

1 **Identification of the genomic mutation in *Epha4*^{rb-2J/rb-2J} mice**

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26 **Abstract**

27 The EphA4 receptor tyrosine kinase is involved in numerous cell-signalling activities
28 during embryonic development. EphA4 has the ability to bind to both types of ephrin
29 ligands, the ephrinAs and ephrinBs. The C57BL/6J-Epha4rb-2J/GrsrJ; genetically
30 known as $Epha4^{rb-2J/rb-2J}$ is a spontaneous mouse mutant which arose at The Jackson
31 Laboratory. These mutants exhibited a synchronous hind limb locomotion defect or
32 ‘hopping gait’ phenotype, which is also characteristic of EphA4 null mice. Genetic
33 complementation experiments suggested that $Epha4^{rb-2J}$ corresponds to an allele of
34 EphA4 but details of the genomic defect in this mouse mutant are currently
35 unavailable. We found a single base-pair deletion in exon 9 resulting in a frame shift
36 mutation that subsequently resulted in a premature stop codon. Analysis of the
37 predicted structure of the truncated protein suggests that both the kinase and sterile α
38 motif (SAM) domains are absent. We have also developed a method to ease detection
39 of the mutation through RFLP that will aid in studies where the true genotypes need
40 to be ascertained. The importance of this study is underlined by the numerous
41 isoforms attributed to the Eph-ephrin family and in this case, the identification of the
42 type of mutation enables further functional studies such as protein-protein
43 interactions, immunostaining and gene compensatory studies of the Eph family of
44 receptor tyrosine kinases.

45

46 **Keywords**

47 *EphA4*; hopping gait; spontaneous mutation; rb-2J strain; knockout mouse

48 **Introduction**

49 Erythropoietin hepatocellular carcinoma receptor tyrosine kinases, more commonly
50 known as the Ephs; are members of the largest family of receptor tyrosine kinases
51 (RTKs) which characteristically bind to their ligand ephrins. The mammalian and
52 chick Ephs are divided into two groups based on sequence homologies and affinity for
53 ephrins, the A-type (EphA1-EphA10) and the B-type (EphB1-EphB6) (Pasquale,
54 2008). EphrinAs are known to bind to EphA receptors and ephrinBs bind to EphBs.
55 EphA4 interacts with both ephrinAs and ephrinBs (ephrinB2 and ephrinB3) and
56 ephrinA5 binds to EphB2 (Himanen et al., 2004; Pasquale, 2004). Eph and ephrin
57 signalling play many important roles including remodelling of blood vessels and
58 formation of tissue boundary (Pasquale, 2005, 2008; Wilkinson, 2015). EphA4 has
59 been shown to be involved in cell signalling activities in numerous contexts including
60 axon guidance and development of central nervous system vasculature (Dottori et al.,
61 1998; Kullander et al., 2001).

62 During embryonic development, *EphA4* is expressed at the tips of the closing
63 spinal neural folds, and in the developing forebrain, hindbrain and mesoderm (Abdul-
64 Aziz et al., 2009; Nieto et al., 1992). In adult mice, *EphA4* is highly expressed in the
65 jejunum (Islam et al., 2010) and the brain, mainly in the hippocampus (Greferath et
66 al., 2002; Grunwald et al., 2004; Kullander et al., 2001). Targeted mouse knockouts
67 of the *EphA4* gene display locomotor abnormalities of the hind limb resulting in a
68 rabbit-like hopping movements (Coonan et al., 2001; Herrmann et al., 2010;
69 Kullander et al., 2003; Kullander et al., 2001; Nieto et al., 1992) or clubfoot of the
70 hind limb (Helmbacher et al., 2000). Interestingly, inactivation of the *EphA4* function,
71 which causes the lack of axonal guidance, was reported to promote axonal

72 regeneration and improve functional recovery of a central nervous system injury
73 (Goldshmit et al., 2011).

74 Two spontaneous mutations of the *Epha4* gene; *Epha4*^{rb/rb} (Eph receptor A4;
75 rabbit) and *Epha4*^{rb-2J/rb-2J} (Eph receptor A4; rabbit 2 Jackson) were reported as
76 spontaneous recessive mutants within the C57BL/6J background at The Jackson
77 Laboratory. However, the exact genomic location of the mutated alleles has not been
78 ascertained. Since the phenotype observed in the *Epha4*^{rb-2J/rb-2J} mice was very similar
79 to the targeted knockouts of the *Epha4* gene, complementarity testing was performed
80 by mating a *Epha4*Gt(pGT1TM)38Wcs/+ female (provided by Tessier-Lavigne
81 Laboratory of Stanford University) to an *Epha4*^{rb-2J/+} male. Among the progeny of
82 this cross, 3 pups (1 female and 2 males) out of 7 exhibited hopping gait phenotype
83 without leaning. The information on the *Epha4*^{rb/rb} and *Epha4*^{rb-2J/rb-2J} are from the
84 Mouse Mutant Resource Web Site, The Jackson Laboratory, Bar Harbour, Maine.
85 (<http://mousemutant.jax.org/>) [October 9, 2010]. This finding of genetic
86 complementation strongly suggested that the *rb-2J* mutation lies within the *Epha4*
87 gene.

