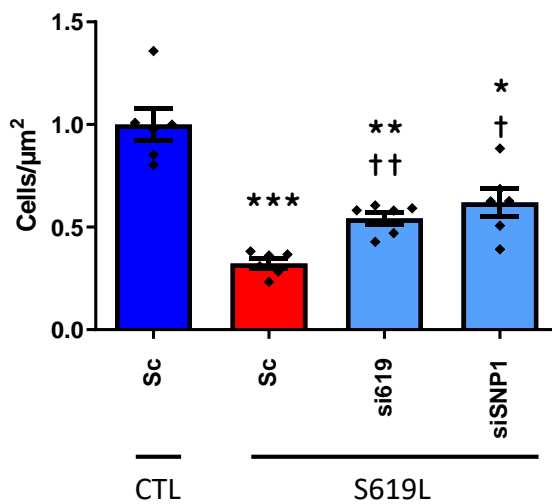
**D** *Bgl*I digestion (siRNA at 100 nM)



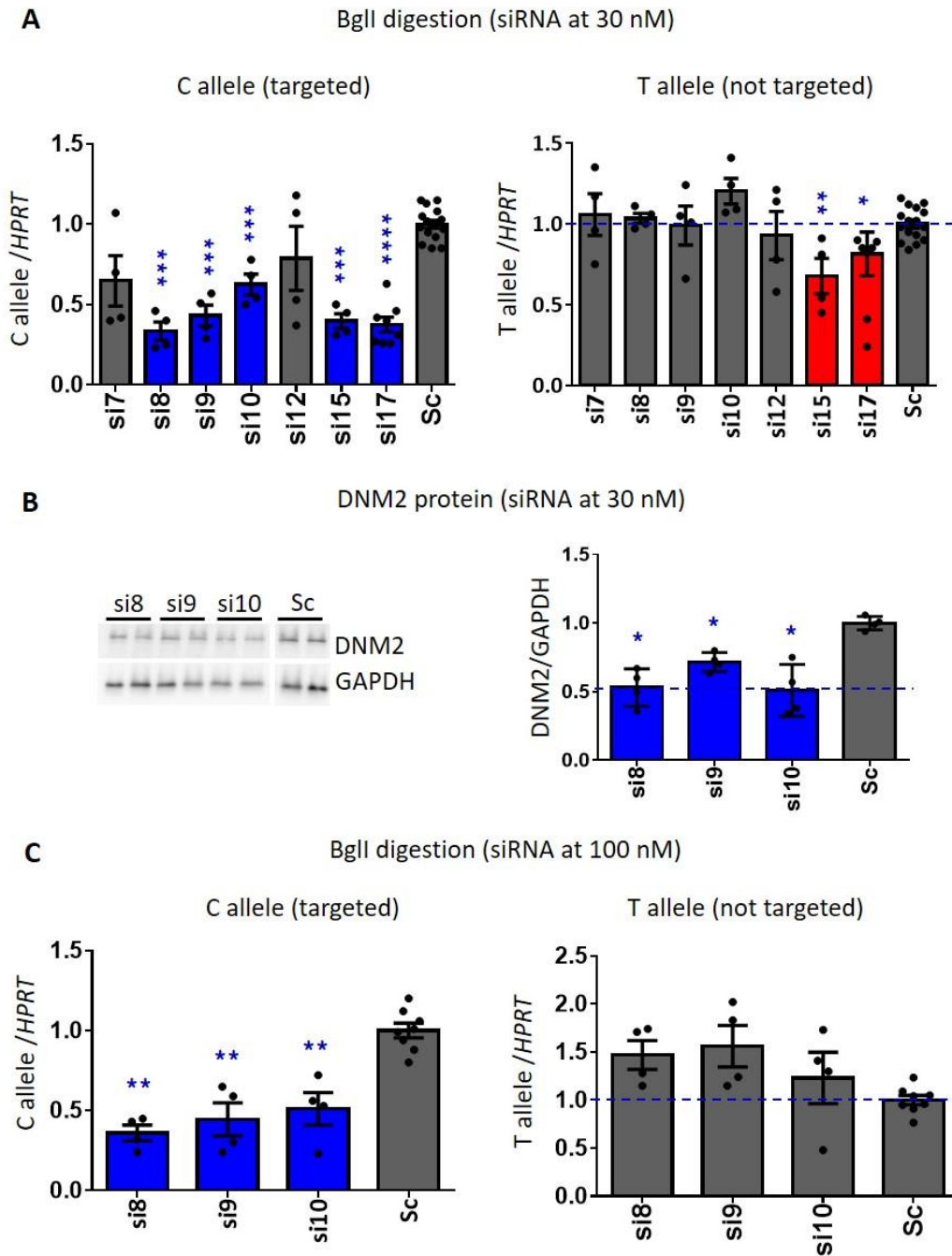
**E** *DNM2* protein (siRNA at 100 nM)





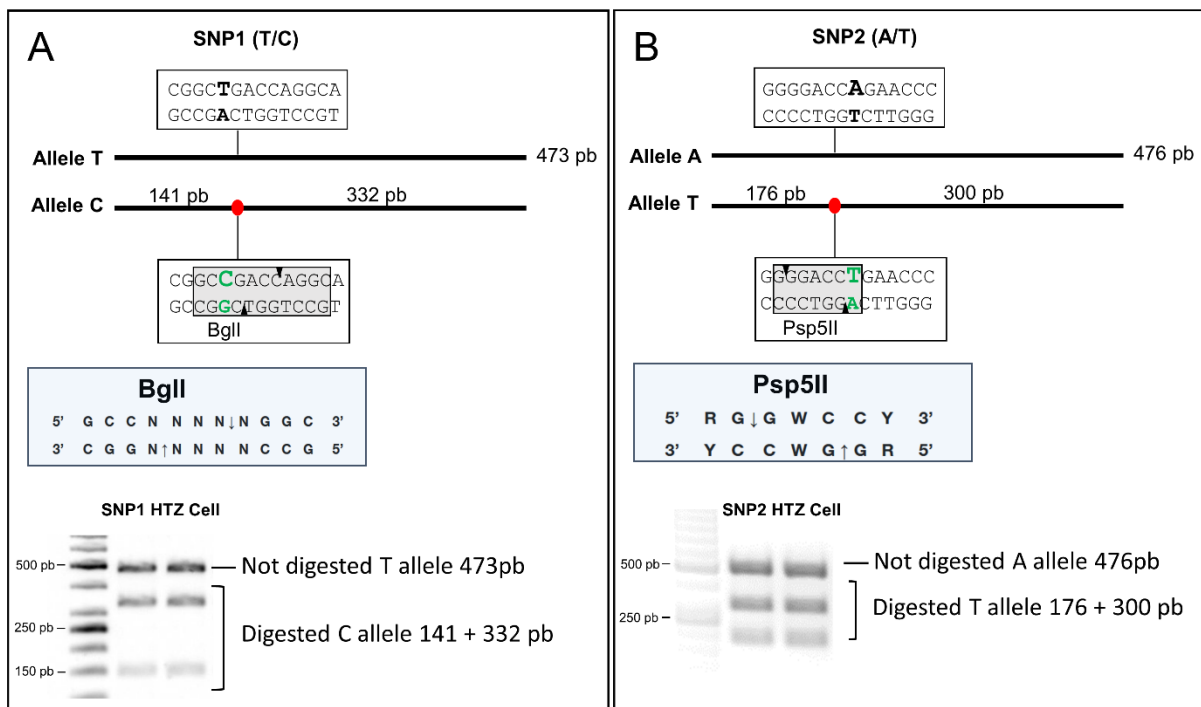
**A** Migration**D** Adhesion**B** siRNA at 30 nM**E** siRNA at 30 nM**C** siRNA at 100 nM**F** siRNA at 100 nM

