

1 Title: **Evidence of a noncoding transcript of the *RIPK2* gene overexpressed in head and**
2 **neck tumor**

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38 **ABSTRACT**

39 Receptor-interacting proteins are a family of serine/threonine kinases, which integrate
40 extra and intracellular stress signals caused by different factors, including infections,
41 inflammation and DNA damage. Receptor-interacting serine/threonine-protein kinase 2 (RIP-
42 2) is a member of this family and an important component of the nuclear factor NF-kappa-B
43 signaling pathway. The corresponding human gene *RIPK2* generates two transcripts by
44 alternative splicing, the full-length and a short transcript. The short transcript has a truncated
45 5' sequence, which results in a predicted isoform with a partial kinase domain but able to
46 transduce signals through its caspase recruitment domain. In this study, the expression of
47 *RIPK2* was investigated in human tissue samples and, in order to determine if both transcripts
48 are similarly regulated at the transcriptional level, cancer cell lines were submitted to
49 temperature and acid stresses. We observed that both transcripts are expressed in all tissues
50 analyzed, with higher expression of the short one in tumor samples, and they are differentially
51 regulated following temperature stress. Despite transcription, no corresponding protein for the
52 short transcript was detected in tissues and cell lines analyzed. We propose that the shorter
53 transcript is a noncoding RNA and that its presence in the cell may play regulatory roles and
54 affect inflammation and other biological processes related to the kinase activity of RIP-2.

56 **Introduction**

57 Unicellular and multicellular organisms are constantly exposed to stressful
58 environments. Chemical and physical stimuli trigger different adaptive responses, which will
59 determine the capability of the organism to maintain internal homeostasis [1].

60 Receptor-interacting proteins (RIP) are a family of serine/threonine kinases, which
61 integrate extra- and intracellular stress signals and share a homologous kinase domain at the
62 N-terminus, but have different C-terminal functional domains [2-4]; RIP-2 (receptor-
63 interacting serine/threonine-protein kinase 2) is a member of the RIP family, which has
64 received attention in the recent years for its role in modulating immune and inflammatory
65 processes [5], and as a sensor of cellular stress [6]. It is expressed at high levels in several
66 normal human tissues [7], as well as in pathological conditions, for example ulcerative colitis
67 [8], triple-negative breast cancers [9,10] and in stressful conditions, such as after
68 hypoxic/ischemic insults [11]. Conversely, lower levels of RIP-2 have been correlated with
69 tumor progression in squamous cell carcinoma (SCC) of the oral cavity [12].

70 RIP-2 is the only member of the RIP family that besides phosphorylating serines and
71 threonines is able to autophosphorylate tyrosine residues [13,14]. Its ATP- and substrate
72 binding sites spread over much of the N-terminal kinase domain, and a caspase recruitment
73 domain (CARD) is present in the C-terminal region [15] (isoform 1, Fig 1A). CARD
74 specifically interacts with the nucleotide-binding oligomerization domain-containing protein 1
75 (NOD1) and NOD2 (also called CARD-4 and CARD-5, respectively), which are intracellular
76 receptors for innate immunity and involved in sensing the presence of pathogens. After
77 activation by bacterial peptidoglycans, NOD1 and NOD2 associate with RIP-2 via CARD-
78 CARD interaction and promote the expression of immune response and inflammatory genes

79 through the nuclear factor-kappa B (NFkB) signaling [16]. NOD1 and NOD2 also cooperate
80 and share redundant roles with Toll-like receptors (TLRs) in detecting bacteria, but there's no
81 consensus on the participation of RIP-2 in TLR signaling (reviewed by [3]). Recently, it has
82 been demonstrated that RIPK2 kinase activity and auto-phosphorylation are not required for
83 NOD2 inflammatory signaling. In fact, NOD2 pathway activation and cytokine production
84 depends on RIP-2 polyubiquitination at several lysines, a process relied on the ubiquitin
85 ligases:RIP-2 kinase domain interaction. Thus, although the kinase domain is not functionally
86 important for NOD2 signaling, it is correlated with NOD2 activation since RIP-2 auto-
87 phosphorylation creates a substrate for ubiquitin ligase binding [17].

88

89 **Fig 1. Diagrams of RIPK2 splice transcripts.** (A) The transcript 1 encodes the longer
90 isoform and (B) the transcript 2 presents skipping of exon 2 and encodes a very short isoform
91 2 [18] and a predicted isoform 3. Arrowheads indicate the positions of the forward primer A
92 and reverse primer B for RT-PCR expression analysis, and horizontal bars below the isoform
93 1 and 2 indicate the epitope region for anti-RIP-2 ab8428, ab57954 and sc8611 used in the
94 present study. (C) 5'UTR (lower case) and codons (capital letters) of exons 1, 3 (gray) and 2
95 (white). Kozak sequences in boxes. First ATGs of full-length isoform and predicted isoform 3
96 in bold. Premature stop codons generated by the frameshift due to exon 2 skipping=dark gray.
97 Kinase/K=protein kinase domain; CARD=caspase recruitment domain; activation loop; ATP
98 binding site; substrate binding site; according to Batch Conserved Domain-Search at NCBI.

99

100 The full-length human *RIPK2* transcript (GenBank accession number NM_003821.5),
101 here named transcript 1 (Fig 1A), has 2588 bps and is composed of 12 exons spanning 33-kb
102 of genomic sequence on chromosome 8q21. In 2004, we suggested an alternative splicing for

103 *RIPK2* transcribing a short variant (AY562996, currently included within the predicted
104 transcript XM_005251092.3) [19], as depicted in Fig 1B. This variant, here named transcript
105 2, has 2389 bps and derives from the skipping of exon 2 (154 bps), which alters the reading
106 frame producing several premature stop codons. However, a potential translation initiation
107 codon AUG (nucleotides 85-87 of exon 3) is in-frame with the downstream *RIPK2* sequence,
108 hence with no subsequent premature termination codon (Fig 1C). Translation from this codon
109 may give rise to an amino-terminal truncated protein (XP_005251149.1, isoform 3 in this
110 study, with 403 residues, predicted molecular weight of 45,582 Da) lacking the first 137
111 amino acids of RIP-2 isoform 1 (NP_003812.1).

112 Alternatively spliced transcript 2 was also studied by Krieg and collaborators [18], who
113 reported a protein product (isoform 2, Fig 1B, top) with extensive truncation of the N-terminal
114 kinase domain and a complete lack of the intermediate domain and CARD due to a frame shift
115 generating a premature stop codon. Krieg et al. also reported that this isoform of RIP-2 lacks
116 the biological effects described for the isoform 1. We here investigated if the use of the
117 downstream alternative translation initiation site may generate an isoform 3 that would keep
118 the original C-terminal structure including CARD, but would present a truncated kinase
119 domain (Fig 1B, bottom), with potential consequences for protein function, and cellular
120 localization if the localization signals were also deleted.

121 Since RIP-2 kinase integrates extra and intracellular stress signals and modulates
122 immune responses [5], we reasoned that physiological and environmental changes, such as
123 hyperthermia and acid stress caused by infections and inflammatory processes, might affect
124 the expression of their transcripts and, therefore, could lead to changes in levels of the
125 isoforms depicted in Fig 1. The use of alternative splicing sites may differ among cell types

126 and phases of development [20-23], or be associated with stress conditions, such as
127 temperature stress [24] and oxidative stress [25].

128 In the present study, the expression of *RIPK2* transcripts and protein products was
129 evaluated in normal human tissues and in SSC samples and, in order to investigate if they are
130 regulated in response to stress conditions, we analyzed their expression upon heat/cold and
131 acid stress in human cancer-derived cell lines.