88 The aim of this study is to understand the nature of the *Epha4*^{rb-2J/rb-2J}
89 mutation and the extent of the mutation in the protein. Therefore it was necessary to
90 define the mutation and determine the exact genotypes of the mice. We also reviewed
91 the impact of the mutation with EphA4 isoforms and other reported EphA4 mutants to
92 better understand the diversity of the EphA4 protein. In addition, we describe a rapid,
93 simple assay for genotyping the C57BL/6J-Epha4rb-2J/GrsrJ mice, which was not
94 previously available and will be invaluable to other groups intending to work with
95 these mice. Therefore, we have addressed the knowledge gap for the C57BL/6J-
96 Epha4rb-2J/GrsrJ mouse in this study.

97 **Materials and methods**

98 The *Epha4*^{rb-2J/rb-2J} strain (003129) was obtained from The Jackson Laboratory and the
99 colony maintained at the Universiti Kebangsaan Malaysia's Animal Biosafety Level 2
100 laboratory. All experimental procedures were approved by the Institutional Animal
101 Care and Use Committee (IACUC) of University of Malaya (approval number
102 PAR/20/09/2011/NMAA).

103

104 ***Epha4*^{rb-2J/rb-2J} knockout mouse and dissection of the hippocampus**

105 As the hopping gait phenotype is only evident in mice at 3 weeks old, mutational
106 analysis was first determined using samples from adult mice in order to correlate the
107 genotype with an affected phenotype. In adult mice, *Epha4* expression is abundant in
108 the hippocampus (Liebl et al., 2003; Murai et al., 2003), therefore hippocampi of two
109 aged matched control and *Epha4*^{rb-2J/rb-2J} mice were isolated (modified protocol)
110 (Fuller & Dailey, 2007). The mice were euthanized by cervical dislocation. After the
111 the midline incision from the foramen magnum was made up to the level of eye
112 sockets, the skull flaps were tilted to break it off using forceps, and the brain was
113 lifted out of the skull vault gently using curved narrow patterned forceps. In the clean
114 petri dish, the brain was cut into half at the midline, and the hemisphere of the brain
115 was held in place by piercing through the rostral end of the brain with Dumont
116 forceps. Then, the brain stem and cerebellum were removed before gently removing
117 the midbrain. The hippocampus is delineated along its length in the midbrain. The
118 hippocampus was flipped over and cut to separate it from the hemisphere and stored
119 in -80°C.

120

121 **RNA Isolation**

122 RNA isolation was carried out by homogenisation in 1 ml of TRIzol (Invitrogen,
123 Carlsbad, CA, USA) to 50 mg of hippocampi tissue, followed by the addition of 0.2
124 ml chloroform according to the manufacturer's instruction. After separation by cold
125 centrifugation, the aqueous phase was placed in a new tube before addition of 0.5 ml
126 of isopropanol and subsequently centrifuged. The pellet was washed with 75%
127 ethanol and left to air-dry for 5-10 minutes. The pellet was then re-suspended in
128 distilled, deionised water.

129

130 **Mutational screening of *Epha4*^{rb-2J/rb-2J}**

131 Seven sets of primers, which amplify overlapping regions of EphA4 cDNA, were
132 designed to allow sequencing of the entire coding region (accession number:
133 NM_007936). After the discovery of location of the putative mutation in *Epha4*,
134 primers for genomic DNA were also designed. All primers were designed using
135 Primer3 (<http://bioinfo.ut.ee/primer3-0.4.0/primer3/>). Primer sequences with
136 overlapping region of *Epha4* cDNA were as following: set 1 flanking exon 1 to 3
137 (668 bp): 5'-CACCTCTTGGCAATGTCTT-3' and 5'-
138 CTTTTCAGGATGTGGGTGCT-3'; set 2 flanking exon 3 to 5 (700 bp): 5'-
139 GACATTGGTGACCGAATCAT-3' and 5'-TCCACTACACACCACAGCAGA-3';
140 set 3 flanking exon 5 to 8 (588 bp): 5'-GGCCGTCAGGACATTTCTTA-3' and 5'-
141 ACTCCACTGTCCTGCTGGTC-3'; set 4 flanking exon 7 to 11 (499 bp): 5'-
142 TCTGACTTCTATGTTTTTCACG-3' and 5'-GAGACTTCCTGAGTGAGGCC-3';
143 set 5 flanking exon 10 to 14 (578 bp): 5'-CGATGCATCCTGCATTAAAA-3' and 5'-
144 CTCAGCCAGTGATGTCTGGA-3'; set 6 flanking exon 13 to 16 (515 bp): 5'-
145 CCGAAGCAGCCTACTACTACC-3' and 5'-TAGAAGCCGTGGTTCACATG-3';

146 and set 7 flanking exon 15 to coding region exon 17 (471 bp): 5'-
147 CTCCCCTGAATTCTCTGCTG-3' and 5'-ATCAGAATTAAACCTGGAGCCA-3'.

