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1 **Polymorphisms in the canine *IL7R* 3'UTR are associated with thymic output in**
2 **Labrador retriever dogs and influence post-transcriptional regulation by microRNA**
3 **185.**

4
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19

20 **Abstract**

21 Interleukin-7 (IL-7) and its receptor (IL-7R) are essential for T cell development in the
22 thymus, and changes in the IL-7/IL-7R pathway have been implicated in age-associated
23 thymic involution which results in a reduction of naïve T cell output. The aim of this study
24 was to investigate the relationship between *IL7* and *IL7R* genetic variation and thymic output
25 in dogs. No single nucleotide polymorphisms (SNPs) were identified in the canine *IL7* gene,
26 but a number were present in the canine *IL7R* gene. Polymorphisms in the *IL7R* exon 8 and
27 3'UTR were found to be associated with signal joint T cell receptor excision circle (sj-TREC)
28 values (a biomarker of thymic output) in young and geriatric Labrador retrievers.
29 Additionally, one of the SNPs in the *IL7R* 3'UTR (SNP 14 c.1371+446 A>C) was found to
30 cause a change in the seed-binding site for microRNA 185 which, a luciferase reporter assay
31 demonstrated, caused changes in post-transcriptional regulation, and therefore might be
32 capable of influencing IL-7R expression. The research findings suggest a genetic link
33 between *IL7R* genotype and thymic output in dogs, which might impact on immune function
34 as these animals age and provide further evidence of the involvement of IL-7/IL-7R pathway
35 in age-associated thymic involution.

36

37 **Key words**

38 Interleukin-7 receptor; Thymic involution; signal joint T cell receptor excision circle; Canine;

39

40 ¹

41

¹ **Abbreviations**

sj-TREC, signal joint T cell receptor excision circle, TCR, T cell receptor, RTEs, recent thymic emigrants.

42 **1. Introduction**

43 Interleukin 7 (IL-7) and its receptor (IL-7R) play an important role in T cell development, in
44 both primary and secondary lymphoid organs (Fry and Mackall, 2005). In the thymus, IL-7 is
45 produced by the stromal cells (Moore et al., 1993) and its effects are mediated via binding to
46 its cognate receptor expressed on the surface of developing thymocytes (Munitic et al., 2004).
47 The IL-7R is a heterodimer, composed of two subunits, the IL-7 receptor alpha chain (IL-7R)
48 and a common gamma chain (γ /IL2RG), which is shared with other type I cytokine
49 receptors (Rochman et al., 2009). Ligation of the IL-7R generates a number of signals (via
50 phosphorylation of Jak1/Jak3) leading to cell activation (via STATs 1, 3 and 5), proliferation
51 (via ras, raf and ERK1/2) and survival/resistance to apoptosis (via IP3 and Akt/PKB) (Jiang
52 et al., 2005).

53 With increasing age, the thymus undergoes a process of involution, leading to a reduction in
54 the production of naive T cells for recruitment into the peripheral lymphocyte pool (Lynch et
55 al., 2009). This can cause expansion of the existing memory T cell populations (Kilpatrick et
56 al., 2008; Naylor et al., 2005), which in turn can lead to reduced diversity of the T cell
57 repertoire and impairment of immune responses to novel antigens (Naylor et al., 2005).
58 Studies in several species (Douek et al., 1998; Kong et al., 1999; Sempowski et al., 2002;
59 Sodora et al., 2000) have demonstrated that thymic output can be estimated using signal joint
60 T cell receptor excision circles (sj-TRECs) as a biomarker. These small episomal circles of
61 DNA are generated during T cell development, when the T cell receptor (TCR) δ gene
62 segments, positioned within the TCR α locus, are excised as a prelude to VDJ recombination
63 (de Villartay et al., 1988; Hockett et al., 1988). In a recent study (Holder et al., 2016), we
64 have demonstrated that this technique can be applied in dogs, and that there is an age-
65 associated decline in sj-TREC values. This suggests that in dogs, there is a reduction in the

66 number of recent thymic emigrants (RTEs) with increasing age, which is similar to that
67 observed in humans (Douek et al., 1998) and mice (Sempowski et al., 2002).

68 Despite age-associated thymic involution occurring in all vertebrates, and therefore
69 considered an evolutionary conserved event (Shanley et al., 2009), the mechanisms involved
70 in this process still remain to be fully elucidated (Palmer, 2013). In experimental animals, IL-
71 7 expression in the thymus has been shown to decline with age, in parallel with a reduction in
72 the output of naive T cells, associated with thymic involution (Andrew and Aspinall, 2002;
73 Ortman et al., 2002). This has led to the proposal that IL-7 may be a contributing factor
74 towards the aetiology of thymic involution and that it might be possible to use IL-7
75 supplementation as part of a therapeutic strategy for the maintenance of immune competence
76 in old age (Aspinall and Mitchell, 2008). However, there is little published work with respect
77 to IL-7R expression in the aging thymus. In human nonagenarians and their offspring, *IL7R*
78 mRNA expression in peripheral blood samples are associated with familial longevity and
79 healthy aging (Passtoors et al., 2012; Passtoors et al., 2015), suggesting that immune
80 competence in the elderly could be influenced by expression of both IL-7 and its receptor.

81 Genetic variability within the coding region of the *IL7* gene does not seem to be associated
82 with adverse effects/disease (Mazzucchelli et al., 2012), although polymorphisms in the
83 5'UTR of the human *IL7* gene have been associated with susceptibility to multiple sclerosis
84 (Zuvich et al., 2010) and HIV infection (Song et al., 2007). In contrast, polymorphisms in the
85 *IL7R* gene have been linked with a number of human autoimmune diseases (Mazzucchelli et
86 al., 2012), including multiple sclerosis, where they have demonstrated a functional effect by
87 influencing expression of the receptor on the cell surface of thymocytes (Gregory et al.,
88 2007). In multiple sclerosis patients, the polymorphisms in the *IL7R* gene have been
89 associated with the frequency of RTEs, where the number of naive T cells was found to be

90 significantly reduced in those individuals who did not express the 'protective' *IL7R* haplotype
91 (Broux et al., 2010).

92 Companion animals are potentially valuable as comparative and translational models of
93 ageing and disease (Day, 2010). Immunosenescence is likely to occur more rapidly in the
94 canine species, compared with humans, and the differences in longevity apparent in different
95 dog breeds, potentially reflects underlying genetic factors that are involved in ageing and
96 immunological health. The specific aim of the present project was to investigate whether
97 genetic diversity in the *IL7* or *IL7R* genes was associated with differences observed in sj-
98 TREC values in a defined population of Labrador retriever dogs of different ages.

