Short communication

A deletion spanning the promoter and first exon of the hair cycle-specific *ASIP* transcript isoform in black and tan rabbits

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Summary

Black and tan animals have tan-coloured ventral body surfaces separated by sharp boundaries from black-coloured dorsal body surfaces. In the a^t mouse mutant, a retroviral 6 kb insertion located in the hair cycle-specific promoter of the murine *Asip* gene encoding agouti signalling protein, causes the black and tan phenotype. In rabbits, three *ASIP* alleles are thought to exist including an a^t allele causing a black and tan coat colour that closely resembles the mouse black and tan phenotype. The goal of our study was to identify the functional genetic variant causing the rabbit a^t allele. We performed a whole genome sequence-based comparative analysis of the *ASIP* gene in one black and tan and three wildtype agouti-coloured rabbits. The analysis identified 75 a^t-associated variants including an 11 kb deletion. The deletion is located in the region of the hair cycle-specific *ASIP* promoter and thus in a region homologous to the site of the retroviral insertion causing the a^t allele in mice. We observed perfect association of the genotypes at this deletion with the coat colour phenotype in 49 rabbits. The comparative analysis and the previous knowledge about the regulation of *ASIP* expression suggest that the 11 kb deletion is the most likely causative variant for the black and tan phenotype in rabbits.

Keywords *Oryctolagus cuniculus*, pigmentation, coat colour, non-coding, whole-genome sequence, promoter, structural variant

Variation in coat colour is governed by numerous genetic loci that influence the production and/or distribution of pigments (Kaelin and Barsh, 2013). Pigment in mammals is exclusively produced by melanocytes that synthesize two types of melanin. These are yellow to red pheomelanin and dark brown to black eumelanin. The basic coat colour in mammals is determined by the ratio of these two pigment types and regulated by the so-called pigment type switching, an intensively studied signalling process (Barsh, 1996; Cieslak *et al.* 2011).

The melanocortin 1 receptor (MC1R) is expressed in the plasma membrane of melanocytes and can bind two ligands: α-melanocyte stimulating hormone (α-MSH) and agouti signalling protein (ASIP). α-MSH binding leads to activation of MC1R and promotes eumelanin synthesis via the upregulation of cAMP signaling. ASIP is a competitive inhibitor of α-MSH and its binding to MC1R leads to the production of pheomelanin (Barsh *et al.* 2000). ASIP, therefore, plays an important role in the spatial and temporal control of eumelanin and pheomelanin synthesis (Cieslak *et al.* 2011; Kaelin and Barsh, 2013). The use of different alternative promoters of the *ASIP* gene governs the pigment type-switching patterns, as described in mice (Vrieling *et al.* 1994), pigs (Drögemüller *et al.* 2006) and rabbits (Fontanesi *et al.* 2010).

The murine *Asip* gene contains four alternatively used, untranslated exons at its 5'-end, which are under the control of the so-called ventral-specific promoter (exons 1A and 1A') or the hair cycle-specific promoter, which is located further downstream (exons 1B and 1C). Regulated *Asip* expression synchronized with different stages of the hair cycle is responsible for the banded pigmentation in the hairs of *agouti* mice (Vrieling *et al.* 1994). The black and tan phenotype in the a^t mouse mutant is caused by a ~6 kb retroviral-like insertion in the region of the hair cycle-specific promoter (Bultman *et al.* 1994). In black and tan a^t mice, hairs are no longer banded and show a uniformly yellow or uniformly black pigmentation.

In rabbits, three functional *ASIP* alleles with a dominance hierarchy of $A > a^t > a$ have been described (Fontanesi *et al.* 2010). The dominant *A* allele leads to the wildtype or agouti phenotype with three colour zones visible in the individually banded hairs (Fig. 1). The recessive non-agouti *a* allele caused by a frameshift insertion, NM_001122939.1:c.5_6insA,

results in a black fur (Fontanesi *et al.* 2010). The aim of the present study was to identify the causative variant for the a^t allele in black and tan rabbits that lack banding of the hairs, similar to a^t mice (Fig. 1).

We performed whole-genome sequencing of one black and tan and three wildtype agouti-coloured rabbits (ENA accessions are given in Table S1). The resulting fastq-files were mapped to the rabbit reference genome assembly OryCun2.0 (Carneiro *et al.* 2014) and single nucleotide and small indel variants were called. NCBI annotation release 102 was used to predict their functional effects as described previously (Jagannathan *et al.* 2019). The IGV software (Thorvaldsdottir *et al.* 2013) was used for visual inspection of the regions of interest and identification of structural variants.

The black and tan rabbit was homozygous for the alternative allele at 551 variants in the genomic segment containing the *ASIP* gene (NC_013672.1:g.5,423,362-5,631,747). These comprised 395 SNVs, 155 small indels and one structural variant (Table S2). Filtering of those variants, for which at least one of the control genomes was also homozygous for the alternative allele, reduced the list to 49 associated SNVs, 25 small indels, and the structural variant (Table S2). The structural variant represented an ~11 kb deletion (NC_013672.1:g.5,455,408_5,466,123del; Fig. 2). In rabbit, there are currently two *ASIP* transcript isoforms annotated (NCBI annotation release 102). The deletion removes the entire first 5'-untranslated exon of one of these transcripts (NM_001122939.1). Extrapolating from the organization of the murine *Asip* gene, this corresponds to the first exon of the hair cycle-specific transcript.

