

1 Somatic inactivating *PTPRJ* mutations and dysregulated pathways identified in canine melanoma by
2 integrated comparative genomic analysis

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47 **ABSTRACT**

48 Canine malignant melanoma, a significant cause of mortality in domestic dogs, is a powerful
49 comparative model for human melanoma, but little is known about its genetic etiology. We mapped the
50 genomic landscape of canine melanoma through multi-platform analysis of 37 tumors (31 mucosal, 3
51 acral, 2 cutaneous, and 1 uveal) and 17 matching constitutional samples including long- and short-insert
52 whole genome sequencing, RNA sequencing, array comparative genomic hybridization, single nucleotide
53 polymorphism array, and targeted Sanger sequencing analyses. We identified novel predominantly
54 truncating mutations in the putative tumor suppressor gene *PTPRJ* in 19% of cases. No *BRAF* mutations
55 were detected, but activating *RAS* mutations (24% of cases) occurred in conserved hotspots in all
56 cutaneous and acral and 13% of mucosal subtypes. *MDM2* amplifications (24%) and *TP53* mutations
57 (19%) were mutually exclusive. Additional low-frequency recurrent alterations were observed amidst
58 low point mutation rates, an absence of ultraviolet light mutational signatures, and an abundance of
59 copy number and structural alterations. Mutations that modulate cell proliferation and cell cycle control
60 were common and highlight therapeutic axes such as MEK and MDM2 inhibition. This mutational
61 landscape resembles that seen in *BRAF* wild-type and sun-shielded human melanoma subtypes. Overall,
62 these data inform biological comparisons between canine and human melanoma while suggesting
63 actionable targets in both species.

64

65 **AUTHOR SUMMARY**

66 Melanoma, an aggressive cancer arising from transformed melanocytes, commonly occurs in pet
67 dogs. Unlike human melanoma, which most often occurs in sun-exposed cutaneous skin, canine
68 melanoma typically arises in sun-shielded oral mucosa. Clinical features of canine melanoma resemble
69 those of human melanoma, particularly the less common sun-shielded human subtypes. However,
70 whereas the genomic basis of diverse human melanoma subtypes is well understood, canine melanoma
71 genomics remain poorly defined. Similarly, although diverse new treatments for human melanoma
72 based on a biologic disease understanding have recently shown dramatic improvements in outcomes for
73 these patients, treatments for canine melanoma are limited and outcomes remain universally poor.
74 Detailing the genomic basis of canine melanoma thus provides untapped potential for improving the
75 lives of pet dogs while also helping to establish canine melanoma as a comparative model system for
76 informing human melanoma biology and treatment. In order to better define the genomic landscape of
77 canine melanoma, we performed multi-platform characterization of 37 tumors. Our integrated analysis
78 confirms that these tumors commonly contain mutations in canine orthologs of human cancer genes
79 such as *RAS*, *MDM2*, and *TP53* as well mutational patterns that share important similarities with human
80 melanoma subtypes. We have also found a new putative cancer gene, *PTPRJ*, frequently mutated in
81 canine melanoma. These data will guide additional biologic and therapeutic studies in canine melanoma
82 while framing the utility of comparative studies of canine and human cancers more broadly.

83

84 **INTRODUCTION**

85 Human melanoma is of increasing clinical concern. It is one of a few cancers with rising
86 incidence, while five-year survival for patients with metastatic disease has until recently remained low
87 (15-20%) due to a dearth of curative systemic therapies(1). Discovery of frequent activating *BRAF*
88 mutations in melanoma and treatment with selective inhibitors of this mutant kinase has led to
89 dramatic responses in the setting of metastatic disease(2-4). However, not all *BRAF*-mutant melanomas
90 respond to targeted therapy and responses that do occur are often brief and followed by the emergence
91 of drug-resistant disease(5). Moreover, targeted treatment options in melanoma subtypes without
92 activating *BRAF* mutations are limited. New treatment paradigms such as immunotherapy, drug

93 combinations, and alternative dosing strategies may circumvent resistance and broaden the scope of
94 precision medicine in melanoma(6-9), but rapid preclinical study of such regimens requires access to
95 robust models that recapitulate complex tumor features such as intratumoral genomic heterogeneity
96 and tumor-host interactions. Meanwhile, few animal models exist for uncommon molecular or
97 histological melanoma subtypes such as *BRAF* wild-type (*BRAF*_{wt}) or mucosal melanoma.

98 Naturally-occurring canine cancers are increasingly recognized as meeting a need for complex
99 cancer models that develop gradually amidst interactions with host stroma and immune system(10-16).
100 Spontaneous canine malignant melanomas, which are almost universally *BRAF*_{wt} and for which the
101 mucosal subtype is the most prevalent clinically significant form, may fill a specific gap in models of
102 *BRAF*_{wt} and rare histological melanoma subtypes(11). Human mucosal melanoma is an aggressive
103 histological subtype that is predominantly *BRAF*, *RAS*, and *NF1* wild type (Triple Wild Type or TWT) with
104 occasional mutations in *KIT* or *NRAS* and carries a five-year survival rate between 12.3% and 35.3% (17-
105 26). Study of this subtype is limited by its low prevalence, only 1-2% of human melanomas in the United
106 States, with as few as 1,500 cases per year(27). On the other hand, canine malignant melanoma
107 accounts for up to 100,000 yearly cancer diagnoses in the United States, occurring most commonly in
108 the oral mucosa, but also arising in cutaneous and acral epithelium(28-31).

109 Canine malignant melanoma is highly prevalent, closely mirrors human melanoma clinically and
110 pathologically, and is extremely aggressive, with median survival for oral cases being a mere 200
111 days(32-36). However, little is known about its genetic etiology. It is predominantly *BRAF*_{wt} with
112 frequent copy number alterations of regions of canine chromosomes (CFA) 13, 17, 22, and 30, alongside
113 frequent *MYC* amplifications and deletions of *CDKN2A*. Targeted sequencing studies, though limited,
114 have shown that it infrequently bears alterations in other known drivers of human melanoma(32, 36-
115 42). It has been shown that CFA 30 aberrations are characteristic of canine oral melanoma and complex
116 copy number profiles on this chromosome homologous to the same profiles on human chromosome
117 (HSA) 15 in human mucosal melanoma are suggestive of rearrangements that may drive this melanoma
118 subtype (41). Despite the very low prevalence of *BRAF* mutations, immunohistochemistry (IHC) has
119 shown that the mitogen-activated protein kinase (MAPK) and/or phosphoinositide 3-kinase (PI3K)
120 pathways are activated in 52-77% of cases(32, 36-40). These data hint at underlying mutations driving
121 these pathways that could guide future biological exploration and therapeutic development in the
122 canine and human diseases.

123 We therefore set out to map the genomic landscape of canine melanoma using a combination of
124 massively parallel whole genome sequencing (WGS), array-based platforms and targeted sequencing to
125 identify somatic changes driving these cancers. Here we report the identification of recurrent
126 inactivating mutations in the candidate tumor suppressor gene *PTPRJ* in addition to frequent *RAS*
127 mutations, and mutually-exclusive *MDM2* and *TP53* alterations. We thereby define the genomic
128 landscape of these cancers and identify similarities between melanoma subtypes across species while
129 highlighting subtype-specific aberrations that may be used to guide future research.

130

131 RESULTS

132 **Patterns of mutation identified by whole genome analysis of canine melanoma.** We undertook
133 comprehensive analysis of acquired genetic alterations in a discovery cohort of seven melanomas and
134 matched germlines from six dogs (two tumors were derived from one dog) using WGS for detection of
135 subtle sequence alterations alongside long-insert WGS (LI-WGS, see Materials and Methods)(43) for
136 sensitive detection of structural variants. We then performed copy number and targeted gene analyses
137 in an additional 27 tumors and three melanoma cell lines (**Table 1**). Tumors (all primary tumors except
138 one acral metastasis) and matching whole blood were collected through the Van Andel Research

139 Institute from dogs undergoing surgery at specialty veterinary clinics and immediately snap frozen.
140 Diagnosis of melanoma was confirmed by two independent board certified veterinary pathologists in
141 addition to staining for three melanocytic differentiation markers where tissue was available(36, 44).
142 Diverse breeds are represented in this cohort with enrichment for Cocker Spaniels and Golden
143 Retrievers (five dogs of each breed), an equal ratio of male and female dogs and a median age at
144 resection of 11 years. Clinicopathologic characteristics for this cohort are described in **Supplementary**
145 **Table 1** and **Supplementary Figure 1**.

146 For WGS and LI-WGS respectively a median of 38/11-fold sequence coverage and 209/155-fold
147 physical coverage was achieved (**Supplementary Table 2**). Read alignment was performed using the
148 canine reference genome CanFam 3.1 and stringent criteria were used to call somatic sequence variants
149 intersecting Seurat, Strelka and Mutect (Materials and Methods). A total of 31,053 somatic single
150 nucleotide variants (SNVs) and small insertions and deletions (indels) were found with a median of 4,223
151 genome-wide SNVs (range 1,880-6,342) and 316 indels (range 88 - 655) and a median mutation rate of
152 2.03 mutations per callable haploid megabase (range 0.97-3.14, **Table 2**). The genome-wide SNV
153 spectrum showed C:G>T:A transitions to be most prevalent, at a median of 27.09% of total SNVs
154 followed by T:A>C:G transitions (median of 21.19%) and C:G>A:T transversions (median 15.74% ,
155 **Supplementary Figure 2A**). Despite the prevalence of C:G>T:A transitions, most occurred in CpG
156 dinucleotides and were not enriched at dipyrimidines (median 22.5%). Therefore, a canonical UV
157 signature was not present in any of these cases (**Supplementary Figure 2B**)(45, 46). We additionally
158 looked for *TERT* promoter mutations, which have been reported in 71% of human cutaneous
159 melanomas and are associated with UV damage(47), but no mutations were found within one kilobase
160 of the *TERT* transcription start site. While no single mutation was represented at greater than 4% of the
161 SNV population, C:G>T:A in GCG trinucleotides was the most common mutation (median 6.7%) followed
162 by C>T in ACG (median 2.6%) and C>A in TCT (median 2.5%) (**Supplementary Figure 2C**). No evidence of
163 localized hypermutation (kataegis) was identified in these tumors(48).