132

133 **Material and methods**

134 **Samples and cell lines**

135 Nine samples of normal human tissues removed at autopsy (brain, testis, heart, lung,
136 stomach, kidney, larynx, liver and tongue) and 16 matched tumor/resection margin samples of
137 oral SCC were used to evaluate the expression of the two transcripts of the *RIPK2* gene. RIP-2
138 protein levels were analyzed in another set of 19 matched tumor/resection margin of oral and
139 laryngeal SCC.

140 For the stress experiments, we used the human cell lines FaDu (HTB-43, derived from
141 SCC of the hypopharynx), and SiHa (HTB-35, derived from cervix SCC). The cell lines were
142 cultured in Minimum Essential Medium (MEM, 552, Cultilab), supplemented with 10% fetal
143 bovine serum (FBS, 63, Cultilab), 10 mM non-essential amino acids (M7145, Sigma), 2 mM
144 L-glutamine (687, Cultilab), 1 mM sodium pyruvate (P5280, Sigma), 1.5 g/L sodium
145 bicarbonate (S5761, Sigma), penicillin (100 units/mL) and streptomycin (90 µg/mL) (1012,
146 Cultilab), in a humidified atmosphere with 5% CO₂ at 37°C. The study protocol was approved
147 by the National Committee of Ethics in Research (CONEP 1763/05, 18/05/2005, and CONEP
148 128/12, 02/03/2012) and informed consent was obtained from all patients enrolled.

149

150 **Temperature and acid stresses**

151 Prior to stress experiments, cell lines were grown to 80-90% confluence and cell cycle
152 synchronized in serum-free medium for 24 h. For temperature stress, cells were maintained in
153 medium plus 10% FBS at 40°C, 17°C or 5°C for 3 h (eight replicates for each condition). Six
154 control replicas were also cultured in medium plus 10% FBS at 37°C for 3 h. Acidic shock
155 was performed by maintaining the cultures (four replicas) in an atmosphere with elevated CO₂
156 (10% CO₂) for 24 h or 72 h. Four control replicas were cultured in a humidified atmosphere
157 with 5% CO₂ at 37°C for the same time period. After the incubation period, cells were
158 immediately lysed by adding TRIzol (15596026, ThermoFisher), and stored at -80°C until
159 RNA extraction.

160

161 **RNA extraction and cDNA synthesis**

162 Total RNA from tissue samples and cell lines was obtained following the TRIzol
163 protocol. Integrity of the RNA was confirmed by agarose gel electrophoresis, and the purity
164 and concentration were determined using the NanoDrop ND-1000 spectrophotometer (Thermo
165 Fisher). One microgram of total RNA was converted to cDNA using the High Capacity cDNA
166 Reverse Transcription kit (4368813, Thermo Fisher), according to the manufacturer's
167 instructions.

168

169 **Detection of RIPK2 transcripts by polymerase chain reaction**

170 The PCR amplification of *RIPK2* transcripts was performed using the oligonucleotides
171 5'-CGCCTCTGGCACTGTGTCGT-3' (forward primer A) and 5'-
172 CGTGACTGTGAGAGGGACAT-3' (reverse primer B). The PCR reaction was carried out in

173 a total volume of 25 μ L containing 1X PCR buffer, 1 mM $MgCl_2$, 2 μ M of each *RIPK2*
174 primer, 2 μ M *GAPDH* primers, 5 mM dNTPs mix, 1 U Taq DNA polymerase (EP0402,
175 ThermoFisher) and 50 ng of cDNA. After pre-incubation for 5 min at 94°C (initial
176 denaturation), the amplification was carried out through 35 cycles at 94°C for 50 s, 58°C for
177 40 s, 72°C for 50 s, and 72°C for 10 min, using a thermal cycler (9700 GeneAmp PCR
178 System, Applied Biosystems). PCR primers for the endogenous control gene *GAPDH* were
179 GAPDHF (5'-ACCCACTCCTCCACCTTTGA-3') and GAPDHR (5'-
180 CTGTTGCTGTAGCCAAATTCGT-3'). The expected lengths for PCR amplicons were 101
181 base pairs (bps) for *GAPDH* and 456 or 302 bps for *RIPK2* transcripts. Amplicons were
182 separated on 2% agarose gels, bands were quantified by densitometry using Image J software,
183 and sequenced in both directions after being isolated from the gels. The sequences were
184 analyzed using BLAST similarity search against the non-redundant database available from
185 the National Center for Biotechnology Information (NCBI) [26].

186

187 **Evaluation of *RIPK2* transcripts by relative quantification using RT-qPCR**

188 The expression of *RIPK2* transcripts in matched tumor/resection margin samples and in
189 cell lines following stress treatment was investigated by quantitative PCR (qPCR). Reactions
190 were performed in triplicate using an ABI Prism 7500 Sequence Detection System (Applied
191 Biosystems). The primers were manually designed and optimized for RT-qPCR using basic
192 parameters for PCR primer design. The final sequences were 19-24-bp long, with 30-70% GC
193 content and producing a short amplicon size (66-104 bps), as follows: *RIPK2* transcript 1
194 forward 5'- AGAAGCTGAAATTTTACACAAAGC-3' and reverse 5'-
195 CCATTTGGCATGTATTCAGTAAC-3'; *RIPK2* transcript 2 forward 5'-
196 TGCTCGACAGAAAACCTGAATATC-3' and reverse 5'-

197 AAGGAGGAGTCATATTGTGCAG-3'; *GAPDH* forward 5'-
198 ACCCACTCCTCCACCTTTGA-3' and reverse 5'-CTGTTGCTGTAGCCAAATTCGT-3';
199 *TUBA1C* forward 5'- TCAACACCTTCTTCAGTGAAACG-3' and reverse 5'-
200 AGTGCCAGTGCGAACTTCATC-3'; *ACTB* forward 5'-GGCACCCAGCACAATGAAG-3'
201 and reverse 5'-CCGATCCACACGGAGTACTTG-3'. All primers were purchased from
202 Invitrogen. Briefly, reactions were carried out in a total volume of 20 μ L, with 10 μ L SYBR
203 Green PCR Master Mix (4385612, ThermoFisher), 250 nM of each primer and 20 ng cDNA.
204 The PCR conditions were 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for
205 15 s, 58°C for 10 s, 60°C for 1 min. Following PCR, dissociation curve analyses were
206 performed to confirm the single gene product. Adequate internal control reference genes were
207 selected using the geNorm algorithm [27] and *TUBA1C* (stress assays) and *ACTB*
208 (tumor/margin samples) were selected. The relative expression ratio (fold-change) of the
209 target genes was calculated according to Pfaffl [28]. Statistical analysis was carried out by a
210 two-tailed unpaired t test using GraphPad Prism (GraphPad Software). Values were Log₂
211 transformed and those below -1 indicated down-regulation in gene expression while values
212 above 1 represented up-regulation in test samples compared with control samples.