## Supplementary Figures, Tables and Legends



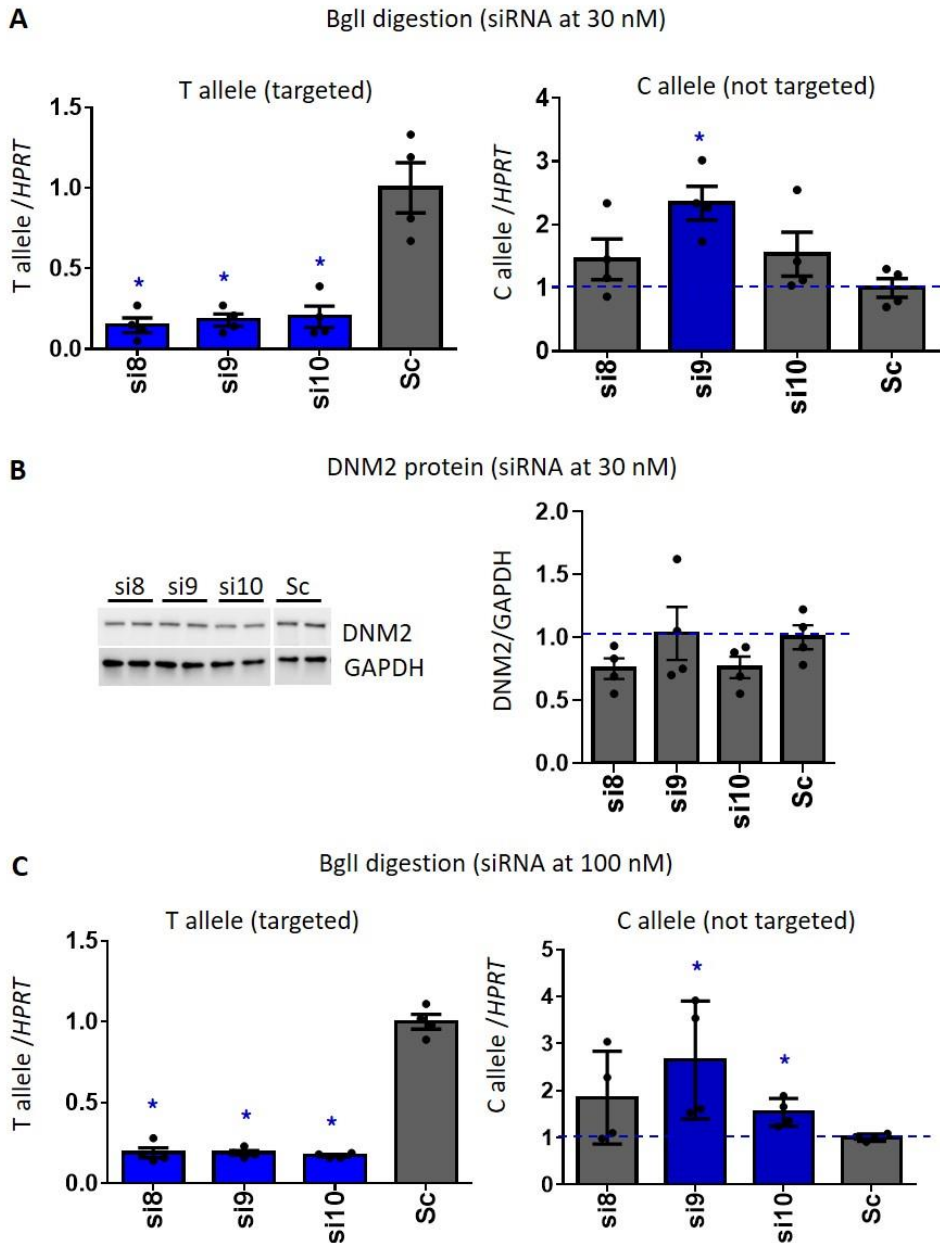
**Supplementary Figure 1: Allele-specific siRNA against the C version of SNP1. A.** Expression of the C and T alleles 48 hours after siRNA transfection at 30 nM. Quantification of the 2 alleles after BglI digestion of the RT-PCR product and normalized to *HPRT* ( $n \geq 4$  per condition). **B.** DNM2 western blot and quantification of the signal by densitometry after siRNA

transfection at 30 nM concentration. GAPDH was used as a loading control ( $n \geq 4$ ). C. Expression of the C and T alleles 48 hours after siRNA transfection at 100 nM. Quantification of the 2 alleles after BglI digestion of the RT-PCR product and normalized to *HPRT* ( $n \geq 4$  per condition). Data information: In scatter plots, the bars are mean values and error bars indicate SEM. \*\*\* $P < 0.001$ , \*\* $P < 0.01$  and \* $P < 0.5$  using a two-tailed Mann–Whitney *U*-test relative to the scramble siRNA (Sc).