99

100 **2. Material and methods**

101 **2.1 Study samples**

102 Blood samples from crossbreed dogs (n=6) and Labrador retrievers (n=100) were identified
103 in the clinical sample archive of the Royal Veterinary College, University of London. EDTA
104 blood had been archived following completion of diagnostic testing, with ethical approval
105 (approval number URN2016/1475) and informed owner consent for their use in clinical
106 research. The Labrador retriever dogs were categorised into young (<2 years, n=30), middle
107 aged (5-7 years, n=30) and geriatric (≥ 10 years, n=40) age groups. Genomic DNA was
108 extracted from blood samples using the GenElute Blood Genomic Kit (Sigma-Aldrich, Poole,
109 UK) according to the manufacturer's instructions.

110

111 **2.2 Amplification and sequencing of canine *IL7* and *IL7R* genes**

112 Genomic DNA samples from six crossbreed dogs were used to amplify selected regions of
113 the canine *IL7* (NM_001048138.1) and *IL7R* (XM_005619397.2) genes. Gene specific
114 primers were designed (Supplementary Table A1) using sequence information the NCBI
115 Entrez nucleotide sequence database (Genbank) (www.ncbi.nlm.nih.gov/Entrez) and the
116 Ensembl canine genome assembly version CanFam 3.1
117 (www.ensembl.org/Canis_familiaris/index.html).

118 PCR reactions were carried out using Immolase DNA polymerase (Bioline, London, UK)
119 according to the manufacturer's instructions. Thermocycling conditions consisted of an initial
120 polymerase activation at 95°C for 10 min, followed by 35 cycles of 94°C for 40 s, 55 or 60°C
121 for 30 s and 72°C for 1 or 2 min with a final extension step of 72°C for 10 min (G Storm GS1
122 thermocycler, Gene Technologies Ltd, Essex, UK).

123 The PCR products generated were separated by agarose gel electrophoresis, purified using
124 the GenElute Gel Extraction Kit (Sigma-Aldrich), and then submitted for sequencing (Source
125 Bioscience, Nottingham, UK). Single nucleotide polymorphisms (SNPs) were identified
126 using CLC Main Workbench version 6.0.2 (CLC bio, Aarhus, Denmark).

127 Following initial SNP discovery in the six cross breed dogs, a region of the *IL7R* exon 8 and
128 3'UTR, containing seven polymorphisms (*IL7R* SNPs 9-15) identified as being in linkage
129 disequilibrium, was amplified and sequenced using genomic DNA samples from the
130 Labrador retrievers (n=100).

131

132 **2.3 Real-time quantitative PCR (qPCR) for sj-TREC**

133 Genomic DNA samples from the 100 Labrador retrievers were used to quantify sj-TREC
134 expression by real-time qPCR as previously described by Holder et al. (Holder et al., 2016).

135 Briefly, the samples were initially amplified in a pre-quantification PCR reaction using
136 Immolase DNA polymerase (Bioline), according to the manufacturer's instructions, which
137 contained primers located upstream (sense) and downstream (antisense) of those used for the
138 subsequent qPCR (Supplementary Table A1). Thermocycling conditions for the pre-
139 quantification PCR were as follows: 95°C for 10 min, followed by 10 cycles of 94°C for 40 s,
140 60°C for 30 s and 72°C for 1 min with a final extension step of 72°C for 10 min (G Storm
141 GS1 thermocycler, Gene Technologies Ltd, Essex, UK).

142 A multiplex real-time qPCR was then performed, using the StepOne Real-Time PCR System
143 (Applied Biosystems 2010 Life Technologies Corporation, Grand Island, USA), to quantify sj-
144 TREC and albumin expression in samples that had undergone the pre-quantification PCR. The
145 qPCR reactions, containing gene specific primers and Taqman probes (Appendix A), were
146 performed using SensiFAST Probe Hi-ROX qPCR Mix (Bioline) according to the
147 manufacturer's instructions. The reaction conditions were as follows: 95°C for 5 min, followed
148 by 40 cycles of 95°C for 10 s and 65°C for 60 s. Fluorescent readings were taken after each
149 cycle.

150 To enable quantification of target DNA in the test samples, standard curves were generated
151 from serial dilutions of a recombinant plasmid DNA, containing partial sequences for both
152 canine sj-TREC and albumin. Sj-TREC values were corrected for the pre-amplification, and
153 normalised for numbers of white blood cells (WBC) (estimated from albumin qPCR values)
154 using the following equation:

155

$$156 \quad \text{sj-TREC}/1 \times 10^5 \text{ WBC} = \frac{\text{sj-TREC (copies}/\mu\text{l)}}{102.4} \times \frac{1 \times 10^5}{(\text{Albumin (copies}/\mu\text{l)} \times 10) \div 2}$$

157

158 **2.4 Generation of miRNA target recombinant constructs**

159 A region of the canine *IL7R* 3'UTR, containing SNPs 11-15, was examined for the presence
160 of miRNA seed-binding sites, using the Target Mining function on the miRDB website
161 (www.mirdb.org) (Wong and Wang, 2015). SNP 14 (c.1371+446A>C) was identified as
162 causing a change to the seed-binding site for cfa-miR-185.

163 An oligonucleotide pair (sense and antisense) was designed to contain the miRNA target
164 sequence for cfa-miR-185 (Supplementary Fig. A1). The oligonucleotide pair was designed
165 so that they would dimerise and could be ligated into the dual-luciferase miRNA target
166 expression vector, pmirGLO (Promega), following digestion with *PmeI* and *XbaI*. An internal
167 *NotI* site was included to allow confirmation of ligation into the vector, which also contains a
168 *NotI* site at position 93. The sense and anti-sense oligonucleotides were both diluted to 1
169 nmol/ μ l and 1 μ l of each was added to 18 μ l Oligo Annealing Buffer (Promega). This
170 mixture was then heated at 90°C for 3 min before being transferred to a water bath for 15 min
171 at 37°C. The annealed oligonucleotide dimers were then ligated into pmirGLO vector
172 (Promega), which had previously been linearized by restriction digestion with *PmeI* and
173 *XbaI*, using the LigaFast™ Rapid DNA Ligation System (Promega) according to the
174 manufacturer's instructions.

175 A 280 bp region of the *IL7R* 3'UTR (containing SNPs 11-15) representing the two haplotypes
176 was amplified by PCR (see Supplementary Table A1 for primers) and purified by gel
177 extraction (GenElute™ Gel Extraction Kit, Sigma-Aldrich). These were ligated into to
178 pmirGLO, using the *PmeI* and *XbaI* restriction sites and the LigaFast™ Rapid DNA Ligation
179 System (Promega).