213

214 **Protein sequence alignment and homology modeling procedures**

215 Homology modeling of RIP-2 isoforms 1 and 3 was carried out using the MODELLER
216 software that performs modeling by satisfaction of spatial restraints [29]. Six homologues with
217 structures available in the Protein Data Bank (codes 2GSF, 1JPA, 1K2P, 1U59, 1UWH,
218 2EVA) used as templates were selected through a non-redundant BLASTp search [26]. Two
219 putative conserved domains with statistical significance were detected: TyrKc and S_TKc,
220 which correspond, respectively, to the catalytic domains of tyrosine and serine/threonine

221 protein kinases, and include the leucine L10-threonine T296 sequence. These six templates
222 share sequence identities of 27.1% (Eph receptor tyrosine kinase, PDB code 1JPA) to 30%
223 [Transforming growth factor-beta (TGF-beta)-activated kinase 1 - TAK1, PDB code 2EVA]
224 with RIP-2. Analyses were performed using pairwise alignments via the AMPS (Alignment of
225 Multiple Protein Sequences) package [30]. Previous to the modeling, a final multiple
226 alignment was obtained by analyzing the superposition of the six structures regarding the
227 alpha-carbons of the residues, using the INSIGHT II program, version 2005 (Accelrys Inc,
228 San Diego, CA, USA), which allowed refine the previous alignment obtained from the AMPS
229 MULTALIGN module. The information of secondary structure in the template sequence was
230 incorporated into this previous alignment using the MULTALIGN module of the AMPS
231 package, with the restriction that all insertions and deletions were limited to regions outside
232 the common core of alpha-helices and beta-sheets. A gap penalty of 1000 was fixed to any
233 deletion or insertion inside a secondary structure element. The alignment obtained was edited,
234 investigating and considering the aligned residues, which were close in the space, as
235 visualized in the structural superposition. This procedure resulted in a final alignment that is
236 different from the one based on the Dayhoff matrix (PAM 250) used in AMPS.

237 NetPhosK 1.0 server [31] was used for phosphorylation site analyses. The algorithm
238 produces neural network predictions of kinase-specific eukaryotic protein phosphorylation
239 sites. Currently, NetPhosK covers the following kinases: PKA, PKC, PKG, CKII, Cdc2, CaM-
240 II, ATM, DNA PK, Cdk5, p38 MAPK, GSK3, CKI, PKB, RSK, INSR, EGFR, and Src.

241

242 **Western blot**

243 Western blot analysis aimed at detecting isoforms 1 and 3. The antibodies used were: (a)
244 polyclonal anti-RIP2 (ab8428, Abcam), immunogenic peptide corresponding to amino acids

245 11/30 of human RIP-2 (which are only present in isoform 1), N-terminal domain, diluted
246 2:1000 or 3:1000; (b) monoclonal anti-RIP2 (ab57954; Abcam), immunogenic peptide
247 corresponding to amino acids 431-541, C-terminal domain, 3 µg/mL; (c) polyclonal anti-
248 RICK (C-19) (sc8611, Santa Cruz), immunogenic peptide corresponding to C-terminal
249 domain according to the manufacturer's datasheet, diluted 1:200; (d) monoclonal anti-beta-
250 actin (A5441, Sigma-Aldrich) diluted 1:5000. The antibodies mapping at C- and N-terminus
251 of RIP-2 are depicted in Fig 1 (isoforms 1 and 3).

252 In brief, protein samples (30 µg) were loaded onto 12% resolving gel with 5% stacking
253 gel (SDS-PAGE) in denaturing conditions at 120V for 80 min. The molecular weight ladder
254 used was the PageRuler Prestained Protein Ladder (#26616; Thermo Scientific). The proteins
255 were then transferred electrophoretically (162.5 mA per blot 70 min; Mini Protean 3 Cell,
256 BioRad) to polyvinylidene fluoride (PVDF) membrane (IPVH00010, Immobilon-P, Millipore)
257 with transfer buffer (25 mM Tris, 0.2 M glycine, 20% v/v methanol; Merck, Germany).
258 Western blotting was performed using the Amersham ECL Select Western Blotting Detection
259 Reagent (RPN2235, GE Healthcare, Life Sciences), according to the manufacturer's protocol.
260 The immunoreactive proteins were visualized using horseradish peroxidase-coupled secondary
261 antibody (074-1506, 074-1806, KPL, Kirkegaard & Perry Laboratories Inc., Gaithsburg, MD,
262 USA) and enhanced chemiluminescence reagent (Amersham ECL Select kit, RPN2235, GE
263 Healthcare). The Fusion FX5 system (Vilber Lourmat) was used for the acquisition of the
264 signal. The PVDF membranes were also submitted to chromogenic staining using the Western
265 Breeze kit (Invitrogen). The blots were then scanned and analyzed (Gel Logic HP 2200
266 imaging system; Carestream Health Inc./Kodak Health Group, Rochester, NY, USA).

267

268 **Results**

269 **Identification and expression patterns of *RIPK2* transcripts**

270 Alternatively spliced transcripts 1 and 2 of *RIPK2* were co-expressed in normal tissue
271 samples from brain, testis, heart, lung, stomach, kidney, larynx, liver and tongue (Fig 2A).
272 Both transcripts were also detected in tumor and tumor free surgical resection samples from
273 patients with oral SCC as illustrated by 5 matched tumor/surgical margin samples in Fig 2B.
274 Quantitative real time PCR showed no difference in expression between full length transcript
275 1 and their respective surgical margins, whereas a significant higher level of shorter transcript
276 2 was observed ($p=0.03$) (as illustrated by 11 paired samples in Fig 2C).

277

278 **Fig 2. *RIPK2* mRNA expression in normal tissues, oral SCC samples and cell lines under**
279 **stress conditions. (A-B)** Conventional PCR products from *RIPK2* transcript 1 (456 bps),
280 transcript 2 (302 bps), and *GAPDH* (101 bps) in: (A) normal human tissues: 1=brain, 2=testis,
281 3=heart, 4=lung, 5=stomach, 6=kidney, 7=larynx, 8=liver, 9=tongue; (B) samples from
282 patients with oral cancer: T=tumor; M=resection margin; L=100-bp fragment size marker. (C-
283 E) RT-qPCR products. (C) Log₂ fold-change of *RIPK2* transcripts showing that transcript 2
284 has a higher expression than transcript 1 in tumors normalized with matched resection margins
285 ($p=0.03$, unpaired t test). *ACTB* was used as the expression reference. (D) Expression of
286 *RIPK2* transcript 1 and (E) of transcript 2 in FaDu cells maintained at 5°C, 17°C or 40°C for 3
287 h, normalized with control cells at 37°C (calibrator sample). Temperature stress induced a
288 significant increase in transcript 2 expression level at lower temperatures and a decrease at a
289 higher temperature ($p<0.0001$, unpaired t test), but no effect on transcript 1 expression.
290 *TUBA1C* was used as the expression reference. Values were log₂ transformed (y-axis) so that

291 all values below -1 indicate down-regulation in gene expression while values above 1
292 represent up-regulation. The error bar represents the mean \pm S.E.M (standard error of the
293 mean). Significant differences: $p < 0.05$.

294

295 **Splicing patterns of *RIPK2* under stress conditions**

296 To investigate whether alternative splicing of *RIPK2* is induced or inhibited by stress
297 conditions, two cell lines (FaDu and SiHa) were exposed to severe temperature stress (40°C,
298 17°C and 5°C), and acid stress (atmosphere of 10% CO₂). Acid stress resulted in no effect on
299 the expression of both transcripts (data not shown), whereas heat/cold stress induced a
300 significant increase in shorter transcript 2 expression level at lower temperatures and a
301 decrease at a higher temperature ($p < 0.0001$), but no effect on full-length transcript 1
302 expression. This result was only observed in FaDu cells and suggests that distinct regulatory
303 mechanisms may interfere in the alternative splicing of *RIPK2* and that this may be tissue or
304 context dependent.

