

1 **A non-coding regulatory variant in the 5'-region of the *MITF* gene is associated**
2 **with white spotted coat in Brown Swiss cattle**

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20 **Summary**

21 Recently, the Swiss breeding association reported an increasing number of white
22 spotted cattle in the Brown Swiss breed which is normally solid brown coloured. A
23 total of 60 Brown Swiss cattle with variably sized white abdominal spots, facial
24 markings and depigmented claws were collected for this study. A genome-wide
25 association study using 40k SNP genotypes of 20 cases and 1619 controls enabled
26 us to identify an associated genome region on chromosome 22 containing the *MITF*
27 gene encoding the melanogenesis associated transcription factor. Variants at the
28 *MITF* locus have been reported before to be associated with white or white-spotted
29 phenotypes in other species such as horses, dogs and mice. Whole genome
30 sequencing of a single white spotted cow and subsequent genotyping of 172 Brown
31 Swiss cattle revealed two significantly associated completely linked single nucleotide
32 variants (rs722765315 and rs719139527). Both variants are located in the 5'-
33 regulatory region of the bovine *MITF* gene and comparative sequence analysis
34 showed that the variant rs722765315, located 139 kb upstream of the transcription
35 start site of the bovine melanocyte specific *MITF* transcript, is situated in a multi-
36 species conserved sequence element which is supposed to be regulatory important.
37 Therefore we hypothesize that rs722765315 represents the most likely causative
38 variant for the white spotting phenotype observed in Brown Swiss cattle. Presence of
39 the mutant allele in heterozygous or homozygous state supports a dominant mode of
40 inheritance with incomplete penetrance and results in a variable extent of coat colour
41 depigmentation.

42 **Keywords**

43 Coat colour, depigmentation, *Bos taurus*, *MITF*, melanocyte

44 **Introduction**

45 White spotting coat colour phenotypes are well known in many species. At present,
46 there are already eight genes known to be associated with white coat colour
47 phenotypes or leucism (Reissmann & Ludwig 2013; Fleck et al. 2016). In Hereford
48 cattle, the spotted locus was initially mapped to the KIT proto-oncogene receptor
49 tyrosine kinase (*KIT*) gene on chromosome 6 (Grosz & MacNeil 1999). Later on
50 indication for heterogeneity for the proportion of white coat colour in Holstein cattle
51 was observed (Hayes et al. 2010), including association to the *KIT* and
52 melanogenesis associated transcriptions factor (*MITF*) genes (OMIA 000209-9913).
53 Besides other unknown genetic factors, an intronic regulatory single nucleotide
54 variant in bovine *MITF* contributes to the differences between spotted and non-
55 spotted phenotypes in Holstein and Simmental cattle (Fontanesi et al. 2012; Jansen
56 et al. 2013; OMIA 000214-9913). Also in other species like dogs and horses
57 regulatory non-coding *MITF* variants have been described to be associated with
58 white spots on the head and the body (Hauswirth et al. 2012; Körberg et al. 2014;
59 Negro et al. 2017). In cattle like in other species, coding variants in the *MITF* gene
60 cause white coat colour phenotypes associated with eye malformations such as
61 microphthalmia (Wiedemar & Drögemüller 2014; OMIA 001931-9913) or bilateral
62 deafness (Philipp et al. 2011; OMIA 001680-9913). Variants in the human *MITF* gene
63 are associated with Waardenburg syndrome 2A and Tietze syndrome causing
64 deafness due to a lack of melanocytes in the inner ears and pigmentary disturbances
65 in iridis, hair and skin (Liu et al. 1995; Shibahara et al. 2001; Grill et al. 2013;
66 OMIM156845).

67 In Switzerland the so called Original Braunvieh cattle population is ancestral to the
68 worldwide known Brown Swiss population, which was formed in the USA from
69 animals that were obtained in Switzerland between 1869 and 1910 (Hagger 2005).