180

181 **2.5 Dual-Glo miRNA target luciferase reporter assay**

182 Chinese hamster ovary (CHO) cells were transfected with recombinant pmirGLO constructs
183 and/or Mission miRNA mimics (Sigma-Aldrich), which consisted of a targeted miRNA
184 mimic (hsa-miR-185, which demonstrates sequence conservation with cfa-miR-185) and a
185 negative control miRNA mimic (from *Arabidopsis thaliana*). CHO cells were plated in
186 MEM/6% FBS and cultured until they reached 80-90% confluency. The cells were
187 transfected in triplicate with 200ng plasmid DNA and/or 10 pmol miRNA mimics per well
188 using Lipofectamine 2000 (Invitrogen) according to the manufactures instructions.
189 Untransfected cells were used as a negative control.

190 Twenty-four hours after transfection, cells were assayed for both firefly and renilla luciferase
191 activity, using the Dual-Glo Luciferase Assay System (Promega). Briefly, 50 µl Dual-Glo
192 Luciferase Reagent was added to the cells to induce cell lysis and act as a substrate for firefly
193 luciferase. After 15 min incubation on a rotating platform luciferase activity was measured
194 using a luminometer (Spectramax M2, Molecular Devices Ltd, Wokingham, UK). Next, 50
195 µl Dual-Glo Stop & Glo Reagent was added, and after another 15 min incubation
196 luminescence was measured for a second time to obtain a reading for renilla luciferase
197 activity.

198 Luciferase activity (mean firefly luciferase activity/mean renilla luciferase activity) for
199 constructs treated with the miR-185 mimic were compared to those treated with the negative
200 control mimic using the following equation.

201

202
$$\left[\frac{\text{luciferase activity with miR-185 mimic (mean firefly } \div \text{ mean renilla)}}{\text{luciferase activity with negative control mimic (mean firefly } \div \text{ mean renilla)}} \right] \times 100$$

203

204 **2.6 Statistical analysis**

205 Statistical analyses were performed using a commercial software package (SPSS version 23
206 for Windows, IBM). Mann-Whitney U tests were used to compare sj-TREC values between
207 dogs grouped according to age. Associations between IL7R haplotype or genotype frequencies,
208 and sj-TREC levels in different age groups of dog were achieved using Fisher's Exact test. The
209 firefly and *renilla* luciferase activity data generated from three replicate experiments, where
210 CHO cells were transfected with pmirGLO constructs, was calculated as the mean \pm the
211 standard error of the mean (SEM). The variation in normalised luciferase activity
212 (firefly/*renilla*), between transfections with the miR-185 mimic and the negative control
213 mimic, was analysed using an independent two-sample t-test.

214

215 **3. Results**

216 **3.1 Polymorphisms in the canine *IL7* and *IL7R* genes**

217 Variability in the canine *IL7* and *IL7R* genes was initially investigated in DNA samples from
218 crossbreed dogs. No polymorphisms were identified in the coding sequence of the canine *IL7*
219 gene, although a small number of SNPs (n=4) were found in the intronic regions
220 (Supplementary Table B1). Since all these polymorphisms occurred at a relatively low
221 frequency, no further investigation of the canine *IL7* gene was undertaken.

222 Sequencing of canine *IL7R* revealed 15 SNPs, six of which were found to be exonic, while
 223 the other nine were intronic (Table 1). Of the six SNPs located in the coding region, four
 224 were synonymous while the remaining two (c.956A>G, c.1145C>T) resulted in amino acid
 225 substitutions (Glu-Gly and Ser-Phe, respectively). Based on the genetic variation identified in
 226 the cross-breed dogs, it was decided further studies would focus on *IL7R* exon 8 (containing
 227 the coding sequence for the C-terminal region and the 3'UTR). This region was selected
 228 because it contained nine of the 15 SNPs identified, including the two non-synonymous
 229 SNPs. Additionally, it was determined that SNPs 9-15 were in linkage disequilibrium, giving
 230 rise to the following haplotypes: haplotype 1 (SNPs 9-15 CGTAAAT), haplotype 2 (SNPs 9-
 231 15 TAGGGCC).

232

233 **Table 1. *IL7R* gene polymorphisms identified in six crossbreed dogs.**

Name	Exon	Position on chromosome 4	SNP ID	SNP	F	Type	Amino acid change
SNP1	2-3	72,659,664	c.221+136G>C	G-S-C	5:1:0	Intronic	n/a
SNP2	4	72,641,688	c.402T>C	T-Y-C	2:3:0	Exonic Synonymous	Asp- Asp
SNP3	4-5	72,641,521	c.537+32A>C	A-M-C	4:1:0	Intronic	n/a
SNP4	5	72,639,560	c.543T>G	T-K-G	2:2:2	Exonic Synonymous	Val- Val
SNP5	7-8	72,637,664	c.795-110T>C	T-Y-C	3:2:1	Intronic	n/a
SNP6	7-8	72,637,331	c.870+148G>C	G-S-C	3:2:1	Intronic	n/a
SNP7	8	72,637,009	c.956A>G	A-R-G	5:1:0	Exonic Non-synonymous	Glu- Gly
SNP8	8	72,636,820	c.1145C>T	C-Y-T	5:1:0	Exonic Non-synonymous	Ser- Phe
SNP9	8	72,636,813	c.1152C>T	C-Y-T	3:2:1	Exonic Synonymous	Ser-Ser

SNP10	8	72,636,732	c.1233G>A	G-R-A	3:2:1	Exonic Synonymous	Thr- Thr
SNP11	8	72,637,155	c.1371+284T>G	T-K-G	3:2:1	3'UTR	n/a
SNP12	8	72,637,293	c.1371+423A>G	A-R-G	3:2:1	3'UTR	n/a
SNP13	8	72,637,302	c.1371+432A>G	A-R-G	3:2:1	3'UTR	n/a
SNP14	8	72,637,316	c.1371+446A>C	A-M-C	3:2:1	3'UTR	n/a
SNP15	8	72,637,325	c.1371+455T>C	T-Y-C	3:2:1	3'UTR	n/a

234 F; genotype frequency

235

236 In Labrador retriever dogs, the non-synonymous SNPs were present at low frequency, similar
 237 to that seen in the crossbreed dogs (Fig. 1A). At SNP 7, only two dogs were homozygous for
 238 the minor G allele and 10 dogs were heterozygous, while at SNP 8 the minor T allele was
 239 only present in two heterozygous dogs. For SNPs 9-15, which are in linkage disequilibrium,
 240 more genetic variation was observed in this breed, with 22 dogs homozygous for haplotype 1
 241 (CGTAAAT), 40 heterozygous dogs (YRKRRMY) and 38 dogs homozygous for haplotype 2
 242 (TAGGGCC). These haplotypes were also found to be in Hardy-Weinberg equilibrium
 243 (P=0.074). When *IL7R* haplotype frequencies were analysed, no significant differences in
 244 haplotype or genotype frequencies (P>0.05) were identified comparing the different age
 245 groups (Fig. 1B and 1C).