305

306 **Protein sequence alignment and homology modeling procedures**

307 The model built for the catalytic domain of isoforms 1 and 3 showed good
308 stereochemical quality (Fig 3 – kinase domain present only in isoform 1 inside dashed-box;
309 superposition of the isoform 1 and 3 models outside the box). Despite overall low sequence
310 identity among the complex structures of the homologue RIP-2 proteins, the active sites are
311 structurally similar and reasonably well conserved. The model built for the isoform 1 is 137
312 residues larger than the one obtained for the isoform 3, and includes leucine L10 to threonine
313 T296 of the overall sequence. Regarding the phosphorylation sites predicted for the isoform 1,
314 the NetPhosK 1.0 server pointed out 10 sites, which are absent in the isoform 3 sequence:

315 serines at positions 8, 25, 29, 33, 58, 76, 102, and threonines at positions 12, 31, 95. The study
316 of Dorsch's group [32] suggested that serine S176 is an important autophosphorylation site for
317 RIP-2, and this phosphorylation can be used to monitor the activation state of RIP-2. Fig 3
318 shows the superposition of the two models, as well as the localization of serine S176, which
319 seems to be conveniently accessible to the solvent and to phosphorylation. The lysine K47 in
320 the conserved ATP-binding site and the critical polyubiquitination site lysine K209 are also
321 shown in Fig 3.

322

323 **Fig 3. Homology modeling of the catalytic domain of RIP-2 isoforms.** Superposition of the
324 models built for the RIP-2 isoforms 1 and 3 (ribbon diagram colored in magenta and green,
325 respectively), with the kinase domain highlighted by the dashed-box (present only in isoform
326 1), and the superposition of the isoform 1 and 3 models outside the box. Isoform 3 lacks the
327 first 137 amino acids of RIP-2 and, consequently, the residue critical for kinase activity of
328 RIP-2 (lysine K47). Ten phosphorylation sites (serine and threonine residues, respectively)
329 predicted for isoform 1 and absent in isoform 3 sequence are indicated in blue and cyan inside
330 the dashed box. Serine S176 is shown in yellow stick, and is indicated to be conveniently
331 accessible to the solvent and to phosphorylation. Lysines K47 and K209 are also shown in
332 colored sticks.

333

334 **Immunodetection of RIP-2 isoforms**

335 Western blot analysis detected RIP-2 protein in extracts derived from FaDu cell line
336 and from human tumors and their resection margins (Fig 4). The best results were obtained
337 with the N-terminal domain anti-RIP2 ab8428 and C-terminal anti-RICK sc8611 antibodies,
338 whereas ab57954 antibody yielded weak or non-specific bands. In cell line samples, Western

339 blot demonstrated a single immunoreactive band at ~61 kDa, consistent with the molecular
340 weight of the isoform 1 (61,195 Da). When cells were exposed to heat/cold stress, a lower
341 expression of the RIP-2 isoform 1 was observed in FaDu cells maintained at low temperatures
342 compared with control cells at 37°C (Fig 4A).

343 Isoform 3 (predicted molecular weight of 45,582 Da) was not detected both in cell
344 lines and normal or neoplastic tissue samples (Fig 4A, 4B), even after mass spectrometry
345 analysis of Western blot band corresponding to the region around 45 kDa (data not shown).
346 The result indicates that, despite the detection of transcripts 1 and 2 and the proteins predicted
347 *in silico* for the isoforms, alternatively spliced transcript 2 does not seem to be translated into
348 protein in normal human tissues, cancer samples or cell lines analyzed.

349

350 **Fig 4. RIP-2 expression in cell lines under stress conditions and in oral SCC samples.**

351 Western blot illustrating (A) lower expression of the RIP-2 isoform 1 (~61 kDa) in FaDu cells
352 maintained at low temperatures compared with control cells at 37°C (anti-RIP-2 ab8428
353 against N-terminus); and (B) an apparent decreased expression of the RIP-2 isoform 1 in
354 tumor (T) than in resection margin (M) samples (anti-RIP-2 sc8611 against C-terminus). (A-
355 B) No band that corresponds to isoform 3 was observed. Data were normalized by beta-actin.
356 L = Protein Ladder.

357

358 **Discussion**

359 In the present study, *RIPK2* transcripts 1 and 2 were co-expressed in normal tissue
360 samples from brain, testis, heart, lung, stomach, kidney, larynx, liver and tongue. Our stress
361 experiments showed that one of the transcripts (transcript 2) is up-regulated at low

362 temperatures compared with the control group (at 37°C), whereas the opposite occurs at 40°C.
363 We tested two distinct SCC-derived cell lines, but this effect was seen only in FaDu cell line.
364 This finding may be tissue/context dependent. In fact, literature has already referred that a
365 mild hypothermic condition appears to induce or to inhibit synthesis of specific proteins when
366 compared with control cells, an effect that is cell line dependent (reviewed by [33]. It's well
367 known that both prokaryotic and eukaryotic organisms respond to cold stress reducing
368 transcription, translation, and metabolic processes, except in the case of cold-shock proteins
369 [34].

370 Many studies are available on alternative splicing regulation by temperature and other
371 extrinsic agents. For example, Gemignani and collaborators [35] also observed a shift in
372 splicing of a mutated human b-globin gene affected by temperature *in vitro*, which led the
373 authors to propose temperature changes as a treatment for β -thalassemia. More recently,
374 Farashahi Yazd's group [24] described a novel spliced variant of *OCT4* gene significantly
375 elevated under heat-stress conditions, and proposed a potential role of OCT4B1 transcript and
376 protein in mediating temperature response.

377 Yan and collaborators [36] found evidence that hyperthermia induces Toll-like receptors
378 expression and TLR signaling-mediated activation of NFKB and MAPK pathways, resulting
379 in increased synthesis of pro- and anti-inflammatory cytokines. These data suggest that fever
380 may modulate innate immune responses by TLR pathway and, although the role of *RIPK2* in
381 this signaling remains controversial [37-40], they provide a possible link to *RIPK2* expression
382 changes depending on the temperature variation.

383 Considering these data, we hypothesize that, under stress conditions, a putative
384 mechanism may induce higher or lower expression of the exon 2-containing transcript of

385 *RIPK2*. Since the presumed isoform 3 has a truncated kinase domain but is potentially able to
386 mediate NF κ B activation via CARD, the balance of isoforms 1 and 3 might affect signaling
387 pathways related to its kinase activity. For instance, hyperthermia may increase the alternative
388 splicing kinetics or alter transcript stability and affect ERK pathways. A dominant-negative
389 mechanism by the truncated isoform should not be excluded [41]. DNA-damaging or altered
390 expression/subcellular distribution of RNA processing regulators [42] caused by temperature
391 changes may also be responsible for the abnormal accumulation of alternatively spliced
392 transcripts. These hypotheses obviously require experimental confirmation.

393 In the present study, *RIPK2* transcripts also showed a different expression pattern in
394 samples from head and neck squamous cell carcinoma patients, with alternatively spliced
395 transcript 2 exhibiting higher expression in tumors compared to their respective surgical
396 margins. Differences in alternative splicing between tumor and normal samples have been
397 described in the literature. For example, Gracio et al. [43], using ExonArray analysis of breast
398 cancer and normal breast tissue samples, identified more than 200 genes with splicing
399 differences associated with clinical outcome. Bjørklund et al. [44] obtained similar results by
400 RNA-seq analysis in primary breast tumors for five genes. The large study of Kahles et al.
401 analyzed 32 cancer types, including head and neck cancers, using RNA and whole-exome
402 sequencing data and observed many differences in alternative splicing events in cancer
403 compared with normal cells [45]. Specifically in regard to head and neck cancer, several other
404 groups have described genes with differential expression of spliced variants [46-50]; among
405 others). However, as far as we know, this is the first report showing differential expression of
406 spliced transcripts of *RIPK2* gene between tumor and normal tissues.