70 Original Braunvieh cattle have a solid brown coloured coat with a light stripe around
71 the muzzle and black claws. In the Original Braunvieh cattle breed, two rarely
72 occurring dominant inherited coat colour variations are known as colour-sided (Durkin
73 et al. 2012; OMIA 001576-9913) and belted (Drögemüller et al. 2009; Awasthi Mishra
74 et al. 2017; OMIA 001469-9913). With the introduction of artificial insemination in the
75 1960's, the Original Braunvieh population was introgressed with Brown Swiss
76 individuals from North America resulting in today's Braunvieh population that
77 represents one of the two main dairy breeds in Switzerland. The use of American
78 Brown Swiss sires in Braunvieh cattle is still common and thus leads to Braunvieh
79 animals with various levels of Brown Swiss genes (Stergiadis et al. 2015.) In recent
80 years, more frequently Brown Swiss cattle with white spots on the abdomen and/or
81 on the head (Figure 1) have been reported to the national breeding association.
82 These white spotted animals, although accepted in the herd book, do not comply with
83 the official breed standards and are therefore not desired. The aim of this study was
84 to understand the molecular genetic cause for this unusual coat colour variation in
85 Brown Swiss cattle.

86 **Material and Methods**

87 A total of 60 Brown Swiss animals with white spotted coat were sampled as cases.
88 First of all, we genotyped all animals for the two recently described DNA variants
89 associated with depigmentation phenotypes in Brown Swiss cattle: the colour-sided-
90 associated *KIT* variant by direct PCR testing as described before (Durkin et al. 2012),
91 and the belt-associated variant on chromosome 3 by indirect haplotype estimation
92 (Awasthi et al. 2017). A total of 1639 animals (20 cases and 1619 controls) have
93 been genotyped for 40'636 SNPs and were used for GWAS as described in the
94 supplementary data. Genotyping data can be retrieved at

95 <https://www.animalgenome.org/share/tmp/KIG1531815029.zip.gz>. After whole
96 genome sequencing of one case and subsequent variant filtering against 26
97 genomes of solid coloured cattle , including 11 Original Braunvieh, a total of 57
98 private variants remained. We then filtered these variants against the current variant
99 repository of 2332 sequenced cattle of the 1000 bull genome project. The finally
100 remaining two private variants (rs722765315 and rs719139527) were genotyped in
101 all animals (Table S1). The genome data corresponding to roughly 20x coverage of
102 the genome was made freely available under study accession no. PRJEB18113 at
103 the European Nucleotide Archive (sample accession SAMEA19313668). Additional
104 details on the methodology are provided in the supplementary data.
105 In addition, we checked if the two private variants are situated in highly conserved
106 sequence domains with the PhastCons tool (Margulies et al. 2003) and searched
107 also for regulatory elements from the ENCODE data at the UCSC Genome Browser
108 (<https://genome.ucsc.edu/>).

109 **Results and Discussion**

110 Among the sampled Brown Swiss cattle collected for this study the quantity and
111 quality of abdominal spots, facial marking and depigmented claws varied significantly
112 (Figure S1). Visible eye abnormalities or signs of deafness in animals with white
113 spotted coat have not been observed or reported. A single animal with white spotted
114 coat was heterozygous for the colour-sided-associated *Cs6 KIT* variant (Durkin et al.
115 2012; Figure S2), whereas the belt associated structural variant in the 5'-flanking
116 region of the *TWIST2* gene (Awasthi et al. 2017) was absent from all white spotted
117 animals in this study. Analysing the pedigree data of the sampled cattle, we identified
118 a common ancestor for all cases born in the year 1959 (Figure S3).
119 We performed a genome-wide association study (GWAS) with 20 Brown Swiss cattle
120 with white spotted coat and 1619 Brown Swiss controls. The most significant

121 association was located on chromosome 22 about 612kb upstream of the *MITF* gene
122 (Figure 2, Table S2). The marker with the strongest association was
123 ARSBFGLNGS21229 with a p-value of 3.35×10^{-38} at chromosome 22 position
124 32,573,751 (Bos_taurus_UMD_3.1.1). Further significantly associated markers were
125 found distributed over almost the entire chromosome 19 and on chromosome 6, 35
126 Mb away from the *KIT* gene (Figure 2; Table S2). We could not identify plausible
127 functional candidate genes close to any of these markers. We therefore assumed
128 that the associated SNPs on chromosomes 6 and 19 either represent false positive,
129 spurious association signals or indicate genetic heterogeneity.