246

247 **3.2 Polymorphisms in canine *IL7R* exon 8 are associated with sj-TREC values in** 248 **Labrador retriever dogs**

249 Measurement of sj-TREC in the Labrador retriever samples, which had previously been used
 250 to sequence the *IL7R* exon 8 polymorphisms, revealed significant differences when

251 comparing young and middle aged animals ($P < 0.0001$), and also comparing middle aged and
252 geriatric dogs ($P < 0.005$), indicating an age-associated decline in sj-TREC levels (Fig. 2).

253

254 The Labrador retrievers were further sub-divided as having high or low sj-TREC values
255 compared to the median for that particular age group. Median sj-TREC values were 109.5,
256 25.0 and 3.5 sj-TRECs/ 1×10^5 WBC for the young, middle aged and geriatric groups,
257 respectively (Fig. 2). Significant differences were observed in the haplotype and genotype
258 frequencies for *IL7R* SNPs 9-15 comparing dogs with high and low sj-TREC values in both
259 the young ($P = 0.009$ and $P = 0.0049$) and geriatric ($P = 0.005$ and $P = 0.026$) age groups (Fig. 3A
260 and 3B). In the young dogs, *IL7R* SNPs 9-15 haplotype 1 was associated with high sj-TREC
261 values and *IL7R* SNPs 9-15 haplotype 2 was associated with low sj-TREC values. In contrast,
262 in the geriatric dogs, *IL7R* SNPs 9-15 haplotype 2 was associated with having high sj-TREC
263 values, while no association with having low sj-TREC values was found. This suggests that
264 polymorphisms in the *IL7R* gene are associated with thymic output.

265

266 **3.3 Polymorphisms in canine *IL7R* 3'UTR influence post-transcriptional regulation by** 267 **miRNA-185**

268 Since a number of the SNPs identified in the canine *IL7R* gene were located in the 3'UTR, it
269 was decided to investigate whether these polymorphisms influence miRNA binding sites,
270 potentially impacting on post-transcriptional regulation of mRNA expression. Analysis of the
271 canine *IL7R* 3'UTR identified three putative canine specific miRNA binding sites (Table 2).
272 One of these (cfa-miR-185) had its seed-region binding site in an area of the *IL7R* 3'UTR
273 containing a SNP (SNP 14: c.1371+446A>C).

274

275 **Table 2. miRNA seed binding sites in canine *IL7R* 3'UTR.**

miRNA name	Species	miRNA sequence	Seed region location in <i>IL7R</i> 3'UTR
cfa-miR-185	Canine	UGGAGAGAAAGGCAGUCCUGA	1371+443
cfa-miR-508a	Canine	UACUUGAGAGGGUGACAUUCAUAGA	1371+609
cfa-miR-8793	Canine	UCUGAAGCUUUAGCAGGCCCCGAGG	1371+189

276

277 A dual luciferase reporter assay was employed to assess the functional consequences of the
 278 *IL7R* c.1371+446A>C polymorphism, in terms of miRNA regulation. Recombinant plasmid
 279 DNA constructs were designed that contained the target sequence for cfa-miR-185
 280 (pmirGLO/miR-185; positive control), or a 280 bp region of each of the *IL7R* 3'UTR
 281 haplotypes (pmirGLO/*IL7R* 3'UTR Hap1 and pmirGLO/*IL7R* 3'UTR Hap2), located
 282 downstream of the firefly luciferase coding sequence and within its 3'UTR.

283 CHO cells transfected with recombinant pmirGLO constructs and/or miRNA mimics were
 284 assayed for both Renilla luciferase activity (Fig. 4A), to estimate transfection efficiency, and
 285 firefly luciferase activity (Fig. 4B), which is the primary reporter gene. Compared with the
 286 negative control miRNA mimic, there was a reduction in firefly luciferase activity when
 287 pmirGLO/miR-185 and pmirGLO/*IL7R* 3'UTR Hap1 were co-transfected with the miR-185
 288 mimic (Fig. 4B), which suggests this miRNA is capable of binding to the target sequences in
 289 these particular constructs. When firefly luciferase activity was normalised against renilla
 290 luciferase activity, the observed effect was more pronounced for the positive control
 291 construct (pmirGLO/mir-185; P=0.001) and the construct containing the *IL7R* 3'UTR
 292 haplotype 1 (P<0.05), whereas there was no significant reduction for the construct containing
 293 haplotype 2 (Fig 4C). This suggests that miRNA-185 preferentially binds to the *IL7R* 3'UTR

294 sequence containing the haplotype 1 polymorphisms, impacting on mRNA half-life and
295 causing reduced expression of the reporter protein.

296

297 **4. Discussion**

298 This study was designed to characterise polymorphisms in the canine *IL7* and *IL7R* genes,
299 and to examine the influence these might have on thymic output as estimated by measuring
300 sj-TREC values in a population of Labrador retriever dogs. There was limited variability in
301 the canine *IL7* gene, however sequencing of the canine *IL7R* gene revealed a number of
302 SNPs. Of particular interest were a group of polymorphisms, located in exon 8 and the
303 3'UTR of the canine *IL7R* gene, which were found to be in linkage disequilibrium. Haplotype
304 and genotype frequencies for this group of SNPs (9-15) were found to be associated with sj-
305 TREC levels in both young and geriatric Labrador retriever dogs. One of the SNPs identified
306 in the 3'UTR of the *IL7R* gene was found to alter a miRNA binding site, which, by use of a
307 luciferase reporter assay, was shown to influence protein expression.

308 The polymorphisms in the *IL7R* gene consisted of 9 SNPs located in non-coding intronic
309 regions and six in the coding region, two of which were non-synonymous (SNP7 and SNP8).
310 SNP7 (c.956A>G) was found to cause an amino acid change from glutamic acid to glycine at
311 residue 319, while SNP8 (c.1145C>T) caused a change from serine to phenylalanine at
312 residue 382. Since both of these changes occur at the C-terminus of the intracellular domain
313 of the IL-7R it is possible that they might affect downstream signalling of the receptor.
314 Mutations in the Box 1 motif of the intracellular domain have been shown to effect the
315 binding and function of the tyrosine kinase JAK1 (Jiang et al., 2005). However, as it is the
316 membrane-proximal part of the intracellular domain which appears to be most important for

317 signalling in cytokine receptors (Jiang et al., 2005), it seems unlikely that SNPs 7 and 8 will
318 have any influence on signalling. In addition, given the relatively low minor allele frequency
319 of these particular SNPs in the Labrador retriever population, this suggests that they are
320 unlikely to be making a substantial contribution to the variability seen in thymic output in
321 age-matched dogs of this particular breed.