We confirmed the deletion by PCR followed by Sanger sequencing and genotyped 49 rabbits with different coat colours (Table S1). Out of 19 black and tan rabbits analysed, 17 carried the deletion in a homozygous state. The remaining two black and tan rabbits were heterozygous for the deletion but also heterozygous for the single base insertion causing recessive black and thus presumably compound heterozygous a^t/a. As the *a* allele is recessive to a^t, rabbits with an a^t/a genotype are phenotypically black and tan. None of the 30 rabbits

that were not black and tan carried the deletion in a homozygous state (Table S1). These genotyping results are therefore compatible with a causative role for this deletion.

We note, however, that the rabbit genome assembly still contains some small gaps in the region of the *ASIP* gene. If the causative variant for black and tan is located in a gap of the genome reference assembly or if it is located outside of the genomic interval defined by the flanking *EIF2S2* and *AHCY* genes, we would have missed it in our analysis.

In conclusion, based on comprehensive whole-genome sequencing data we report 75 variants whose genotype distribution was compatible with a causative role in the black and tan phenotype. None of these variants affected the coding sequence of the *ASIP* gene. It is very intriguing that the only associated structural variant in rabbits, an ~11 kb deletion, was in the same region of the *ASIP* gene as the retroviral insertion in the murine a^t allele (Fig. 2). The deletion removed the transcription start site and the first untranslated exon of the presumable hair cycle-specific transcript isoform suggesting that this is the most likely causative variant for the black and tan phenotype. Definitive proof of the causality will require further experiments, e.g. by introducing the rabbit deletion into an agouti (wildtype) genetic background via CRISPR/Cas9-mediated genome editing or by performing comprehensive transcript analyses in black and tan rabbits.

Acknowledgments

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Figure legends

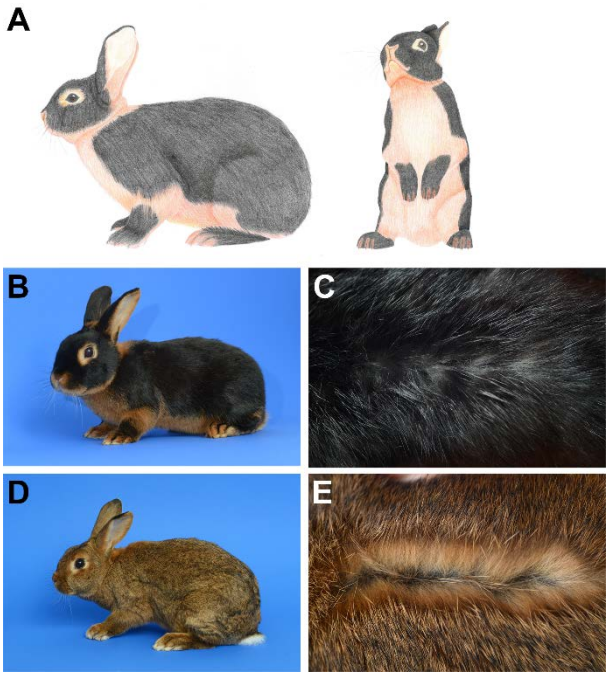

Figure 1 Black and tan coat colour phenotype. (A) Schematic representation of a black and tan rabbit. (B) Photo of a black and tan rabbit. (C) Close-up view of the coat on the back of the same black and tan rabbit. The hair on this rabbit does not show any banding. It is uniformly black on the back and uniformly yellow in the tan areas. (D) Photo of a rabbit with the agouti (wildtype) coat colour. (E) Close-up view of the coat on the back of the same agouti rabbit. The hairs were parted to demonstrate the banding of the individual hairs.

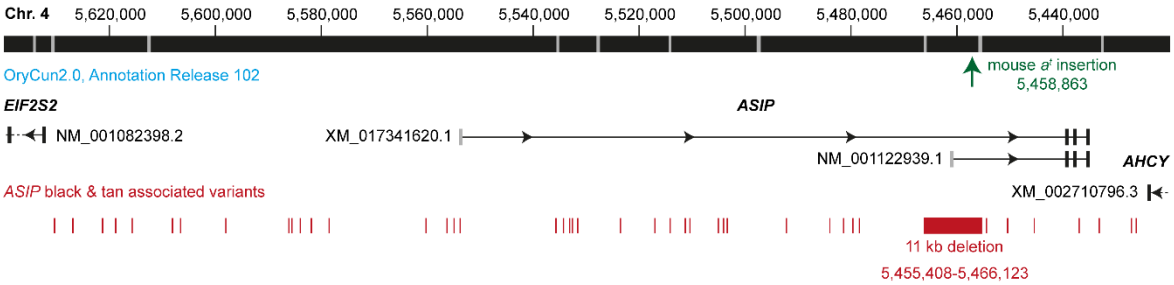

Figure 2 Genomic organization of the rabbit *ASIP* locus and location of variants associated with the black and tan phenotype. On the top, chromosome 4 is represented by a solid black bar. Grey areas represent gaps in the genome reference assembly. The position corresponding to the insertion site of the 6 kb retroviral-like sequence in the murine $a^{t\text{-}2Gso}$ allele is indicated with a dark green arrow (Bultman et al. 1994). The NCBI annotation of the region is indicated. The two annotated *ASIP* transcripts correspond to transcripts 1A and 1C reported previously (Fontanesi *et al.* 2010). We considered variants in the interval between *EIF2S2* and *AHCY* as candidate variants for the black and tan phenotype. The positions of 75 variants that were private to the black and tan rabbit and absent from four agouti-coloured rabbits are indicated in red.

Supporting Information

Table S1 Detailed information about studied rabbits.

Table S2 List of variants of a sequenced black and tan rabbit in the *ASIP* region.