164
165 **Somatic coding mutations identified in canine melanoma.** Tumors assessed by whole-genome analysis
166 displayed an abundance of somatic SVs and copy number variants (CNVs), with a modest burden of SNVs
167 in coding regions (**Figure 1A and 1B**). The landscape of somatic mutations in the full cohort of 37 tumors
168 based on multi-platform analysis is shown in **Figure 1C**. Circos plots depicting somatic alterations in each
169 tumor in the discovery cohort are shown in **Supplementary Figure 3**. Of the genome-wide SNVs
170 described above, a median of 26 nonsynonymous single-base substitutions and indels occurred within
171 coding regions (nsSNVs, range 14-42) with a median nonsynonymous: synonymous mutation ratio of 2.3
172 (range 1.9-3.9) (**Figure 1B**). We additionally performed RNA sequencing in this cohort, aligning with
173 TopHat and utilizing IGV to manually validate expressed sequence variants (Materials and Methods).
174 Eighty-five percent of nsSNVs (all but 28) identified by WGS were confirmed by their presence in two or
175 more sequencing platforms (**Supplementary Table 3**).

176 A number of mutations in orthologs of human cancer genes were present in a single tumor each
177 and include: *ATF6*, *EPAS1*, *FAT2*, *FAT4*, *FOXA3*, *FOXO1*, *GAB2*, *HRAS*, *KIT*, *KRAS*, *MMP21*, *NRAS*, *PBX1*, and
178 *XPO1*. Although no recurrent SNVs were seen in the discovery cohort, three genes were mutated in two
179 cases: *FAT4*, *LRFN2*, and *PTPRJ*. Of these, only *PTPRJ* was validated in multiple platforms in both cases.
180 Both cases containing somatic *PTPRJ* mutations were mucosal (ND10-166 and ND10-376) and both
181 putatively contained two hits. To determine the prevalence of mutations in a panel of genes whose
182 orthologs are known to play a role in human melanomagenesis, as well as the *PTPRJ* gene mutated in
183 two cases, we performed targeted Sanger sequencing of all protein-coding regions of *BAP1*, *BRAF*, *CDK4*,
184 *GNA11*, *GNAQ*, *KIT*, *KRAS*, *MDM2*, *MITF*, *NF1*, *NRAS*, *PTEN*, *PTPRJ*, and *TP53* in the expanded cohort.

185 *BRAF*, *CDK4*, *GNAQ*, *MDM2*, *MITF*, and *NF1* were all found to be universally wild-type whereas putative
186 pathogenic mutations were discovered in *BAP1*, *GNA11*, *KIT*, *KRAS*, *NRAS*, *PTEN*, *PTPRJ*, and *TP53* as
187 described below and in **Supplementary Table 4**.

188

189 **Somatic copy number and structural variants identified in canine melanoma.** Somatic CNVs in the
190 discovery cohort were identified by analysis of short-insert whole genome sequencing (SI-WGS) using
191 established methods (Materials and Methods). A median of 27 focal CNVs (range 4-68), two focal
192 amplifications with a \log_2 ratio ≥ 2 (range 0-61), and eight focal deletions with a \log_2 ratio ≤ 0.2 (range 3-
193 41) were identified in the discovery cohort (**Table 2 and Supplementary Table 5**) comprising 0%-10% of
194 the genome (**Table 2**). CNVs were additionally identified in this cohort utilizing Illumina CanineHD
195 BeadChip Single Nucleotide Polymorphism (SNP) arrays and Agilent SurePrint G3 Canine Genome CGH
196 microarrays as previously described(41, 49) (Materials and Methods) with a high platform concordance
197 (**Supplementary Figure 4**). CNV analysis was then expanded to a total of 37 melanomas through SNP
198 arrays in an additional 30 cases in the prevalence cohort (**Table 1 and Supplementary Table 5**). Altered
199 regions were assessed by GISTIC(50) for statistically significant frequency and amplitude (G-score >1.0
200 and $Q < 0.05$). Ten significant regions were identified including losses within CFA 1, 11, 15, and X, as well
201 as gains in CFA10, 11, 13, 30, and X (**Supplementary Table 6**). Nine of 10 GISTIC regions contained genes
202 and included gains in orthologs of the human cancer genes *MDM2* and *CDK4*. Additional cancer driver
203 alterations (homozygous deletions of tumor suppressor genes or focal amplifications of oncogenes)
204 included *CDKN2A* homozygous deletion (3%) and *KIT* focal amplification (8%) (**Supplementary Table 7**).

205 Somatic SVs including translocations, inversions, and duplications, were identified in the
206 discovery cohort, based on calls from Delly(51) in LI-WGS (Materials and Methods). Between 9 and 65
207 predicted SVs were identified in each tumor (median 34) and were predominantly inversions (**Table 2**
208 **and Supplementary Table 8**). No recurrent rearrangements were present. Notable alterations in human
209 cancer gene orthologs impacted by SVs in single cases include an *ARHGEF12* inversion, a *BIRC3*
210 inversion, a *CLPTM1L-TERT* translocation, a *DDIT3* inversion, a *MYO5A* translocation, and a *TCF12*
211 inversion. However, two regions of CFA10 and 30 were found to contain somatic SVs in two or more
212 tumors. CFA10 rearrangements occurred in five of seven cases, four of which bore alterations in the
213 region spanning 10 – 12 Mb (also a significant GISTIC region from CNV analysis). CFA30 SVs were also
214 present in three tumors with alterations occurring within a region spanning 15-24 Mb (also
215 encompassing a GISTIC region) in each case. Complex chromosomal rearrangements reminiscent of
216 chromothripsis were observed in four tumors (ND09-345, ND10-370, ND10-361, and ND10-441), with
217 chained or clustered breakpoints localized to a subset of chromosomes in regions that also contained
218 copy-number oscillations(52) (**Supplementary Figure 3**). Gene fusions were also identified in RNAseq
219 data using the TopHat-Fusion software package(53) and IGV verification (Materials and Methods and
220 **Supplementary Table 8**). Three fusions were identified in two tumors (*OSBPL11-NFKB1* and *DGKA-*
221 *ABCC5* in ND09-345, and *RPTOR-TIMP2* in ND10-376) for which translocations were validated in LI-WGS
222 on IGV inspection. No *BRAF* fusions were identified.

223

224 ***BRAF*, *RAS*, *NF1*, and *KIT* mutations.** Approximately 90% of human cutaneous melanomas are driven in
225 part by *BRAF*, *RAS*, *NF1*, and *KIT* mutations that confer constitutive mitogenic signaling through the
226 MAPK pathway(24, 45, 54). However, these alterations are far less common in human mucosal and acral
227 melanomas(20, 22, 23, 55-57). No somatic alterations in *BRAF* were identified within any platform in our
228 canine melanoma cohort. However, *RAS* family members, whose protein products are predicted to
229 share 100% sequence identity with their human orthologs, were the most commonly mutated genes in
230 aggregate, occurring in 24% of cases in human-conserved hotspots (**Figure 1C and 2A**). *NRAS* codon 61

231 (Q61R/H/K) and *KRAS* codon 12 (G12C) mutations occurred each in four cases while a single case bore
232 an *HRAS* Q61R mutation (nine total RAS mutations). All three acral and two cutaneous cases bore *NRAS*
233 or *KRAS* mutations, while only 4/31 (13%) of mucosal cases bore an *NRAS*, *KRAS*, or *HRAS* mutation.
234 Although *NF1* copy number losses occurred in six cases, no homozygous deletions or truncating
235 mutations were identified (**Supplementary Table 7**). *KIT* mutations were present in one cutaneous and
236 two mucosal tumors (**Supplementary Tables 3 and 4**). In the cutaneous case, the mutation results in a
237 glutamine (Q) to arginine (R) change in codon 396, notably a site of variation between canine and
238 human orthologs, a change that is not predicted to be damaging by PROVEAN, and may constitute a
239 germline SNP, but germline DNA was not available in this case(58). *KIT* mutations in the mucosal cases
240 included an in-frame deletion of amino acids 560-562, a likely damaging mutation in a commonly
241 mutated region of the human ortholog, as well as an aspartic acid (D) to valine (V) change in codon 815
242 corresponding to the most common hotspot D816V mutations occurring in the kinase domain of *KIT* in
243 human cancers (**Supplementary Figure 5**)(59). Copy number gains encompassing *KIT* were also present
244 in 10 samples (eight mucosal, one acral, and one cutaneous – Jones, 17CM98, ND10-104, ND10-158,
245 ND10-365, ND10-370, ND10-376, ND10-361, ND10-363, and ND10-441), although no focal amplifications
246 were identified (**Supplementary Table 7**).

247
248 ***PTPRJ* Mutations.** The most commonly mutated gene in this cohort was the putative tumor suppressor
249 gene *PTPRJ*, not previously shown to have frequent inactivating point mutations in cancer (**Figure 1C**
250 **and 2C**). *PTPRJ* (also known as density-enhanced phosphatase 1 (DEP-1) or CD148) is a protein tyrosine
251 phosphatase receptor originally discovered by virtue of its overexpression in dense cultures of human
252 lung fibroblasts(60). It has since been shown to be frequently involved in allelic loss or loss of
253 heterozygosity (LOH) in human cancers and mouse models(61, 62) and to potentially play a role in
254 oncogenesis in diverse cancer types, but somatic homozygous deletions or truncating mutations have
255 yet to be described in cancer from any species and its tumor suppressor status remains controversial(63-
256 71). Canine and human orthologs share 70% sequence identity with a highly conserved C terminus
257 containing the protein tyrosine phosphatase catalytic domain that is nearly 100% identical between
258 species (**Supplementary Figure 6**). Sequencing of *PTPRJ* across all 37 tumors revealed nine mutations in
259 seven cases (all mucosal), comprising 19% of all tumors and 23% of mucosal cases. Six frameshifts or
260 stop gains were discovered in addition to two splice site mutations, a C-terminal 10-amino acid deletion,
261 and a single predicted damaging missense mutation. Two cases – ND10-190 and ND10-376 – contained
262 two mutations each, consistent with putative bi-allelic inactivation of a tumor suppressor gene. Further,
263 LOH was evident by analysis of adjacent SNPs in WGS data in case ND10-166 bearing the M110fs
264 mutation (**Supplementary Table 10**). Consistent with this finding, the *PTPRJ* frameshift in the ND10-166
265 tumor occurred at an allele ratio of 61% in DNA and 100% in RNA.