322 No polymorphisms were identified within exon 6 of the *IL7R* gene. Polymorphisms in this
323 particular exon have been reported to be associated with susceptibility to human autoimmune
324 diseases, such as multiple sclerosis, rheumatoid arthritis and type 1 diabetes mellitus
325 (Mazzucchelli et al., 2012), through an amino acid change at residue 244 from isoleucine to
326 threonine. In multiple sclerosis, this polymorphism has been shown to have a functional
327 effect on receptor expression, caused by alternative splicing of exon 6, leading to exon
328 skipping (Gregory et al., 2007). It is possible that the canine *IL7R* exon 6 is less variable than
329 its human orthologue, or that polymorphisms were not identified in the relatively small
330 number of crossbreed dogs used for the initial SNP discovery phase of the study. Crossbreed
331 dogs were selected because they are likely to demonstrate increased genetic diversity
332 compared with pure-breed dogs (Kennedy et al., 2002). However, crossbreed dogs are not
333 necessarily representative of the dog population as a whole (Kennedy et al., 2002) and there
334 might be polymorphisms which are only present in some pedigree breeds. Sequencing of
335 *IL7R* in pedigree dogs affected with autoimmune disease, or in an autoimmune high risk
336 breed (such as the Cocker spaniel) might identify further polymorphisms.

337 A series of seven SNPs (SNPs 9-15), located in the coding region and 3'UTR of *IL7R* exon 8,
338 which were found to be in linkage disequilibrium and had a higher minor allele frequency
339 than the non-synonymous SNPs, were considered more suitable for further investigation into
340 the relationship between *IL7R* and thymic output. Since the formation of sj-TREC occurs

341 specifically in the thymus and this DNA does not replicate, sj-TREC has been used as a
342 biomarker for thymic output/RTEs in a wide range of species, including humans (Douek et
343 al., 1998), mice (Sempowski et al., 2002), primates (Sodora et al., 2000), chickens (Kong et
344 al., 1999) and pigs (Vallabhajosyula et al., 2011). In Labrador retriever dogs an age-
345 associated decline in sj-TREC values was observed which appear to be bi-phasic in nature,
346 with differences occurring between different age groups, and has been previously described
347 (Holder et al., 2016). This is similar to that seen in humans, where the greatest decline in sj-
348 TRECs occur between the teenage years and middle age (40-50 years) (Geenen et al., 2003),
349 subsequently, sj-TREC values show a slow decline between the 6th and 9th decades of life
350 before decreasing significantly in the 10th decade (Mitchell et al., 2010).

351 When Labrador retrievers were further sub-divided according to sj-TREC values, an
352 association with the *IL7R* haplotype and genotype frequencies was identified in both the
353 young and geriatric dogs. Polymorphisms in the coding region of the human *IL7R* gene have
354 previously been shown to be associated with the frequency of RTEs (as measured by sj-
355 TREC) in patients affected with multiple sclerosis (Broux et al., 2010). In the current canine
356 study, the polymorphisms associated with sj-TREC are located in the 3'UTR and might
357 therefore be regulating receptor expression through epigenetic mechanisms. Studies
358 investigating polymorphisms in the genes for interleukin-23 receptor (Zheng et al., 2012) and
359 tumour necrosis factor alpha receptor 2 (Puga et al., 2005) have demonstrated that SNPs in
360 the 3'UTR are capable of reducing receptor expression by altering post-transcriptional
361 regulation leading to increased degradation of mRNA transcripts.

362 The association between sj-TREC and *IL7R* genotypes also suggests a relationship between
363 IL-7R expression and thymic involution. Human longitudinal studies have shown that sj-
364 TREC values decline by an average of 3% of the baseline level per year (Kilpatrick et al.,

365 2008), while a recent study in dogs suggests that differences in sj-TREC levels observed
366 between individual young Labrador retrievers might be indicative of the rate of thymic
367 involution (Holder et al., 2016). In mouse strains which undergo rapid thymic involution, as
368 determined by an earlier decline in sj-TREC values, developing thymocytes were found to
369 have increased expression of IL-7R, compared to those mouse strains demonstrating a slower
370 rate of thymic involution (Wang et al., 2006).

371 The associations between sj-TREC values and *IL7R* haplotypes were not consistent for the
372 young and geriatric Labrador retrievers, which suggests that the influence of the IL-7/IL-7R
373 system on thymic output in the dog changes with age. This might be expected, since in
374 laboratory rodents expression of IL-7 in the thymus declines with age (Andrew and Aspinall,
375 2002; Ortman et al., 2002), such that, following thymic involution, there will be reduced
376 amounts of IL-7 available for T cell development. Therefore, with advancing age, high
377 expression of IL-7R might play a role in ensuring thymocytes are sensitive to the limited
378 supply of IL-7 in the thymus, so that some level of thymic output is maintained. However,
379 without longitudinal data from individual dogs it is not possible to determine the exact
380 influence of the polymorphisms on sj-TREC values.

381 If the polymorphisms identified in the 3'UTR of the canine *IL7R* gene have a biological
382 impact on receptor expression, this is likely due to differences in post-transcriptional
383 regulation of the mRNA, possibly by miRNAs. Therefore, the *IL7R* 3'UTR sequence was
384 interrogated for miRNA seed-region binding sites. The seed region of a miRNA (nucleotides
385 2 to 8 at the 5'end) determines binding of the miRNA to its target mRNA by Watson-Crick
386 pairing to the regulatory sequence in the 3'UTR of the gene (Bartel, 2009). Several of these
387 sites were identified in the canine *IL7R* 3'UTR, and one of these (cfa-miR-185) was found to
388 be located in an area containing a polymorphism (SNP 14: c.1371+446A>C).

