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This is the final peer-reviewed author's accepted manuscript (postprint) of the following publication:

Published Version:

Di Palma, S., Porcellato, I., Capaccia, C., Guelfi, G., Lo Giudice, A., Brachelente, C., et al. (2026). Rac1 signaling-associated genes are upregulated in nodal metastasis of canine oral mucosal melanoma. *VETERINARY PATHOLOGY*, 63(2), 193-203 [10.1177/03009858251386913].

Availability:

This version is available at: <https://hdl.handle.net/11585/1038265> since: 2026-01-20

Published:

DOI: <http://doi.org/10.1177/03009858251386913>

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1 **Rac1 signaling-associated genes are upregulated in nodal metastasis of canine oral**
2 **mucosal melanoma**

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5

6 **ABSTRACT**

7 Oral mucosal melanomas (OMMs) are the most frequent oral malignancy in dogs,
8 characterized by aggressive local behavior and high metastatic rate. The mechanisms that
9 drive canine OMM metastasis are still largely unknown, providing for limited therapeutic
10 approaches once the disease has spread to metastatic sites.

11 The objective of this investigation was to evaluate the differences in gene expression
12 between canine primary OMMs and their matched nodal metastases.

13 Transcriptional profiling of formalin-fixed, paraffin-embedded biopsies of four canine OMMs
14 and their respective lymph node biopsies was performed using exon microarrays.

15 Confirmation of the differential expression of selected genes was subsequently sought by
16 quantitative RT-PCR (RT-qPCR) on 13 paired samples (primary tumor-metastatic lymph
17 node). Results highlight the activation of pathways associated with actin cytoskeleton
18 organization, cellular motility and migration. In particular, microarray assay indicated
19 increased expression, in lymph node metastases, of genes (including ELMO1, VAV3 and
20 DOCK2) associated with Rac1 signaling-regulated cell migration. The differential
21 expression of several genes was validated by RT-qPCR.

22 Overall, the results of this investigation point to a significant role for Rac1 signaling in the
23 pathogenesis of OMM metastasis to regional lymph nodes. The Rac1 signaling-associated
24 genes highlighted herein are indeed involved in the activation of cellular migration and
25 one, or more, may represent a future therapeutic target to prevent OMM-metastatic
26 dissemination.

27

28 **Keywords:** canine oral mucosal melanoma; gene expression profiling; exon microarray;
29 RT-qPCR; Rac1, GTP binding protein; nodal metastasis.

30

31 INTRODUCTION

32 Oral melanocytic tumors are common in dogs, accounting for up to 17% of all oral
33 neoplasms⁴³ and approximately 30-40% of all oral malignancies.⁶⁰ In general, canine oral
34 malignant melanomas (OMMs) are characterized by aggressive biologic behavior and
35 shorter median survival time, when compared to their cutaneous counterpart. For dogs
36 treated with surgery alone, the survival rate is less than 35%, and local recurrence is
37 frequent (about 10% of cases) even when surgical margins are clean.³ Metastases to
38 regional lymph nodes and distant organs are frequently reported (in up to 74% of cases).⁴⁵
39 Tissue invasion and metastasis are biological hallmarks of malignant tumors. Metastasis is
40 defined by the spread of neoplastic cells to sites physically discontinuous with the primary
41 tumor. Tumor cells can reach these sites by penetrating blood vessels, lymphatics, and
42 body cavities.

43 In general, metastatic dissemination appears to be facilitated by pro-metastatic genetic
44 and epigenetic changes in the genome of the primary tumor, and interactions between
45 neoplastic cells and other cells (such as immune cells, fibroblasts, and endothelial cells) in
46 both the initial and distant microenvironments.¹⁵

47 The identification of pro-metastatic genetic and epigenetic events, in addition to those
48 driving tumor development, and understanding of the pathways underpinning the
49 metastatic cascade in cancer, is critical to discover molecular targets for the prevention
50 and treatment of distant metastases.^{5,66} In melanomas, neoplastic cells display plasticity
51 and highly migratory behavior, facilitating dissemination to distant sites; these
52 characteristics are likely attributed to a neural crest-like reprogramming of neoplastic

53 melanocytes.²⁵ Indeed, malignant melanoma cells reactivate pro-migratory and pro-
54 invasive pathways, leading to aberrant dissemination.²⁴ Nevertheless, the
55 pathomechanisms involved in local invasion and metastasis of both human and canine
56 mucosal melanoma remain poorly understood.^{5,6}

57 Recently, gene expression profiling has proven to be a powerful tool for the identification of
58 genes involved in metastasis in canine OMM.⁵ It was shown that primary OMMs that
59 metastasized were characterized by reduced expression of CXCL12, a protein that
60 normally stimulates the migration and activation of hematopoietic progenitor cells,
61 endothelial cells, and several leukocytes,¹⁰ and increased expression of APOBEC3A,
62 when compared to non-metastasizing primary OMMs. Similarly, in human head/neck
63 melanomas APOBEC3A overexpression has been identified, supporting its role in the
64 increase of mutational load, and in promoting inter- and intratumoral heterogeneity.²

65 However, genomic analysis of the metastatic process in canine OMMs is still in its infancy.
66 In another recent study, podoplanin, a cell-surface protein that modulates signal
67 transductions that regulate cell proliferation, differentiation, migration, invasion, epithelial-
68 to-mesenchymal transition,⁶² was also identified as a driver protein for ameboid invasion in
69 both human and canine mucosal melanoma, hence promoting tumor progression and
70 metastasis.⁵⁷

71 The aim of the present study was to investigate the molecular basis of canine OMM lymph
72 node metastasis, evaluating differences in gene expression between primary OMMs and
73 matched regional lymph nodal metastases by microarray mRNA profiling and quantitative
74 RT-PCR.

75

76 **MATERIALS AND METHODS**

77 **Case selection and sample collection**

78 Formalin-fixed paraffin-embedded (FFPE) samples of canine primary OMMs and regional
79 lymph node metastases from 13 dogs were retrospectively selected from diagnostic
80 histopathology archives [Pathology Department at the Animal Health Trust (UK) and
81 Department of Pathobiology at the University of Utrecht (the Netherlands)]. Tissue
82 samples were surgically excised from dogs (between 2012 and 2018) for treatment and
83 staging purposes. The confirmed diagnosis of both primary OMMs and metastatic OMMs
84 was obtained by microscopic examination of H&E-stained sections by a board-certified
85 pathologist (SDP). Histological evidence of lymph node metastasis was defined as the
86 presence of large groups of neoplastic cells showing a mass effect on the lymphoid tissue,
87 or the presence of small cohesive groups (>5 cells per high power field/400x) of
88 pleomorphic cells containing melanin pigment. One single representative block was
89 selected from each of the primary OMMs and their lymph node metastasis. Each
90 neoplastic area was outlined using a fine-point permanent marker. A piece of Parafilm
91 large enough to cover the region of interest was placed on the H&E-stained slide. Using a
92 fine-point permanent marker, the entire tissue and the region of interest within the tissue
93 were outlined on the parafilm. The marked parafilm was then transferred to the
94 corresponding FFPE tissue block, matching the outline with the shape of the OMM tissue
95 in the block. Using the tip of a permanent marker, shallow but visible indentations were
96 drawn along the outline of the region of interest. Three tissue cores were extracted from
97 the highlighted area of each FFPE block using a 3.0 mm Miltex Biopsy punch with plunger
98 (Agar Scientific, UK).