**Supplementary Figure 2: RT-PCR assays for identification of SNP allele-specificity in fibroblasts.**

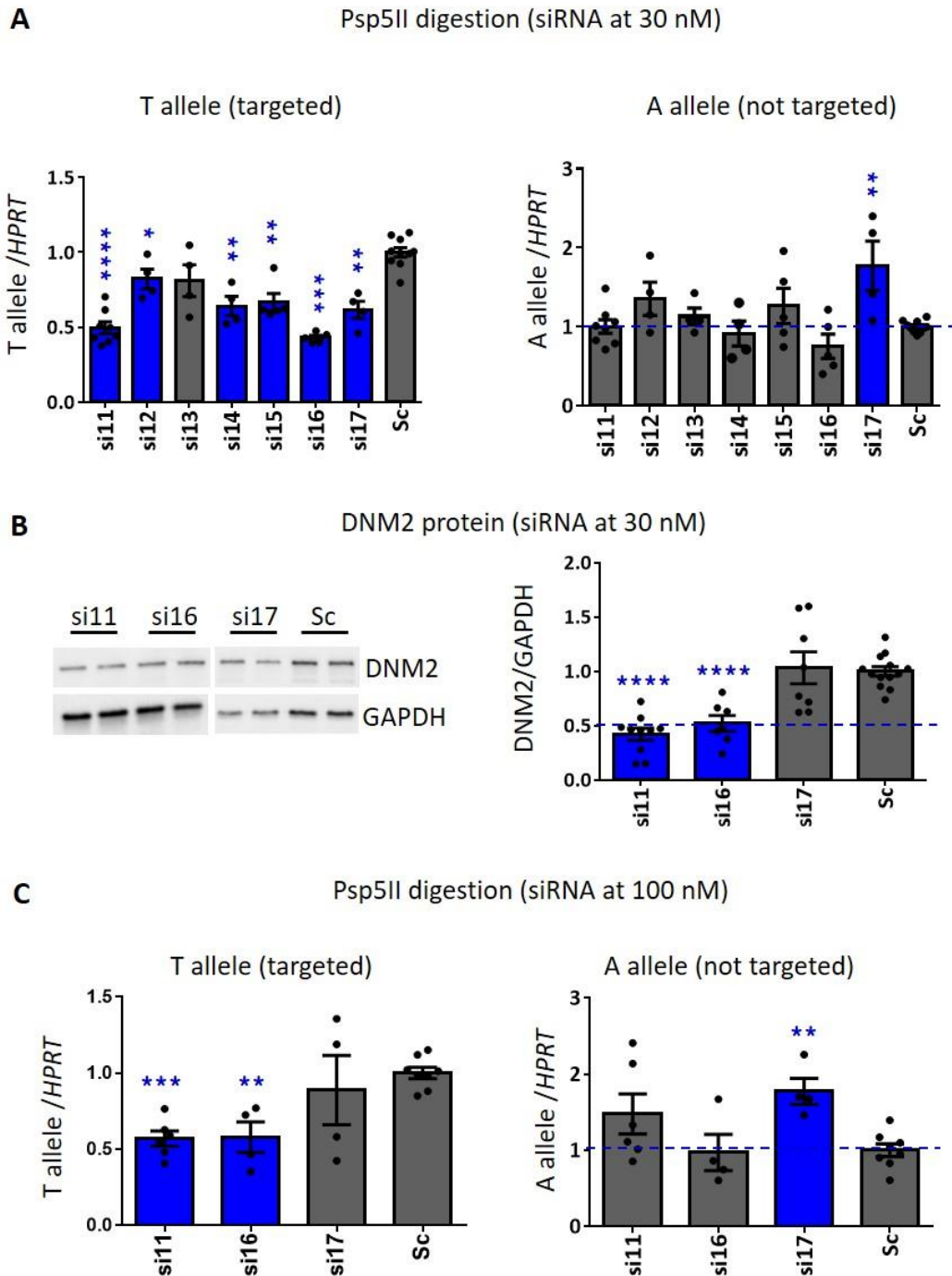
Schematic representation of the PCR fragment amplifying the SNP1 (A) and SNP2 (B) regions. The restriction sites used to discriminate the two alleles of each SNP are positioned on the PCR fragments and their nucleotide sequence is shown. The nucleotide variation for each SNP is in bold, in green when the enzyme recognized the site and in black when the recognition is abolished. The size of the digested and undigested PCR amplicons are indicated as well as the canonical recognition sequence for the enzyme BglI and Psp5II. Below, agarose gel electrophoresis of digested PCR amplicons after BglI (left) or Psp5II (right) digestion for cells heterozygous for these SNPs are shown. The enzymatic digestion allows the discrimination between both version of each SNP alleles and quantification of signal associated with both alleles. The associated-signal for the digested allele is obtained by the sum of the signal for the two bands. Length of each band in base-pairs (bp) is indicated on the right.



**Supplementary Figure 3: Allele-specific siRNA against the T version of SNP1. A.** Expression of the T and C alleles 48 hours after siRNA transfection at 30 nM. Quantification of the 2 alleles after BglI digestion of the RT-PCR product and normalized to *HPRT* ( $n \geq 4$  per condition). **B.** DNM2 western blot and quantification of the signal by densitometry after siRNA transfection at 30 nM concentration. GAPDH was used as a loading control ( $n \geq 4$ ). **C.** Expression of the T and C alleles 48 hours after siRNA transfection at 100 nM. Quantification of the 2 alleles after BglI digestion of the RT-PCR product and normalized to *HPRT* ( $n \geq 4$  per

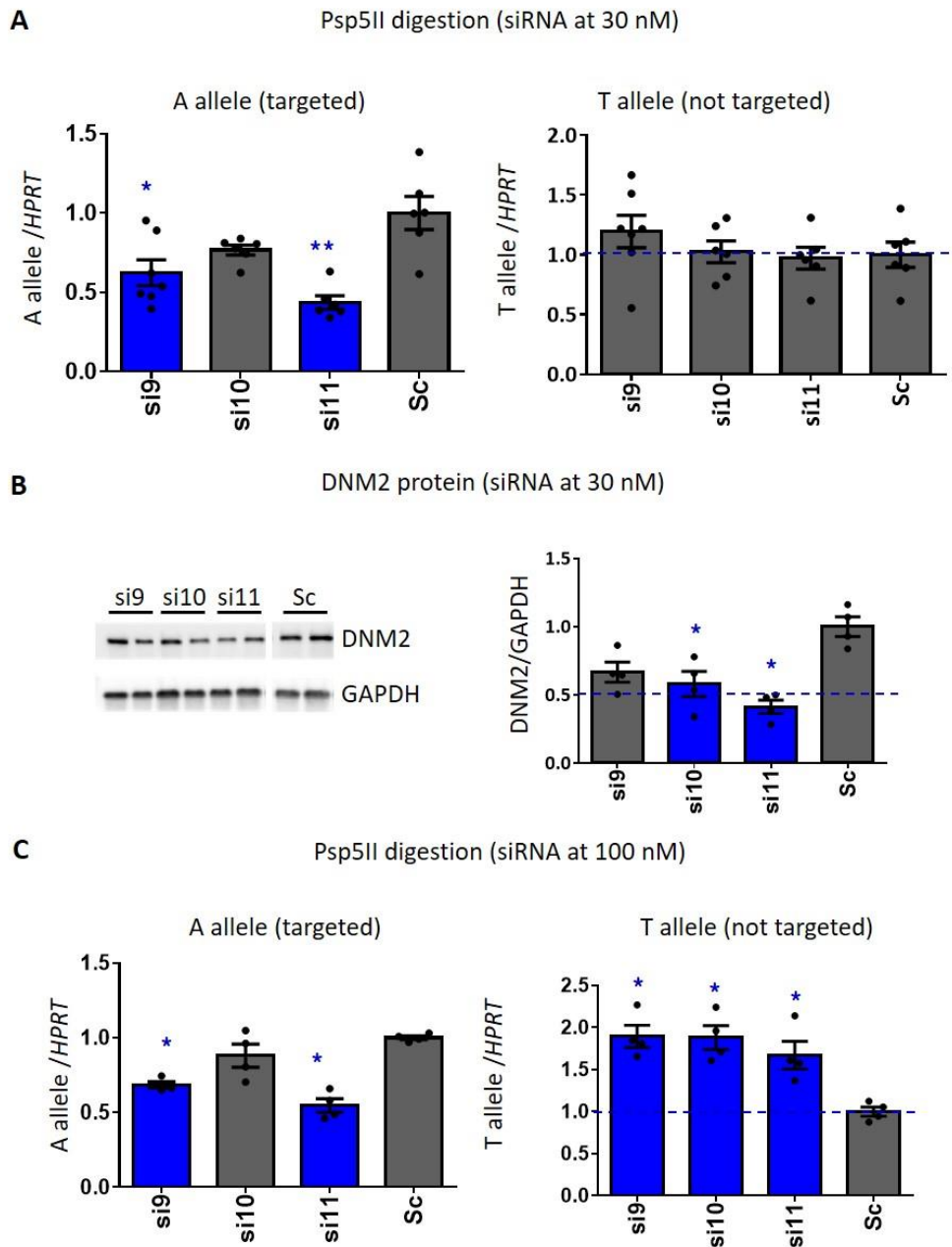


condition). Data information: In scatter plots, the bars are mean values and error bars indicate SEM. \* $P < 0.5$  using a two-tailed Mann–Whitney  $U$ -test relative to the scramble siRNA (Sc).