148 RT-PCR was performed using Transcriptor One-Step RT-PCR kit (Roche
149 Diagnostics, Mannheim, Germany). The transcriptor enzymes mix contains
150 transcriptor reverse transcriptase, expand system and protector RNase inhibitor. The
151 1x reaction buffer including Tris, MgCl₂, 1.5 mM dNTPs and additives for hot start
152 PCR. We generated cDNA and amplified 50 ng of total RNA in a 50 ul of reaction
153 mixture consisting of 0.4 uM of each primer. Glyceraldehyde 3-phosphate
154 dehydrogenase (*GAPDH*) and Phosphoglycerate kinase 1, (*Pgk1*) mRNA were used
155 as internal controls. The reverse transcription and amplification condition was set as
156 follows: cDNA generation at 50°C for 30 minutes, and 94°C for 7 minutes, followed
157 by 35 cycles of denaturation at 94°C for 10 seconds, annealing at optimised
158 temperature for 30 seconds, extension at 68°C for 1 minute, with final extension at
159 68°C for 7 minutes. Resulting PCR products were analysed by electrophoresis on 1%
160 agarose gels containing ethidium bromide.

161 RT-PCR products from *Epha4*^{+/+}, *Epha4*^{rb-2J/+} and *Epha4*^{rb-2J/rb-2J} samples
162 were purified using QIAquick PCR purification kit or QIAquick gel extraction kit
163 (Qiagen, Valencia, CA, USA). All the purification steps were according to the
164 manufacturer's directions. Subsequently, 10 ul of 30 ng/ul single band PCR products
165 were sent for Sanger sequencing through a commercial company.

166

167 **Genomic DNA Isolation and PCR**

168 While the mutational screening was performed on the coding transcripts, to enable
169 ease of genotyping from the genomic DNA, we developed a method to genotype the
170 mutation from genomic DNA. Tail or ear clips of adult mice were obtained and DNA

171 extraction for genotyping. The DNA extraction described previously (Sambrook et al.,
172 1989). The tissues were lysed in SNET buffer containing 20 mM Tris-Cl pH 8.0
173 (Sigma), 5 mM EDTA pH 8.0 (Sigma), 400 mM NaCl (Sigma), 1% (w/v) SDS
174 (Sigma) and sterilised by filtration through 0.45 µm nitrocellulose filter before the
175 addition of 20 mg/ml Proteinase K (Sigma). The tissues were incubated in 55°C until
176 the tissue was completely lysed. The DNA was isolated by using 1:1 of phenol
177 solution (Sigma), followed by 2:1 of absolute ethanol precipitation and 70% ethanol
178 washing step. After left to air-dry, the pellet was re-suspended in distilled, deionised
179 water.

180 The genomic DNA amplification of samples were performed with 0.6mM of
181 EphA4 primers spanning the mutation (accession number: NC_000067.6; forward P1:
182 5'-GTAACATGTGCACTGCCTATCC-3' and reverse P2: 5'-
183 CACAGGCATATTAACCAACACTTC-3') in a 50 ul total reaction of DreamTaq
184 Green DNA polymerase (ThermoScientific), 1x buffer DreamTaq Green buffer
185 including 2 mM MgCl₂, and 0.2 mM dNTP. The amplification condition was set as
186 follows: 95°C for 4 minutes, followed by 35 cycles of denaturation at 95°C for 1
187 minute, annealing at 59°C for 1 minute, extension at 72°C for 1 minute, with final
188 extension at 72°C for 10 minutes. The expected size of amplicon is 258 bp.
189 Subsequently, 40 ul of 30 ng/ul single band PCR products were sent for DNA
190 purification and Sanger sequencing through a commercial company.

191

192 **PCR- RFLP (Restriction Fragment Length Polymorphism)**

193 We employed PCR-RFLP to rapidly genotype the novel mutation in more samples.
194 New primers were designed based on the *Epha4*^{rb-2J/rb-2J} exon 9 nucleotide sequence
195 (<http://bioinfo.ut.ee/primer3-0.4.0/primer3/>) to introduce mutations into the amplified

196 DNA at nucleotide 1799 and 1800 (AA→TC) of the gene to generate an *Xho*I
197 recognition site CTCGAG in mutant samples. The forward primer sequence (P3) is
198 5'- TACAGCAAAGCGAAACTTCGA-3' (the altered sequence was underlined) and
199 reverse primer sequence (P2) is 5'-CACAGGCATATTAACCAACACTTC-3'. We
200 amplified 50ng of genomic DNA in a 20 µl of reaction mixture consisting of 0.5µM
201 of each primer and 1X LightCycler® 480 Probe Master containing FastStart Taq
202 DNA Polymerase, dNTP mix and 6.4mM MgCl₂ (Roche Diagnostics, Mannheim,
203 Germany). The amplification condition was set as follows: 95°C for 10 minutes,
204 followed by 35 cycles of denaturation at 95°C for 10 seconds, annealing at 53°C for
205 30 seconds, extension at 72°C for 10 seconds, with final extension at 72°C for 7
206 minutes. Approximately 15µl of amplicon was digested with 20U of *Xho*I (New
207 England Biolabs, Ipswich, MA, USA) according to manufacturer's protocol followed
208 by separation on 4% agarose gel. The fragments generated were a single 149bp for the
209 homozygote mutant, a single 166bp band for wildtype and two bands of 149bp and
210 166bp bands for heterozygous profiles.