99

100 **RNA isolation, purification and quantification**

101 Total RNA was isolated from all three tissue cores obtained for each FFPE primary OMM
102 and matched lymph node metastasis using the RecoverAll Total Nucleic Acid Isolation Kit
103 (ThermoFisher Scientific, Paisley, UK). OMM RNA samples were further purified (i.e. to

104 remove melanin) by spin column filtration (OneStep PCR Inhibitor Removal Kit; Zymo
105 Research, Freiburg, Germany). Total RNA concentration was measured by
106 spectrophotometry (NanoDrop 1000 Spectrophotometer, ThermoFisher Scientific),
107 followed by fluorometry assay (Quant-iT RiboGreen RNA Assay Kit, ThermoFisher
108 Scientific).

109

110 **Global gene expression profiling**

111 *RNA amplification, labelling and microarray hybridization*

112 Fragmented, biotinylated single-stranded cDNA was prepared from 29 ng of each of 4
113 FFPE primary OMMs and matched metastasis RNA samples using the GeneChip WT Pico
114 Reagent Kit (ThermoFisher Scientific).

115 A mixture of 4 unlabeled synthetic RNAs was added (at the beginning of the procedure) to
116 each tumor RNA sample to act as 'labelling controls', and a mixture of 4 labelled bacterial
117 DNAs was added (at the end of the procedure) to each labelled tumor cDNA sample to act
118 as hybridization controls. Each cDNA was individually hybridized to an array in a Canine
119 Gene 1.1 ST Array Strip (ThermoFisher Scientific), in a proprietary hybridization cocktail
120 (ThermoFisher Scientific). Array strip washing and streptavidin- phycoerythrin staining
121 were undertaken by the GeneAtlas System (ThermoFisher Scientific) Fluidics Station, and
122 array scanning by the GeneAtlas System (ThermoFisher Scientific)

123

124 *Microarray data analysis*

125 Exon-level probe set expression values were generated by quantile normalization, log₂
126 transformation and signal summarization, performed using the RMA algorithm²⁹,
127 implemented within 'Affymetrix Expression Console Software 1.3' (ThermoFisher
128 Scientific). 'Outlier arrays' were considered to be those that had any single sample quality,
129 labelling quality and hybridization quality metric value ≥ 2 standard deviations away from

130 the mean of the metric value for all the arrays.⁷³ Outlier arrays were excluded, and
131 processing of the raw probe-level signal intensity data was repeated to generate both
132 quantile normalized and log2-transformed exon and gene-level probe set expression
133 values. Gene-level probe sets ('Transcript clusters') with 'crosshyb_type' = 1 (unique
134 hybridization target) and 'category' = 'main' annotations⁷⁴, and for which at least 10% of its
135 exons was 'present' (detection above background p-value <0.01⁷⁵) in at least ≥ 2 of the
136 primary OMMs and ≥ 2 of the primary OMM lymph node metastases, were considered to
137 be expressed and used for subsequent analyses.

138 The similarity between the global expression profiles of primary and lymph node OMMs
139 was visualized by hierarchical clustering performed using WebMeV.¹⁹ Transcript clusters
140 displaying statistically significant differential expression between primary OMM and lymph
141 node metastases were identified by moderated T-test (p-value < 0.05) performed using the
142 R Limma package.^{37,72} P-values were adjusted by permutation testing⁹ to compensate for
143 "chance" p-values of 0.05 obtained due to multiple testing. Transcription clusters
144 demonstrating a >2-fold difference in median expression level between primary OMM and
145 lymph node metastases were subject to further analysis.

146

147 *Functional annotation enrichment analysis*

148 The biological processes and pathways overrepresented amongst genes with different
149 expression, comparing primary OMMs and OMM lymph node metastases, were identified
150 using DAVID.^{17,27} The functional annotations associated with differentially expressed
151 genes were compared with those ascribed to all Transcript clusters ('crosshyb_type' = 1
152 and 'category' = 'main') for which the expression of at least 10% of its exons was detected
153 above background in $\geq 2\%$ of the tumors in the primary OMMs and metastatic OMMs
154 cohort, and over-represented biological processes and pathways identified.

155

156 **Quantitative RT-PCR analysis**

157 *cDNA synthesis*

158 Total RNA (80 ng) was reverse transcribed in 20 µL reactions using the iSCRIPT™ cDNA
159 Synthesis Kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions.⁵²
160 Controls reactions without reverse transcriptase (RT-) were included to enable checking
161 for genomic DNA contamination.

162

163 *Gene-specific preamplification and quantitative PCR (qPCR)*

164 To increase the sensitivity of qPCR analysis, for each cDNA sample a 20 µL
165 preamplification reaction was performed using 3 µL of cDNA diluted 1:10 (equivalent to 0.4
166 ng of RNA), 1 µL of TaqMan Gene Expression Assay (Table 1), 10 µL of SsoAdvanced™
167 Preamp Supermix (Bio-Rad, Hercules, CA, USA). Preamplification reactions were run for 3
168 min at 95°C, followed by 10 cycles of 15 s at 95°C and 4 min at 58 °C.

169 QPCR amplification was performed using 1 µL of preamplification reaction product, 10 µL
170 of SsoAdvanced Universal Probes Supermix (Bio-Rad, Hercules, CA), 1 µL of TaqMan
171 Gene Expression Assay (Table 1), and RNase-free water to a final volume of 20 µl. QPCR
172 cycling conditions included an initial denaturation step of 15 s at 95°C, followed by 40
173 cycles at 95°C for 5 s and at 60°C for 30 s. RT- controls were enrolled in the qPCR to
174 check for potential genomic DNA contamination. Both preamplification and amplification
175 reactions were run in 96-well optical plates on a StepOne Plus Real-time PCR instrument
176 (Applied Biosystems, California, USA). Three technical replicate qPCR reactions were
177 performed for each preamplified cDNA sample, and the mean Cq value calculated from
178 values determined using StepOne Software v2.3 (Applied Biosystems, California, USA).
179 QPCR amplification efficiency was assessed as previously described.⁴⁰ The 'Livak method'
180 was used to calculate normalized value ($2^{-\Delta Cq}$) of the target genes³⁸.

181

182 *Selection of endogenous control reference genes for relative quantification of gene*
183 *expression*

184 Following the MIQE guidelines⁸, qPCR analysis requires data normalization with the most
185 stably expressed endogenous control (EC) genes to minimize data variation that can mask
186 or exaggerate biological changes. Three candidate genes were chosen as a potential EC:
187 ACTB, GAPDH and RPS18. Candidate ECs expression stabilities were examined with four
188 algorithms, comparative ΔCq method⁵⁸, BestKeeper⁵⁰, NormFinder¹, and GeNorm⁶⁷. The
189 RefFinder tool⁷¹ was used to compare and integrate the output data of the four algorithms,
190 and rank the candidate ECs in the order of the decreasing stability of their expression
191 across all the RNA samples²¹.

192

193 *Statistical analysis*

194 Statistical analysis was performed on normally distributed $2^{-\Delta Cq}$ values. Gene
195 expression data were analyzed using an unpaired t-test to compare the normalized
196 expression value ($2^{-\Delta Cq}$) of selected genes in metastatic OMM *versus* primary OMM
197 cases. Statistical analyses were performed with GraphPad Prism 9 (GraphPad, San
198 Diego, CA, USA). Statistical significance occurred when $P < 0.05$.