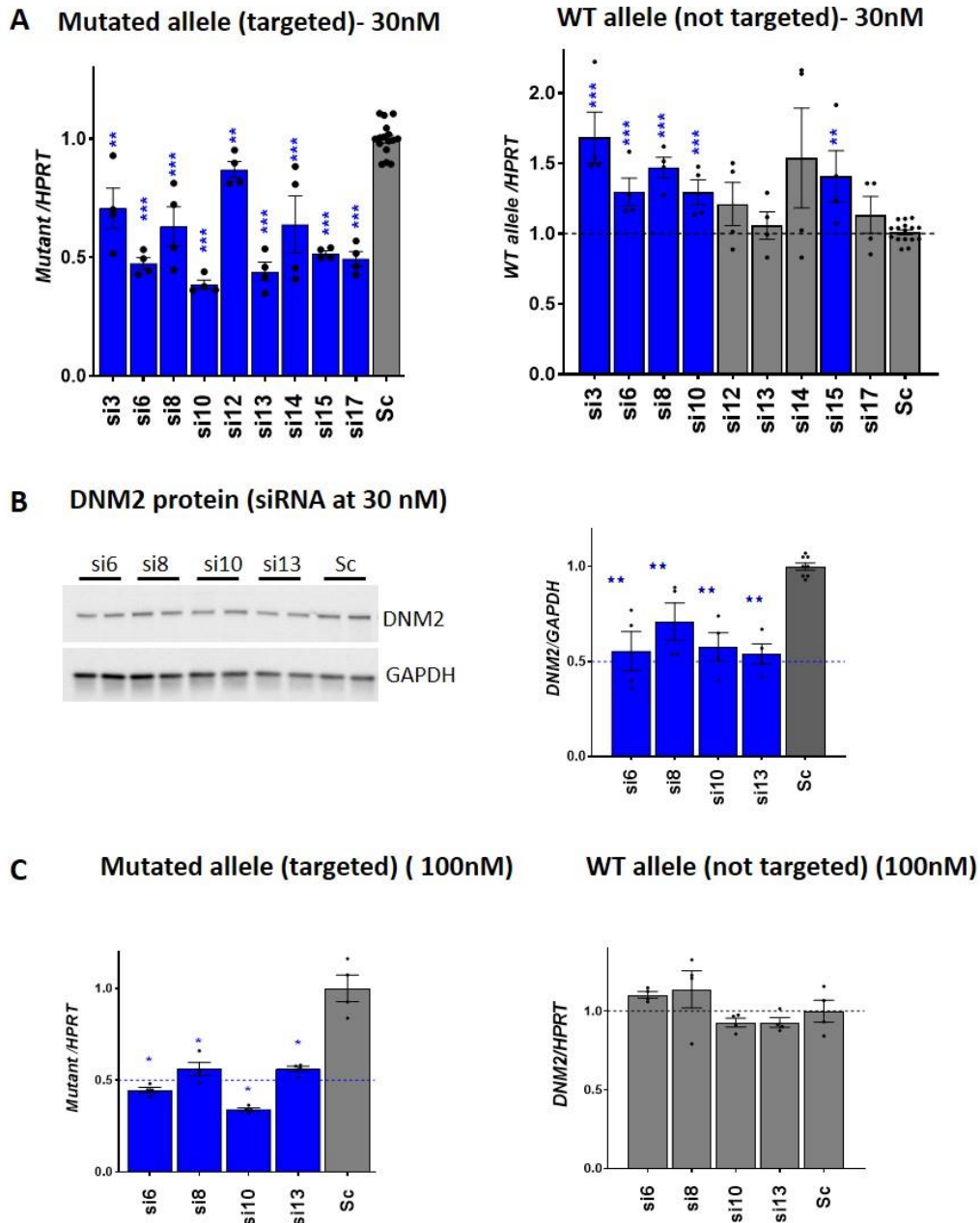
**Supplementary Figure 4: Allele-specific siRNA against the T version of SNP2.** **A.** Expression of the T and A alleles 48 hours after siRNA transfection at 30 nM. Quantification of the 2 alleles after Psp5II digestion of the RT-PCR product and normalized to *HPRT* ( $n \geq 4$  per condition). **B.** DNM2 western blot and quantification of the signal by densitometry after siRNA transfection at 30 nM concentration. GAPDH was used as a loading control ( $n \geq 4$ ). **C.**

Expression of the T and A alleles 48 hours after siRNA transfection at 100 nM. Quantification of the 2 alleles after Psp5II digestion of the RT-PCR product and normalized to *HPRT* ( $n \geq 4$  per condition). Data information: In scatter plots, the bars are mean values and error bars indicate SEM. \*\*\*\*P < 0.0001, \*\*\*P < 0.001, \*\*P < 0.01 and \*P < 0.05 using a two-tailed Mann–Whitney *U*-test relative to the scramble siRNA (Sc).



**Supplementary Figure 5: Allele-specific siRNA against the A version of SNP2.** **A.** Expression of the A and T alleles 48 hours after siRNA transfection at 30 nM. Quantification of the 2 alleles after Psp5II digestion of the RT-PCR product and normalized to *HPRT* ( $n \geq 4$  per condition). **B.** DNMT2 western blot and quantification of the signal by densitometry after siRNA transfection at 30 nM concentration. GAPDH was used as a loading control ( $n \geq 4$ ). **C.** Expression of the T and A alleles 48 hours after siRNA transfection at 100 nM. Quantification

of the 2 alleles after Psp5II digestion of the RT-PCR product and normalized to *HPRT* ( $n \geq 4$  per condition). Data information: In scatter plots, the bars are mean values and error bars indicate SEM. \*\* $P < 0.01$  and \* $P < 0.05$  using a two-tailed Mann–Whitney *U*-test relative to the scramble siRNA (Sc).