211

212 **Western Blot Analysis**

213 To determine the effect of the mutation, immunoblot analysis was performed using
214 anti-EphA4 (EphA4 Antibody S-20; sc-921) and anti-GAPDH (Santa Cruz
215 Biotechnology) on wildtype, heterozygous and mutant protein samples isolated from
216 the hippocampi of 31-day old mice. Protein amounting to 25ug isolated from the
217 hippocampi was mixed with 2µl of 1M DTT in a final volume of 20µl. Protein
218 samples were heated to 100°C for 10 minutes then immediately placed on ice prior to
219 gel loading. Life Technologies Xcel Surelock Mini Cell system was used with a 4-
220 12% denaturing precast protein gel according to the manufacturers guidelines.

221 Samples were run at 200V for 1 hour. Protein was then transferred using Life
222 Technologies Xcel Mini Cell transfer system per manufacturers guidelines. Protein
223 was transferred at 35V for 3 hours with transfer apparatus submerged in ice
224 bucket. Blot was then blocked with 5% milk in TBS-T buffer (50mM Tris Base,
225 150mM NaCl, 01% Tween 20, pH 7.5) for 1 hour. After blocking, anti-EphA4
226 primary antibody was diluted to 1:100 in 5% milk in TBS-T buffer and incubated
227 with blot overnight at 4°C. Anti-GAPDH primary antibody was added after 24 hours
228 incubation with anti-EphA4 at a dilution of 1:50,000 and was incubated for an
229 additional 1 hour at 4°C. Blot was washed 5 times with TBS-T buffer for 5 minutes
230 per wash with agitation. Secondary antibody (Life Technologies; A24531) was
231 diluted 1:10,000 in 5% milk in TBS-T buffer and incubated with blot for 1 hour at
232 room temperature. Blot was washed 5 times with TBS-T buffer for 5 minutes per
233 wash with agitation. Secondary antibody was detected by mixing equal volumes of
234 ECL reagents (Pierce; 34077) and exposing the blot to the mixed ECL reagents for 5
235 minutes. The blot was imaged on a BioRad chemidoc station.

236

237 **Results**

238 The *Epha4*^{rb-2J/rb-2J} mouse is commercially available from The Jackson Laboratory.
239 However, the mutation was not known and there is no clear protocol on how to
240 genotype these mice. Knowing the exact genotype is crucial when investigating the
241 effect of the loss of the gene in the affected mice. Therefore, in this study we
242 determined the precise mutation in the *Epha4*^{rb-2J/rb-2J} strain, confirming that *Epha4*
243 loss of function is the cause of the phenotype in this strain and facilitating further
244 experimental studies.

245

246 **A single nucleotide deletion in *Epha4*^{rb-2J/rb-2} results in a truncated**
247 **EphA4 protein**

248 The *Epha4* gene is 6,328bp in size located on mouse chromosome 1
249 (Ensembl; release 80, May 2015). The sequences of the translated regions, contained
250 within exon 1-17, of the *Epha4*^{rb-2J/rb-2J} strain were compared against the annotated
251 *Epha4* gene deposited in GenBank and with control C57BL/6J mice (accession
252 number: NM_007936). A single nucleotide deletion (del1802) was located in exon 9
253 at 77,390,062 on mouse chromosome 1 and confirmed by sequencing of genomic
254 DNA. This deletion is predicted to result in a frame shift and creation of a premature
255 stop codon (Figure 1A). The resultant protein is therefore predicted to consist of the
256 wildtype sequence up to amino acid 582 (E582), followed by a series of six altered
257 amino acids and a stop codon (Figure 1B). The mutation lies within a conserved
258 protein region in several organisms including human, orang-utan, frog, pig, rat and
259 chick (Table 1).

260 This truncated protein lacks a further 390 amino acids compared to the full-
261 length wildtype protein, which has 986 amino acids (Figure 1B). The protein structure
262 encoded by *Epha4*^{rb-2J/rb-2J} was predicted using Simple Modular Architecture Research
263 Tool, SMART (<http://smart.embl-heidelberg.de>). The predicted structure of the
264 truncated proteins lacks the kinase and sterile alpha motif (SAM) domains (Figure
265 2E).

266 Western blot analysis (Figure 3) revealed an intact 110kDa band in samples
267 from both wildtype and heterozygous mice. This band was absent in the homozygous
268 mutant mice. A second band at 104kDa was detected in samples from all genotypes.
269 We hypothesise that the lower molecular band is an alternate uncharacterised isoform.