199

200 **Results**

201 ***Total RNA yields and purity***

202 Total RNAs isolated from OMM FFPE biopsies were of high purity, with OD260/280nm
203 ratios ranging from 1.9 - 2.0, and OD260/230nm ratios from 1.9 to 2.2. Total RNA yields
204 varied between 50 and 100 ng.

205

206 ***Tumors included in differential gene expression analysis***

207 In order to define changes in the gene expression profiles of primary OMMs associated
208 with the process of metastatic dissemination to a regional lymph node, comparative global
209 gene expression analysis of biopsies of canine primary OMMs (4) and their lymph node
210 metastases (4) was performed. The use of matched primary and metastatic tumors
211 intended to minimise potential confounding effects of tumor genetic heterogeneity.
212 The microarray expression data for one lymph node metastasis sample ('3S') was
213 excluded from the analysis because it was an "outlier array" (see Materials and Methods),
214 having an 'all_probeset_rle_mean' quality metric value 2.24 standard deviations away from
215 the mean of the metric value of all the arrays.

216 The gene-level probe set expression values obtained for 4 primary and 3 metastatic
217 OMMs were compared for 9,964 Transcript clusters (category = main; crosshyb_type = 1).
218 A Transcript cluster was considered to be present if $\geq 10\%$ of its exons were present both in
219 ≥ 2 primary OMMs and ≥ 2 metastatic OMMs. Unsupervised hierarchical clustering of the 4
220 primary OMMs and 3 OMM lymph node metastases on the basis of the expression levels
221 of the 9,964 genes suggested a higher degree of similarity between unrelated OMM lymph
222 node metastases, and between different primary OMMs, respectively, than between a
223 primary OMM and its derivative lymph node metastasis (Figure 1).

224

225

226 ***Genes displaying differential expression between primary and metastatic OMMs***

227 In total, 501 Transcript Clusters (genes) displayed a statistical difference in expression (p-
228 value < 0.05) between primary and metastatic OMMs, after permutation testing-adjustment.
229 A > 2 -fold change (median primary/median metastatic expression value or median
230 metastatic/median primary expression value) was considered to be authentic and more
231 likely to be reproducibly measurable. Greater than 2-fold differences in expression
232 between the primary and OMM metastases were exhibited by 151 genes, with 13

233 displaying increased expression and 16 decreased expression, respectively, in the lymph
234 node metastases (Figure 2 and Supplementary Table 1).

235

236 ***Functional annotation enrichment analysis:***

237 The frequencies of functional annotations assigned to 148 of the 158 Transcription
238 Clusters that were differentially expressed between the primary and metastatic OMMs, and
239 which had unique Ensembl canine gene IDs, were compared with those associated with all
240 the Transcript Clusters expressed by the OMMs (9,123 of 9,964 which had associated
241 Ensembl canine gene IDs). Over-represented amongst the genes exhibiting differential
242 expression were 4 Gene Ontology Consortium biological processes and 4 KEGG
243 pathways potentially involved in the metastatic process (Table 2).

244

245 ***RT-qPCR assessment of differential gene expression***

246 The relative expression levels of selected genes were derived using the normalized value
247 $2^{-(\text{Cq target gene} - \text{Cq reference gene})}$ values. ACTB was selected as the best
248 endogenous control, with respect to GAPDH and RPS18, by utilizing four mathematical
249 approaches: comparative ΔCq method, BestKeeper, NormFinder, and GeNorm (Table 3).²¹
250 RT-qPCR analysis featured 13 pairs of primary OMMs and their lymph node metastases,
251 including the 4 sample pairs subject to global gene expression profiling, and 10
252 subsequently collected primary tumor: lymph node metastasis sample pairs. The analysis
253 sought to confirm the differential expression of 7 selected genes directly associated with
254 Rac1 activation (Figure 3). The statistically significant >2-fold increased median expression
255 of CORO1A, ITGA4 and VAV3 in the lymph node metastases determined by microarray-
256 based transcriptional profiling was validated by RT-qPCR. RT-qPCR assay also confirmed
257 the 'direction' of the >2-fold difference in the median expression of ELMO1 (increased
258 expression in OMM lymph node metastases), and SPARC and MMP2 (both decreased

259 expression in OMM lymph node metastases), in accordance with the results of the
260 microarray analysis, although the differences in expression did not reach statistical
261 significance. DOCK2 exhibited statistically significant >2-fold decreased median expression
262 in the lymph node metastases, in contrast to the >2-fold increased median expression
263 observed in the lymph node metastases in the 4 sample-pair microarray investigation.

264

265 **DISCUSSION**

266 OMM is the most common malignant cancer of the oral cavity in the dog,^{22,46} with frequent
267 metastases and poor prognosis. The propensity of OMMs to metastasise is likely to be
268 associated with pro-metastatic changes in gene expression in primary tumor cells.⁵

269 Identification of these alterations holds the key to pinpointing potential targets for the
270 control and treatment of OMM metastatic dissemination. Thus, this study aimed to identify
271 potentially metastasis-associated differences in gene expression between canine primary
272 OMMs and their relative regional lymph nodal metastases.

273 Several of the genes exhibiting differential expression between primary and metastatic
274 OMMs are associated with cell motility, and are directly and/or indirectly involved in the
275 regulation of the Rac1 signaling-associated genes.^{32,35,39,41} Variation in the expression of
276 these genes are also frequently correlated with highly aggressive tumor subtypes in
277 humans.^{11,14,35,39}

278 Rac1 (Ras-related C3 botulinum toxin substrate 1) is the major ubiquitous isoform of Rac
279 expressed in mammalian tissue, as part of the Rho family of small GTPases, which
280 controls assembly of the cellular actin cytoskeleton. Lamellipodia are actin polymerisations
281 at the leading edge of cells, representing the main force for cell migration, and their
282 formation is under the control of Rac1. Indeed, loss of Rac1 in tissues and cell cultures
283 leads to the loss of lamellipodia and a general reduction of migration speed.³³ The results

284 of our study support the hypothesis that activation of downstream effectors and upstream
285 regulators of Rac1 may facilitate canine OMM cell migration to a regional lymph node.
286 This study highlighted the increased expression in canine OMM lymph node metastases of
287 a number of genes involved with cell migration-associated Rac1 signaling.

288 *VAV3* is a member of the *VAV* gene family. *VAV* proteins activate pathways leading to actin
289 cytoskeletal rearrangements, mainly acting as a guanine nucleotide exchange factor for
290 RhoG, RhoA and Rac1. Elevated *VAV3* expression has been linked to prostate cancer
291 progression and post-treatment recurrence,³⁶ to glioblastoma cell migration, invasion and
292 proliferation and also to a shorter overall survival⁴², lymphatic metastasis and perineural
293 invasion in gastric cancer.⁶⁴ Microarray and RT-qPCR-determined increased expression in
294 lymph node metastases indicate that this gene may also be involved in canine OMM
295 progression.

296 Coronin1A (encoded by the *CORO1A* gene) plays a pivotal role in the cytoskeleton in
297 highly motile cells, contributing to the formation of plasma membrane protrusions essential
298 for cell locomotion. Notably, this protein is particularly enriched in haematopoietic tissues.⁴⁷
299 Overexpression of *CORO1A* prompts the translocation of Rac1 to the plasma membrane,
300 favouring its activation.¹² We hypothesize that the increased expression of *CORO1A* may
301 be associated with the acquisition of a highly motile phenotype in OMM cells, as previously
302 suggested in human mammary tumors.³¹

303 Integrin alpha 4 (*ITGA4*) mediates cell-cell adhesion important in immune function. *ITGA4*
304 is also widely expressed in neural crest cells, leukocytes, striated and smooth muscle, and
305 neurons, and mediates their migration, most likely co-localising and activating Rac1⁴⁸. A
306 recent study on human cutaneous melanoma suggests that *ITGA4* is closely related to the
307 occurrence and development of melanoma, and that it can promote the metastasis of
308 melanoma by favoring the aggregation of melanoma cells in the lymphatic system.⁴⁹

309 Moreover, overexpression of ITGA4 has been demonstrated in human gastrointestinal
310 stromal tumors, where it is associated with an unfavourable prognosis⁵³.