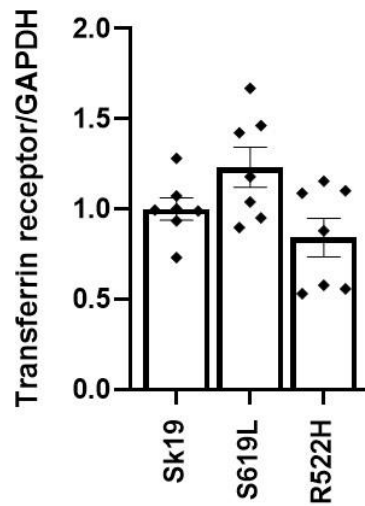
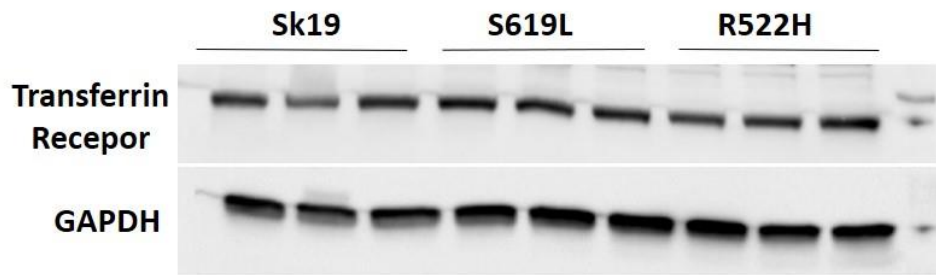


**Supplementary Figure 6: Identification of allele-specific siRNA against the S619L mutated allele.** **A.** Expression of the mutated and wild-type alleles 48 hours after siRNA transfection at 30 nM. Quantification of the 2 alleles after BgII digestion of the RT-PCR product and normalized to *HPRT* ( $n \geq 4$  per condition). **B.** DNM2 western blot and quantification of the signal by densitometry after siRNA transfection at 30 nM concentration. GAPDH was used as a loading control ( $n \geq 4$ ). **C.** Expression of the mutated and wild-type alleles 48 hours after siRNA transfection at 100 nM. Quantification of the 2 alleles after BgII digestion of the RT-

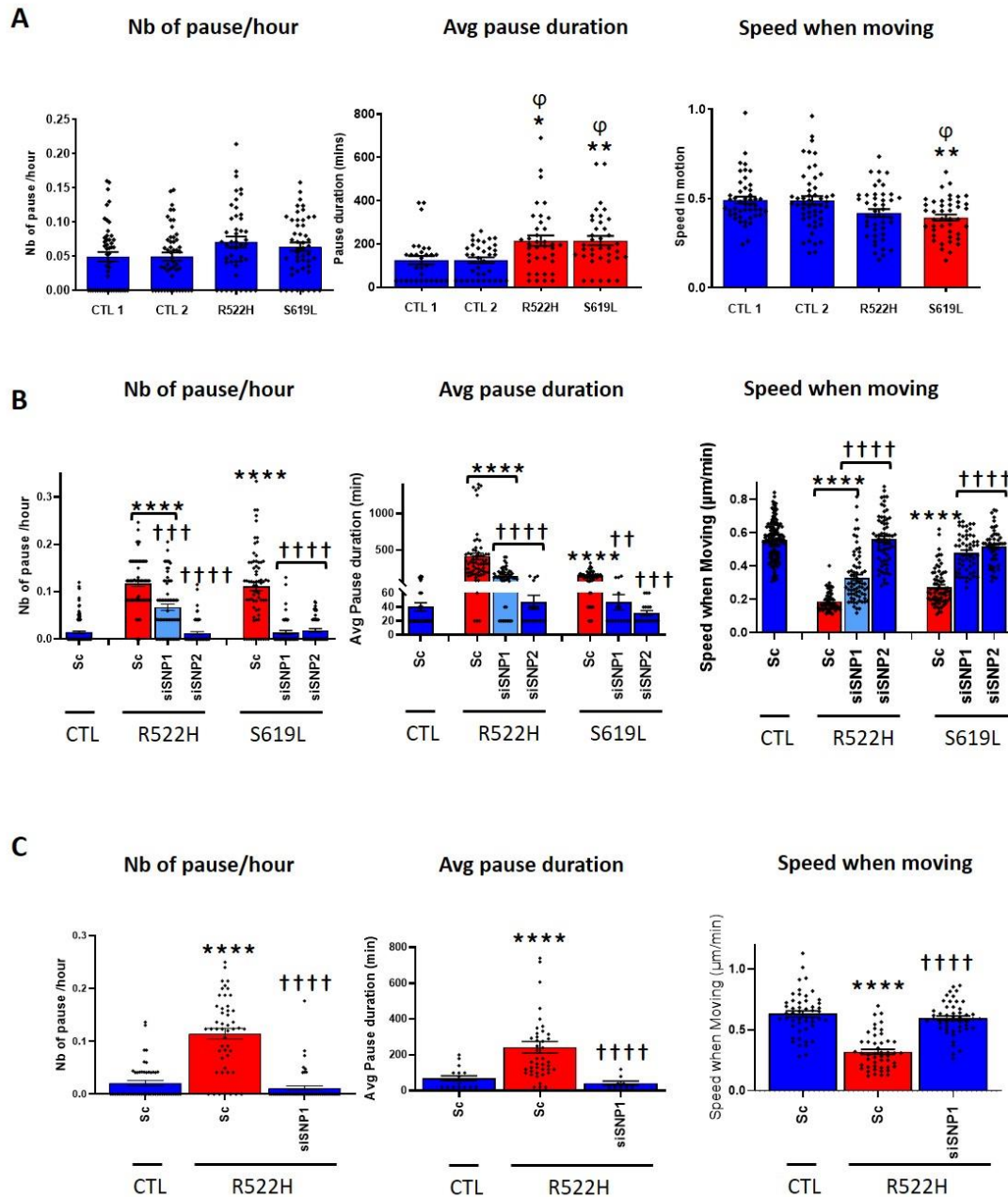
PCR product and normalized to *HPRT* ( $n \geq 4$  per condition). Data information: In scatter plots, the bars are mean values and error bars indicate SEM. \*\*\* $P < 0.001$ , \*\* $P < 0.01$  and \* $P < 0.05$  using a two-tailed Mann–Whitney *U*-test relative to the scramble siRNA (Sc).







**Supplementary Figure 8:** Transferrin receptor western blot and quantification of signal by densitometry in control and mutant cells. GAPDH was used as a loading control (n=7). Data information: In scatter plots, the bars are mean values and error bars indicate SEM. Kruskal-Wallis was non-significant.



**Supplementary Figure 9: Skypad Analysis of cell motility and maximum speed.** Cell motility analyses were performed using Skypad software, showing the number of pauses per cell per hour tracking, the average duration of pause (in minutes), and the average speed when moving for each cell. All these parameters were plotted for (A) cells under basal conditions, (B) cells 48 hours after transfection at a concentration of 30 nM siRNA and (C) cells 48 hours after transfection at a concentration of 100 nM siRNA. The histograms represent mean  $\pm$  SEM. Statistical analysis are performed using Kruskal-Wallis, followed by Dunn's test relative to

control cells 1 (\*\* P<0.01, \* P<0.05) or control cells 2 ( $\varphi$  P<0.05) in A, or to control cells transfected with scramble siRNA in B and C (\*\*\*\* P<0.0001), or to patient cell lines transfected with scramble siRNA in B and C (†††† P<0.0001, ††† P<0.001, †† P<0.01). Sc: Scramble.