270 There are no reported isoforms of EphA4 with only exon 9 (containing the truncating
271 mutation) spliced out.

272

273 **An RFLP-PCR assay was developed to rapidly genotype the mice**

274 In addition to provide a means to identify mice carrying the deletion on exon 9,
275 we analysed C57BL/6J-*Epha4^{rb-2J}/GrsrJ* mouse colony with and without hopping
276 gait characteristics using an inexpensive PCR-RFLP analysis (see Materials and
277 Methods). The analysis showed *Epha4^{+/+}* mice with wildtype profile (a single 166 bp
278 band), *Epha4^{rb-2J/+}* mice with heterozygous profile (two bands at 149bp and 166bp)
279 and *Epha4^{rb-2J/rb-2J}* mice with mutant profile (a single 149bp band) (Figure 4). All the
280 RFLP analyses were subsequently confirmed by DNA sequencing. All mutant mice
281 were characterised with hopping gait features whereas both wildtype and
282 heterozygous mice were apparently normal.

283

284 **Genotypes obtained correlate with the EphA4 phenotype**

285 Among 63 samples that were sequenced, 18 were wildtype (*Epha4^{+/+}*), 35
286 were heterozygous (*Epha4^{rb-2J/+}*) and 10 were mutants (*Epha4^{rb-2J/rb-2J}*). The analysis
287 showed that the deletion found on exon 9 of *Epha4^{rb-2J/rb-2J}* mice was 100% in
288 concordance with the features observed in the mutant mice.

289

290 **Discussion**

291 The central pattern generators (CPGs) are the neuronal networks that generate and
292 coordinate rhythmic limb movement. The hopping gait phenotype displayed by the
293 *Epha4* mutant was discovered due to CPG neurons aberrantly crossing the midline of

294 the spinal cord (Kullander et al., 2003). The crossing generates the synchronous
295 locomotion of the hind limbs through reciprocal over-excitation of the CPG neurons.

296 Previous studies on the understanding of the functional domains of EphA4
297 receptor in the mouse revealed the requirement of kinase function in axon guidance
298 and its formation (Dufour et al., 2006; Egea et al., 2005; Kullander et al., 2001). The
299 cytoplasmic domains of Eph receptor are the juxtamembrane (JM) domain, kinase
300 domain, sterile- α -motif (SAM) domain and PDZ domain. In functional studies, the
301 hopping gait phenotype only appeared in *EphA4* mutants (*EphA4^{KD}*) with a defective
302 kinase domain (Kullander et al., 2001) and in mutants (*EphA4^{GFP}*) with the absence of
303 the entire functional cytoplasmic domains (Egea et al., 2005). Another *EphA4* mutant
304 (*EphA4^{EE}*) displayed normal alternating gait when two mutations of tyrosine residues
305 (Y596E and Y602E) were introduced in the JM domain (Egea et al., 2005). Despite
306 the decrease of auto-phosphorylation in the mutant, there was an increase of basal
307 kinase activity almost similar or higher than ephrin-activated EphA4. This would
308 explain the requirement of kinase activity for normal functioning CPG neurons and
309 normal alternating gait. The findings support the hypothesis that tyrosine residues in
310 the JM domain regulate EphA4 kinase activity. The mutation in *Epha4^{rb-2J/rb-2J}* is
311 located at E582 prior to the major auto-phosphorylation sites. The deletion resulted in
312 a frameshift, leading upon translation to altered six encoded codons followed by a
313 stop codon. The *Epha4^{rb-2J/rb-2J}* protein is truncated, lacking the tyrosine auto-
314 phosphorylation sites in the JM domain, the kinase domain, SAM domain and PDZ
315 domain. The phenotype of the *Epha4^{rb-2J/rb-2J}* mouse differs from the other EphA4
316 mutants that have been published in the past in that it not only hops but also leans.
317 Therefore, variability in phenotypic representations exists from the same gene
318 knockouts (Table 2).

319 The kinase activity of the *Epha4*^{rb-2J/rb-2J} protein is expected to be low to almost
320 non-existent similar to previously reported mutants. Truncation of EphA4 was
321 indicated by the western blot analysis, which showed the absence of the full length
322 EphA4 protein (110kDa) in homozygote mutant mice. A second band similar to
323 smaller isoform reported in UniProt
324 (<http://www.uniprot.org/uniprot/?query=EphA4&sort=score>) at 104kDa was detected
325 in wildtype, heterozygous and mutant EphA4 mice. However, this band is most likely
326 an unknown isoform, which has exon 9, spliced out but it still possesses the epitope
327 located at the SAM domain, which is detected by the Santa Cruz antibody.

328 There exists 7 isoforms of the EphA4 protein (available from UniProt; Table
329 3). It seems likely that the *Epha4*^{rb-2J/rb-2J} protein exists in two spliced variations. The
330 first being that the full length version is mutated and lacks the kinase domain;
331 therefore the signal from the full length version is not picked up by the western blot in
332 Figure 3 but possesses an unknown shorter isoform at 104 kDa (similar in size but
333 differs from Figure 2C). The truncated full length version of *Epha4*^{rb-2J/rb-2J} is 63 kDa
334 in size when analysed using SMART prediction (Figure 2E) and it is similar in size to
335 the 63 kDa short isoform of EphA4 (Figure 2D). Ephs and ephrins are known to carry
336 spliced variations of its protein as evidenced in both man (Finne et al. 2004) and
337 mouse (Holmberg et al. 2000).