311 Dock2 (Dedicator Of Cytokinesis 2) is a member of the CDM family of proteins, known to
312 regulate the actin cytoskeleton by functioning upstream of Rac1.⁶⁵ DOCK2 activates Rac1
313 and regulates the actin cytoskeleton through a self-inhibiting interaction with ELMO1.^{13,55}
314 Proteins encoded by *ELMO1* interact with dedicator of cytokinesis proteins to promote
315 phagocytosis and cell migration³⁰. Mutation-effected aberrant activation of *ELMO1* and
316 *DOCK2* has been reported in human oesophageal adenocarcinoma, a highly invasive
317 tumor prone to early metastasis.¹⁸ RT-qPCR assay did not confirm the >2-fold increase in
318 median DOCK2 expression in OMM lymph node metastases determined by microarray
319 gene expression profiling.

320 Although several RAC1 signaling-associated genes displayed increased expression in
321 OMM lymph node metastases, RT-qPCR assay suggested that this was not the case for
322 MMP2, and SPARC, respectively. Downregulation of SPARC expression is related to the
323 Rac1 signaling-associated pathways. Secreted protein acidic and cysteine-rich (SPARC,
324 also known as osteonectin) is a matrix-associated protein, usually playing a role in bone
325 mineralisation, regulating cell interaction with the extracellular milieu during development
326 and in response to injury.⁷ SPARC expression has been associated with an aggressive,
327 mesenchymal-like phenotype in a variety of human cancers, including melanoma. In
328 particular, its expression in melanoma cells was associated with decreased E-cadherin
329 and increased N-cadherin expression levels suggesting that this protein may regulate
330 epithelial–mesenchymal transition⁵¹. However, there is evidence that SPARC is a
331 permissive factor for Rac1 that becomes fully active only when SPARC levels are low;
332 indeed, suppression of SPARC expression induces the formation of lamellipodia
333 extension.⁵⁴ The potentially decreased SPARC expression in canine OMM lymph node
334 metastases demonstrated herein is conceptually consistent with the latter finding, although

335 further investigation of a possible role for SPARC in canine OMM metastasis is warranted.

336 Matrix metalloproteinase 2 (MMP-2) is an enzyme able to cleave components of the
337 extracellular matrix, precisely gelatin type I and collagen types IV, V, VII and X. It has been
338 shown that Membrane Type 1 Metalloprotease (MT1-MMP), highly expressed in neural
339 crest cells, mediates human melanoma cell invasion through the activation of its target
340 MMP-2 and, in turn, MMP-2 activation is required to sustain Rac1 activity and promote cell
341 migration.⁵⁶

342 Rac1 is also implicated in epithelial-mesenchymal transition (EMT), inducing
343 downregulation of E-cadherin and upregulation of N-cadherin, vimentin, and SNAIL1 in
344 human cancers.⁷⁰ Melanocytes do not belong to the epithelial lineage and therefore the
345 term EMT cannot be formally attributed to the progression of malignant melanoma.

346 However, various studies have demonstrated that melanoma cells display EMT-like
347 phenotype switching, both in humans and dogs.^{34,59,68} Indeed, differentiated melanocytes
348 do express E-cadherin and loss of this protein, which represents a hallmark of EMT in
349 epithelial tumors, is also evident in late-stage malignant melanomas, especially in nodal
350 metastases⁴⁴.

351 RAC1 has been identified as an oncogene in human cutaneous melanoma.⁶¹ In particular,
352 the p.P29S hot spot mutation likely destabilises RAC1 favouring its active GTP-bound
353 state.²⁶ Intriguingly, both RAC1^{P29S} mutant melanoma cells and RAC1 wild type melanoma
354 cells reduce their proliferation by downregulation of Rac1.²⁸ However, RAC1 mutations
355 have not been found in human mucosal melanomas, or in primary and metastatic canine
356 OMMs.⁶⁹ Beyond the p.P29S missense mutation, Rac1 is activated by other molecular
357 mechanisms in human cutaneous melanoma.¹⁶ Some of these mechanisms may be
358 similar to the putative Rac1 activation-promoting gene expression changes identified in the
359 current study. This study has demonstrated that canine OMM lymph node metastases are
360 characterized by increased expression (relative to primary OMMs) of a set of genes

361 involved in cell motility, in particular genes involved in Rac1 signaling. The potential key
362 role of Rac1 and its upstream activators and downstream effectors in the modulation of
363 critical elements of metastasis has been highlighted, as it has been done previously in
364 human medicine.³ It is possible that melanoma cells are able to disseminate to distant
365 sites due to re-acquisition of plastic and highly migratory behaviour characteristic of their
366 embryonic precursors.²³ Rac1 activation could be one of these mechanisms as it is
367 essential for lamellipodia formation. Indeed, lamellipodia formation is also used by
368 dendritic cells to interact with the endothelium, polarise, and actively crawl toward the
369 draining lymph node.⁶³

370 The main limitation of this study is the relatively low number of primary OMMs and lymph
371 node metastasis biopsy pairs analysed. Fine needle aspiration (FNA) remains a mainstay
372 diagnostic technique when evaluating for metastasis to regional lymph nodes in animals,
373 being less invasive compared to surgical excision and frequently preferred to histological
374 examination of the lymph node, despite the latter technique being considered the gold
375 standard for the identification of nodal metastasis²⁰. Moreover, dogs with OMM are often
376 inoperable at diagnosis, hence, cytology would be the only viable option to provide a
377 complete staging. Therefore, there are only a limited number of instances where both a
378 primary OMM and a regional lymph node biopsy are available. A further constraint is that it
379 was necessary to use FFPE tissue biopsies. Degradation of RNA by formalin fixation is
380 well known, and probably mainly affects the accurate quantification of genes expressed at
381 low levels.

382 The role of Rac1 signaling in tumorigenesis, angiogenesis, invasion, and metastasis has
383 been demonstrated for several different human tumor types³, and it may have a similarly
384 important role in canine OMM development and progression. Targeting Rac1 and its
385 regulatory network would be beneficial for cancer treatment, but this has unfortunately
386 proved to be difficult. Rac1 signaling is involved in many processes of normal cell

387 physiology, including cellular plasticity, migration and invasion, cellular adhesions, cell
388 proliferation, apoptosis, reactive oxygen species (ROS) production and immune
389 responses.⁴ Therefore, targeting Rac1 in a clinical setting might bear undesirable side
390 effects. Investigation of both the downstream effectors and the upstream regulators of
391 Rac1 could indicate the best unimodal/multimodal therapeutic approaches for canine
392 OMMs. At this stage, additional validation in different preclinical cancer models is needed
393 to supplement the striking *in vitro* evidence of the potential benefits of targeting Rac1.⁴¹

394

395 **Ethics statement**

396 This study (Project number 59-2016) was approved by the Animal Health Trust (AHT)
397 Ethics Committee. Informed, written consent was obtained from the owners of dogs whose
398 oral melanoma and lymph nodal biopsies were included in this study. A melanoma/lymph
399 node biopsy could be withdrawn from the study at any time. Patient treatment was
400 unaffected by the study.