**Supplemental Table 1: Mean +/- SEM values for each of the data figures**

Figure 2													
Figure 2A	si5	si6	si7	si8	si9	si10	si11	si12	si13	si14	si15	si17	Sc
Number of values	4	4	4	4	4	4	4	4	4	4	4	8	16
Mean	0.2325	0.6325	0.6175	0.5525	0.6568	0.502	0.7993	0.6875	0.1575	0.2125	0.6975	0.4564	1,000
SEM	0.03376	0.1924	0.0832	0.06223	0.0843	0.1006	0.1156	0.06115	0.01797	0.01887	0.1256	0.05897	0.0332

Figure 2B	si7	si8	si9	si10	si12	si15	si17	Sc
Number of values	4	4	4	4	4	4	8	16
Mean	0.5225	0.33	0.4325	0.535	0.8075	0.6075	0.5388	1,000
Std. Error of Mean	0.03351	0.06258	0.02869	0.08312	0.0833	0.03092	0.09127	0.01049

Figure 2C	si8	si9	si10	Sc
Number of values	4	4	4	8
Mean	0.4468	0.4095	0.2573	1,000
Std. Error of Mean	0.04338	0.03833	0.02381	0.02677

Figure 2D	si8	si9	si10	Sc
Number of values	4	4	4	8
Mean	0.2415	0.2723	0.426	1,000
Std. Error of Mean	0.0129	0.03026	0.02013	0.02044

Figure 2E	si8	si9	si10	Sc
Number of values	4	4	4	4
Mean	0.525	0.6975	0.57	1,000
Std. Error of Mean	0.08617	0.03351	0.09806	0.02944

Figure 3				
Figure 3A	si8	si9	si10	Sc
Number of values	4	4	4	4
Mean	0.4475	0.4975	0.31	1,000
Std. Error of Mean	0.004787	0.0225	0.03764	0.1033

Figure 3B	si8	si9	si10	Sc
Number of values	4	4	4	4
Mean	0.0975	0.075	0.12	1,000
Std. Error of Mean	0.01493	0.00866	0.01472	0.01472

Figure 3C	si8	si9	si10	Sc
Number of values	4	4	4	4
Mean	0.5925	0.5325	0.405	1,000
Std. Error of Mean	0.02562	0.06524	0.02901	0.02041

Figure 3D	si8	si9	si10	Sc
Number of values	4	4	4	4
Mean	0.1175	0.08	0.1175	1,000
Std. Error of Mean	0.01887	0.01472	0.01031	0.01826

Figure 3E	si8	si9	si10	Sc
Number of values	4	4	4	4
Mean	0.6575	0.745	0.625	1,000
Std. Error of Mean	0.04553	0.04787	0.06397	0.0072

Figure 4													
Figure 4A	si6	si7	si8	si9	si10	si11	si12	si13	si14	si15	si16	si17	Sc
Number of values	4	4	4	4	4	8	4	4	4	5	5	4	10
Mean	0.8119	0.9516	0.877	0.8476	0.8549	0.5609	0.4369	0.4824	0.3192	0.675	0.4414	0.5906	1,000
Std. Error of Mean	0.2225	0.2927	0.1336	0.04379	0.1502	0.07189	0.09511	0.115	0.1523	0.09761	0.07344	0.07959	0.05327

Figure 4B	si11	si12	si13	si14	si15	si16	si17	Sc
Number of values	8	4	4	4	5	5	4	10
Mean	0.4902	0.7327	0.7687	0.6897	0.6928	0.5171	0.4229	1,000
Std. Error of Mean	0.03832	0.113	0.1	0.07701	0.1114	0.06743	0.05742	0.03475

Figure 4C	si11	si16	si17	Sc
Number of values	10	5	7	10
Mean	0.4415	0.4147	0.4751	1,000
Std. Error of Mean	0.05982	0.06817	0.03278	0.07782

Figure 4D	si11	si16	si17	Sc
Number of values	6	4	4	8
Mean	0.5147	0.5885	0.504	1,000
Std. Error of Mean	0.04336	0.07434	0.136	0.05306

Figure 4E	si11	si16	si17	Sc
Number of values	6	4	4	7
Mean	0.6546	0.5707	0.6243	1,034
Std. Error of Mean	0.08467	0.08649	0.02727	0.05652

Figure 5				
<b>Figure 5A</b>	si9	si10	si11	Sc
Number of values	6	6	7	6
Mean	0.6791	0.5452	0.4306	1,000
Std. Error of Mean	0.05002	0.03654	0.04105	0.0371
<b>Figure 5B</b>	si9	si10	si11	Sc
Number of values	7	6	6	6
Mean	0.5729	0.7305	0.4633	1,000
Std. Error of Mean	0.14	0.06196	0.06051	0.03249
<b>Figure 5C</b>	si9	si10	si11	Sc
Number of values	4	4	4	4
Mean	0.6879	0.4889	0.5238	1,000
Std. Error of Mean	0.07457	0.0683	0.02817	0.03623
<b>Figure 5D</b>	si9	si10	si11	Sc
Number of values	4	4	4	4
Mean	0.3413	0.4174	0.3089	1,000
Std. Error of Mean	0.02837	0.04186	0.02852	0.02661
<b>Figure 5E</b>	si9	si10	si11	Sc
Number of values	5	5	6	4
Mean	0.8055	0.709	0.5812	1,000
Std. Error of Mean	0.07197	0.04763	0.03067	0.0779

Figure 6																
<b>Figure 6A</b>	si3	si4	si5	si6	si7	si8	si9	si10	si11	si12	si13	si14	si15	si16	si17	Sc
Number of values	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	16
Mean	0.7047	0.363	0.278	0.4223	0.3638	0.6021	1.084	0.5671	1.057	0.7508	0.4498	0.7734	0.4371	0.2285	0.4614	1,000
Std. Error of Mean	0.03991	0.004007	0.03304	0.01582	0.01962	0.02255	0.06726	0.04148	0.01654	0.01885	0.01325	0.04102	0.05429	0.04761	0.05063	0.01678
<b>Figure 6B</b>	si3	si6	si8	si10	si12	si13	si14	si15	si17	Sc						
Number of values	4	4	4	4	4	4	4	4	4	16						
Mean	0.42	0.3698	0.4373	0.3013	0.5843	0.5244	0.4319	0.386	0.4471	1,000						
Std. Error of Mean	0.02542	0.01877	0.04498	0.01641	0.02175	0.02139	0.02484	0.04506	0.03414	0.008212						
<b>Figure 6C</b>	si6	si8	si10	si13	Sc											
Number of values	4	4	4	4	4											
Mean	0.5075	0.5633	0.4978	0.4223	1,000											
Std. Error of Mean	0.05842	0.02949	0.03185	0.04182	0.06843											
<b>Figure 6D</b>	si6	si8	si10	si13	Sc											
Number of values	4	4	4	4	4											
Mean	0.4042	0.5027	0.368	0.6063	1,000											
Std. Error of Mean	0.01004	0.03794	0.005713	0.02057	0.003268											
<b>Figure 6E</b>	si6	si8	si10	si13	Sc											
Number of values	6	6	5	6	10											
Mean	0.5767	0.7617	0.666	0.5867	1,000											
Std. Error of Mean	0.04072	0.04534	0.04057	0.0424	0.02186											

