Two *MC1R* loss of function alleles in cream coloured Australian Cattle Dogs and white Huskies

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Running title: Canine *MC1R* variants

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Summary

Loss of function variants in the *MC1R* gene cause recessive red or yellow coat colour phenotypes in many species. The canine MC1R:c.916C>T (p.Arg306ter) variant is widespread and found in homozygous state in many uniformly yellow or red coloured dogs. We investigated cream coloured Australian Cattle Dogs whose coat colour could not be explained by this variant. A genome-wide association study with 10 cream and 123 red Australian Cattle Dogs confirmed that the cream locus indeed maps to *MC1R*. Whole genome sequencing of cream dogs revealed a single nucleotide variant within the MITF binding site of the canine *MC1R* promoter. We propose to designate the mutant alleles at MC1R:c.916C>T as e^1 and at the new promoter variant as e^2. Both alleles segregate in the Australian Cattle Dog breed. When we considered both alleles in combination, we observed perfect association between the *MC1R* genotypes and the cream coat colour phenotype in a cohort of 10 cases and 324 control dogs. Analysis of the *MC1R* transcript levels in an e^1/e^2 compound heterozygous dog confirmed that the transcript levels of the e^2 allele were markedly reduced with respect to the e^1 allele. We further report another *MC1R* loss of function allele in Alaskan and Siberian Huskies, which is caused by a 2 bp deletion in the coding sequence, MC1R:c.816_817delCT. We propose to term this mutant allele e^3. Huskies that carry two copies of *MC1R* loss of function alleles have a white coat colour.

Keywords: *Canis lupus familiaris*, pigmentation, melanocyte, heterogeneity, non-coding, transcription, *e2*, *e3*

Introduction

Melanocytes, the only pigment producing cell type in mammals, are able to synthesize two types of pigment: yellow-reddish pheomelanin and dark brown or black eumelanin. Pigment type switching governs which of the two types of pigment is produced at a given point in time (Barsh et al. 2000). The melanocortin receptor 1 (MC1R), a G-protein coupled receptor with seven transmembrane domains, is one of the key regulators of pigment type switching (Mountjoy et al. 1992). MC1R activation prompts the melanocyte to produce eumelanin, while MC1R inhibition leads to the production of pheomelanin (Barsh et al. 2000). A huge number of functional genetic variants at the *MC1R* gene is known in many different species, which either lead to dominant black coat colour phenotypes (gain of function alleles) or recessive red or yellow phenotypes (loss of function alleles; Robbins et al. 1993). As coat colour variation has already been studied long before the rise of molecular genetics, *MC1R* alleles are still named in reference to the *extension* locus *(E)* from classical genetics (Wright, 1917; Robbins et al. 1993).

In dogs, three mutant *MC1R* alleles in addition to the wildtype E^+ allele were characterized on the molecular level: $E^M > E^G > E^+ > e$. The most dominant allele E^M caused by the amino acid exchange p.Val264Met is found in dogs with a black mask such as e.g. Leonbergers or Malinois (Schmutz et al. 2003). The E^G allele caused by p.Gly78Val is found in “grizzle” Salukis or “domino” Afghan Hounds (Dreger & Schmutz, 2010). Finally, the recessive loss of function allele *e*, caused by the p.Arg306ter variant is found in yellow or red coloured dogs such as e.g. yellow Labrador Retrievers, red Irish Setters, and many others (Everts et al. 2000; Newton et al. 2000).

The standard coat colours of the Australian Cattle Dog are red or black and tan (“blue”) with varying degrees of mottling and/or speckling (Figure 1). This variation in the base coat colour is most likely controlled by the dominant a^y and recessive a^t alleles at the *agouti (A)* locus or *ASIP* gene (Berryere et al. 2005). Occasionally cream coloured dogs are born from red or blue parents. The cream colour does not correspond to the breed standard and cream coloured dogs cannot be registered. The first goal of this study was to investigate the

molecular basis of the cream coat colour phenotype in Australian Cattle Dogs. The second goal of this study was to investigate the genotype phenotype correlation of a 2 bp deletion in *MC1R*, which was previously presented during a conference (Schmutz & Loechel, 2015).