266
267 ***MDM2* Amplifications and *TP53* Mutations.** Inactivation of the p53 network is a critical step in
268 tumorigenesis in nearly all cancers(72). Both truncating *TP53* mutations and amplifications of *MDM2*, a
269 negative regulator of p53, are key routes to p53 inactivation(73). Although *TP53* mutations and *MDM2*
270 amplifications in human melanoma less common(23-25, 45, 54, 56), 16/37 (43%) of the cases in our
271 cohort of canine melanoma bore focal amplifications of *MDM2* or truncating *TP53* mutations (**Figure**
272 **1C**). A recurrent focal amplification on CFA10 was identified by whole genome analysis in three of seven
273 tumors in the discovery cohort with extended SNP array analysis in the prevalence cohort revealing an
274 additional eight tumors bearing these amplifications (minimal region 10.9-11.8 Mb) (**Figure 1C and 2C**).
275 In total, 11/38 cases (29%) bore this amplification involving seven genes, with *MDM2* being the likely
276 amplification target (**Figure 2B**). All such amplifications occurred in mucosal melanomas (11/31, 35%).

277 *CDK4*, a cancer gene 10 Mb proximal to *MDM2* in both human and canine genomes and often the target
278 of bipartite amplification alongside *MDM2*(74, 75), was co-amplified in three of these cases. Twenty
279 tumors were additionally assessed for *MDM2* expression by IHC (**Supplementary Table 11 and**
280 **Supplementary Figure 7**). Three of five cases with *MDM2* focal amplifications also showed prominent
281 *MDM2* staining while no cases lacking *MDM2* amplifications were positive by IHC.

282 We additionally discovered seven tumors with mutations in *TP53* whose protein product shares
283 80% identity with its human ortholog (**Supplementary Figure 8**). Three of these mutations were
284 truncating – a homozygous T90X in ND10-252, heterozygous K151fs in ND11-201, and a heterozygous
285 Q306X in ND10-564 (**Figure 2D and Supplementary Table 4**). Of the three missense mutations, R145C
286 and R270H were predicted to be damaging. R145C occurred in two tumors and R270H in a single tumor,
287 with both mutations confirmed somatic through analysis of matched germline DNA. Codon 270 in canine
288 *TP53* is homologous to codon 282 in human *TP53*, the fifth most common hotspot for mutations in
289 human cancer(59). The missense G290R variant is a likely SNP. It occurs in a tumor for which matched
290 germline DNA is unavailable and it is predicted to be neutral, although it has not been previously
291 described(76-78). In keeping with findings in other cancers, no sequence mutations were present in
292 *MDM2* and *MDM2* amplifications were mutually exclusive with *TP53* mutations. Further, *TP53* and
293 *MDM2* alterations were mutually exclusive with *RAS* mutations in all but one case (ND10-748, **Figure 1**).

294
295 **Pathway dysregulation in canine melanoma.** Common genomic subtypes of human cutaneous
296 melanoma (*BRAF*, *RAS* (N/H/K), and *NF1* in 90% of cases) that engage oncogenic signaling through the
297 MAPK pathway are less common in human non-cutaneous melanoma and in canine malignant
298 melanoma (24% of cases here, **Figure 1C**). Therefore, to undertake unbiased identification of pathways
299 contributing to canine melanomagenesis, we performed pathway analysis using WGS data from the
300 discovery cohort. We generated a list of all genes bearing nonsynonymous mutations, lying within
301 chromosomal breakpoints or significant CNV regions from GISTIC (n=1047) in order to determine
302 enrichment of these mutated genes within specific KEGG and Reactome pathways (Materials and
303 Methods)(79-81). Network analysis of the affected genes identified 97 pathways with significant
304 Benjamini-Hochberg corrected *P*-values (**Supplementary Table 12**). The most significantly enriched
305 pathways were Insulin Receptor Substrate (IRS)-mediated signaling, and IRS-related events, for which
306 23% (19 genes) of the pathway members are mutated in this cohort. Notably, these pathways converge
307 on MAPK and PI3K mitogenic signaling and contain core pathway members such as *FGFs*, *EIF4G1*, *HRAS*,
308 *KRAS*, *NRAS*, and *RPTOR*. Indeed the majority of the enriched pathways contain members of MAPK, PI3K,
309 or growth factor receptor signaling (e.g. PI3K cascade *P*=0.002, mTOR signaling *P*=0.008, signaling by Rho
310 GTPases *P*=0.012, VEGF signaling *P*=0.017, RAF activation *P*=0.017, melanoma signaling *P*=0.021, RAS
311 signaling *P*=0.031, and MEK activation *P*=0.036) and, in many cases, intersections with *MDM2* signaling.

312 313 **DISCUSSION**

314 Melanoma is a clinically significant disease in dogs, the study of which holds untapped potential for
315 developing clinical approaches to improve the lives of pet dogs while also informing human melanoma
316 biology and treatment. Few treatment options are available for locally advanced or metastatic canine
317 melanoma in part because the molecular etiology is still largely unknown. Similarly, limited molecular
318 understanding of rare sun-shielded and *BRAF*^{wt} subtypes of human melanoma has constrained clinical
319 innovation. In order to identify the molecular alterations underlying canine melanoma, we undertook a
320 comprehensive multi-platform genomic investigation. Our integrated analysis confirms that although
321 these tumors are driven by mutational landscapes distinct from those in human cutaneous melanoma,
322 they share important similarities with *BRAF*^{wt} and rare histological subtypes of human melanoma.

323 These data not only guide biological and therapeutic studies in canine melanoma, but they also lend
324 further support for the use of the naturally occurring canine model in comparative studies of human
325 cancers.

326 This study builds on knowledge of the cytogenetic landscape of canine melanoma(41) to provide
327 a comprehensive view of numbers and types of somatic coding mutations in this cancer. Given the
328 dearth of genomic data for canine melanoma, we initially focused here on collecting primary tumors
329 from diverse breeds. Although numbers were too small to power such analyses, we saw no significant
330 breed-associated alterations in this cohort. Breed-specific somatic mutational landscapes have been
331 shown to occur for other canine cancers such as lymphoma(82). Future expanded study of breed-
332 specific cohorts will be critical for further understanding the role of germline variation in shaping
333 somatic cancer landscapes across species. It will also be important to further define subtype differences
334 in expanded cohorts of canine acral and cutaneous tumors as well as benign and precursor lesions.

335 Overall, the genomic landscapes of human melanoma vary by anatomic site and degree of sun
336 exposure(22, 26, 57). Cutaneous sun-exposed melanoma is characterized both by high point mutation
337 frequencies linked to UV damage(45) and also only modest burdens of structural variation. In contrast,
338 sun-shielded and non-cutaneous melanomas harbor a low point mutation, but high structural mutation
339 burden. Here, we establish that the canine malignant melanoma genome landscape resembles that
340 reported in human sun-shielded melanoma. Canine melanoma of all subtypes in our discovery cohort is
341 likely sun-shielded, including cutaneous tumors which occur in densely hair-bearing skin, although
342 cropping or shaving during summer months may in some cases increase UV exposure. In keeping with
343 this status, WGS in this cohort provides a deep view of genome-wide mutation burden revealing low
344 point mutation frequencies (median 2.03 somatic mutations per Mb) similar to that seen in human acral
345 and mucosal melanoma WGS data from Hayward *et al.* 2017 (**Figure 3A**)(26). This low point mutation
346 burden relative to human sun-exposed melanoma has potential bearing on expected responses to
347 immunotherapy such as anti-CTLA4 and anti-PD1 checkpoint blockade. Numerous studies have shown a
348 clear positive correlation between mutation burden, abundance of neoantigens, and clinical benefit in
349 human melanoma and other cancers(83, 84). Nonetheless, other molecular determinants of response to
350 immunotherapy exist beyond simply mutation burden and the activity of such agents in canine
351 malignant melanoma remains to be determined. Notably, CNV and SV burden from our WGS in canine
352 malignant melanoma was markedly lower than all subtypes as described in Hayward *et al.* (**Figure 3B**
353 **and 3C**) (26).

354 WGS additionally provides a deep view of genome-wide mutation signatures. High point
355 mutation burden in sun-exposed cutaneous melanoma is understood to result from UV-induced over-
356 representation of C>T transitions occurring in dipyrimidines versus non-dipyrimidines. UV-induced C>T
357 mutations occurring in dipyrimidines comprise a low proportion of total SNVs in our cohort (25%),
358 reflective of human sun-shielded cutaneous, mucosal and acral melanoma, in contrast to 85-90% of C>Ts
359 occurring in dipyrimidines in human sun-exposed melanoma (**Figure 3C**)(24, 26, 45, 55, 56, 85). This
360 lends support for a non-UV etiology of canine melanoma.

361 The genome-wide SNV spectrum further revealed that C>T transitions in CpGs were the most
362 common sequence alterations (**Supplementary Figure 2A**). These mutations correlate with age in
363 human cancers and are due to spontaneous deamination of 5-methylcytosine(46). Enrichment for these
364 mutations in canine melanoma is not surprising given that the largest risk factor for cancer in humans
365 and dogs is biological (not chronological) age(86-91) and that the mean age of these dogs at the time of
366 surgical resection was 13 years (range: 10 – 16). Relative to the average number of human somatic
367 mutations, these data provide further evidence that not only cancer incidence, but also mutational
368 burden increases with biological, rather than chronological, age(92). Commonly observed mutational

369 patterns in human melanoma such as kataegis were not observed, although four tumors exhibited
370 clustered or chained translocations suggestive of breakage-fusion-bridge events due to telomere crisis
371 or of chromothripsis, in which one or a few chromosomes undergo punctuated shattering and
372 reassembly events(52). Such events have been linked to poor outcome in human melanoma(93) and
373 may be enriched in tumors with p53 dysfunction or those that lack means to extend telomeres(94, 95).
374 Notably, we show here that *MDM2* and mutually exclusive *TP53* alterations are common in canine
375 melanoma. Similarly, inactivating p53 mutations have been found in human mucosal and acral
376 melanoma, suggesting p53 pathway dysregulation may be crucial in non-UV induced melanoma
377 development. Further, UV-induced *TERT* promoter mutations are common in human cutaneous
378 melanoma, and, although they are rare in sun-shielded subtypes, these subtypes have been shown to
379 bear enrichment for other types of mutation that drive *TERT* overexpression such as SVs and CNVs(57).
380 The cutaneous tumors in this cohort do not bear somatic *TERT* promoter mutations or other known
381 genetic lesions that would enable telomere extension. Thus, telomere crisis and the survival of
382 structurally aberrant genomes may play a significant role in the molecular etiology of canine and non-UV
383 induced human melanoma.