401

402 **REFERENCES**

- 403 1. Andersen CL, Jensen JL, Ørntoft TF. Normalization of real-time quantitative reverse
404 transcription-PCR data: a model-based variance estimation approach to identify genes suited for
405 normalization, applied to bladder and colon cancer data sets. *Cancer Res.* 2004;**64**(15):5245–5250.
- 406 2. Argyris PP, Naumann J, Jarvis MC, et al. Primary mucosal melanomas of the head and neck
407 are characterised by overexpression of the DNA mutating enzyme APOBEC3B. *Histopathology.*
408 2023;**82**(4):608–621.
- 409 3. Bid HK, Roberts RD, Manchanda PK, Houghton PJ. RAC1: An emerging therapeutic option
410 for targeting cancer angiogenesis and metastasis. *Molecular Cancer Therapeutics.* 2013:
- 411 4. Bosco EE, Mulloy JC, Zheng Y. Rac1 GTPase: A “Rac” of all trades. *Cellular and*
412 *Molecular Life Sciences.* 2009:
- 413 5. Bowlt Blacklock KL, Birand Z, Selmic LE, et al. Genome-wide analysis of canine oral
414 malignant melanoma metastasis-associated gene expression. *Sci Rep.* 2019;**9**(1):6511.
- 415 6. Brachelente C, Cappelli K, Capomaccio S, et al. Transcriptome Analysis of Canine
416 Cutaneous Melanoma and Melanocytoma Reveals a Modulation of Genes Regulating Extracellular
417 Matrix Metabolism and Cell Cycle. *Sci Rep.* 2017;**7**(1):6386.
- 418 7. Bradshaw AD, Sage EH. SPARC, a matricellular protein that functions in cellular
419 differentiation and tissue response to injury. *J Clin Invest.* 2001;**107**(9):1049–1054.
- 420 8. Bustin SA, Benes V, Garson JA, et al. The MIQE Guidelines: Minimum Information for
421 Publication of Quantitative Real-Time PCR Experiments. *Clin Chem.* 2009;**55**(4):611–622.

- 422 9. Camargo A, Azuaje F, Wang H, Zheng H. Permutation – based statistical tests for multiple
423 hypotheses. *Source Code Biol Med.* 2008;**3**(1):15.
- 424 10. Cambier S, Gouwy M, Proost P. The chemokines CXCL8 and CXCL12: molecular and
425 functional properties, role in disease and efforts towards pharmacological intervention. *Cell Mol*
426 *Immunol.* 2023;**20**(3):217–251.
- 427 11. Cannon AC, Uribe-Alvarez C, Chernoff J. RAC1 as a Therapeutic Target in Malignant
428 Melanoma. *Trends Cancer.* 2020;**6**(6):478–488.
- 429 12. Castro-Castro A, Ojeda V, Barreira M, et al. Coronin 1A promotes a cytoskeletal-based
430 feedback loop that facilitates Rac1 translocation and activation. *EMBO J.* 2011.
- 431 13. Chang L, Yang J, Jo CH, et al. Structure of the DOCK2–ELMO1 complex provides insights
432 into regulation of the auto-inhibited state. *Nat Commun.* 2020;**11**(1):3464.
- 433 14. Chen M, Li H, Xu X, et al. Identification of RAC1 in promoting brain metastasis of lung
434 adenocarcinoma using single-cell transcriptome sequencing. *Cell Death Dis.* 2023;**14**(5):1–14.
- 435 15. Chiang AC, Massagué J. Molecular Basis of Metastasis. *N Engl J Med.* 2008;**359**(26):2814–
436 2823.
- 437 16. Colón-Bolea P, García-Gómez R, Casar B. RAC1 Activation as a Potential Therapeutic
438 Option in Metastatic Cutaneous Melanoma. *Biomolecules.* 2021;**11**(11):1554.
- 439 17. Dennis G, Sherman BT, Hosack DA, et al. DAVID: Database for Annotation, Visualization,
440 and Integrated Discovery. *Genome Biol.* 2003;**4**(5):P3.
- 441 18. Dulak AM, Stojanov P, Peng S, et al. Exome and whole-genome sequencing of esophageal
442 adenocarcinoma identifies recurrent driver events and mutational complexity. *Nat Genet.* 2013.
- 443 19. Eisen MB, Spellman PT, Brown PO, Botstein D. Cluster analysis and display of genome-
444 wide expression patterns. *Proc Natl Acad Sci U S A.* 1998;**95**(25):14863–14868.
- 445 20. Grimes JA, Matz BM, Christopherson PW, et al. Agreement Between Cytology and
446 Histopathology for Regional Lymph Node Metastasis in Dogs With Melanocytic Neoplasms. *Vet*
447 *Pathol.* 2017.
- 448 21. Guelfi G, Capaccia C, Santoro MM, Diverio S. Identification of Appropriate Endogenous
449 Controls for Circulating miRNA Quantification in Working Dogs under Physiological Stress
450 Conditions. *Animals.* 2023;**13**(4):576.
- 451 22. Guth AM, Dow S. *Withrow and MacEwen’s Small Animal Clinical Oncology. Withrow and*
452 *MacEwen’s Small Animal Clinical Oncology, 5/e.* Elsevier 2013.
- 453 23. Hendrix MJC, Seftor EA, Seftor REB, Kasemeier-Kulesa J, Kulesa PM, Postovit LM.
454 Reprogramming metastatic tumour cells with embryonic microenvironments. *Nat Rev Cancer.*
455 2007;**7**(4):246–255.
- 456 24. Hendrix MJC, Seftor EA, Seftor REB, Kasemeier-Kulesa J, Kulesa PM, Postovit L-M.
457 Reprogramming metastatic tumour cells with embryonic microenvironments. *Nat Rev Cancer.*
458 2007;**7**(4):246–255.
- 459 25. Heppt MV, Wang JX, Hristova DM, et al. MSX1-Induced Neural Crest-Like
460 Reprogramming Promotes Melanoma Progression. *J Invest Dermatol.* 2018;**138**(1):141–149.
- 461 26. Hodis E, Watson IR, Kryukov G V., et al. A landscape of driver mutations in melanoma.
462 *Cell.* 2012;**150**(2):251–263.
- 463 27. Huang DW, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene
464 lists using DAVID bioinformatics resources. *Nat Protoc.* 2009;**4**(1):44–57.
- 465 28. Immisch L, Papafotiou G, Gallarín Delgado N, et al. Targeting the recurrent Rac1P29S
466 neoepitope in melanoma with heterologous high-affinity T cell receptors. *Front Immunol.*
467 2023;**14**:1119498.
- 468 29. Irizarry RA, Bolstad BM, Collin F, Cope LM, Hobbs B, Speed TP. Summaries of
469 Affymetrix GeneChip probe level data. *Nucleic Acids Res.* 2003;**31**(4):e15.
- 470 30. Jiang J, Liu G, Miao X, Hua S, Zhong D. Overexpression of engulfment and cell motility 1
471 promotes cell invasion and migration of hepatocellular carcinoma. *Exp Ther Med.* 2011;**2**(3):505–
472 511.