130 We sequenced the genome of a Brown Swiss animal with white spotting coat colour
131 phenotype and detected a total of 2005 variants in a region of 1.3 Mb spanning the
132 *MITF* gene on chromosome 22. We analysed the interval from the end of the *FOXP1*
133 gene, located upstream of *MITF*, to the beginning of the *FRMD4B* gene, the
134 downstream neighbouring gene of *MITF*. None of these variants affected the protein
135 coding region of *MITF*. Visual inspection of the bam-files did not reveal any
136 indications for the presence of structural variants. Variant filtering identified two
137 private variants (g.31,908,435G>A (rs722765315), and g.32,054,240T>A
138 (rs719139527)) located in the 5'-region of the *MITF* gene which occurred exclusively
139 in 18 Brown Swiss and 2 Danish Red cattle (Table S3). As the Danish Red breed
140 was introgressed with Brown Swiss individuals (Sørensen et al. 2005), these two
141 variants are most likely private to Brown Swiss cattle and their crossbred offspring.

142 Subsequently, we genotyped these two variants in 172 animals and observed perfect
143 linkage disequilibrium between the two variants (Table 1). Interestingly, the single
144 animal carrying the colour-sided-associated *Cs6* *KIT* variant was also heterozygous
145 for the two *MITF* variants (Table S4). There was a highly significant difference in
146 *MITF* allele frequencies between Brown Swiss cattle with white spotted coat and

147 controls using a standard chi-square test (Table 1). The presence of homo- and
148 heterozygotes in the cohort of white spotted animals supports a dominant mode of
149 inheritance. However, approximately 51% (35/68) of solid coloured cattle were either
150 homo- or heterozygous for the mutant alleles, indicating reduced penetrance.
151 Interestingly, there is no perfect correlation between the phenotype and the genotype
152 at the two identified *MITF* variants (Table 1; Table S4). We observed heterozygous
153 animals that had a similar proportion of depigmented coat as homozygous animals.
154 Extended white spots on the abdomen, the head and the legs were seen in
155 heterozygous and in homozygous mutant cattle (Figure 3). On the other hand, some
156 homozygous animals were found, which showed only a little white spot on the head.
157 The white spotting was not always left/right symmetrical and our analysis was
158 hampered by the fact that for some animals we had only one photo showing one
159 side. The varying degrees of white spotting in selected homo- and heterozygous
160 mutant animals are illustrated in Figure 3. Furthermore, three out of the 60 sampled
161 Brown Swiss animals with white spotted coat were genotyped homozygous wild type
162 at the two identified *MITF* variants (Table 1). These animals were closely related to
163 each other (paternal half-sibs) and showed a range of white spots of varying size on
164 the ventral abdomen. As they could not be explained by the presence of the two
165 *MITF* variants seen in all other cases we postulate that further genetic heterogeneity
166 contributes to white spotting phenotypes in Brown Swiss cattle. This hypothetical
167 heterogeneity might be allelic or it might involve other coat colour loci. During
168 genotyping of control animals we identified four Brown Swiss sires as heterozygous
169 for the two identified *MITF* variants which showed no genealogical relationship to the
170 abovementioned sire from 1959 (shown in Figure S3). Therefore we conclude, that
171 the mutation event has probably occurred earlier than 1959. We could not identify a
172 potential founder animal due to missing records of that time.