384 Our comprehensive analysis of canine melanoma reveals that most canine melanomas bear a
385 low coding mutation burden and are also less structurally complex than human melanoma. Two WGS
386 approaches coupled with array-based platforms have enabled deep interrogation of these changes,
387 complementing recent cytogenetic analyses of this tumor type(41). Significant copy number gains on
388 CFA10 and 30 that have been reported as a defining signature of these lesions are recapitulated in this
389 dataset (**Supplementary Table 6**). Our multi-platform approach was also able to further elucidate
390 complex chromosomal rearrangements present in these regions. Both regions are involved in multiple
391 intra- and inter-chromosomal structural events across this cohort (**Supplementary Table 8**). Additionally,
392 focal amplification of the CFA10 10-12MB region encompasses *MDM2*, a gene which is known to drive
393 human cancers and is currently being explored as a drug target in TP53 wild type tumors(96). CNVs
394 associated with canine melanoma also include gain of CFA13 and loss of CFA22. While not statistically
395 significant via GISTIC in this cohort, both events are present in individual samples. Overall, extensive
396 copy number and structural variation suggest high levels of large-scale chromosome instability, i.e. gain
397 and loss of whole chromosomes or chromosome arms. Intriguingly, mutually exclusive focal
398 amplification of *MDM2* or inactivating mutation in *TP53* have been shown to be enriched in *BRAF*-,
399 *NRAS*-, and *NF1*-wild-type human melanoma, although human *TP53*-mutant melanomas tend to also
400 display higher mutation burden and presence of C>T transitions(97). Taken together the high degree of
401 structural complexity, the lack of *TERT* mutations or telomere-lengthening mechanisms, and the
402 frequency of *MDM2/TP53* mutations all suggest that chromosome instability plays a key role in canine
403 melanomagenesis.

404 In the discovery cohort, putatively pathogenic somatic mutations in orthologs of human cancer
405 genes were present in a single tumor each including *ATF6*, *EPAS1*, *FAT2*, *FAT4*, *FOXA3*, *FOXO1*, *GAB2*,
406 *HRAS*, *KIT*, *KRAS*, *MMP21*, *NRAS*, *PBX1*, and *XPO1* (**Supplementary Table 3**). Of the 14 melanoma
407 hallmark genes evaluated in the prevalence cohort (including *PTPRJ*), an additional 24 putatively
408 pathogenic somatic mutations were identified in seven genes – *NRAS*, *TP53*, *PTPRJ*, *KIT*, *KRAS*, *GNA11*,
409 and *BAP1* (**Supplementary Table 4**). Overall, across discovery and prevalence analyses, RAS gene family
410 members were the genes most commonly bearing somatic SNVs, occurring in 24% of cases (**Figure 1C**
411 **and 2A**), followed by *TP53* and *PTPRJ* mutations each in 19% of cases, *KIT* in 8% and *PTEN* in 5%.
412 Combined, these mutations most commonly impact proliferative and cell cycle/apoptosis pathways in
413 patterns similar to those observed in human melanoma (**Figure 3D**). These findings also suggest that
414 both MAPK pathway inhibition (via MEK inhibitors) or p53 pathway inhibition (via MDM2 inhibitors) may

415 be of equal relevance in canine melanoma as they are in human(38).

416 The oncogenic MAPK pathway is critically important in many cancers given its central role in
417 conveying extracellular signals to the nucleus in order to regulate cancer hallmarks including
418 proliferation, invasion, metastasis, and angiogenesis. The majority of human cutaneous melanomas are
419 driven in part by constitutive activation of the MAPK pathway through mutation of genes such as *BRAF*,
420 *NRAS*, *NF1*, *KIT*, *GNAQ*, and *GNA11*, often in a mutually exclusive pattern(98). The high frequency of
421 these mutations has motivated the TCGA classification of these tumors according to MAPK mutation
422 status: *BRAF* (~50% of cases), *RAS* (~30%), *NF1* (~15%), and TWT (~10%)(97). These genomic categories
423 are correlated with clinical, pathological, molecular, and biological features of melanoma and thus may
424 comprise distinct subtypes. However, less common histological subtypes of melanoma such as mucosal,
425 acral, and uveal melanoma bear unique mutation spectra that are not uniformly centered on canonical
426 activating mutations in the MAPK pathway. Correspondingly, it has been shown that *BRAF* mutations are
427 exceedingly rare in predominantly oral canine malignant melanoma and, to date, few alterations in
428 other MAPK members have been discovered. These findings were recapitulated in our cohort, which
429 showed no canonical *BRAF* or *NF1* mutations. Nonetheless, MAPK and/or PI3K signaling have been
430 shown to be activated in nearly all cases(99). Additional mutations impacting the MAPK and PI3K
431 pathways include three *KIT* mutations, two *PTEN* mutations, and one *GNA11* mutation. In total, 35% of
432 mucosal and 43% of all canine melanomas bear an alteration impacting the MAPK pathway (**Figures 1C**
433 **and 3D**). Prior to our studies described here, the mutations underlying such activation have remained
434 largely unknown.

435 Here we show a complete absence of somatic *BRAF* mutations (SNVs, CNVs, translocations, or
436 fusions encompassing the *BRAF* locus) in canine malignant melanoma in keeping with prior studies(32,
437 37, 41, 100). We also did not uncover truncating SNVs in or homozygous deletions of *NF1*. A higher
438 proportion of our cohort bear *RAS* mutations than the 6-13% previously described(32, 99), although
439 prior studies have focused almost exclusively on *NRAS* exons one and two. All three major RAS family
440 members are highly conserved (100% protein identity) between canine and human. In humans, of these
441 family members, malignant melanomas predominantly bear *NRAS* mutations with only very rare *KRAS*
442 and *HRAS* mutations. In our cohort, we found four *NRAS* codon 61 alterations (11%), four *KRAS* G12C
443 mutations and one *HRAS* Q61R mutation. Further, four of these RAS alterations (two *NRAS*, one *KRAS*,
444 and one *HRAS* mutation) occur in mucosal tumors, a frequency of 13% in this subtype. However, in our
445 cohort all three acral tumors and both cutaneous tumors had detectable *RAS* alterations (three *KRAS*
446 and two *NRAS* mutations). This unusual pattern of *RAS* mutation in canine melanoma may reflect
447 important differences in biological, tissue, and species specificities of RAS family members.

448 These data point to the genomic lesions underlying MAPK and PI3K activation in a substantial
449 proportion of canine melanomas, and to subtle genetic differences in disease subtypes within and
450 across species. Most striking is the discovery of a putative novel tumor suppressor gene, *PTPRJ*, a
451 receptor-type protein tyrosine phosphatase, which has been genetically and functionally implicated in
452 cancer (61, 62), but for which clear genetic mechanisms of inactivation have yet to establish its definitive
453 role as a canonical tumor suppressor gene. *PTPRJ* consists of an extracellular domain with eight
454 fibronectin III motifs, a transmembrane domain, and an intracellular catalytic domain. It was originally
455 cloned from HeLa cells and characterized by overexpression and hyper-activation in dense cultures of
456 fibroblasts, by regulation of contact inhibition, and by its role in regulation of cancer cell proliferation
457 and invasion(60, 101-106). Early genetic studies of quantitative trait loci for mouse cancer susceptibility
458 with homologous regions in human cancers pointed to recurrent *PTPRJ* deletions, LOH, and missense
459 mutations in small cohorts of colorectal (49%), lung (50%), and breast (78%) carcinomas in addition to a
460 correlation between *PTPRJ* LOH and colorectal cancer progression(61, 62). Additional sequencing studies

461 in larger cohorts have identified nonsynonymous SNPs in the extracellular fibronectin repeats associated
462 with risk of developing thyroid, colorectal, head and neck squamous cell, and esophageal cancers(67, 70,
463 107-109). More recently, a subclonal K1017N missense mutation in the non-catalytic cytoplasmic
464 domain of PTPRJ was identified in a primary breast tumor with significant enrichment in a brain
465 metastases and patient-derived xenograft(110). PTPRJ substrates that may mediate its tumor
466 suppressive potential include ERK1/2, Akt, various receptor tyrosine kinases, and Src kinases(42, 111-
467 115). However, *Ptprj* knockout mice have normal development with no cancer predisposition and thus
468 inactivation of this gene does not appear to be sufficient to induce tumorigenesis(65). Across all TCGA
469 studies published to date, the frequency of mutations and/or homozygous deletions appears to be low
470 (400 altered cases), although truncating mutations have been found to comprise 31 of the 257
471 mutations identified alongside 56 missense mutations predicted to be of medium or high impact
472 (**Supplementary Figure 9 and Supplementary Table 13**)(116, 117). Only 10 mutations are present in the
473 TCGA human cutaneous melanoma data set (a single homozygous deletion and nine missense
474 mutations) with two missense mutations in desmoplastic melanoma and no detectable mutations in
475 uveal melanoma. However, a related receptor-type protein tyrosine phosphatase, *PTPRD*, is thought
476 play a role in regulation of STAT3 signaling and has been frequently implicated as a tumor suppressor in
477 human cancers through inactivating somatic mutation, focal deletion or methylation in glioma,
478 melanoma, neuroblastoma, colorectal, liver, head and neck, and lung cancers(118-121). In human
479 cutaneous melanoma, *PTPRD* is deleted or truncated in 9-12% of cutaneous cases, but has not been
480 determined to occur at high frequency in rare histological subtypes(49, 55, 56, 119, 122).