407 At the protein level, decreased expression of the RIP-2 isoform 1 (immunoreactive band
408 at ~61 kDa) was detected at low temperatures, disagreeing with our findings for full-length

409 transcript 1, which showed no change at the same condition compared to the control. A
410 divergent pattern was also observed for transcript 1 and the corresponding isoform 1 in
411 neoplastic tissues, the former showing higher and the second lower levels in tumor samples
412 compared with resection margins. This apparent discordance can be explained by the fact that
413 protein abundance may differ from mRNA expression profile, mainly due to post-
414 transcriptional control of gene expression or protein half-lives [51,52]. In addition, confirming
415 our results from immunodetection assays, Wang and collaborators [12] also found reduced
416 levels of RIP-2 in oral SCCs using immunohistochemical techniques.

417 In spite of using three distinct antibodies, another divergent result between RNA and
418 protein expression was the absence of isoform 3, which suggests that translation of *RIPK2* into
419 isoform 3 may not happen, at least in cell and tissue types analyzed in this study.

420 If the predicted alternative isoform 3 of RIP-2 is present in other conditions and tissue,
421 then it should exhibit some impaired functions related to its kinase domain, including NFKB
422 activation [5] [15,17,53], regulation of ERKs, p38 kinases, and own degradation [5,17,37,54].

423 Recently, Brady et al. identified a dominant-negative isoform of the translation initiation
424 factor eIF-2B created by a hypoxia-mediated intron retention that inhibits translation and
425 increases survival of neoplastic cells [50]. Dasgupta and collaborators [55] also described a C-
426 terminal fragment of a member of RIP family, RIP-1, which can activate signaling events
427 including NFKB and TNF pathways. They concluded that short RIP-1 with an aberrant N-
428 terminal affects the long isoform levels and may represent a new regulation mechanism.

429 Albeit exhibiting some differences, RIP-1 and -2 participate in the same regulatory pathway
430 and therefore RIP-2 isoform also may have a similar function and modulate full-length RIP-2
431 under different conditions.

432 It is tempting to speculate that the Krieg ORF [18] could act as an uORF (upstream
433 ORF) and repress translation of the downstream isoform 3 ORF, as cited for several stress
434 response mRNAs by the literature [56,57]. The short intercistronic region between both ORFs
435 should not be favorable for translation reinitiation due to an insufficient ribosomal scanning
436 time necessary for reacquisition of the ternary complex (eIF2-GTP-Met-tRNA_i) and for the
437 downstream AUG recognition [58]. Translation reinitiation also depends on the Kozak
438 context. Using numeric scores based on translation initiation site efficiencies determined in
439 mammalian cells by Noderer et al.[59], both full-length and short RIP-2 transcripts have good
440 efficiency values (90 and 107, respectively), but the predicted ratio of the initiation occurring
441 at the second site compared to the first one is low (< 0.005), which may justify the absence of
442 isoform 3.

443 As RIP kinases play a critical role in integrating stress signals, the elucidation of the
444 factors that take part in the regulation of these proteins is of major importance. The alternative
445 transcripts and protein isoforms may be tissue and context dependent and related to important
446 disease responses [41].

447 Although the *RIPK2* transcript 2 has a coding potential, at present there is no direct
448 evidence that it is translated in the isoform 3 or that it regulates biological processes, by
449 competing with other molecules or modulating stress responses. Even without clarifying these
450 issues, the present study raises many questions about RNA biology that may stimulate further
451 functional investigation on the molecular mechanisms underlying *RIPK2* splicing regulation
452 and their links to physiological and environmental changes.

453

454 **Conclusions**

455 In conclusion, *RIPK2* transcripts 1 and 2 are expressed in different tissues and
456 modulated by temperature, as determined by quantitative PCR assays. As far as we know, this
457 is the first report showing splicing imbalances between tumor and normal samples of *RIPK2*
458 gene, an important immune and inflammatory modulator. Despite transcription, no
459 corresponding protein for the short isoform was detected in tissues and cell lines analyzed,
460 which suggests that the balance of both transcripts may play regulatory roles and affect
461 inflammation and other biological processes related to the activity of RIP-2.