389 To test the hypothesis that polymorphisms in the canine *IL7R* 3'UTR affect mRNA stability,
390 via the action of miRNA, an in-vitro model system was established. Our results revealed that
391 there was a significant reduction with the miR-185 mimic when cells were transfected with
392 the *IL7R* 3'UTR haplotype 1 compared with haplotype 2, where no reduction was seen. This
393 suggests that the miR-185 mimic is capable of binding to the *IL7R* 3'UTR haplotype 1
394 sequence expressing the C allele of SNP14, where there is complete pairing with the miR-185
395 seed site. However, in the haplotype 2 sequence expressing the A allele, where this change
396 causes incomplete pairing with the seed site, the miR-185 mimic seemingly has no effect.
397 These research findings indicate that the polymorphisms in the *IL7R* 3'UTR are potentially
398 capable of altering protein expression, through post-transcriptional modification of mRNA by
399 miR-185.

400 It is important to acknowledge that this is a model system and does not necessarily indicate
401 this interaction would occur in a canine cellular environment. Transfection of the reporter
402 constructs into T cell lines might provide information on whether endogenous miRNAs are
403 capable of acting on the *IL7R* 3'UTR sequences, while transfecting T cell lines with the miR-
404 185 mimic and measuring changes in endogenous IL-7R expression would also provide
405 valuable functional evidence. However, *in vivo* studies or *ex vivo* analysis of T
406 cells/thymocytes from dogs of defined genotype would be required to provide conclusive
407 evidence that the *IL7R* 3'UTR polymorphisms are influencing expression of the receptor.

408 In conclusion, this study has demonstrated that variation in the *IL7R* gene is associated with
409 thymic output in the dog, and that this might be mediated through changes in mRNA
410 stability, leading to altered receptor expression. This provides further evidence of the general
411 premise that the IL-7/IL-7R pathway might be playing an important role in the regulation of
412 thymic output, and therefore age-associated thymic involution.

413

414 **Figure captions**

415 **Figure 1. Analysis of *IL7R* exon 8 polymorphisms in Labrador retriever dogs.** Sequence-
416 based typing was used to genotype 100 Labrador retrievers for polymorphisms in exon 8 of
417 the *IL7R* gene (A). The sequencing data was then analysed to generate haplotype (B) and
418 genotype (C) frequencies for *IL7R* SNPs 9-15 in the Labrador retrievers categorised into
419 young (<2 years, n=30) middle aged (5-7 years, n=30) and geriatric (\geq 10 years, n=40) age
420 groups. Differences in the haplotype and genotype frequencies between the different age
421 groups were compared using Fisher's Exact Test, and found not to be significant.

422

423 **Figure 2. Measurement of sj-TREC values in Labrador retrievers by real-time qPCR.**

424 Sj-TREC values were determined in gDNA samples from 100 Labrador retrievers, previously
425 used to sequence the *IL7R* exon 8. These values were normalised against white blood cell
426 numbers, estimated by measuring the albumin copy number in each sample concurrently, in
427 multiplex qPCR. The dogs were categorised into young (<2 years, n=30) middle aged (5-7
428 years, n=30) and geriatric (\geq 10 years, n=40) age groups. Each dog is represented by a circle
429 within their age group. A trend line is shown at the median for each age group. P values were
430 calculated using the Mann-Whitney U test.

431

432 **Figure 3. Polymorphisms in *IL7R* exon 8 are associated with sj-TREC values in young
433 and geriatric Labrador retrievers.** Sequencing data was analysed to generate (A) haplotype
434 and (B) genotype frequencies for *IL7R* SNPs 9-15 in Labrador retriever dogs. The dogs were
435 categorised into young (<2 years, n=30), middle aged (5-7 years, n=30) and geriatric (\geq 10

436 years, n=40) age groups, and then further subdivided into high or low sj-TREC groups based
437 on their relationship with the median for that age group. P values were generated using a two-
438 tailed Fisher's exact test.

439

440 **Fig 4. Polymorphisms in *IL7R* 3'UTR influence post-transcriptional regulation by**
441 **miRNA-185.** CHO cells were co-transfected with pmirGLO constructs and miRNA mimics.
442 After 24 hrs, (A) renilla luciferase and (B) firefly luciferase activity was measured using the
443 Dual-GLO luciferase assay system. Results are the mean \pm SEM of triplicate wells. (C)
444 Normalised luciferase activity (firefly/renilla) in cells co-transfected with miR-185 mimic
445 was calculated relative to results obtained with the negative control mimic. A trend line is
446 positioned at the percentage luciferase activity for transfections with the negative control
447 mimic. P values were calculated using an independent two sample Student's t test. *P<0.05,
448 ***P<0.001.

449

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453

454 **Author contributions**

455 AH, DP, RA and BC conceived and designed the experiments. AH, GJ and FS performed the
456 experiments. AH, GJ, FS and BC analysed and interpreted the results. AH, DP, RA and BC
457 wrote the manuscript.