481 Here, we present the first report of recurrent somatic truncating mutations in *PTPRJ* in a
482 naturally occurring cancer. We have discovered seven cases (19%) of canine melanomas bearing somatic
483 *PTPRJ* mutations. Canine and human *PTPRJ* orthologs share 70% sequence identity with a highly
484 conserved C-terminus containing the protein tyrosine phosphatase catalytic domain (**Supplementary**
485 **Figure 6**). Sequencing of *PTPRJ* across all 38 tumors revealed nine mutations in seven cases (seven
486 mucosal and one uveal) comprising 19% of all tumors and 23% of mucosal cases. Six frameshifts or stop
487 gains were discovered in addition to one splice site mutation, a C-terminal 10-amino acid deletion, and a
488 single predicted damaging missense mutation. Two cases – ND10-190 and ND10-376 – contained two
489 mutations each, consistent with bi-allelic inactivation of a tumor suppressor gene. Further, loss of
490 heterozygosity (LOH) was evident by analysis of adjacent SNPs in WGS data in case ND10-166 bearing
491 the M110fs mutation (**Supplementary Table 10**). Although regional LOH on chromosome 18 was
492 observed by SNP array in three of six cases bearing single mutations in *PTPRJ*, these regions were not
493 observed to directly overlap the coding region of *PTPRJ*. Overall, the enrichment for *PTPRJ* truncating
494 mutation in canine malignant melanoma bears intriguing implications both for a previously
495 underappreciated role for this gene in human melanoma (e.g. through as-yet understudied roles for
496 hemizygous deletion(123) and/or epigenetic modifications) and for the possibility of unique mechanisms
497 of tumorigenesis across species.

498 Through deep integrated genomic analysis combining WGS, LI-WGS, RNA sequencing, aCGH,
499 SNP arrays, and targeted Sanger sequencing we have determined that canine melanoma is driven by
500 extensive chromosomal instability and frequent dysregulation of MAPK and cell cycle/apoptosis
501 pathways. In keeping with prior comparative melanoma studies that have incorporated histology,
502 targeted sequencing, and aCGH(32, 36, 38, 41), this work highlights the striking resemblance of canine
503 malignant melanoma to sun-shielded, *BRAF*wt subtypes of human melanoma. Finally, we have
504 additionally discovered a putative novel tumor suppressor that may reflect unique species-specific
505 biology and/or may highlight a tumor suppressive axis more subtly altered and as-yet underappreciated
506 in human melanoma. This work bears immediate relevance for development of improved diagnostic and

507 treatment approaches in canine malignant melanoma and provides further evidence to credential the
508 naturally occurring canine melanoma model for study of relevant genomic subsets of human melanoma.
509

510 **MATERIALS AND METHODS**

511 *Clinical samples, histopathology and sample assessment*

512 Tumor samples and whole blood were obtained under institutional review protocols at the Van Andel
513 Research Institute in collaboration with local specialty veterinary clinics. Material was collected at
514 surgery, immediately snap frozen, and preserved in optimal cutting temperature (OCT) compound.
515 Patient matched control DNA was obtained from peripheral blood mononuclear cells. Each resected
516 tumor was evaluated by a board certified pathologist (BD) to estimate tumor content and extent of
517 tissue heterogeneity. Diagnosis of malignant melanoma was histologically confirmed according to
518 criteria defined by the American College of Veterinary Pathologists in addition to criteria recently
519 established by comparative analyses of canine and human melanoma focusing on architecture,
520 pigmentation, and the presence of differentiation markers(32, 99, 124).

521

522 *Immunohistochemistry*

523 Two tissue microarrays (TMAs), designated Dog MEL A TMA and Dog MEL B TMA, consisted of 96
524 individual dogs and 131 tissue samples placed in duplicate and two tissue samples placed in
525 quadruplicate (272 array spots). Multiple tumors from nine dogs were present on the array and multiple
526 samples from varying sites within the same tumor were present for twelve dogs. Additionally, non-
527 melanoma stromal or control normal tissues were included. TMAs were H&E-stained and evaluated via
528 routine immunohistochemical procedures for melanoma cocktail (anti-melan A, anti-melanosome, and
529 anti-tyrosinase), and antibodies to vimentin, MDM2 and p53. Samples scoring positive for MDM2
530 staining were then confirmed for positive staining with melanoma cocktail and re-evaluated for p53
531 staining. Positive staining was counted if at least one of the two duplicate samples could be evaluated
532 for both MDM2 and melanoma cocktail on the TMA. Antibodies were purchased from Santa Cruz
533 Biotechnology or Cell Marque. A total of 98 dogs and 189 spots/samples (132 tumors) met these criteria
534 for evaluation for MDM2 protein expression by IHC. Of these 98 dogs, 18 dogs (17%) had melanocytic
535 tumors positive for MDM2 staining in 33 spots/samples (25%). MDM2 staining was predominantly
536 cytoplasmic highest intensity at junction between epithelial and subepithelial (submucosa, dermis).
537 Staining was observed in both malignant pigmented and amelanotic melanoma and benign
538 melanocytomas. Most intense staining (4+ cytoplasmic and nuclear) was observed in a benign cutaneous
539 melanocytoma from a boxer that had additionally a malignant melanoma (negative for MDM2 staining
540 on the array) and multiple cutaneous mast cell tumors.

541

542 *Nucleic acid extraction from tumor tissue and blood*

543 Tissue was disrupted and homogenized in Buffer RLT plus (Qiagen AllPrep DNA/RNA Mini Kit), using the
544 Bullet Blender™, Next Advance, and transferred to a microcentrifuge tube containing Buffer RLT plus
545 and 1.6 mm stainless steel beads or 0.9 mm–2.0 mm RNase free stainless steel beads. Blood leukocytes
546 (buffy coat) were isolated from whole blood by centrifugation at room temperature and resuspended in
547 Buffer RLT plus. All samples were homogenized, centrifuged at full speed, and lysates were transferred
548 to Qiagen AllPrep spin columns. Genomic DNA and RNA were then purified following the manufacturer's
549 protocol. DNA was quantified using the Nanodrop spectrophotometer and quality was assessed from
550 260/280 and 260/230 absorbance ratios. RNA was analyzed on the Agilent Bioanalyzer RNA 6000 Nano
551 Chip to validate RNA integrity (RIN≥7.0).

552

553 *Library construction and next generation sequencing*

554 Three µg of genomic DNA from each sample was fragmented to a target size of 300–350 base pairs (bp).
555 Overhangs in the fragmented samples were repaired and adenine bases were ligated on. Diluted paired

556 end Illumina adapters were then ligated onto the A-tailed products. Following ligation, samples were run
557 on a 3% TAE gel to separate products. Ligation products at 300 bp and 350 bp were selected for each
558 sample, isolated from gel punches, and purified. 2× Phusion High-Fidelity PCR Master Mix (Finnzymes;
559 catalog#F-531L) was used to perform PCR to enrich for these products. Enriched PCR products were run
560 on a 2% TAE gel and extracted. Products were quantified using Agilent's High Sensitivity DNA chip
561 (catalog#5067-4626) on the Agilent 2100 Bioanalyzer (catalog#G2939AA).

562 Long insert whole genome libraries were constructed as previously described(125) with the
563 following modifications: 1100ng inputs were used; following DNA fragmentation, a bead purification
564 was performed at a 1:1.8 (sample volume: bead volume) ratio; a 1% size selection gel was used; and
565 during library enrichment, 10 PCR cycles was used. Libraries were clustered onto Illumina V3 flowcells
566 (San Diego, CA) using Illumina's TruSeq PE Cluster Kit V3 (cat#PE-401-3001) and sequenced for paired
567 100bp reads using Illumina's TruSeq SBS Kit V3 (cat#FC-401-3002, n=3) on the Illumina HiSeq.

568 10 ng of total RNA was used to generate whole transcriptome libraries for RNA sequencing.
569 Using the Nugen Ovation RNA-Seq System (cat#7100-08), total RNA was used to generate double
570 stranded cDNA, which was amplified using Nugen's SPIA linear amplification process. Amplified cDNA
571 was input into Illumina's TruSeq DNA Sample Preparation Kit – Set A (cat#FC-121-1001) for library
572 preparation. In summary, 1 µg of amplified cDNA was fragmented to a target insert size of 300 bp and
573 end repaired. Samples were then adenylated and indexed paired end adapters were ligated. Ligation
574 products were run on a 2% TAE gel and size selected at 400 bp. Ligation products were isolated from gel
575 punches and purified. Cleaned ligation products were input into PCR to enrich for libraries. PCR products
576 were cleaned and quantified using the Agilent Bioanalyzer.

577 Tumor and normal libraries were prepared for paired end sequencing as described above.
578 Clusters were generated using Illumina's cBot and HiSeq Paired End Cluster Generation Kits (catalog#PE-
579 401-1001) and sequenced on Illumina's HiSeq 2000 using Illumina's HiSeq Sequencing Kit (catalog#FC-
580 401-1001).

581 *Next generation sequencing data analysis*

582 BCL to FASTQ file conversion was performed using Illumina's BCL converter tool. Read alignment was
583 performed with BWA (Burrows-Wheeler Aligner)(126) using the canine reference genome CanFam 3.1.
584 Aligned BAMs were indel (insertion/deletion) realigned and recalibrated using GATK(127, 128) and
585 duplicate pairs marked using Picard (<http://broadinstitute.github.io/picard/>). Variants were called using
586 Strelka(129), Seurat(130) and MuTect(131) and calls were annotated according to dbSNP 139, SNPs on
587 the Illumina CanineHD BeadChip, and SnpEff-3.5(132). Final somatic SNVs were called by at least 2/3
588 callers. LI-WGS data were utilized for CNV and SV detection. For CNV detection, read depths at every
589 100 bases across sequenced regions were determined. Next, normalized \log_2 fold-changes between
590 tumor and normal were calculated and a smoothing window applied. Tumor allele frequencies of known
591 heterozygous germline SNPs were utilized to evaluate potential false positives and correct biases.
592 Finally, the Circular Binary Segmentation algorithm(133) was used to correct \log_2 fold-changes. For
593 mutation burden metrics, a focal CNV is included if the \log_2 change is $\geq |2|$. SV detection was
594 performed utilizing Delly(51). A minimum tumor allele ratio of 0.10 and a minimum quality score of 20 is
595 required for an SV to be called.

596
597 RNA sequencing data in FASTQ format from the Illumina HiSeq was checked for quality using
598 cycle-by-cycle quality plots and biases, such as GC content. Reads were aligned to the canine reference
599 genome CanFam 3.1 using the TopHat spliced aligner to generate alignment files in BAM
600 format(134). These data were utilized for validation of expressed sequence variants in IGV. Gene fusions
601 were also identified in RNAseq data using the TopHat-Fusion software package(53) and IGV verification.