338

339 **Conclusions**

340 We identified a single nucleotide deletion of adenine in exon 9 of EphA4 in the
341 *Epha4*^{rb-2J/rb-2J} mouse mutant. This deletion results in a frameshift, which is predicted
342 to cause premature truncation of the protein, and lack of key cytoplasmic domains.

343 This data is important in validating experiments that utilises the *Epha4*^{rb-2J/rb-2J}

344 knockout mouse to look at the function of the *EphA4* gene. Furthermore, this study
345 underlines the numerous isoforms of the *EphA4* gene and the variability in phenotype
346 arising from which isoform becomes the major gene product.

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357

358 **Conflict of Interest**

359 The authors report no conflicts of interest. The authors alone are responsible for the content
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361

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453 **Tables**

454 **Table 1.** *Epha4*^{rb-2J/rb-2J} mutation lies within a conserved region. Alignment of
 455 *Epha4*^{rb-2J/rb-2J} protein sequences of different organisms including human, rat and *Xenopus*,
 456 revealed the spontaneous mutation was in a conserved region.

Organism	Amino acids
<i>Epha4</i> wildtype (mouse)	AKQEADDEEKHL.....
<i>Epha4</i> mutant (mouse)	AKQEQMKRNI-stop
Human	AKQEADDEEKHL.....
Orangutan	AKQEADDEEKHL...
<i>Xenopus</i>	AKQEADDEEKHL....
Pig	AKQEADDEEKHL....
Rat	AKQEADDEEKHL....
Chick	AKQEADDEEKHL...

457 **Table 2. Phenotypes of *EphA4* mutation mice based on position of mutation**

Position of mutation	<i>EphA4</i> mutations	Type of knockout and method	Phenotype and anatomical defects	References
Exon 1 knockout and exone 3 frame shift mutation	<i>EphA4</i>	Replacement vector; lac-Z reporter fusion	Hind limb phenotype (“club foot”) high penetrance Loss of dorsal hindlimb innervation (peroneal nerve); absent anterior commissure	Helmbacher et al. 2000
Ligand binding domain (exon 3)	<i>EphA4</i> ⁰	Gene replacement <i>pgk-neo</i>	Kangaroo-like (ROO) hopping gait. Abnormal corticospinal tract (CST) axons; absent anterior commissure	Dottori et al. 1998; Coonan et al., 2001
Extracellular region (exon 3)	<i>EphA4</i> ^{EGFP}	Insertion of EGFP (reporter)	Hopping gait	Grunwald et al. 2004
Deletion of exon 3	<i>EphA4 conditional</i> ; <i>EphA4</i> ^{Flox}	Knock-in mCFP reporter gene; targeted (floxed/Frt)	Hind limb hopping gait	Herrmann et al. 2010
Deletion of exon 3	<i>EphA4 null</i>	Knock-in mCFP reporter gene	Hind limb hopping gait	Herrmann et al. 2010
Entire intracellular is missing	<i>EphA4</i> ^{GFP}	Entire intracellular part was replaced by green fluorescent protein (GFP)	Hopping gait	Egea et al., 2005
Fibronectin domain (abberant amino acids after position 439._ and truncation at position 442. T>C intron 6 at position 113891 mutation (NC_000067). Possibility, splicing of exon 6 and frameshift that create stop codon early in exon 7.	<i>Frog</i>	Chemical induced (ENU)	Hopping gait	Milstein et al., 2010
Juxtamembrane domain (Y596E and Y602E)	<i>EphA4</i> ^{EE}	Knock-in strategy (similar to Kullander 2001), replacement vector—glutamic acid residues replace juxtamembrane tyrosines	<u>Normal alternating gait. No discernible phenotype.</u> Abnormal thalamocortical topography; and partly defective central paatern generator (CPG) rhythmicity.	Egea et al., 2005

Juxtamembrane domain (Y596F and Y604F)	<i>EphA4</i> ^{2F}	EphA4 Knock-in strategy (targeting vectors)	Hopping gait	Kullander et al. 2001
Kinase domain and truncated at E582. Deletion adenine at 1802 (NM_007936)	<i>EphA4</i> ^{rb-2J}	Spontaneous mutation	Hopping gait and leaning	Mohd-Zin et al., this study; Cook et al., 2004
Kinase domain (K653M)	<i>EphA4</i> ^{KD}	EphA4 Knock-in strategy (targeting vectors); kinase dead	Hopping gait Signalling mutants: abnormal signalling mutants hopping gait; anterior commissure	Kullander et al. 2001
SAM domain (905-974 amino acids deletion); leaving 12 last amino acids residues intact	<i>EphA4</i> ^{ΔSAM}	EphA4 Knock-in strategy (targeting vectors)	<u>Normal alternating gait</u>	Kullander et al. 2001
PDZ-binding motif and 12 last amino acids of EphA4 gene	<i>EphA4</i> ^{ΔPBM}	Knock-in	Not described	Dufour et al. 2006
Not described	<i>EphA4</i> ^{PLAP}	Gene trapped, insertion of PLAP vector	Hopping kangaroo gait Guidance defects in the CST (crossing defects of axons) and anterior commissure. However, low expression of <i>EphA4</i> in CST neurons.	Leighton et al., 2001
Not described	<i>PGK-cre;EphA4</i> ^{lx}	Recombination of PGK-Cre and EphA4 ^{lox/lox}	Kangaroo-like (ROO) hopping gait. Abnormal formation of anterior commissure and significant reduction of CST axons	Filosa et al. 2009
Not described	<i>Conditional EphA4; EphA4</i> ^{lx}	Targeted (floxed/frt)	<u>Normal alternating gait</u> EphA4 expression in control EphA4 ^{lx/lx} mice is reduced to 15–20% compared to +/+ mice	Filosa et al. 2009
Not studied	<i>EphA4</i> ^{rb}	Spontaneous mutation	Normal alternating gait	Cook et al, 2004