Material and Methods

Ethics statement

All animal experiments were performed according to the local regulations. The dogs in this study were examined with the consent of their owners. The study was approved by the "Cantonal Committee For Animal Experiments" (Canton of Bern; permits 75/16 and 38/17).

Animals and DNA isolation

Samples from 10 cream coloured and 324 non-cream coloured Australian Cattle dogs were used for the study. The phenotype of the cream coloured dogs was evaluated based on photographs. The coat colour phenotype of the control dogs was either based on the pedigree record and/or as reported by the owner. Genomic DNA was extracted from EDTA blood samples using the Maxwell RSC Whole Blood DNA kit, used with the Maxwell RSC Instrument (Promega). Samples from 50 Huskies were used. These were either Siberian Huskies, an AKC and FCI recognized breed or Alaskan Huskies, which represent working dogs without official pedigree registrations that are bred for sled racing. Fifteen Huskies were white and 35 had a significant amount of black, grey, brown, or red hair and were classified as non-white. We additionally used DNA samples from 628 dogs of diverse breeds that had been submitted to the Vetsuisse Biobank.

SNP genotyping, GWAS, and haplotype analyses

Genotyping of 10 cream and 123 non-cream Australian Cattle Dogs was done on Illumina CanineHD BeadChips containing either 173,662 or 220,853 SNVs by GeneSeek/Neogen.

The SNV genotype data are publicly available at https://www.animalgenome.org/repository/pub/BERN2017.1010/. PLINK v1.09 (Chang et al. 2015) was used to perform basic quality filtering operations on the dataset. First, all genotypes from markers with call rates < 100% were removed. Then, the pruned genotypes were used to perform an allelic genome-wide association study (GWAS). The analysis comprising MDS plot, QT score, principal component analysis (PCA) and a mixed model approach was done using the GenABEL library (Aulchenko et al. 2007) and the hglm package (Ronnegard et al. 2010) in the R environment. During the quality control step, uninformative SNPs with a minor allele frequency below 1%, and SNPs deviating strongly from Hardy-Weinberg equilibrium (p-value of 0.000001) were excluded. All individuals had call rates >95%. The final dataset for GWAS consisted of 133 dogs and 67,918 markers. Haplotypes were phased with Shapeit (Delaneau et al 2014).

Sanger sequencing

All primer sequences are listed in Table S1. The entire coding sequence of the canine *MC1R* gene was amplified as two overlapping genomic PCR products. A third amplicon was generated for the promoter region. PCR products were directly sequenced on an ABI 3730 capillary sequencer (Applied Biosystems) after treatment with exonuclease I and shrimp alkaline phosphatase. Sequence data were analysed with Sequencher 5.1 (GeneCodes).

Whole genome sequencing

Illumina TruSeq PCR-free libraries with insert sizes of 350 bp were prepared from two cream and one blue Australian Cattle Dog. We also prepared libraries from one Alaskan and one Siberian Husky. The libraries were sequenced using 2 x 150 bp reads on an Illumina HiSeq 3000 instrument. Single nucleotide and small indel variants with respect to the CanFam3.1 canine reference genome assembly were called as described (Bauer et al. 2017). The variants were compared to previously obtained genome sequences. In total, 3 wolf and 188

dog genomes were analysed (Table S2). The functional effect of variants was predicted using SNPeff (Cingolani et al. 2012) and the NCBI annotation release 104.

RNA isolation and RT-PCR

Total RNA from a skin biopsy of an e^1/e^2 Australian Cattle Dog dog with cream coat colour was isolated using QIAzol and RNeasy spin columns according to the manufacturer's recommendations (Qiagen). RNA samples were treated with RNase-free DNase to remove contaminations with genomic DNA. Reverse transcription was carried out using an oligo-dT primer, and Superscript® IV reverse transcriptase according to the manufacturer's recommendations (Invitrogen). PCR was performed with 2 µl of the synthesized cDNA and the primer pair for the 3'-end of the *MC1R* gene (Table S1). As *MC1R* consists of a single exon, the same primers can be used for amplification on genomic DNA and cDNA. A control reaction on the isolated RNA without reverse transcriptase was run in parallel to ensure that the RNA did not contain any genomic DNA contamination. The cDNA amplicon was sequenced with the internal primer CACTATCCTGCTGGGCATTT.