- 473 31. Kim D-H, Bae J, Lee JW, et al. Proteomic analysis of breast cancer tissue reveals
474 upregulation of actin-remodeling proteins and its relevance to cancer invasiveness. *Proteomics Clin*
475 *Appl.* 2009;**3**(1):30–40.
- 476 32. ten Klooster JP, Leeuwen I v, Scheres N, Anthony EC, Hordijk PL. Rac1-induced cell
477 migration requires membrane recruitment of the nuclear oncogene SET. *EMBO J.* 2007;**26**(2):336–
478 345.
- 479 33. Li A, Ma Y, Yu X, et al. Rac1 Drives Melanoblast Organization during Mouse Development
480 by Orchestrating Pseudopod- Driven Motility and Cell-Cycle Progression. *Dev Cell.* 2011.
- 481 34. Li FZ, Dhillon AS, Anderson RL, McArthur G, Ferrao PT. Phenotype Switching in
482 Melanoma: Implications for Progression and Therapy. *Front Oncol.* 2015;**5**.
- 483 35. Liang J, Oyang L, Rao S, et al. Rac1, A Potential Target for Tumor Therapy. *Front Oncol.*
484 2021;**11**.
- 485 36. Lin KT, Gong J, Li CF, et al. Vav3-Rac1 signaling regulates prostate cancer metastasis with
486 elevated Vav3 expression correlating with prostate cancer progression and posttreatment
487 recurrence. *Cancer Res.* 2012.
- 488 37. Liu W, Peng Y, Tobin DJ. A new 12-gene diagnostic biomarker signature of melanoma
489 revealed by integrated microarray analysis. *PeerJ.* 2013;**2013**(1):1–23.
- 490 38. Livak KJ, Schmittgen TD. Analysis of Relative Gene Expression Data Using Real-Time
491 Quantitative PCR and the 2– $\Delta\Delta$ CT Method. *Methods.* 2001;**25**(4):402–408.
- 492 39. Ma N, Xu E, Luo Q, Song G. Rac1: A Regulator of Cell Migration and a Potential Target
493 for Cancer Therapy. *Molecules.* 2023;**28**(7):2976.
- 494 40. Mandara M t., Reginato A, Foiani G, De Luca S, Guelfi G. Gene Expression of Matrix
495 Metalloproteinases and their Inhibitors (TIMPs) in Meningiomas of Dogs. *J Vet Intern Med.*
496 2017;**31**(6):1816–1821.
- 497 41. Marei H, Malliri A. Rac1 in human diseases: The therapeutic potential of targeting Rac1
498 signaling regulatory mechanisms. *Small GTPases.* 2017;**8**(3):139–163.
- 499 42. Miao R, Huang D, Zhao K, et al. VAV3 regulates glioblastoma cell proliferation, migration,
500 invasion and cancer stem-like cell self-renewal. *Mol Med Rep.* 2023;**27**(4):94.
- 501 43. Mikiewicz M, Paździor-Czapula K, Gesek M, Lemishevskiy V, Otrocka-Domagala I.
502 Canine and Feline Oral Cavity Tumours and Tumour-like Lesions: a Retrospective Study of 486
503 Cases (2015-2017). *J Comp Pathol.* 2019;**172**:80–87.
- 504 44. Miller AJ, Mihm MC. mechanisms of disease Melanoma. *N Engl J O F Med.* 2006.
- 505 45. Munday JS, Löhr CV, Kiupel M. Tumors of the Alimentary Tract. *Wiley Online Library.*
506 John Wiley & Sons, Ltd 2016:499–601.
- 507 46. Munday JS, Löhr C V., Kiupel M. Tumors of the Alimentary Tract. In: *Tumors in Domestic*
508 *Animals.* John Wiley & Sons, Inc. 2016:500.
- 509 47. Nal B, Carroll P, Mohr E, et al. Coronin-1 expression in T lymphocytes: insights into protein
510 function during T cell development and activation. *Int Immunol.* 2004;**16**(2):231–240.
- 511 48. Nishiya N, Kiosses WB, Han J, Ginsberg MH. An α 4 integrin-paxillin-Arf-GAP complex
512 restricts Rac activation to the leading edge of migrating cells. *Nat Cell Biol.* 2005;**7**(4):343–352.
- 513 49. Nurzat Y, Su W, Min P, Li K, Xu H, Zhang Y. Identification of Therapeutic Targets and
514 Prognostic Biomarkers Among Integrin Subunits in the Skin Cutaneous Melanoma
515 Microenvironment. *Front Oncol.* 2021;**11**:751875.
- 516 50. Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP. Determination of stable housekeeping
517 genes, differentially regulated target genes and sample integrity: BestKeeper – Excel-based tool
518 using pair-wise correlations. *Biotechnol Lett.* 2004;**26**(6):509–515.
- 519 51. Podhajcer OL, Benedetti LG, Girotti MR, Prada F, Salvatierra E, Llera AS. The role of the
520 matricellular protein SPARC in the dynamic interaction between the tumor and the host. *Cancer*
521 *Metastasis Rev.* 2008;**27**(4):691–705.
- 522 52. Porcellato I, Brachelente C, Guelfi G, et al. A Retrospective Investigation on Canine
523 Papillomavirus 1 (CPV1) in Oral Oncogenesis Reveals Dogs Are Not a Suitable Animal Model for

524 High-Risk HPV-Induced Oral Cancer. *PLOS ONE*. 2014;**9**(11):e112833.

525 53. Pulkka OP, Mpindi JP, Tynninen O, et al. Clinical relevance of integrin alpha 4 in
526 gastrointestinal stromal tumours. *J Cell Mol Med*. 2018;**22**(4):2220–2230.

527 54. Salvatierra E, Alvarez MJ, Leishman CC, et al. SPARC controls melanoma cell plasticity
528 through Rac1. *PLoS ONE*. 2015.

529 55. Sanui T, Inayoshi A, Noda M, et al. DOCK2 regulates Rac activation and cytoskeletal
530 reorganization through interaction with ELMO1. *Blood*. 2003.

531 56. Shaverdashvili K, Wong P, Ma J, Zhang K, Osman I, Bedogni B. MT1-MMP modulates
532 melanoma cell dissemination and metastasis through activation of MMP2 and RAC1. *Pigment Cell
533 Melanoma Res*. 2014;**27**(2):287–296.

534 57. Shinada M, Kato D, Motegi T, et al. Podoplanin Drives Amoeboid Invasion in Canine and
535 Human Mucosal Melanoma. *Mol Cancer Res*. 2023;**21**(11):1205–1219.

536 58. Silver N, Best S, Jiang J, Thein SL. Selection of housekeeping genes for gene expression
537 studies in human reticulocytes using real-time PCR. *BMC Mol Biol*. 2006;**7**(1):33.

538 59. Silvestri S, Porcellato I, Mechelli L, et al. E-Cadherin Expression in Canine Melanocytic
539 Tumors: Histological, Immunohistochemical, and Survival Analysis. *Vet Pathol*. 2020;**57**(5):608–
540 619.

541 60. Smith SH, Goldschmidt MH, McManus PM. A comparative review of melanocytic
542 neoplasms. *Vet Pathol*. 2002;**39**(6):651–678.

543 61. Sondka Z, Dhir NB, Carvalho-Silva D, et al. COSMIC: a curated database of somatic
544 variants and clinical data for cancer. *Nucleic Acids Res*. 2024;**52**(D1):D1210–D1217.

545 62. Suzuki H, Kaneko MK, Kato Y. Roles of Podoplanin in Malignant Progression of Tumor.
546 *Cells*. 2022;**11**(3):575.