173 Comparative *in silico* sequence analysis showed that both variants affect highly
174 conserved nucleotide positions (Figure 4). In humans, rodents, horses, dogs, cats,
175 other ruminants and further diverse species the bovine wild type allele is present, but
176 the PhastCons scores reported at UCSC genome browser of the corresponding
177 human genome positions actually differ: 0.99 for human GRCh38.12
178 chr 3: g.69,792,335 which corresponds to bovine chr 22: g.31,908,435G>A
179 (rs722765315), and 0 for human GRCh38.12 chr 3: g.69,630,671 which corresponds
180 to bovine chr 22: g.32,054,240T>A (rs719139527). Furthermore, extrapolated from
181 human ENCODE data the rs722765315 homologous region contained a DNaseI
182 hypersensitive site and a H3K27ac cluster, which are characteristic hallmarks for
183 transcriptionally active chromatin or enhancer elements (Figure S4; Creighton et al.
184 2010; Rada-Iglesias et al. 2011). In these specific regions of the genome, chromatin
185 has lost its condensed structure, exposing the DNA and making it accessible. These
186 accessible chromatin zones are functionally related to transcriptional activity, since
187 this remodelled state is necessary for the binding of proteins such as transcription
188 factors. Therefore, we speculate that the bovine rs722765315 variant located in the
189 5'-flanking region of the bovine *MITF* gene might alter a putative cis-regulatory
190 element controlling the regulation of *MITF* expression during development and thus
191 cause the depigmentation phenotype.

192 In conclusion, we identified two single nucleotide variants in the non-coding 5'-region
193 of the *MITF* gene which are in complete linkage disequilibrium and private to the
194 Brown Swiss cattle breed. The mutant alleles are associated with variably expressed
195 white spotting and appear to act in a dominant manner with incomplete penetrance.
196 Based on evolutionary conservation and annotated epigenetic marks, we rate
197 rs722765315 to be more likely to represent the true causative variant.

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201 photos of the animals. We are grateful to Swissgenetics, SelectStar and Fischer
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289 **Tables**

290 **Table 1** Genotypes of 172 Brown Swiss cattle at two variants (rs722765315 and
 291 rs719139527) in the 5'-region of the *MITF* gene.

292 (Allele frequencies using standard chi-square test: p-value = 5.175e⁻¹⁰. Please note
 293 that the samples were not selected randomly, most likely the statistics is biased
 294 because of the targeted search for cattle with white spotted coat.)

Phenotype	White spotted	Solid coloured	Unknown	Total
Number of animals	60	68	44	172
wt/wt	3	33	24	60
wt/var	20	13	10	43
var/var	37	22	10	69

295

296 **Legends to figures**

297 **Figure 1.** Coat colour phenotype of a solid coloured (A) and a Brown Swiss cow with
298 white spotted coat (B). The region, where the most cases show a white coat colour is
299 inguinal, on the ventral belly and lateral on the abdomen in front of the hind limbs (B).
300 Most of the cases have also a white spot on the head. Additional, white claws at the
301 hind limbs and a white end of the tail were rarely observed (B).

302 **Figure 2.** Genome-wide association study comparing Brown Swiss cattle with white
303 spotted coat (n = 20) to solid coloured Brown Swiss cattle (n = 1619). (A) The green
304 line indicates the Bonferroni corrected significance threshold for significant
305 association ($\alpha=0.05$; $-\log_{10}p = 5.88$). The red line indicates the Bonferroni corrected
306 significance with a $-\log_{10}p = 6.58$ ($\alpha=0.01$). (B) The quantile-quantile (QQ) plot shows
307 the distribution of SNP markers under the null hypothesis and the curve in the left
308 under edge indicates that these markers are stronger associated with the feature
309 than it would be expected by chance.