458 Different type of mutations showed most with hind limb hopping gait phenotypes (bold) and few with normal alternating gait phenotypes

459 (underlined).

460 **Table 3**

UniProt ID***	Size of transcript (bp)*	Protein (aa, amino acids)	Size (Da)	Aligned to Q03137 (position)	Domain(s)
Q03137 Long isoform (Experimental evidence at protein level)	6328	986 aa	109,814	1-986 aa	Ligand binding domain (30-209 aa), cysteine rich domain (191-325 aa), fibronectin type III domain (328-439 aa & 440-537 aa), juxtamembrane domain, protein kinase domain (621-882 aa), SAM domain (911-975 aa), PDZ-binding motif (984-986 aa)**
Q03137-2 Shorter isoform (No experimental confirmation available)	No information	936 aa	103,984	Missing 783-832 aa (missing protein kinase domain). Missing whole exon 14.	Ligand binding domain, fibronectin type III domain, juxtamembrane domain, SAM domain
Q3V1W9 Short isoform (Experimental evidence at transcript level)	No information	572 aa	63,034 Da	Missing whole exon 9-17 (missing juxtamembrane domain, kinase domain, SAM domain and PDZ-binding motif)	Ligand binding domain, fibronectin type III domain
A0A087WRH4 (Experimental evidence at protein level)	614	117 aa	13,206	833-949 aa (whole exon 15&16)	SAM domain
A0A087WQW6 (Experimental evidence at protein level)	584	177 aa	19,075	328 to 473 aa (exon 5&6)	Fibronectin type III
Q99KA8 (Experimental evidence at transcript level)	No information	927 aa	103,444	Missing 1-59 aa (ligand binding domain is not affected)	Ligand binding domain, fibronectin type III domain, juxtamembrane domain, protein kinase domain, SAM domain
A0A087WQZ6 (Protein Predicted)	3043	38 aa	4,137	1-38 aa (missing the rest of domains)	No information

461 *Information obtained from Ensembl (http://asia.ensembl.org/Mus_musculus/Gene/Summary?db=core;g=ENSMUSG00000026235;r=1:77367185-77515088)462 **Information obtained from UniProt (<http://www.uniprot.org/uniprot/Q03137#showFeatures>)

463 ***Note: (taken from UniProt last modified January 8, 2015)

464 The value '**Experimental evidence at protein level**' indicates that there is clear experimental evidence for the existence of the protein. The criteria include partial or
465 complete Edman sequencing, clear identification by mass spectrometry, X-ray or NMR structure, good quality protein-protein interaction or detection of the protein by
466 antibodies.

467 The value '**Experimental evidence at transcript level**' indicates that the existence of a protein has not been strictly proven but that expression data (such as existence of
468 cDNA(s), RT-PCR or Northern blots) indicate the existence of a transcript.

469 The value '**Protein Predicted**' is used for entries without evidence at protein, transcript, or homology levels.

470 Legends

471 **Figure 1. A single nucleotide deletion in exon 9 of *Epha4*^{rb-2J/rb-2J}**

472 **A**, The deletion of adenine at nucleotide 1,802 or 1,803 in exon 9 of *Epha4* gene (indicated
473 by an arrowhead). **B**, Nucleotide and deduced amino acid sequence of the *Epha4*^{+/+} and
474 *Epha4*^{rb-2J/rb-2J}. The deletion in *Epha4* (highlighted by the red box) resulted in a frame shift of
475 downstream codons and a premature termination.

476

477 **Figure 2. A schematic representation of Eph receptor, *Epha4*^{+/+} and *Epha4*^{rb-2J/rb-2J}** 478 **structure**

479 **A**, A general structure of an Eph receptor. **B**, The predicted structures using Simple Modular
480 Architecture Research Tool, SMART for *Epha4*^{+/+}. **C**, SMART structure of EphA4 isoform at
481 104 kDa lacking kinase domain but possesses SAM domain. **D**, SMART structure of EphA4
482 isoform at 63 kDa which is truncated downstream of the juxtamembrane domain. **E**, The
483 *Epha4*^{rb-2J/rb-2J} structure by SMART showing truncation downstream of the juxtamembrane
484 domain, lacking the kinase and SAM domains.