Reference sequences

All analyses were performed with respect to the CanFam 3.1 genome reference assembly. Numbering within the canine *MC1R* gene refers to the mRNA accession NM_001014282.2 and the protein accession NP_001014304.2.

Results

Candidate gene analysis of the MC1R coding sequence in Australian Cattle Dogs

All 10 available cream coloured Australian Cattle Dogs had two non-cream parents suggesting autosomal recessive inheritance of the cream coat colour. We therefore hypothesized that the cream colour in Australian Cattle Dogs is caused by a loss of function variant in the *MC1R* gene. We genotyped the 10 cases for the known MC1R:c.916C>T (p.Arg306ter) variant and observed all three genotypes at this variant in the cream coloured dogs. One dog was homozygous for the variant allele, four were heterozygous, and five were homozygous wildtype. As these data suggested allelic heterogeneity, we sequenced the entire coding sequence of *MC1R* in all the cases. However, this analysis did not reveal additional loss of function variants within the *MC1R* coding sequence.

Genome-wide association study (GWAS)

As the initial candidate gene analysis had not revealed the hypothesized additional *MC1R* allele, we switched to a hypothesis-free approach and performed a genome-wide allelic association study with 10 cream (cases) and 123 red (control) Australian Cattle Dogs. GWAS revealed a single significant association signal close to the *MC1R* gene (Figure 2). The best associated marker BICF2S23624769 at Chr5:63,661,161 had a P-value of 5.3×10^{-9} after correction for population stratification (Pc1df). As the GWAS confirmed association to the *MC1R* locus, we expanded our search for the candidate causative variant to the presumed non-coding regulatory regions of *MC1R*. Phasing of the *MC1R* haplotypes of the genotyped dogs revealed only two different haplotypes in the cream dogs. One of these haplotypes contained the T-allele at MC1R:c.916C>T, which from now on we designate as e^1. The presumed second loss of function allele was designated e^2.

Identification of a promoter variant by whole genome sequencing

We obtained whole genome sequences from a cream dog, homozygous for the unknown allele (e^2/e^2), another cream dog, which was compound heterozygous (e^1/e^2) and a blue control dog (E^+/E^+). The sequences were compared to 188 additional publicly available genome sequences from wolves and genetically diverse dogs.

The sequencing data contained 43 variants in the *MC1R* coding sequence and 1 kb each of upstream and downstream regions (Table S3). Only one of the 43 variants showed the genotype distribution expected for the e^2 causative variant, Chr5:63,695,679C>G. This variant is located 430 nucleotides upstream of the start codon within an E-box motif that in humans and mice has been shown to act as binding site for MITF, a key regulatory transcription factor of melanocytes (Figure 3; Figure S1; Steingrimsson et al. 2004). MITF binding sites contain an E-box motif with the consensus sequence CAYGTG. The most frequently observed binding motif in mammals is CATGTG, in which the CATGTG motif must be flanked by a T at the 5'-end or an A at the 3'-end. Alternatively, the palindromic binding site CACGTG functions equally well and has no requirements for additional flanking nucleotides (Aksan & Goding, 1998). The E-box in the canine *MC1R* promoter sequence has the sequence CACGTG. In the e^2 allele the conserved guanine at the fourth position is replaced by a cytosine (CAC<u>C</u>TG).

We genotyped the e^1 and e^2 variants in 334 Australian Cattle Dogs and observed perfect association of the genotypes with the phenotypes. The carrier frequencies for e^1 and e^2 in the Australian Cattle Dogs with standard coat colours were 6% and 12%, respectively (Table 1). The e^2 allele did not occur in 649 dogs from 60 genetically diverse breeds (Table S4).

Demonstration of downregulated MC1R transcription from the e^2 allele

For experimental confirmation of the postulated regulatory effect, we obtained skin RNA from an e^1/e^2 compound heterozygous dog. We amplified genomic DNA fragments containing the e^1 and e^2 variants. Sanger sequencing of these genomic PCR products resulted in the expected 1 : 1 ratio of peaks at the heterozygous position +916 in the electropherogram.