310 **Figure 3.** Illustrative examples of the white spotted phenotype for different
311 rs722765315 and rs719139527 genotypes showing no obvious phenotype/genotype
312 correlation. The first and the last animal in one column are selected as examples with
313 the smallest and the biggest white spot. The drawings are standardised in one row,
314 but the spread of the white spot corresponds to the individual animals selected for
315 these drawings. Different spread of white head markings in homozygous animals is
316 shown in (A), while the differentially depigmented abdomen of homo- and
317 heterozygous animals is shown in (B). Note the extended depigmented hind limbs
318 and claws of two homozygous animal (C).

319 **Figure 4.** (A) The UCSC genome browser screenshot of the bovine UMD3.1.1
320 assembly shows the position of the two identified variants in the 5'-region of the *MITF*
321 gene on bovine chromosome 22. (B) Sanger sequencing electropherograms showing
322 the three observed genotypes at each variant position: rs722765315 and
323 rs719139527. (C) Nucleotide conservation across 14 different mammalian species.
324 Please note that the sequence conservation actually differ as the homologous
325 sequence for rs722765315 is present in all mammalian species shown.

326 **Supporting information**

327 **Appendix S1.** Supplementary methods.

328 **Table S1.** Primer sequences of the two variants (rs722765315 and rs719139527)
329 close to the *MITF* gene.

330 **Table S2.** GWAS results for white spotting in Brown Swiss cattle.

331 **Table S3.** Count of heterozygous and homozygous mutant animals at 57 different
332 variants in the 1000 bull genome project. All variants, which were not Brown Swiss
333 specific, were excluded. This left only two variants highlighted in yellow, which were
334 exclusively present in Brown Swiss and Danish Red, which is known to have some
335 introgression of Brown Swiss animals (Sørensen et al. 2005).

336 **Table S4.** Individual cattle samples of Brown Swiss cattle with the phenotype and
337 their genotypes at the two variant positions near the *MITF* gene. If “no white spots
338 are detected” is written in the coat colour phenotype, we cannot reliably exclude the
339 presence of white spots, because we did not have pictures from all sides of the body.

340 **Figure S1.** Photos of the 20 cases used for GWAS.

341 **Figure S2.** Picture of the heterozygous *KIT*-associated colour-sided Brown Swiss
342 animal, which was heterozygous for the two variants close to the *MITF* gene. In
343 picture (A) we can see a greater spot on the head than usual in colour-sided cattle.
344 Picture (B) shows white the back, which is typical for colour-sided animals.

345 **Figure S3.** Pedigree of the paternal line indicating the white spotted Brown Swiss
346 cattle. The maternal line was omitted for clarity, however we identified a
347 heterozygous tested carrier in every maternal line of a homozygous case. Squares
348 represent males and circles females. Completely filled symbols represent animals

349 with a white spotted phenotype. Animals with no visible spots or unknown status are
350 represented by a white symbol. The black and white symbol represents the *KIT*-
351 associated colour-sided animal, which is also heterozygous for the *MITF* variants
352 rs722765315 and rs719139527. The heterozygous genotype is visible as blue
353 laboratory number and the homozygous variant is marked as red laboratory number.
354 The whole genome sequenced animal is highlighted yellow. All animal defined as
355 cases in the GWAS have a star. In the pedigree 107 animals with genotypes are
356 visible. Wild type tested animals (n = 60) and five heterozygous animals are not
357 shown in the pedigree to avoid confusion. The common ancestor born in 1959 is
358 shown at the top. Four bulls who did not descend from this ancestor were tested
359 heterozygous and have common ancestors born in the 1940s. So we conclude that
360 this mutation event is older than initially suspected.

361 **Figure S4** ENCODE data from the UCSC Genome Browser, which shows the
362 histone H3K27ac mark in the human genome at the homologous position to
363 rs722765315 (picture A) and rs719139527 (picture B). Note that the homologous
364 region to rs722765315 is more conserved and has higher scores for H3K27ac and
365 DNase I hypersensitivity than the homologous region to rs719139527. Therefore, we
366 suggest that the first position (A) is the main variant causing white spotted coat in
367 Brown Swiss cattle breed.