602 Results were reported in tables showing p-values (adjusted for multiple testing) and normalized
603 abundance data in terms of FPKM (fragments per kilo-base of transcript per million mapped
604 reads) which were also examined manually. Gene and transcript annotations were downloaded from
605 ENSEMBL (CanFam 3.1.68) and germline SNPs filtered out using publicly available canine SNP data (71-
606 73).

607

608 *Data access*

609 Next generation sequencing data from this study have been submitted to the NCBI Biosample Database
610 (<http://www.ncbi.nlm.nih.gov/biosample/7196161>) under study ID SUB2752127 and accession numbers
611 SAMN07196161, SAMN07196162, SAMN07196163, SAMN07196164, SAMN07196165, SAMN07196166,
612 and SAMN07196167.

613

614 *Pathway analysis*

615 A list of 1,405 genes with single nucleotide variation or structural variation or copy number variation
616 from the discovery cohort were analyzed using ClueGo4(79), a Cytoscape plug-in, to create a
617 functionally organized pathway network. Kappa scores were then used to measure association between
618 the networks. Functional networks were created with a minimum Kappa score threshold of 0.5 and a
619 minimum of 3 affected genes in every network forming at least 10% of the total associated genes in that
620 particular network. The genes were assigned to the networks based on the predefined pathways from
621 KEGG, REACTOME and Wiki Pathways. 97 pathways were obtained, all with Benjamini-Hochberg
622 corrected P-value <0.05. These pathways were grouped together based on inter-term kappa score and
623 named by the most significant pathway in the respective groups.

624

625 *PCR amplification and Sanger sequencing analysis*

626 PCR amplification of 15 genes (*NRAS*, *KRAS*, *BRAF*, *GNA11*, *GNAQ*, *PTPRJ*, *TP53*, *MDM2*, *BAP1*, *CDK4*,
627 *PTEN*, *c-KIT*, *MITF* and *NF1*) was performed using primers targeting all coding exons as shown in
628 Supplementary Table 9. All amplification reactions were performed using Platinum Taq DNA Polymerase
629 #10966-034 (Life Technologies; Carlsbad, CA). Briefly, each primer pair was mixed with 10 ng of genomic
630 DNA and subjected to the following cycling parameters: 94°C for 2 min., 3 cycles at each temperature:
631 30 sec. at 94°C, 30 sec. at 60-57°C, 45 sec. at 72°C; 25 cycles: 30 sec. at 94°C, 30 sec. at 62°C, 45 sec. at
632 72°C; final extension of 5 min. at 72°C. PCR amplicons were sequenced using M13 forward and reverse
633 primers at the Arizona State University's DNA Lab (Tempe, AZ).

634

635 *Array comparative genomic hybridization*

636 Oligo array CGH (aCGH) was performed by co-hybridization of tumor (test) DNA and a common
637 reference DNA sample, where the latter comprised an equimolar pool of genomic DNA samples from
638 multiple healthy individuals of various breeds. DNA was labeled using an Agilent SureTag Labeling Kit
639 (Agilent Technologies, Santa Clara, CA) with all test samples labeled with Cyanine-3-dCTP and the
640 common reference sample labeled with Cyanine-5-dCTP. Fluorochrome incorporation and final probe
641 concentrations were determined using routine spectrophotometric parameters with readings taken
642 from a Nanodrop1000. Fluorescently labeled test and reference samples were co-hybridized to Canine
643 G3 180,000 feature CGH arrays (Agilent, AMADID 025522) for 40 h at 65 °C and 20 rpm, as described
644 previously(135, 136). Arrays were scanned at 3 µm using a high-resolution microarray scanner
645 (Agilent, Model G2505C) and data extracted using Feature Extraction (v10.9) software. Scan data were
646 assessed for quality by the 'Quality Metrics' report in Agilent's Feature extraction software (v10.5)
647 (Agilent Technologies).

648

649 *SNP array genotyping*

650 SNP genotyping was performed using the Illumina CanineHD array (cat#WG-440-1001). Per
651 manufacturer's protocol, 200ng of DNA was first denatured then neutralized with 0.1N NaOH before
652 amplification at 37°C for 24 hours. The amplified DNA was then enzymatically fragmented and
653 precipitated using 100% 2-propanol before drying for one hour at room temperature. After
654 resuspension the fragmented DNA was then denatured and loaded onto the CanineHD BeadChip and
655 hybridized for 16 hours at 48°C. BeadChips were washed, a single base extension of hybridized primers
656 added followed by multi-layer staining of the primers. Arrays were then washed, coated with the XC4
657 reagent (Illumina) and dried under vacuum for one hour. Coated arrays were read on the HiScan system
658 and data visualized using the Illumina Genome Studio Genotyping 2.0 software with an average sample
659 call rate of 97%.

660

661 *aCGH and SNP array data analysis*

662 For both aCGH and SNP arrays, copy number data were analyzed with NEXUS Copy Number v8.0
663 software (Biodiscovery Inc., CA, USA). For cross-platform comparisons, LI-WGS BAMs were also analyzed
664 utilizing Nexus software. CNVs were identified using a FASST2 segmentation algorithm with a
665 significance threshold of 5.5×10^{-6} . Aberrations were defined as a minimum of three consecutive probes
666 with log2 tumor: reference value of >1.14 (high gain), 1.13 to 0.2 (gain), -0.23 to -1.1 (loss), <-1.1 (big
667 loss). Recurrent CNVs within each subtype were determined within NEXUS using an involvement
668 threshold of 50 %. Significance of these regions was then determined in NEXUS using the GISTIC
669 algorithm (to identify regions with a statistically high frequency of CNVs over background) with a G-
670 score cut off of $G > 1.0$ and a significance of $Q < 0.05$. CNV frequency comparisons amongst sample groups
671 were performed in NEXUS using Fisher's exact test with differential threshold of >50 % and significance
672 $p < 0.05$. Significance of each probe between the two groups was calculated in NEXUS using a Mann-
673 Whitney test for median comparison.

674

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683

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1035 **Tables and Figures**

Table 1. Summary of Genomic Analyses Performed in Canine Melanoma

Analysis platform	Type of alteration detected	Samples analyzed
Discovery cohort		
WGS	Point mutations, copy number, structural alterations	7 tumor and 6 matching normal samples
LI-WGS	Copy number and structural alterations	
mRNASeq	Expressed point mutations and gene fusions	
aCGH	Copy number alterations	
SNP-A	Copy number alterations	
Prevalence cohort		
Targeted Sequencing	Point mutations	27 tumor and 11 matching normal samples, 3 cell lines
SNP-A	Copy number alterations	
Total distinct samples		34 tumor samples, 18 matching normals, 3 cell lines

1036

Table 2. Summary of whole-genome analysis in canine melanoma discovery cohort

Sample Information					SNVs			CNVs				SVs				
Sample	Tumor Type	Breed	Gender	Age at Diagnosis	SNVs	Indels	Mut Rate	CNVs	CNV%	Amp	Del	SVs	CTXs	Invs	Del	Dups
ND09-345	Mucosal	ECS	F	11	4223	264	2.03	41	0.4%	33	8	56	15	17	17	7
ND10-370	Mucosal	LR	M	10	6342	655	3.14	64	2.1%	23	41	65	9	22	21	13
ND10-376	Mucosal	CS	F	16	5085	344	2.48	4	0.3%	0	4	25	2	10	5	8
ND10-166	Mucosal	CS	M	14	3395	316	1.23	68	0.7%	61	7	34	2	11	12	9
ND10-361	Mucosal	CS	M	15	3029	88	1.42	5	0.0%	2	3	24	6	10	3	5
ND10-363	Acral	CS	M	15	4906	323	2.45	11	0.2%	2	9	9	0	2	5	2
ND10-441	Cutaneous	CS	F	11	1880	203	0.97	27	9.9%	0	27	39	8	12	12	7

SNV = somatic single nucleotide variant; Mut Rate = Mutation Rate (SNVs + Indels / Callable Mb)