458

459 **Conflict of interest statement**

460 The authors declare that they have no competing interests.

461

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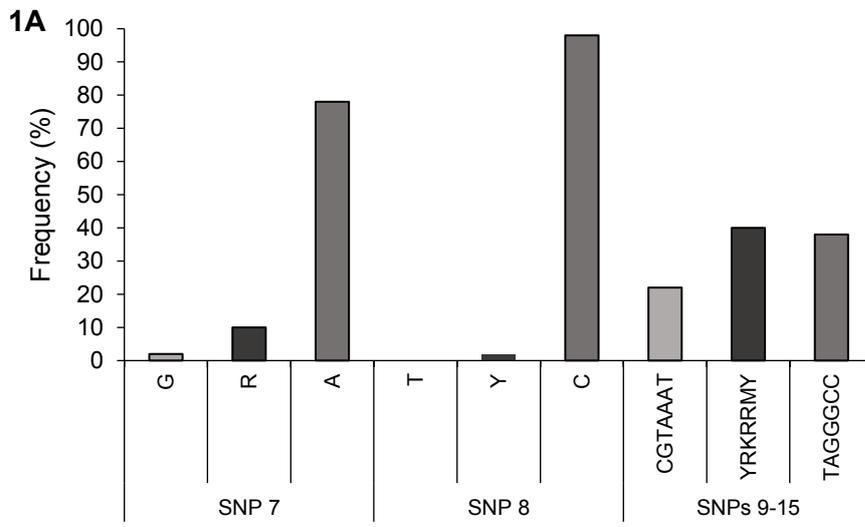
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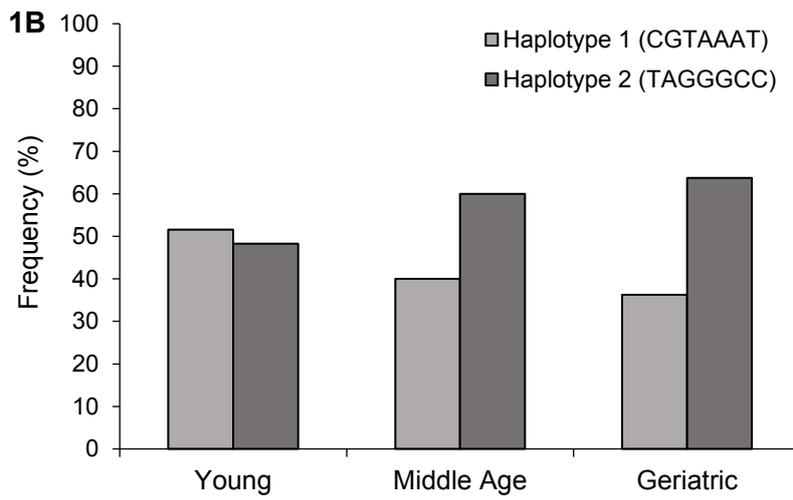
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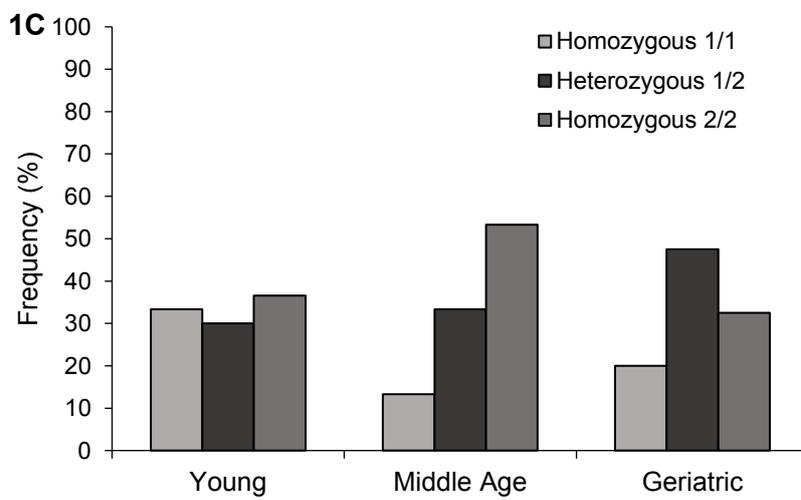
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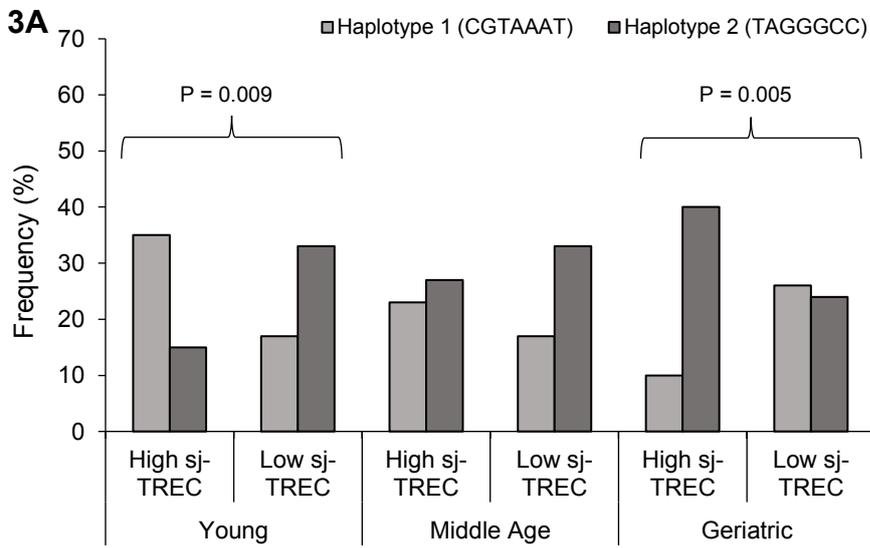
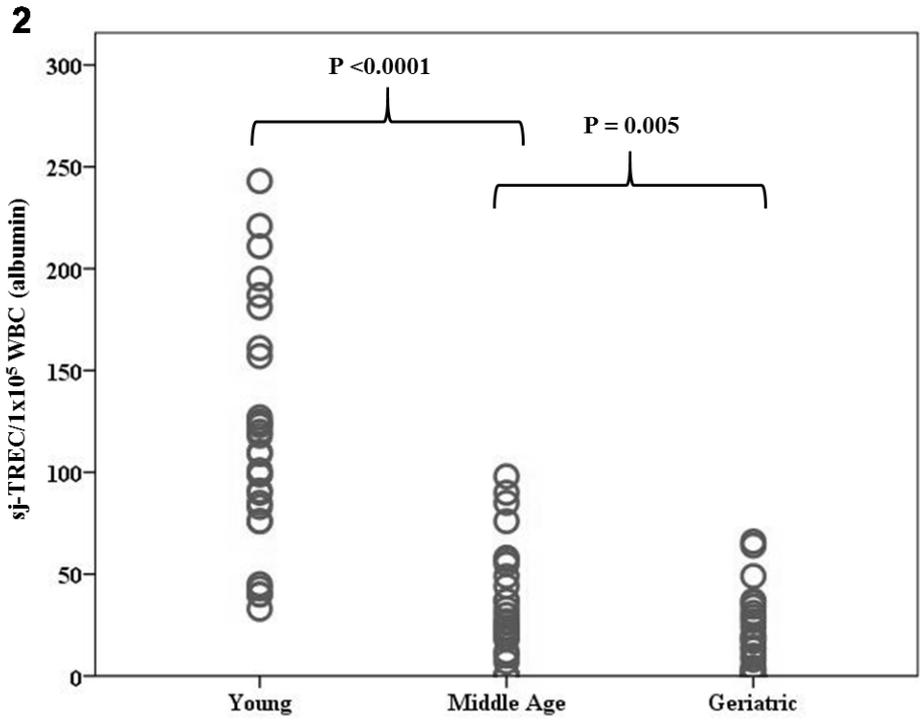
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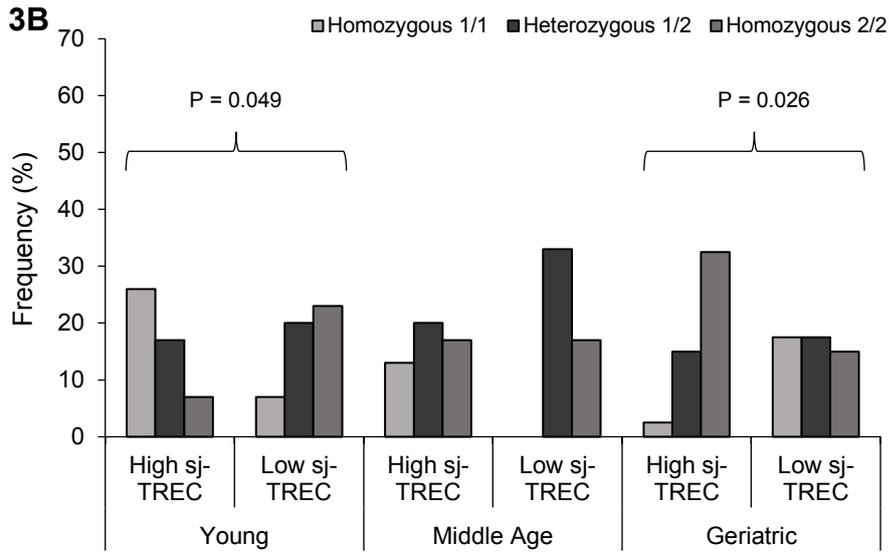