485

486 **Figure 3. EphA4 protein expression**

487 Western blot analysis of EphA4 immunostaining showed that the EphA4 mutant mice lacked
488 the expression of the 110kDa full-length protein but detected a second band similar to EphA4
489 isoform at 104 kDa (UniProt). The 110kDa full-length protein was detected in the EphA4
490 wildtype profile and heterozygous mice.

491

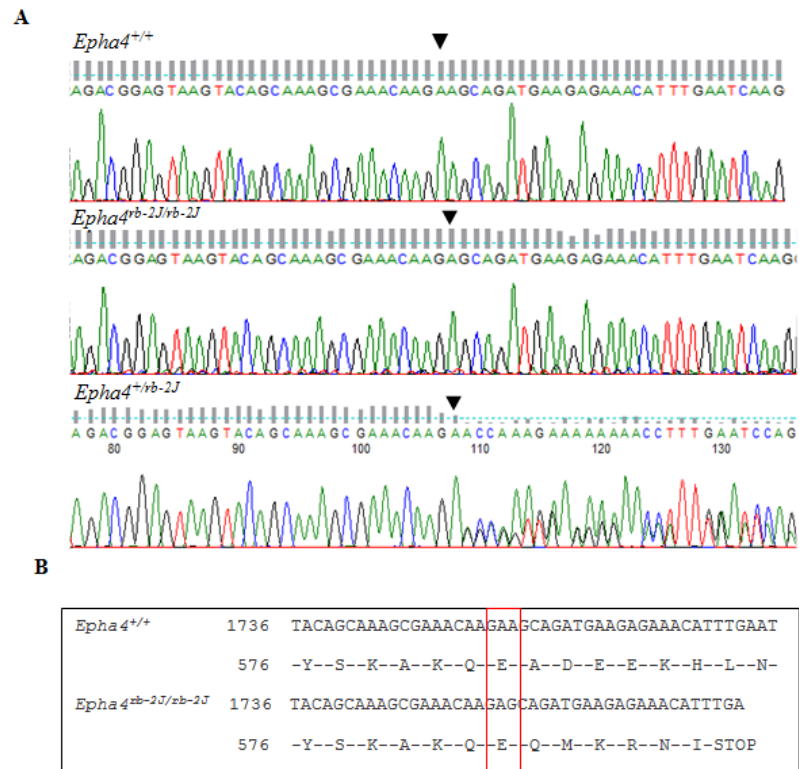
492 **Figure 4. A representative PCR-RFLP analysis of the *Epha4*^{rb-2J/rb-2J} mice genotypes**

493 PCR-RFLP samples were resolved in 4% agarose gel. Lanes 1-3 represent the wildtype
494 profiles (166bp band only), Lanes 4-6 represent the heterozygote profiles (149bp and 166bp

495 bands), Lanes 7-9 represents the homozygote mutant profiles (149bp band only) and Lane 10

496 represents the 50bp ladder.

497



498 Figure 1

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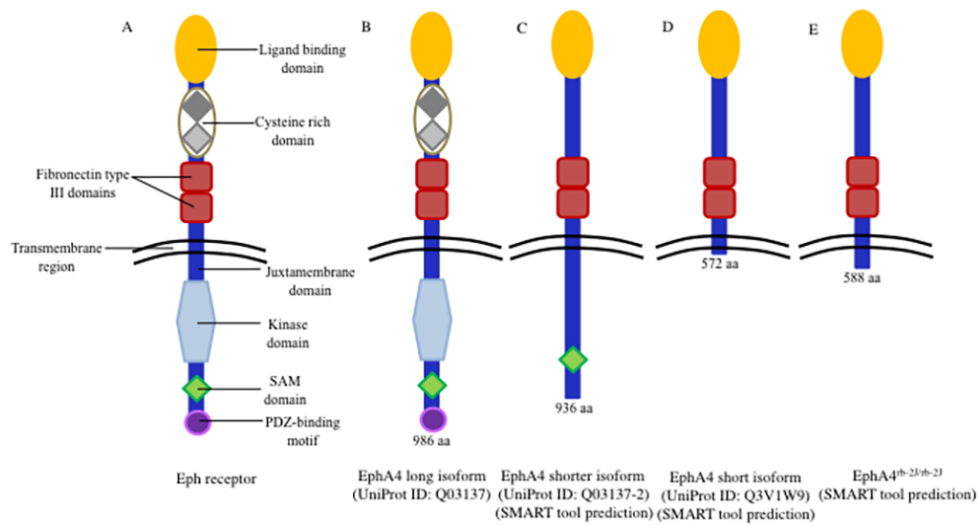
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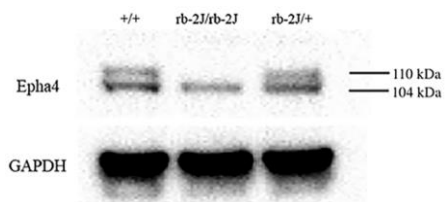


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510 Figure 2

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515 Figure 3

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518 Figure 4

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