Figure 7							
<b>Figure 7A</b>	Control	R522H	S619L				
Number of values	717	633	555				
Mean	0.997	0.8016	1.516				
Std. Error of Mean	0.0295	0.02421	0.04916				
<b>Figure 7B</b>	Control-sc	R522H-sc	R522H-siSNP1	R522H-siSNP2	S619L-sc	S619L-si619	S619L-siSNP1
Number of values	304	323	181	202	281	185	212
Mean	1.305	1.008	1.007	0.858	1.777	1.492	1.247
Std. Error of Mean	0.04644	0.03344	0.05922	0.04976	0.07684	0.1135	0.07072
<b>Figure 7C</b>	Control-sc	R522H-sc	R522H-siSNP1	R522H-siSNP2	S619L-sc	S619L-si619	S619L-siSNP1
Number of values	225	303	354	250	272	252	313
Mean	1.194	0.8706	1.098	0.9513	1.859	1.069	1.16
Std. Error of Mean	0.04256	0.02873	0.03455	0.03055	0.07998	0.06344	0.05475
<b>Figure 7D</b>	Control	R522H	S619L				
Number of values	717	633	556				
Mean	2860	2452	4643				
Std. Error of Mean	72.78	53.66	137.7				
<b>Figure 7E</b>	Control-sc	R522H-sc	R522H-siSNP1	R522H-siSNP2	S619L-sc	S619L-si619	S619L-siSNP1
Number of values	304	323	207	184	281	187	212
Mean	2793	2501	3014	2283	4523	4776	3757
Std. Error of Mean	79.41	58.68	317.7	112,000	173.4	516.6	188.9
<b>Figure 7F</b>	Control-sc	R522H-sc	R522H-siSNP1	R522H-siSNP2	S619L-sc	S619L-si619	S619L-siSNP1
Number of values	240	303	381	232	272	282	312
Mean	2809	2418	2736	2706	4580	3327	3358
Std. Error of Mean	82.35	63.9	81.81	99.41	176.7	131.7	124.7

Figure 8							
<b>Figure 8A</b>	CTL1	CTL2	R522H	S619L			
Number of values	52	51	50	50			
Mean	0.465	0.4694	0.3552	0.3444			
Std. Error of Mean	0.0197	0.02623	0.02234	0.019			
<b>Figure 8B</b>	CTL-Sc	R522H-Sc	R522H-siSNP1	R522H-siSNP2	S619L-Sc	S619L-siSNP1	S619L-siSNP2
Number of values	139	70	69	70	70	55	53
Mean	0.5758	0.1697	0.3209	0.5717	0.2914	0.5007	0.5343
Std. Error of Mean	0.009245	0.007011	0.01647	0.01682	0.01066	0.01495	0.01296
<b>Figure 8C</b>	CTL-Sc	R522H-Sc	R522H-siSNP1				
Number of values	50	50	50				
Mean	0.6516	0.2628	0.6				
Std. Error of Mean	0.02461	0.01513	0.01814				
<b>Figure 8D</b>	Control	R522H	S619L				
Number of values	8	8	8				
Mean	1.000	0.8723	0.4471				
Std. Error of Mean	0.1215	0.1598	0.04734				
<b>Figure 8E</b>	CTL-Sc	S619L-Sc	S619L-si619	S619L-siSNP1			
Number of values	6	6	5	5			
Mean	1.000	0.3227	0.433	0.3858			
Std. Error of Mean	0.06563	0.04377	0.0985	0.06423			
<b>Figure 8F</b>	CTL-Sc	S619L-Sc	S619L-si619	S619L-siSNP1			
Number of values	6	6	6	6			
Mean	1.000	0.3229	0.5432	0.6201			
Std. Error of Mean	0.07941	0.02367	0.03029	0.06797			

**Supplemental Table 2: Mean +/- SEM values for each of the data supplemental figures**

Figure Sup 1								
<b>S1-A-left Pane</b>	si7	si8	si9	si10	si12	si15	si17	Sc
Number of values	4	4	4	4	4	4	8	16
Mean	0.6475	0.335	0.4325	0.625	0.7875	0.3975	0.3763	1,000
Std. Error of Mean	0.1566	0.05635	0.06486	0.06449	0.1988	0.04608	0.04563	0.02451
<b>S1-A-Right Panel</b>	si7	si8	si9	si10	si12	si15	si17	Sc
Number of values	4	4	4	4	4	4	8	16
Mean	1.058	1.035	0.99	1.203	0.9275	0.6775	0.8138	1,000
Std. Error of Mean	0.1298	0.03122	0.1209	0.07889	0.1493	0.1089	0.1351	0.02502
<b>S1-B</b>	si8	si9	si10	Sc				
Number of values	4	4	4	4				
Mean	0.5305	0.716	0.51	1,000				
Std. Error of Mean	0.0681	0.0346	0.09443	0.02483				
<b>S1-C-left</b>	si8	si9	si10	Sc				
Number of values	4	4	4	8				
Mean	0.36	0.445	0.51	1,000				
Std. Error of Mean	0.04916	0.1025	0.1022	0.04555				
<b>S1-C-Right Panel</b>	si8	si9	si10	Sc				
Number of values	4	4	4	8				
Mean	1.47	1.56	1.23	1,000				
Std. Error of Mean	0.1497	0.2151	0.2661	0.04897				

Figure Sup 3				
<b>S3-A-left</b>	si8	si9	si10	Sc
Number of values	4	4	4	4
Mean	0.1475	0.18	0.2	1,000
Std. Error of Mean	0.04589	0.03764	0.06721	0.1555
<b>S3-A-right</b>	si8	si9	si10	Sc
Number of values	4	4	4	4
Mean	1.453	2.34	1.533	1,000
Std. Error of Mean	0.3194	0.2645	0.3489	0.1495
<b>S3-B</b>	si8	si9	si10	Sc
Number of values	4	4	4	4
Mean	1.453	2.34	1.533	1,000
Std. Error of Mean	0.3194	0.2645	0.3489	0.1495
<b>S3-C-left</b>	si8	si9	si10	Sc
Number of values	4	4	4	4
Mean	0.19	0.19	0.1725	1,000
Std. Error of Mean	0.03109	0.01472	0.0075	0.04564
<b>S3-C-right</b>	si8	si9	si10	Sc
Number of values	4	4	4	4
Mean	1.85	2.65	1.538	1,000
Std. Error of Mean	0.4933	0.6286	0.147	0.03764

Figure Sup 4								
<b>S4-A-left</b>	si11	si12	si13	si14	si15	si16	si17	Sc
Number of values	8	4	4	4	5	5	4	10
Mean	0.4966	0.8225	0.8103	0.641	0.6667	0.4293	0.6158	1,000
Std. Error of Mean	0.03955	0.06581	0.1066	0.06474	0.05798	0.0154	0.05759	0.03135
<b>S4-A-right</b>	si11	si12	si13	si14	si15	si16	si17	Sc
Number of values	8	4	4	4	5	5	4	10
Mean	1.002	1.354	1.13	0.9132	1.264	0.7525	1.771	1,000
Std. Error of Mean	0.08623	0.2105	0.1055	0.1582	0.2217	0.1538	0.3127	0.02356
<b>S4-B</b>	si11	si16	si17	Sc				
Number of values	10	7	8	13				
Mean	0.4206	0.5208	1.032	1,000				
Std. Error of Mean	0.05709	0.07277	0.1463	0.04182				
<b>S4-C-left</b>	si11	si16	si17	Sc				
Number of values	6	4	4	8				
Mean	0.5693	0.5779	0.8868	1,000				
Std. Error of Mean	0.05066	0.1006	0.2272	0.0367				
<b>S4-C-right</b>	si11	si16	si17	Sc				
Number of values	6	4	4	8				
Mean	1.476	0.9704	1.773	1,000				
Std. Error of Mean	0.2627	0.2395	0.1695	0.08367				