462

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470

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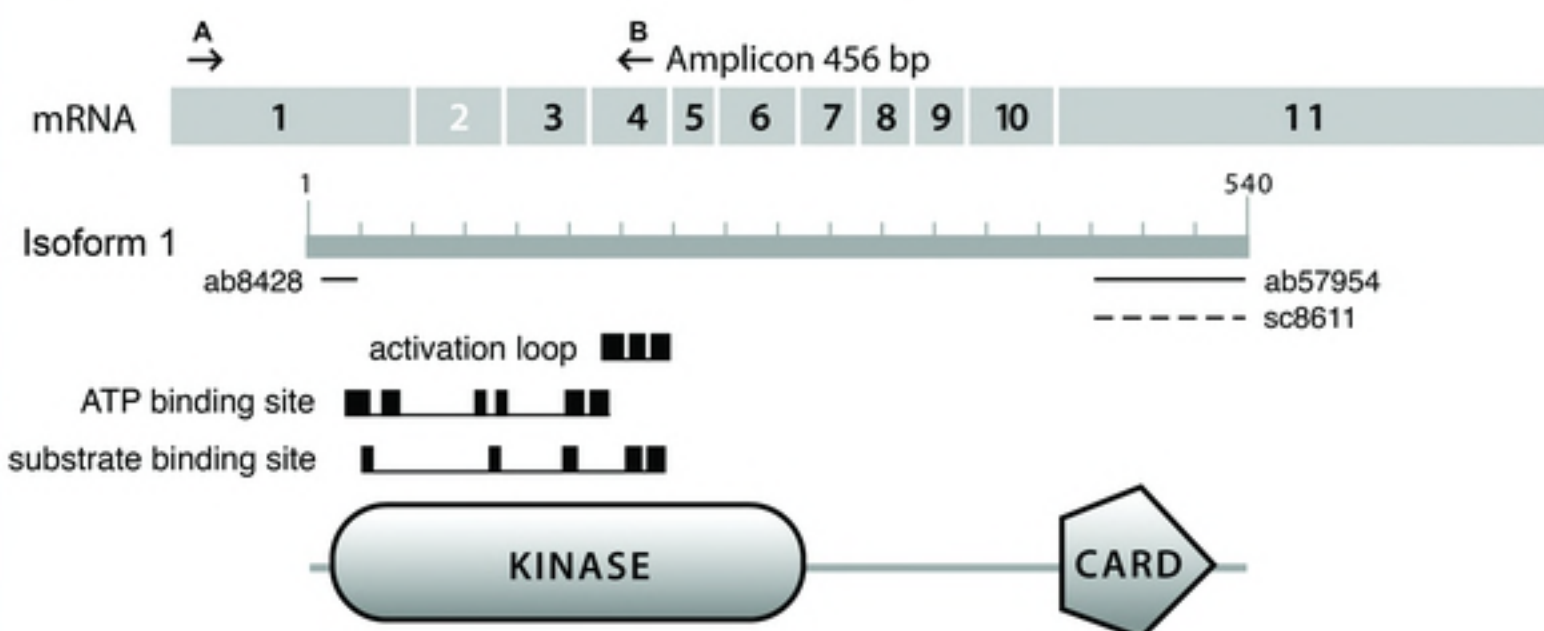
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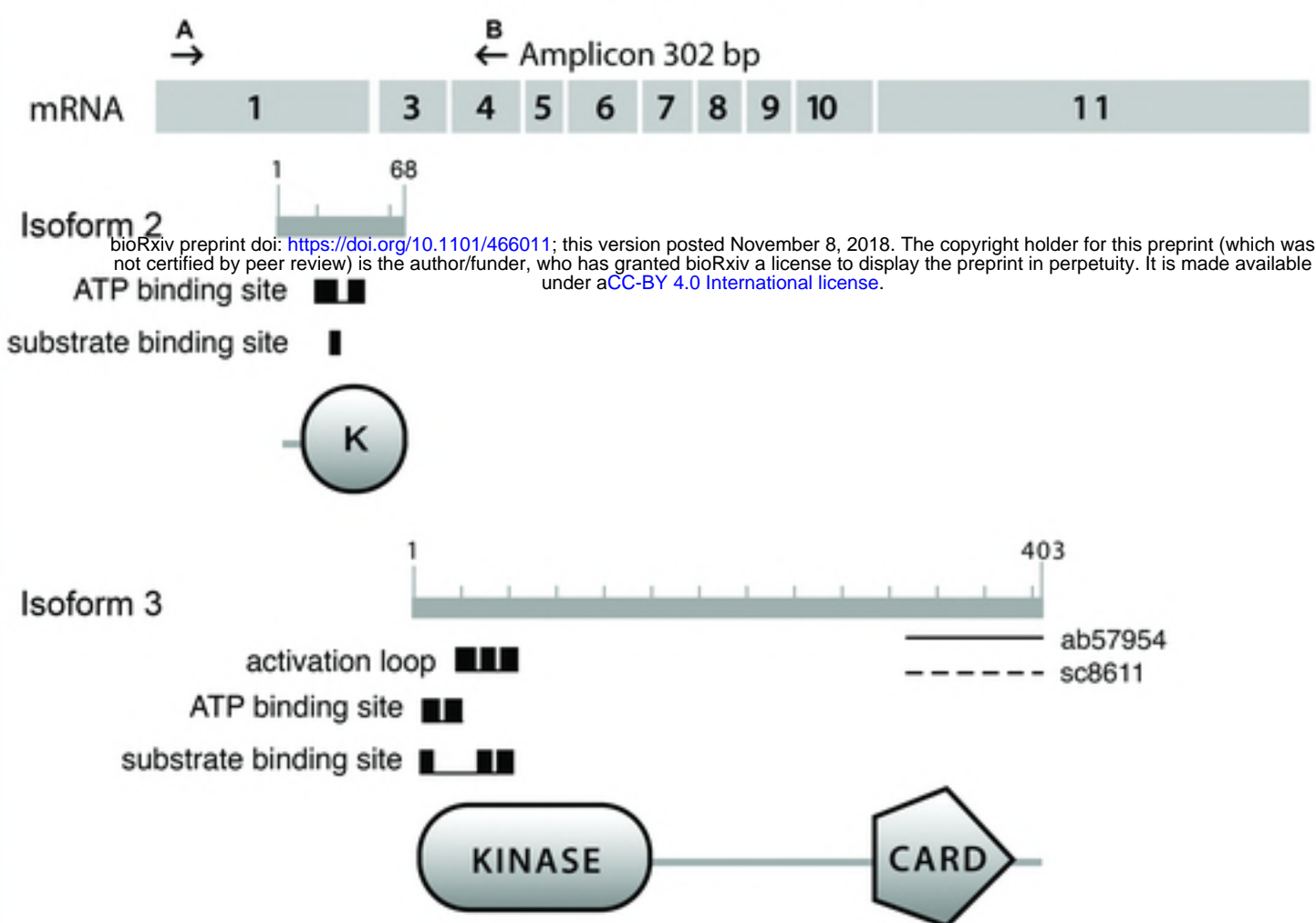
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A *RIPK2* - transcript 1



B *RIPK2* - transcript 2



C

Exon 1

gagctcgggcccggagctggtcgctgcgcgcgcccggggggaggccggggctgagagagaggaagctctttcg
 cggcgctacggcgttggcaccagtctctagaaaagaagtcagctctggttcggagaagcagcggctggcgtgggcat
 ccggggaatgggcccctcgtgacctagtgtgcggggcaaaaagggtcttgcggcctcgtcgtgcagggcgat
 ctgggcgctgagcgcggcgtgggagccttgggagccgcagcagggggcacaccgggaaccggcctgagcg
 ccggggacc **ATG AAC** GGG GAG GCC ATC TGC AGC GCC CTG CCC ACC ATT CCC
 TAC CAC AAA CTC GCC GAC CTG CGC TAC CTG AGC CGC GGC GCC TCT GGC
 ACT GTG TCG TCC GCC CGC CAC GCA GAC TGG CGC GTC CAG GTG GCC GTG
 AAG CAC CTG CAC ATC CAC ACT CCG CTG CTC GAC AG

Exon 2

T GAA AGA AAG GAT GTC TTA AGA GAA GCT GAA ATT TTA CAC AAA GCT AGA
 TTT AGT TAC ATT CTT CCA ATT TTG GGA ATT TGC AAT GAG CCT GAA TTT TTG
 GGA ATA GTT ACT GAA TAC ATG CCA AAT GGA TCA TTA AAT GAA CTC CTA CAT
 AGG

Exon 3

AAA ACT GAA TAT CCT GAT GTT GCT TGG CCA TTG AGA TTT CGC ATC CTG CAT
 GAA ATT GCC CTT GGT GTA AAT TAC CTG **CAC AAT ATG ACT** CCT CCT TTA CTT
 CAT CAT GAC TTG AAG ACT CAG AAT ATC TTA TTG GAC AAT GAA TTT CAT GTT
 AAG