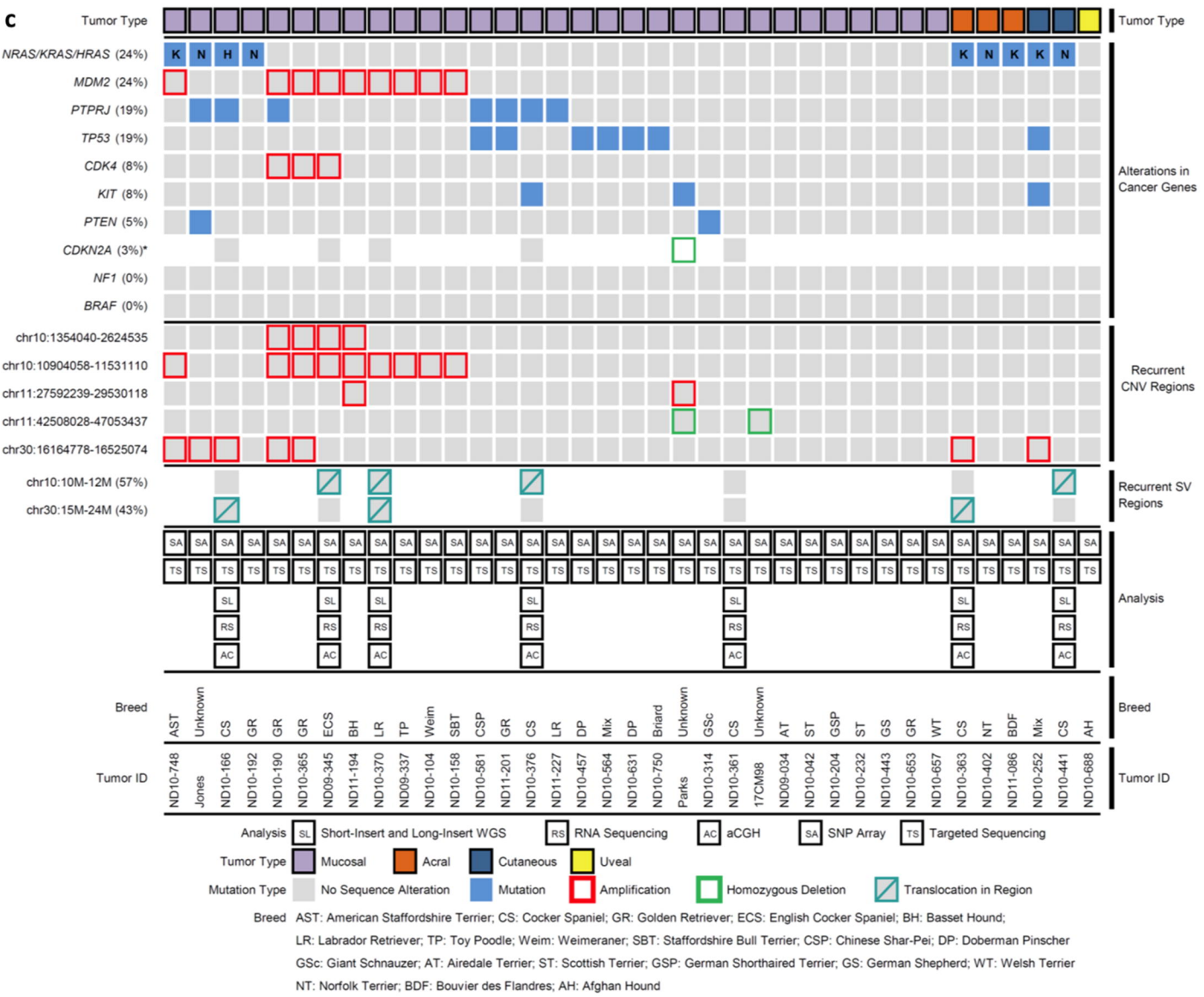
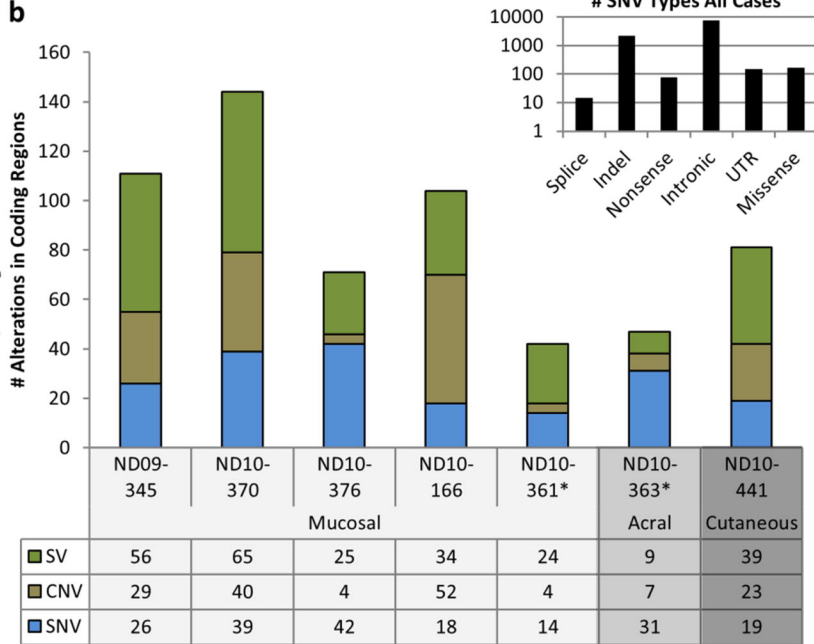
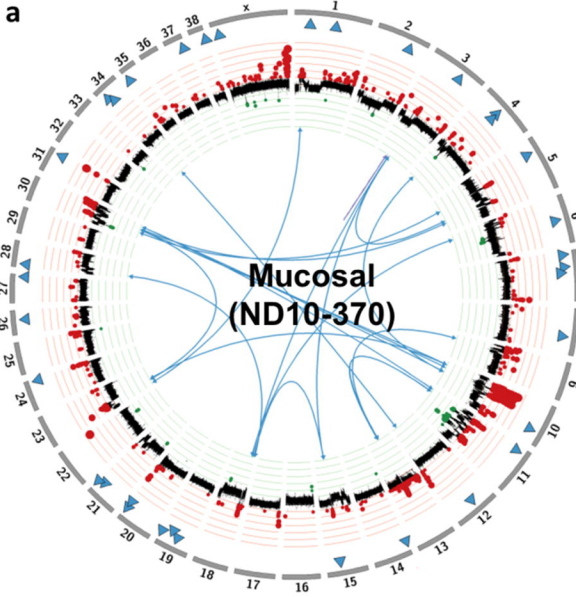
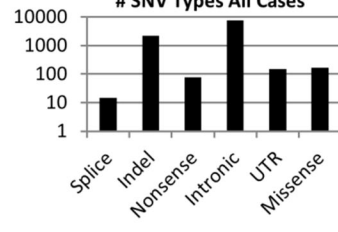
CNV = somatic copy number variant; CNV% = percentage of genome involved in CNVs; Amp = amplification-logratio >=2; Del = deletion-logratio <= -0.6

SV = somatic structural variant from LI; CTX = inter-chromosomal translocation; Inv = inversion; Del=Deletion; Dup = duplication

ECS = English cocker spaniel; LR = Labrador Retriever; CS = Cocker spaniel

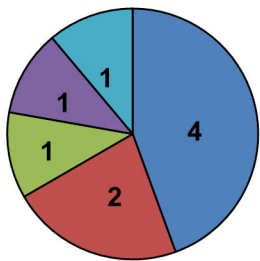
1037

SNV Types All Cases



*Sequence data not available (targeted sequencing not performed for CDKN2A locus)

Figure 1. The mutational landscape of canine melanoma. (a) A representative Circos plot depicting coding SNVs, CNVs, and SVs in a single mucosal melanoma. Outer circle depicts canine chromosome number. Blue triangles are SNVs located within coding regions. The middle circle denotes CNVs with gains (in red) and losses (in green) according to the aberration amplitude. Blue or red lines transecting the plot show translocations. (b) Numbers and types of coding mutations identified by SI-WGS and LI-WGS in the discovery cohort. *ND10-361 and ND10-363 are independent primary tumors from the same dog. (c) Integrated genomic data is presented for 34 canine melanomas and 3 canine melanoma cell lines. Each column represents data from a single tumor. Indication of tumor type (mucosal, uveal, acral, and cutaneous) is displayed above annotation of recurrently-mutated and hallmark genes. Mutations identified by WGS, aCGH, SNP array, and targeted sequencing are presented in order of frequency as are recurrent CNV regions identified by SNP array and GISTIC as well as recurrent regions involved in translocations identified by LI-WGS. Genomic analysis annotation, tumor ID, and figure legend are presented at the bottom of the figure.