**Figure Sup 5**

<b>S5-A-left</b>	<b>si9</b>	<b>si10</b>	<b>si11</b>	<b>Sc</b>
Number of values	7	6	6	6
Mean	0.6242	0.7672	0.4372	1,000
Std. Error of Mean	0.08123	0.03147	0.04225	0.1043

<b>S5-A-right</b>	<b>si9</b>	<b>si10</b>	<b>si11</b>	<b>Sc</b>
Number of values	7	6	6	6
Mean	1.194	1.024	0.9713	1,000
Std. Error of Mean	0.1348	0.09105	0.09153	0.1058

<b>S5-B</b>	<b>si9</b>	<b>si10</b>	<b>si11</b>	<b>Sc</b>
Number of values	4	4	4	4
Mean	0.6666	0.5785	0.4123	1,000
Std. Error of Mean	0.07401	0.09336	0.04964	0.07192

<b>S5-C-left</b>	<b>si9</b>	<b>si10</b>	<b>si11</b>	<b>Sc</b>
Number of values	4	4	4	4
Mean	0.6853	0.8798	0.5466	1,000
Std. Error of Mean	0.02085	0.07736	0.04544	0.01306

<b>S5-C-right</b>	<b>si9</b>	<b>si10</b>	<b>si11</b>	<b>Sc</b>
Number of values	4	4	4	4
Mean	1.896	1.881	1.673	1,000
Std. Error of Mean	0.1313	0.1415	0.1647	0.05589

**Figure Sup 6**

<b>S6-A-left</b>	<b>si3</b>	<b>si6</b>	<b>si8</b>	<b>si10</b>	<b>si12</b>	<b>si13</b>	<b>si14</b>	<b>si15</b>	<b>si17</b>	<b>Sc</b>
Number of values	4	4	4	4	4	4	4	4	4	16
Mean	0.7076	0.4759	0.6316	0.3863	0.8706	0.4412	0.6396	0.5181	0.4948	1,000
Std. Error of Mean	0.08476	0.02392	0.08336	0.01848	0.03363	0.03925	0.1207	0.01135	0.03119	0.01712

<b>S6-A-Rigth</b>	<b>si3</b>	<b>si6</b>	<b>si8</b>	<b>si10</b>	<b>si12</b>	<b>si13</b>	<b>si14</b>	<b>si15</b>	<b>si17</b>	<b>Sc</b>
Number of values	4	4	4	4	4	4	4	4	4	16
Mean	1.683	1.296	1.469	1.293	1.21	1.058	1.538	1.407	1.133	1.01
Std. Error of Mean	0.1801	0.0984	0.07242	0.08906	0.1532	0.09714	0.3552	0.1828	0.1314	0.01745

<b>S6-B</b>	<b>si6</b>	<b>si8</b>	<b>si10</b>	<b>si13</b>	<b>Sc</b>
Number of values	4	4	4	4	8
Mean	0.555	0.71	0.5775	0.54	1,000
Std. Error of Mean	0.1027	0.09823	0.07443	0.05307	0.0188

<b>S6-C-Left</b>	<b>si6</b>	<b>si8</b>	<b>si10</b>	<b>si13</b>	<b>Sc</b>
Number of values	4	4	4	4	4
Mean	0.4457	0.5604	0.3408	0.5611	1,000
Std. Error of Mean	0.01467	0.03664	0.008412	0.01511	0.07281

<b>S6-C-Rigth</b>	<b>si6</b>	<b>si8</b>	<b>si10</b>	<b>si13</b>	<b>Sc</b>
Number of values	4	4	4	4	4
Mean	1.103	1.139	0.9276	0.9281	1,000
Std. Error of Mean	0.02063	0.1184	0.02797	0.03046	0.07003

**Figure Sup 8**

	<b>Sk19</b>	<b>S619L</b>	<b>R522H</b>
Number of values	7	7	7
Mean	1,000	1.231	0.8408
Std. Error of Mean	0.06192	0.1105	0.1061



Figure Sup 9

S9A	Number of pause /hour	CTL 1	CTL 2	R522H	S619L
	Number of values	48	50	46	48
	Mean	0.04923	0.04954	0.07087	0.06394
	Std. Error of Mean	0.006923	0.005995	0.007839	0.006267
	Avg Pause Duration	CTL 1	CTL 2	R522H	S619L
Number of values	30	35	37	37	
Mean	124.9	125	214.7	215.2	
Std. Error of Mean	19.24	13.16	24.63	21.56	
Speed when moving	CTL 1	CTL 2	R522H	S619L	
Number of values	47	51	44	43	
Mean	0.4921	0.4898	0.4195	0.3934	
Std. Error of Mean	0.01908	0.02342	0.02126	0.01709	

S9B	Number of pause /hour	Sc	R522H-Sc	R522H-siSNP1	R522H-siSNP2	S619L-Sc	S619L-siSNP1	S619LsiSNP 2
	Number of values	137	70	69	68	69	52	51
	Mean	0.01477	0.1173	0.06735	0.01271	0.1116	0.01448	0.01846
	Std. Error of Mean	0.002373	0.006164	0.00677	0.003116	0.009134	0.00408	0.00371
	Avg Pause Duration	Sc	R522H-Sc	R522H-siSNP1	R522H-siSNP2	S619L-Sc	S619L-siSNP1	S619LsiSNP 2
Number of values	37	68	53	18	57	15	18	
Mean	40.27	410.1	143.000	46.67	133.9	46.67	31.11	
Std. Error of Mean	5.754	43.51	13.31	10.1	8.693	11.16	3.695	
Speed when moving	Sc	R522H-Sc	R522H-siSNP1	R522H-siSNP2	S619L-Sc	S619L-siSNP1	S619LsiSNP 2	
Number of values	138	63	69	70	68	54	52	
Mean	0.5564	0.187	0.3307	0.5611	0.2746	0.4797	0.5144	
Std. Error of Mean	0.009777	0.007083	0.01826	0.01816	0.01335	0.01531	0.01477	

S9C	Number of pause /hour	Sc	R522H-Sc	R522H-siSNP1
	Number of values	50	49	49
	Mean	0.02111	0.1146	0.01132
	Std. Error of Mean	0.004758	0.009997	0.00447
	Avg Pause Duration	Sc	R522H-Sc	R522H-siSNP1
Number of values	18	43	9	
Mean	70	243.2	42.22	
Std. Error of Mean	13.46	32.41	11.76	
Speed whEn moving	Sc	R522H-Sc	R522H-siSNP1	
Number of values	50	48	50	
Mean	0.6318	0.3191	0.5947	
Std. Error of Mean	0.02458	0.02112	0.01896	