547 63. Tal O, Lim HY, Gurevich I, et al. DC mobilization from the skin requires docking to
548 immobilized CCL21 on lymphatic endothelium and intralymphatic crawling. *J Exp Med*.
549 2011;**208**(10):2141–2153.

550 64. Tan B, Li Y, Shi X, et al. Expression of Vav3 protein and its prognostic value in patients
551 with gastric cancer. *Pathol - Res Pract*. 2017;**213**(5):435–440.

552 65. Terasawa M, Uruno T, Mori S, et al. Dimerization of DOCK2 Is Essential for DOCK2-
553 Mediated Rac Activation and Lymphocyte Migration. *PLoS ONE*. 2012;**7**(9):1–7.

554 66. Valastyan S, Weinberg RA. Tumor metastasis: molecular insights and evolving paradigms.
555 *Cell*. 2011;**147**(2):275–292.

556 67. Vandesompele J, De Preter K, Pattyn F, et al. Accurate normalization of real-time
557 quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol*.
558 2002;**3**(7):RESEARCH0034.

559 68. Veloso ES, Gonçalves INN, Silveira TL, et al. ZEB and Snail expression indicates
560 epithelial-mesenchymal transition in canine melanoma. *Res Vet Sci*. 2020;**131**:7–14.

561 69. Wong K, van der Weyden L, Schott CR, et al. Cross-species genomic landscape comparison
562 of human mucosal melanoma with canine oral and equine melanoma. *Nat Commun*.
563 2019;**10**(1):353.

564 70. Xia L, Lin J, Su J, et al. <p>Diallyl disulfide inhibits colon cancer metastasis by suppressing
565 Rac1-mediated epithelial-mesenchymal transition</p>. *OncoTargets Ther*. 2019.

566 71. Xie F, Xiao P, Chen D, Xu L, Zhang B. miRDeepFinder: a miRNA analysis tool for deep
567 sequencing of plant small RNAs. *Plant Mol Biol*. 2012.

568 72. Yang W, Speed T. Design and Analysis of Comparative Microarray Experiments. *Stat Anal
569 Gene Expr Microarray Data*. 2003.

570 73. QC Metrics for Exon and Gene Design Expression Arrays. A summary based on the
571 Affymetrix Quality Assessment of Exon and Gene Arrays White Paper, [http://static1.1.
572 sqspcdn.com/static/f/1438485/21486054/1359060361517/qc_metrics_exon_gene_qrc.pdf](http://static1.1.sqspcdn.com/static/f/1438485/21486054/1359060361517/qc_metrics_exon_gene_qrc.pdf).

573 74. Affymetrix Exon and Gene Array Glossary,
574 https://www.affymetrix.com/support/help/exon_glossary/index.affx.

575 75. Affymetrix Exon Array Background Correction Revision Date: 2005-09-27, Revision
576 Version: 1.0, [https://assets.thermofisher.com/TFS-](https://assets.thermofisher.com/TFS-Assets/LSG/brochures/exon_background_correction_whitepaper.pdf)
577 [Assets/LSG/brochures/exon_background_correction_whitepaper.pdf](https://assets.thermofisher.com/TFS-Assets/LSG/brochures/exon_background_correction_whitepaper.pdf).
578

579 **FIGURE 1:** Comparison of the global gene expression profiles of primary OMMs and
580 matched lymph node metastases. Hierarchical clustering (average group linkage, distance
581 = 1 - Pearson Correlation Coefficient) of 4 primary OMMs (P) and 3 matched lymph node
582 metastases (M) on the basis of the expression levels of 9,964 genes.

583

584 **FIGURE 2:** Fold differences in the median expression levels (measured by exon
585 microarray) of 501 genes between 4 primary OMMs (P) and 3 matched lymph node
586 metastases (M) are depicted. Red spheres denote 7 genes involved in RAC1 signaling-
587 associated regulation of cell migration, whose differential expression was subsequently
588 assayed by RT-qPCR. The red arrow indicates two genes (CORO1A and DOCK2) that
589 displayed highly similar fold change differences in expression. The dotted line represents a
590 permutation testing-adjusted (PTadj.) t-test p-value of 0.05.

591

592 **FIGURE 3:** PCR normalized expression levels. The figure shows the normalized
593 expression value ($2^{-\Delta Cq}$) of target genes examined in the primary tumor samples (white
594 column) and metastasis samples (gray column). The expression levels of ITGA4, VAV3,
595 and CORO1A genes are significantly higher in metastasis compared to primary tumors.
596 Conversely, DOCK2 shows reduced expression in metastasis compared to primary tumor.
597 ELMO1, SPARC, and MMP2 display no statistically significant difference between the two
598 groups ($p > 0.05$). An unpaired t-test is employed for comparing the two groups of data
599 (metastasis vs primary tumor); * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

600

601

Gene Symbol	Reference Sequence	TaqMan assay ID	Exon boundary	Amplicon (bp)
ITGA4	XM_003502135.1	Cg04426096_m1	20-21	64
SPARC	XM_005619272.4	Cf02661382_m1	5-6	67
CORO1A	XM_547069.7	Cf02706862_m1	9-10	159
VAV3	XM_022420692.1	Cf02705614_m1	11-12	148
DOCK2	XM_546246.5	Cf02703791_m1	15-16	85
ELMO1	XM_005628727.3	Cf00919416_m1	5-6	89
MMP2	XM_014109407.1	Cf01548727_m1	1-2	65
ACTB	NM_001195845.2	Cf04931159_m1	-	52
RPS18	NM_001048082.1	Cf02624916_g1	5-6	99
GAPDH	NM_001003142.2	Cf04419463_gH	5-6	54

603

604 **Table 1.** TaqMan Gene Expression Assays. The table summarizes the TaqMan assays used
605 in the study to detect target genes (white boxes) and candidate normalization control genes
606 (grey boxes). Gene symbol (first column), interrogated transcript sequence (second
607 column), TaqMan probe ID (third column), base co-ordinates of exon-exon junction spanned
608 by the used PCR assay (fourth column), and amplicon length (last column) are listed. All
609 selected probes hybridise to two different exons (except for ACTB) and are specific for *Canis*
610 *familiaris* mRNA transcripts. Design of the TaqMan assay for ITGA4 was based on the
611 *Cricetulus griseus* CHO-K1 cell line ITGA4 mRNA transcript, however, an antibody raised
612 against the *Cricetulus griseus* ITGA4 protein demonstrates 100% cross-reactivity with the
613 *Canis familiaris* ITGA4 protein..