However, when we performed RT-PCR on the RNA sample and sequenced the resulting cDNA product, the e^1 allele was markedly more intense than the e^2 allele. This observed allelic imbalance suggested a massive downregulation of *MC1R* transcription from the allele with the promoter variant in the MITF binding site.

Identification of an e^3 allele in Huskies

The analysis of the genome sequences from an Alaskan and a Siberian Husky revealed an additional frame-shift variant within the *MC1R* coding sequence, MC1R:c.816_817delCT (Figure S1, Table S3). This deletion is predicted to lead to the expression of an altered MC1R, in which the last transmembrane domain and the cytoplasmic C-terminal tail are missing, p.(Ile272MetfsTer22). We propose to designate this variant as e^3. We obtained the e^1 and e^3 genotypes in a cohort of 50 Huskies and observed perfect association of white coat colour with the presence of two *MC1R* loss of function alleles (Table 2). The “white” coat colour in Huskies can be more precisely described as an extremely pale pheomelanistic (yellowish) colour (Figure 5A). Our cohort included a complete family with seven offspring that confirmed the expected co-segregation of e^1 and e^3 with the white coat colour (Figure 5B). We did not detect the e^3 allele in any of the other breeds that we investigated (Table S4).

Discussion

In this study we identified a single nucleotide variant in the *MC1R* promoter as likely cause for a new loss of function allele, termed e^2, which together with the previously reported e^1 allele explains the cream coat colour in Australian Cattle Dogs. We provided functional data that demonstrate a substantial reduction of *MC1R* mRNA levels associated with the e^2 allele. This effect is most likely due to the loss of the binding site for MITF, which has been shown

to be a strong activator of *MC1R* transcription (Aksan & Goding, 1998; Adachi et al. 2000; Aoki & Moro, 2002; Steingrimsson et al. 2004).

Although our data indicated some residual transcription of *MC1R* from the e^2 allele, this is apparently not sufficient to activate eumelanin synthesis in e^2/e^2 dogs. The coat colour of the e^1/e^1 dog in our study was not visibly different from the coat colours of the e^1/e^2 or e^2/e^2 dogs. Therefore, we consider e^1 and e^2 functionally equivalent loss of function alleles.

Our data should help diagnostic laboratories to provide more accurate genetic testing for breeders of Australian Cattle Dogs. We did not observe the e^2 allele outside of Australian Cattle Dogs. However, additional purebred and mixed-breed dogs should be evaluated to determine, if this is truly a breed-specific variant.

We discovered the presence of another loss of function allele in Huskies, e^3, caused by a 2 bp deletion in the coding sequence of *MC1R* by two independent approaches. Initially, the variant was identified by a candidate gene approach and targeted Sanger sequencing (Schmutz and Loechel, 2015). Later on, we found the same variant in whole genome sequence data. In Huskies, both e^1 and e^3 segregate and appear to be functionally equivalent. Loss of *MC1R* function in Huskies leads to an almost completely white coat colour. The fact that black and white Huskies are not black and tan suggests that the pheomelanin pigmentation in Huskies is extremely pale, which is consistent with the nearly white coat colour in *MC1R* deficient Huskies.

In conclusion, we identified a non-coding regulatory *MC1R* promoter variant and an *MC1R* coding deletion as likely causes for the e^2 and e^3 loss of function alleles. Our findings will help to improve genetic testing for coat colours in dogs.

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Conflicts of interest

Rob Loechel, Alexandra Kehl, and Hannes Lohi are affiliated with commercial laboratories marketing genetic tests for dogs.

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Table 1 Association of the *MC1R* genotypes with cream coat colour in a cohort of 334 Australian Cattle Dogs. The mutant alleles at MC1R:c.916C>T and Chr5:63,695,679C>G are designated e^1 and e^2, respectively.