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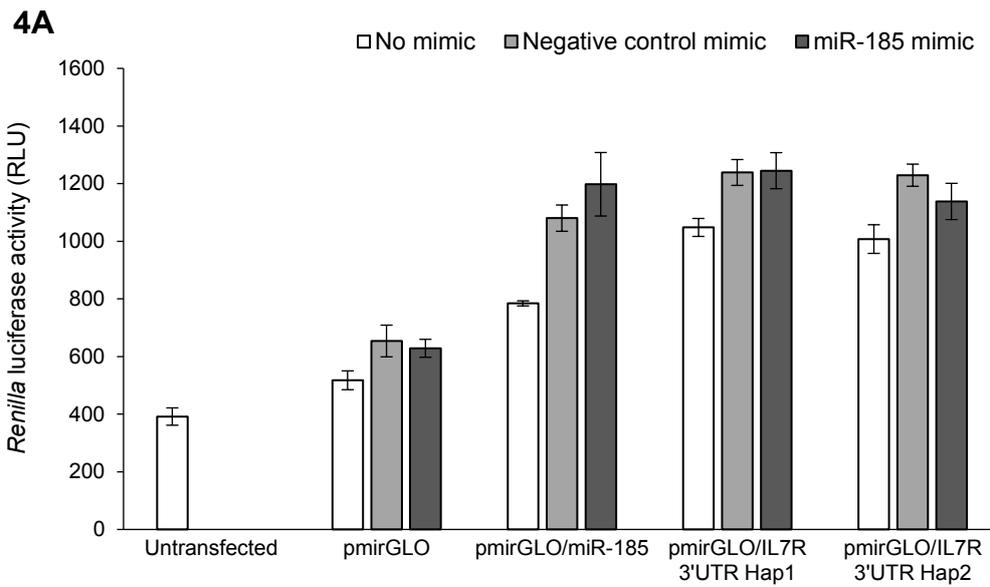
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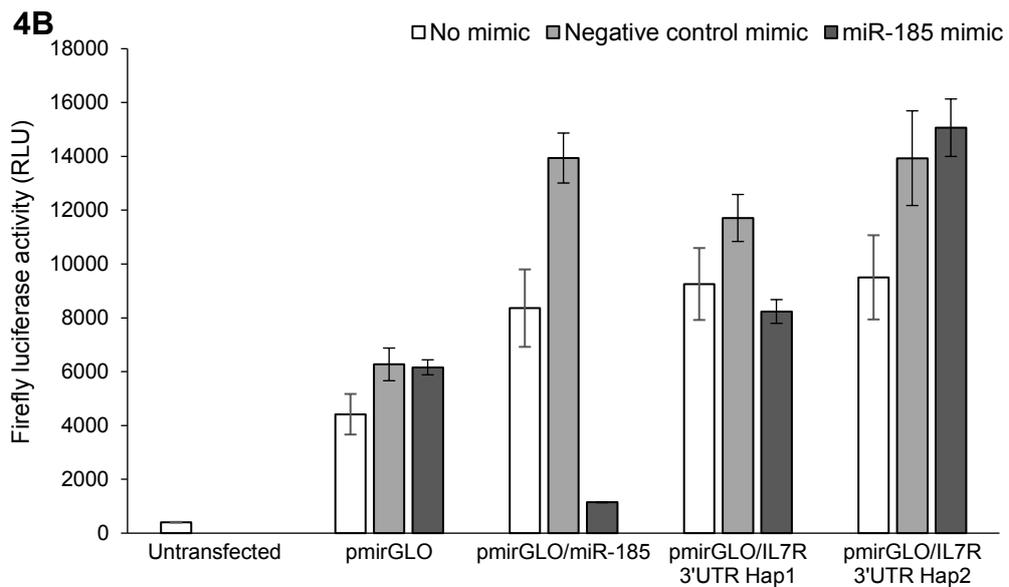
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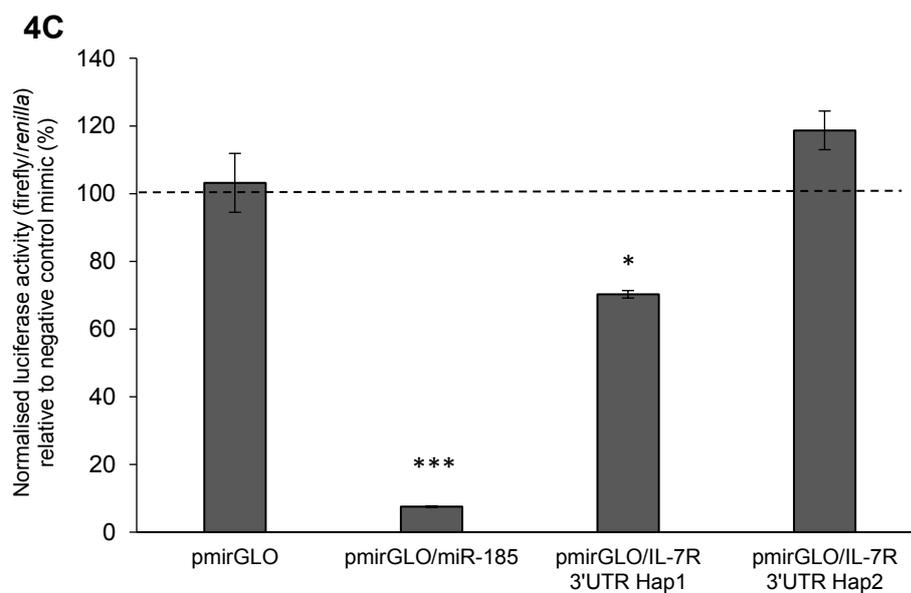


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