614

Functional annotation	Fold enrichment	P-value	Gene expression	
			M>P	P>M
GO:0045785~positive regulation of cell adhesion	15.187	0.015	APBB1IP, VAV3	CYTH3
GO:0030036~actin cytoskeleton organization	7.087	0.017	ELMO1, CORO1A, DOCK2, FMNL1	
GO:0001768~establishment of T cell polarity	47.249	0.041	CCL19, DOCK2	
GO:0007165~signal transduction	4.588	≤0.001	TRAF5, ERBIN, SPOCK2, CDC42SE2, APBB1IP, LNPEP, FYB	SPARC
cfa04514: Cell adhesion molecules	7.002	≤0.0001	PTPRC, CD226, ICOS, PECAM1, DLA-DOB, ITGA4, CD2	
cfa05200: Pathways in cancer	2.741	0.008	TRAF5, IGF1, PLCB2, CDKN1B, STK4, PLCG2, JAK1, LEF1	ADCY2, MMP2
cfa04062: Chemokine signaling pathway	4.951	≤0.001	CCR7, PLCB2, ELMO1, CCL19, CXCL13, DOCK2, VAV3	ADCY2

cfa04670: Leukocyte transendothelial migration	5.001	0.015	<i>PLCG2, PECAM1, VAV3, ITGA4</i>	<i>MMP2</i>
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615

616 **Table 2.** Differentially expressed gene-associated enriched functional annotations. GO:

617 Gene Ontology. M: Metastatic OMMs; P: Primary OMMs.

ALGORITHM	EC RANK		
Delta Cq	ACTB	GAPDH	RPS18
BestKeeper	GAPDH	ACTB	RPS18
NormFinder	ACTB	GAPDH	RPS18
GeNorm	GAPDH ACTB		RPS18
RefFinder comprehensive ranking	ACTB	GAPDH	RPS18

618 **Table 3.** RefFinder ranking stability order. The table shows stability values ranking assigned

619 by RefFinder which integrate the data obtained from the comparative ΔCq method,

620 BestKeeper, NormFinder, and GeNorm algorithms. RefFinder tool generates a

621 comprehensive ranking by ordering the candidate endogenous controls from most stable

622 (first column) to least stable (third column).



623

Figure 1

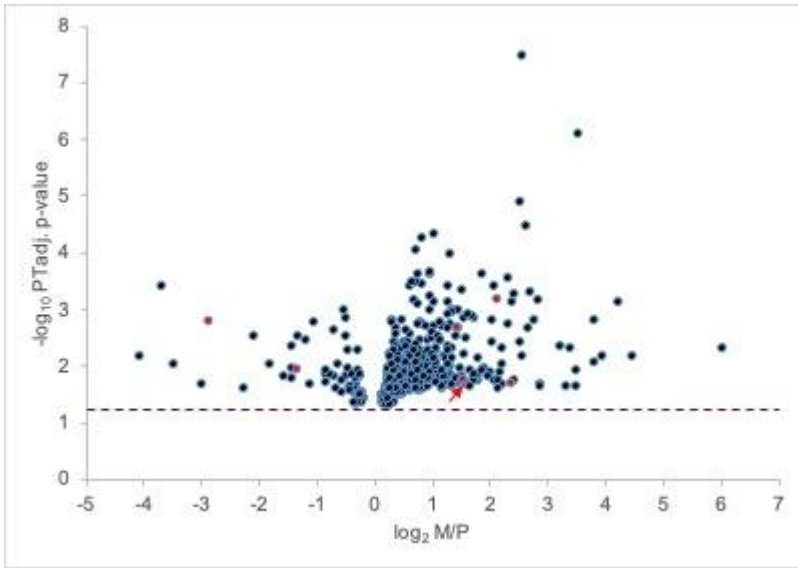
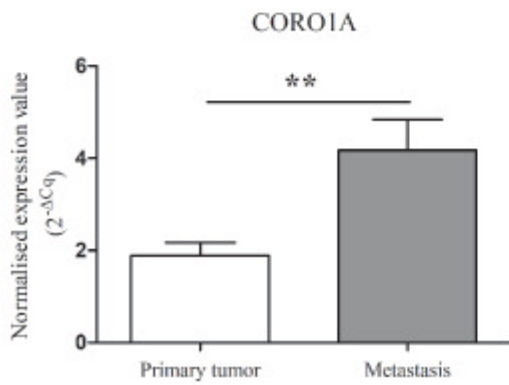
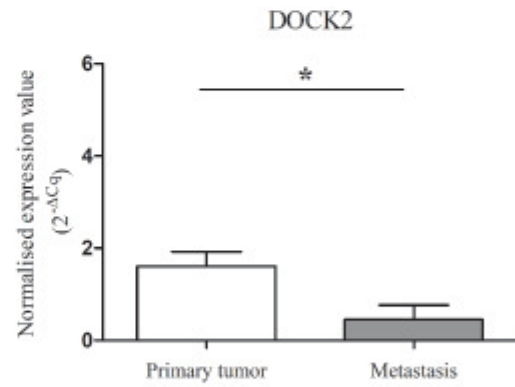
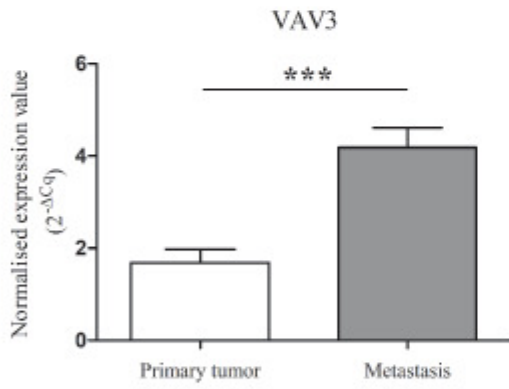
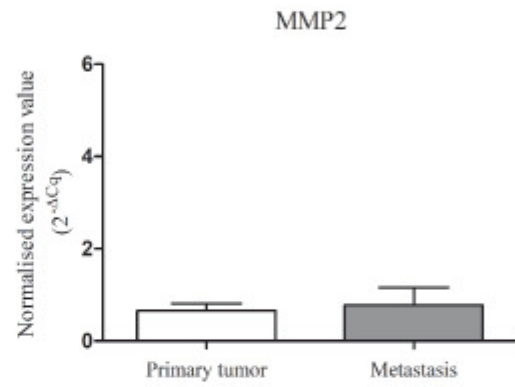
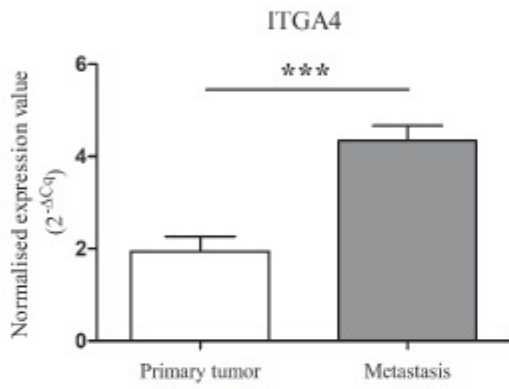
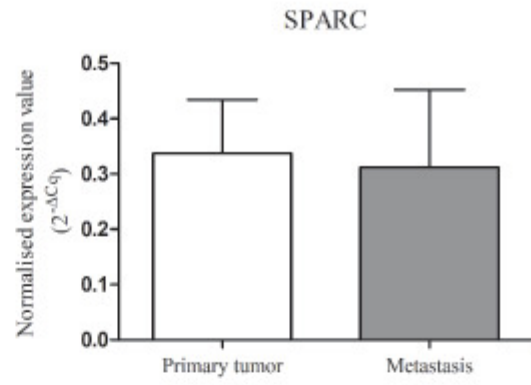
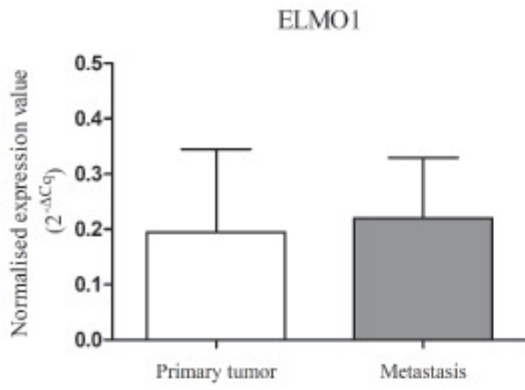


Figure 2

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627 Figure 3

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