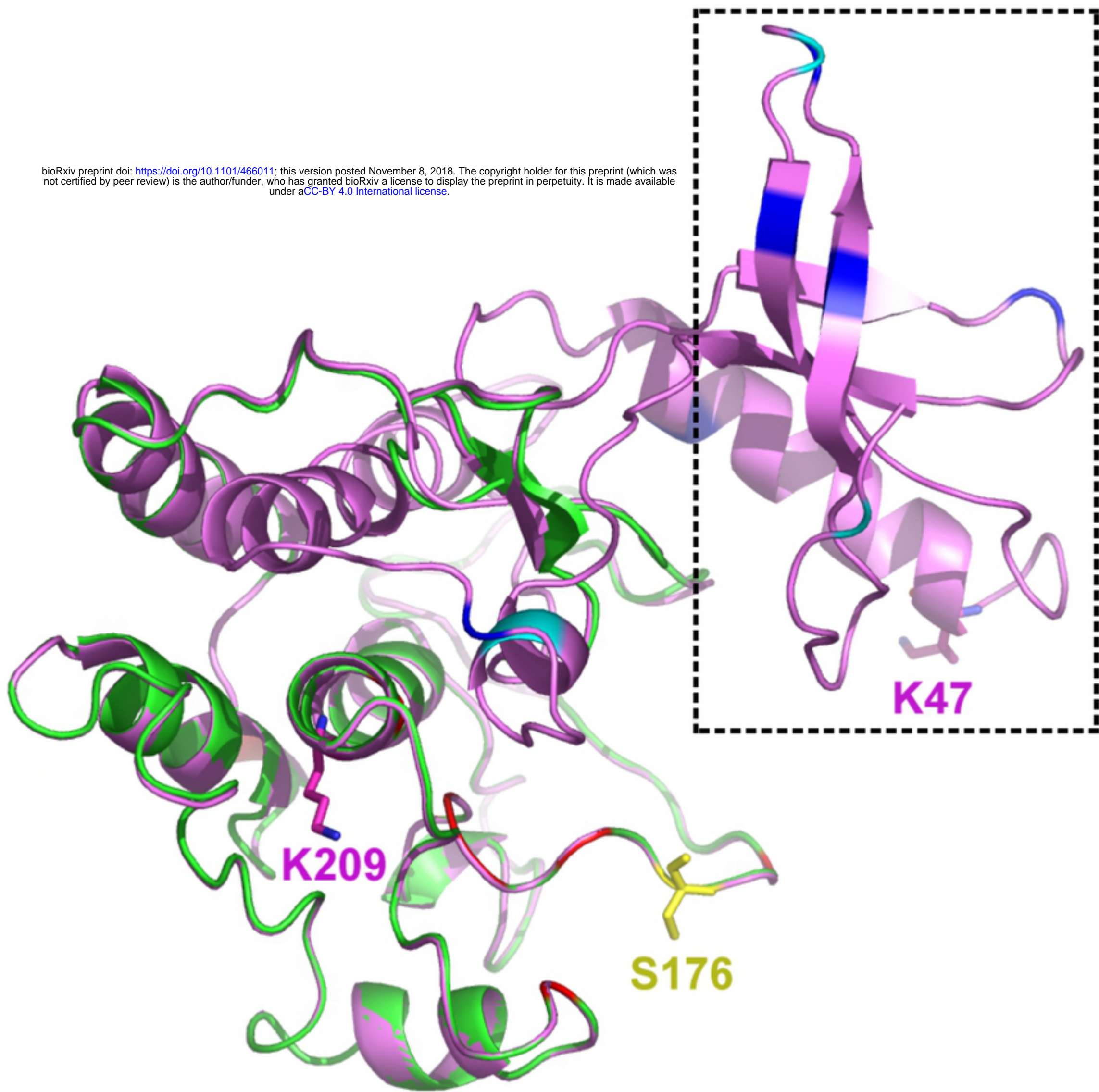


Fig3

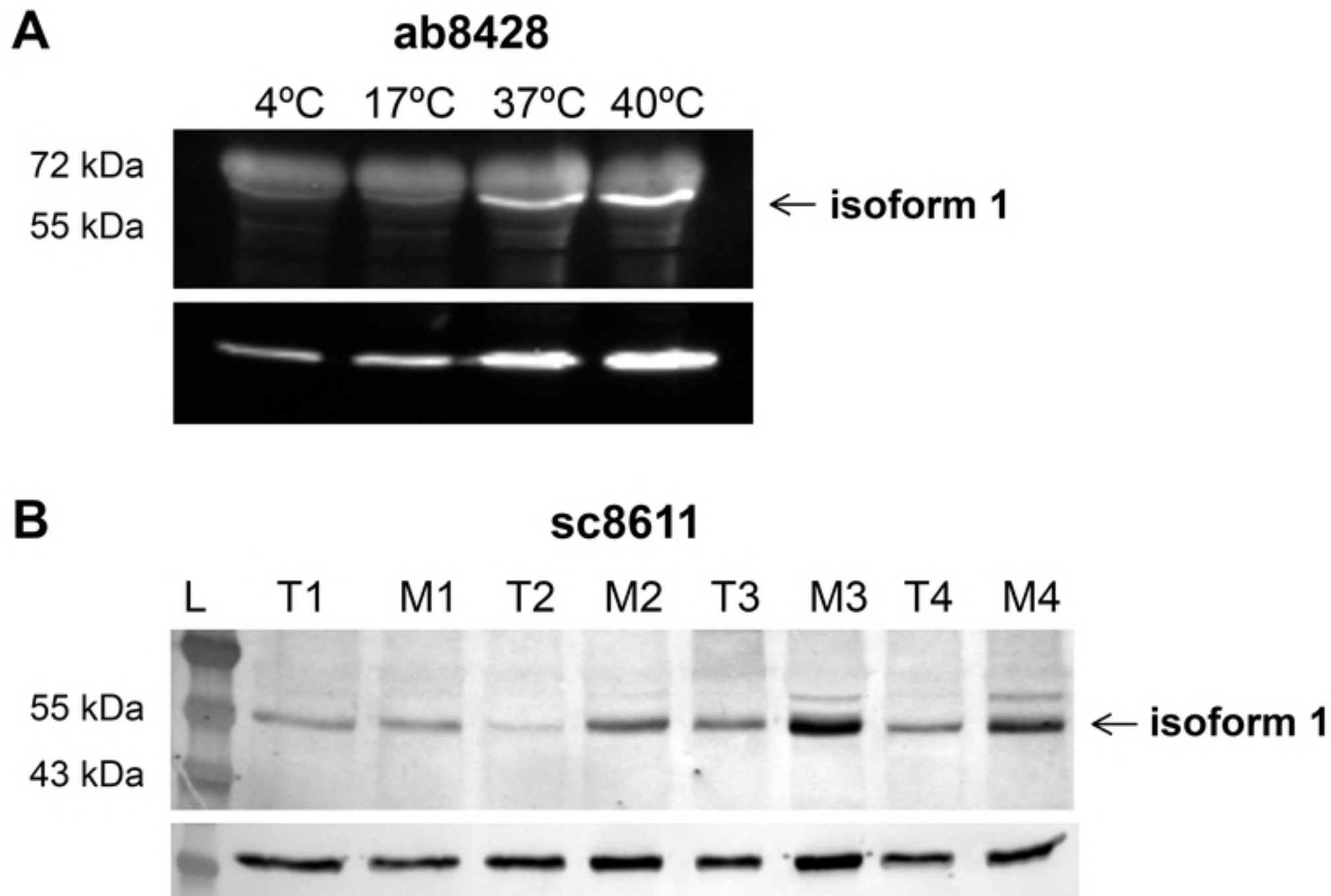


Fig4

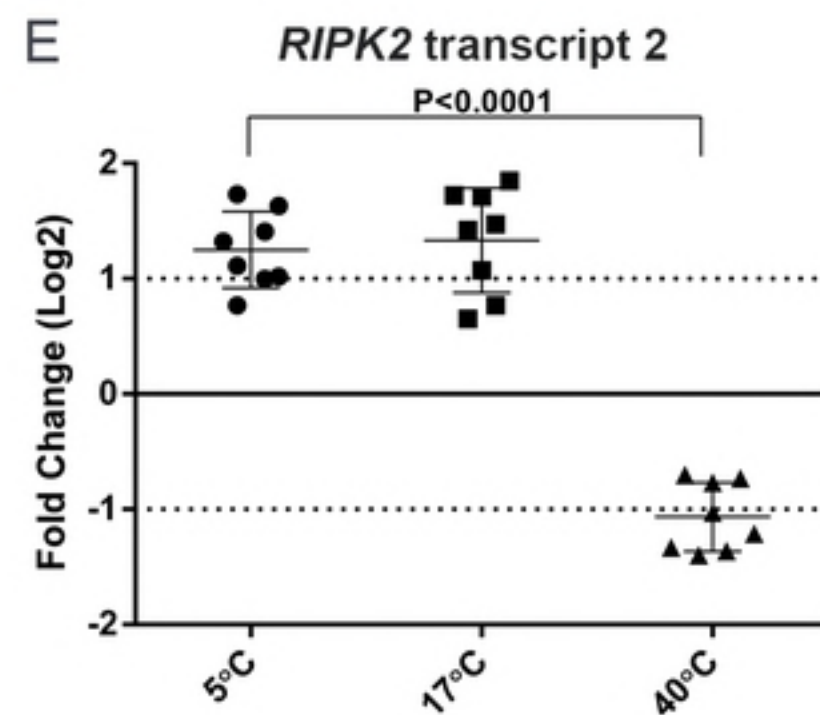