MC1R genotype	*wt/wt*	*wt/e¹*	*wt/e²*	*e¹/e¹*	*e¹/e²*	*e²/e²*
Cream (cases, n = 10))	-	-	-	1	4	5
Non-cream (controls, n = 324)	264	20	40	-	-	-

Table 2 Association of the *MC1R* genotypes with white coat colour in a cohort of 50 Huskies. The mutant alleles at MC1R:c.916C>T and MC1R:c.816_817delCT are designated e^1 and e^3, respectively.

MC1R genotype	*wt/wt*	*wt/e¹*	*wt/e³*	*e¹/e¹*	*e¹/e³*	*e³/e³*
White (cases, n = 15)	-	-	-	4	9	2
Non-white[1] (controls, n = 35)	24	1	10	-	-	-

[1]Dogs with a significant proportion of pigmented hair (black, grey, brown, red) were classified as “non-white”.

Figures and Legends

Figure 1. Coat colour phenotypes of Australian Cattle Dogs. Blue (A) and red (B) are officially recognized coat colours in this breed. (C) A cream coloured Australian Cattle Dog. This colour does not conform to the breed standard.

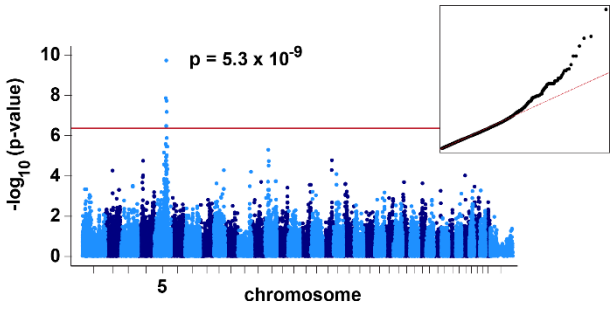

Figure 2. GWAS results. An allelic association study using 10 cream coloured (cases) and 123 red coloured Australian Cattle Dogs (controls) showed a single genome-wide significant signal on chromosome 5 with the best associated marker located close to the *MC1R* gene. Inset: The QQ-plot confirms that the actually observed p-values (shown in black) of the best associated markers are stronger associated with the trait than expected by chance (null hypothesis, red line).

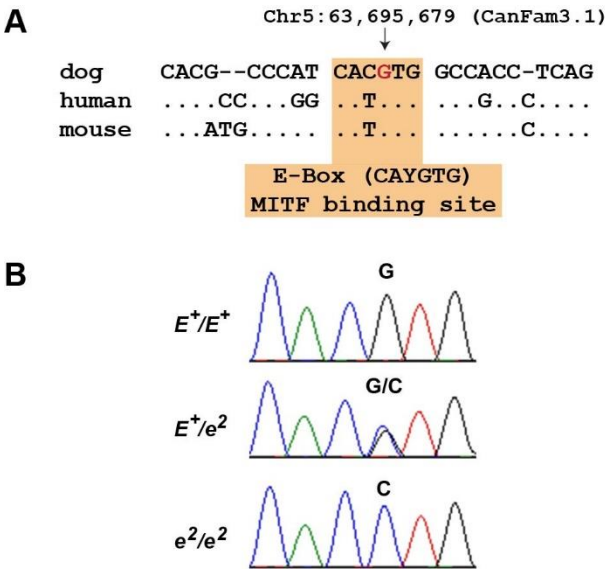

Figure 3. Details of the *MC1R* promoter variant. (A) Sequence context of the Chr5:63,695,679C>G variant located 430 nucleotides upstream of the ATG start codon of the *MC1R* gene. Please note that the dog sequence is given in the orientation of the *MC1R* gene (reverse complementary to the genome reference assembly). There is moderate sequence conservation between dog, human and mouse. The sequences corresponding to the experimentally confirmed human and murine MITF binding sites are highlighted in orange (Adachi et al. 2000; Aoki & Moro, 2002). The G at the variable position is conserved in functional mammalian MITF binding sites (Aksan & Godin 1998). More details are given in supplementary figure 1. (B) Sanger sequencing electropherograms from dogs with the three different genotypes at this variant are shown.