a

- KRAS G12C
- NRAS Q61R
- NRAS Q61H
- NRAS Q61K
- HRAS Q61R

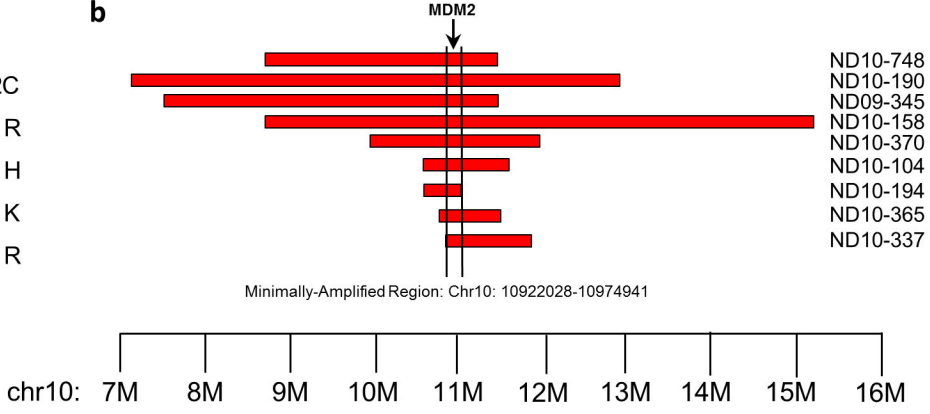
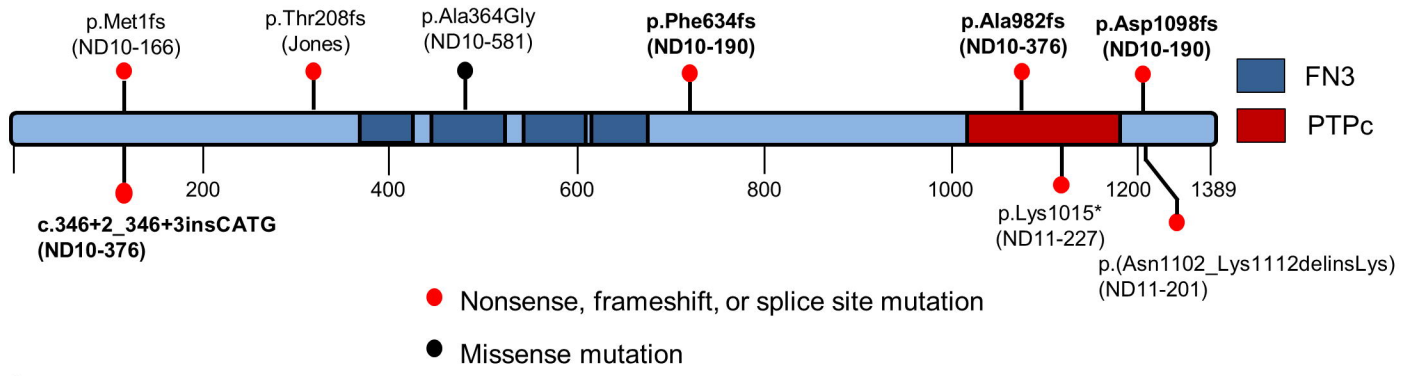
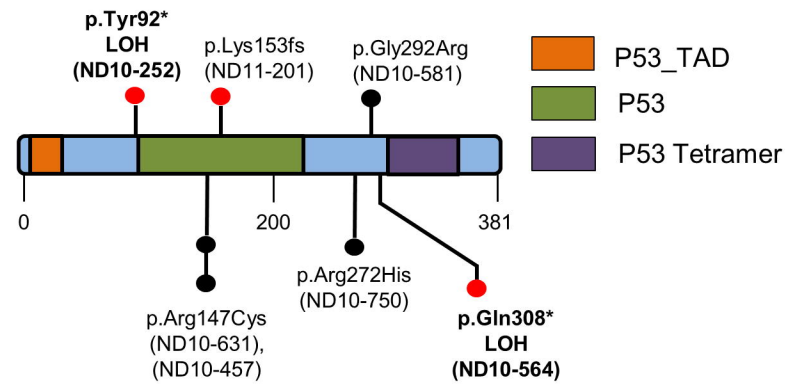
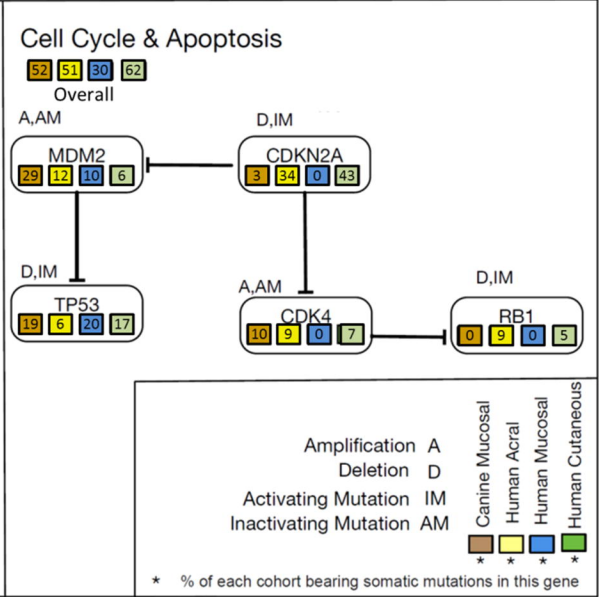
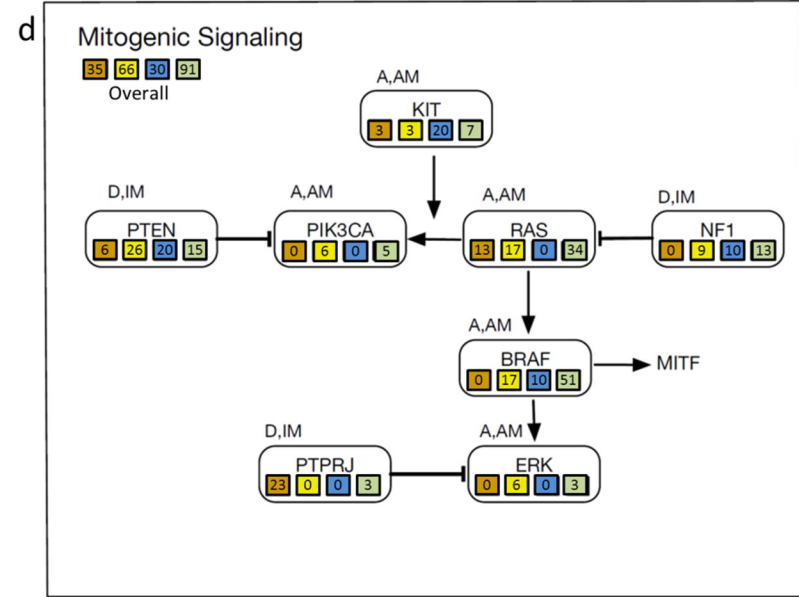
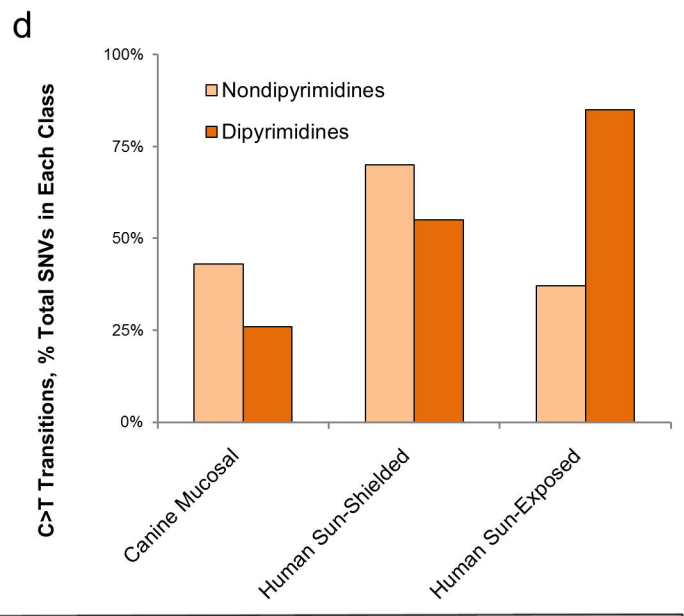
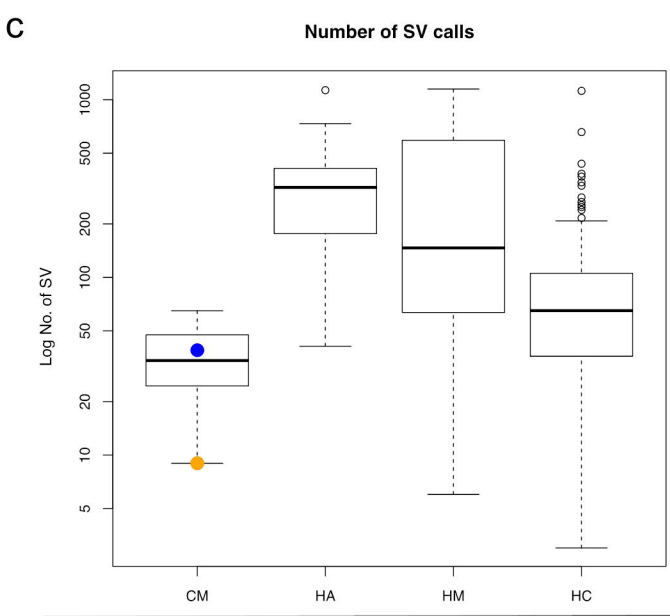
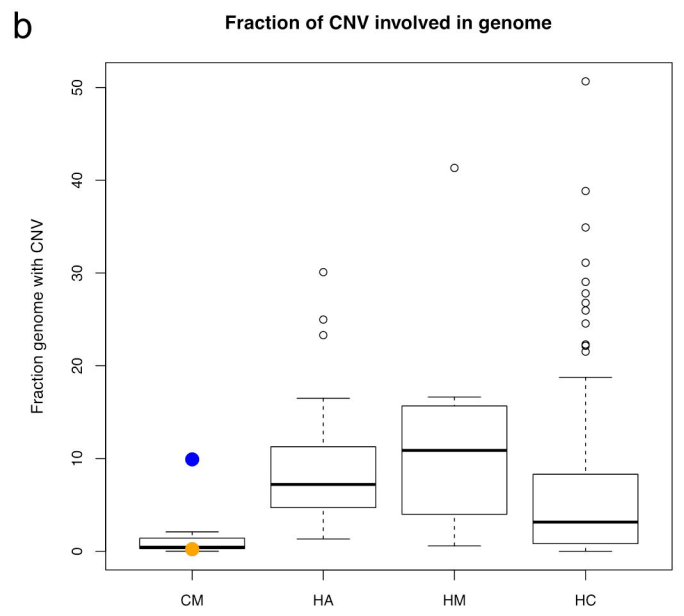
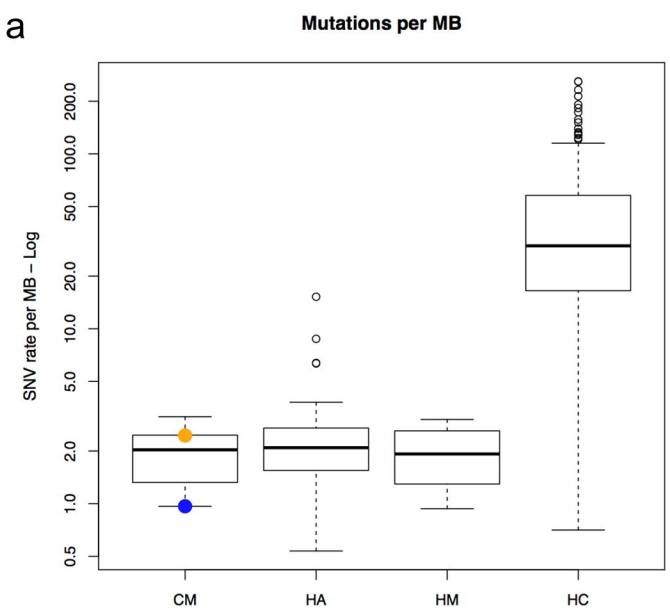
b**c** *PTPRJ***d** *TP53*

Figure 2. Recurrent somatic alterations in canine melanoma. (a) Distribution of *RAS* mutations within the cohort of 37 samples (n=9). (b) Recurrently amplified region on CFA 10 found in nine tumors which is defined by the minimal region surrounding *MDM2*. (c) Location of potentially deleterious mutations present in the putative tumor suppressor *PTPRJ* found through Sanger sequencing of the coding sequence for each tumor. (d) Individual mutations and their locations within *TP53*.



Amplification A
Deletion D
Activating Mutation IM
Inactivating Mutation AM

* Canine Mucosal
* Human Acral
* Human Mucosal
* Human Cutaneous

* % of each cohort bearing somatic mutations in this gene

Figure 3. Key dysregulated pathways in canine and human melanoma. (a) Mutation rate in canine and human melanoma subtypes is shown as somatic SNVs per DNA Mb based on WGS in our discovery cohort compared to WGS data from 140 human cutaneous, 35 acral, and 8 mucosal melanomas (Hayward *et al.* 2017). CM: Canine Mucosal, HA: Human Acral, HM: Human Mucosal, and HC: Human Cutaneous Melanoma. Orange and blue dots in the CM plots represent the individual acral and cutaneous subtypes, respectively, in our discovery cohort. (c) Fraction of copy-number-altered genome in canine melanoma and human melanoma sequencing cohorts. (c) Total number of structural variants identified in canine and human melanoma sequencing cohorts. (d) Comparison of C>T transitions in the major melanoma types in dipyrimidine versus non-dipyrimidines. (d) Overall frequency of mutations in key melanoma pathways in our full cohort of 31 mucosal tumors compared to WGS in other subtypes from Hayward *et al.* 2017. Note that, unlike copy number data, sequence data for CDKN2A, ERK, PIK3CA, and RB1 were only available for the seven tumors in our discovery cohort .