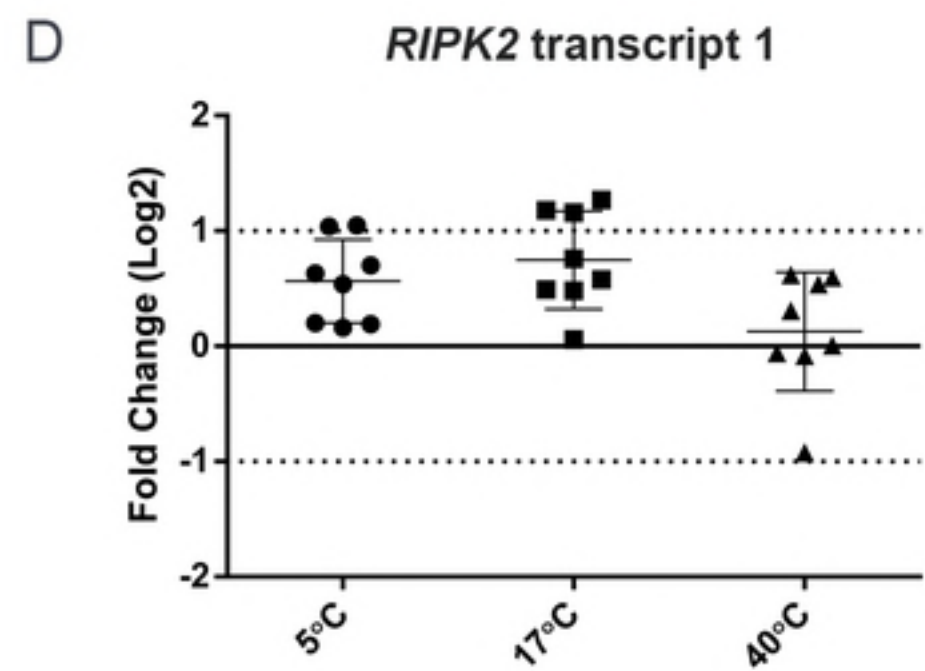
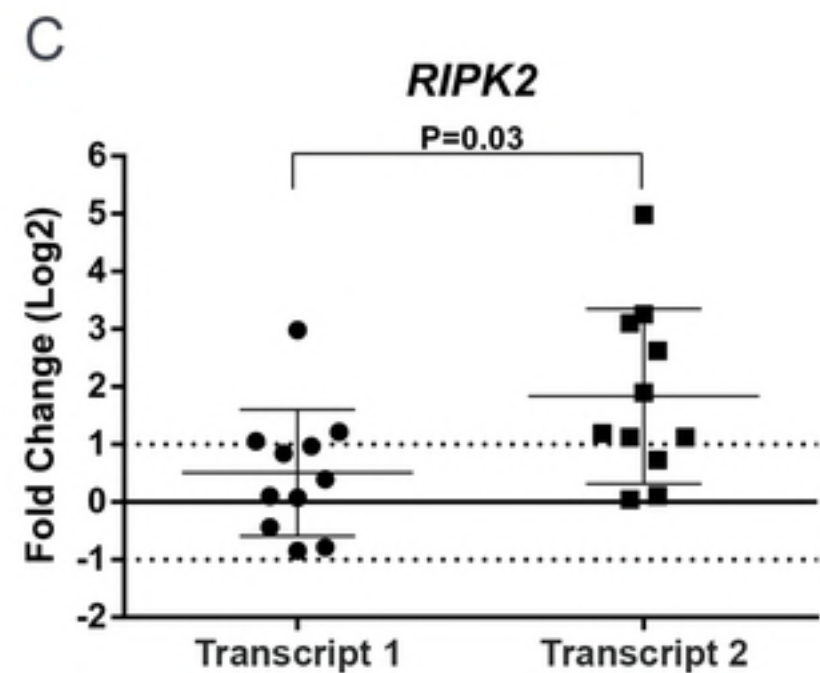
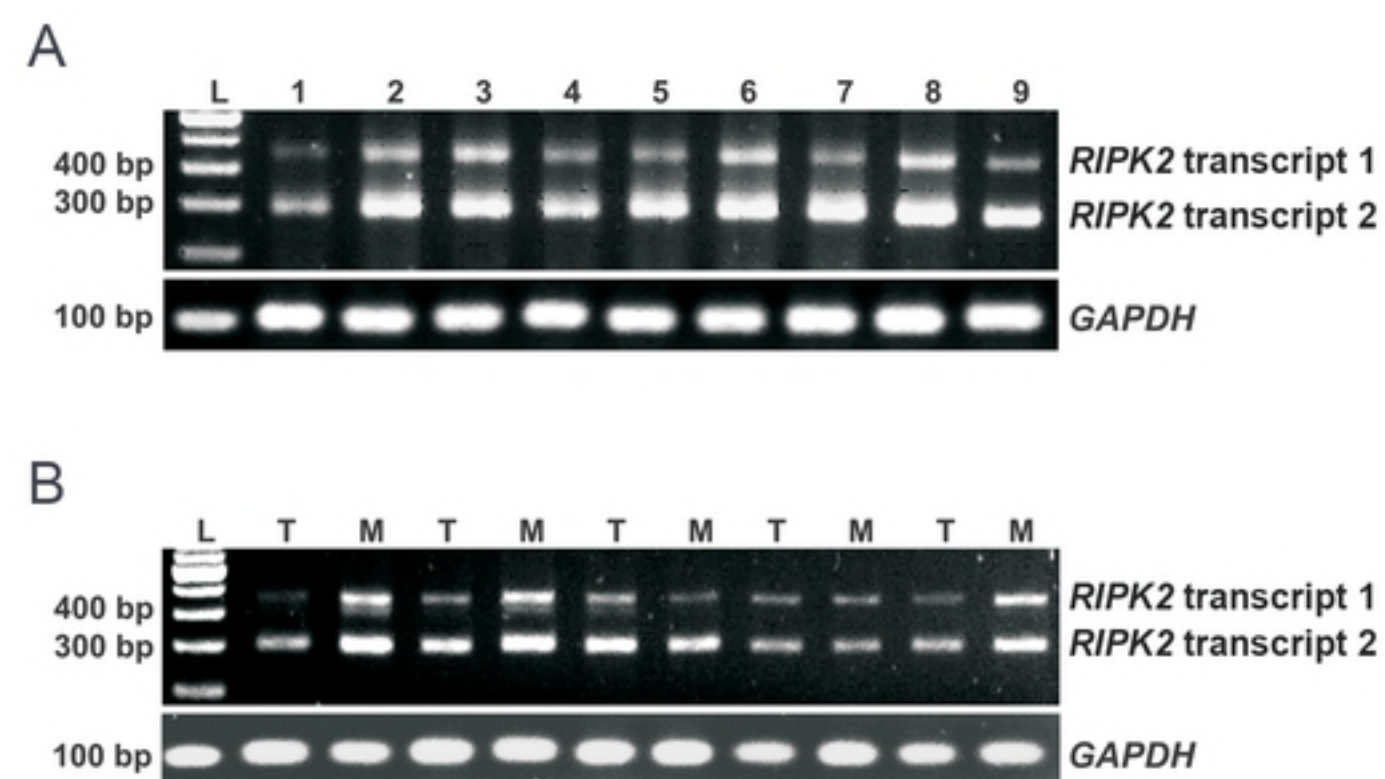


Fig2