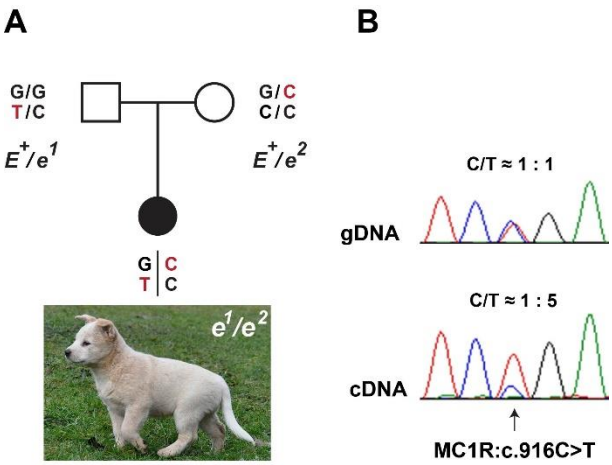

Figure 4. Functional confirmation of the regulatory effect. (A) A cream coloured dog with the compound heterozygous *e1/e2* genotype was selected for the experiment. Both parents of this dog were blue (*ASIP* genotype a^t/a^t in all animals of the trio). The genotypes at both *MC1R* variants are indicated in the pedigree. Variant alleles are shown in red. (B) A Sanger sequencing electropherogram of the MC1R:c.916C>T variant on genomic DNA of the cream coloured dog shows the expected 1 : 1 ratio between the two alleles (area under the peaks). Sanger sequencing of an RT-PCR product derived from skin mRNA with the same primer shows a 1 : 5 ratio of the peak areas originating from the two different alleles. This indicates a marked downregulation of the transcript from the chromosome with the e^2 allele.

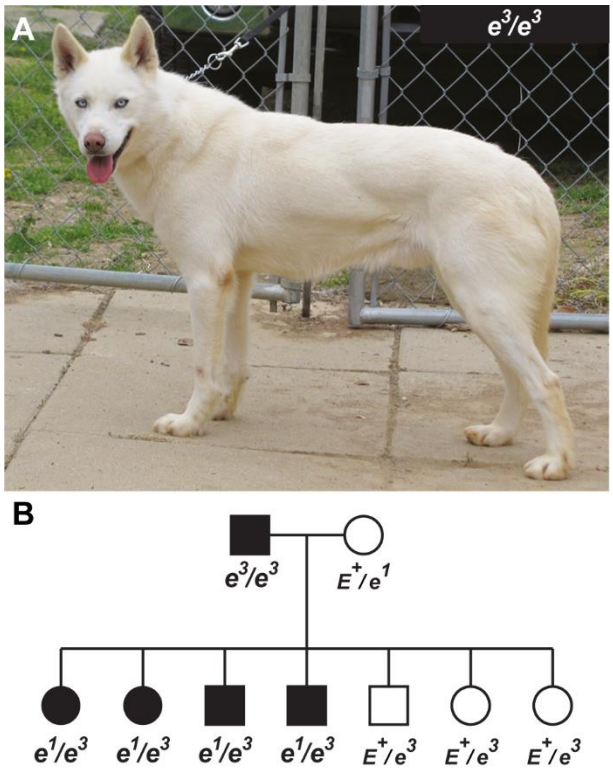

Figure 5. (A) Coat colour phenotype of a Siberian Husky homozygous for the MC1R:c.816_817delCT variant. The dog was registered as white, but had a very pale yellow hue. (B) Cosegregation of *MC1R* loss of function alleles with white coat colour in Huskies. White dogs in this pedigree are indicated with filled symbols, non-white dogs are represented as open symbols. The white dog shown in (A) was mated to a heterozygous non-white female carrying one copy of the e^1 allele. The coat colours in the resulting seven puppies showed the expected genotype-phenotype correlation.

Supplementary Material

Figure S1. Annotated sequence of the canine *MC1R* gene

Table S1. Primer sequences for PCR amplification and Sanger sequencing.

Table S2. Accession numbers of 191 dog/wolf genome sequences.

Table S3. *MC1R* variants detected in 191 genome sequences

Table S4. Genotypes of 334 Australian Cattle Dogs, 50 Huskies, and 628 dogs from different breeds at 3 loss of function variants within the *MC1R* gene.