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SHORT COMMUNICATION

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A missense mutation in the *FZD7* gene is associated with dilution of the red areas of the coat in Montbéliarde cattle

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Summary

Recently, a new genetically autosomal recessive color phenotype emerged in the red pied bovine Montbéliarde breed. It is characterized by a dilution of the red areas of the coat and was denominated 'milca'. A genome-wide homozygosity scan of 106 cases followed by haplotype analysis revealed a candidate region within BTA2 between positions 89.95 and 91.63 Mb. Analysis of whole-genome sequence data generated from milca animals identified a strong candidate variant within the coding region of the *Frizzled-7* gene (*FZD7*). This gene encodes for a G-protein coupled receptor for Wnt signaling proteins. The variant induces a glycine to alanine substitution in the second extracellular loop, p.(Gly414Ala). Cross-species amino acid alignments revealed that this glycine is conserved among orthologs and most paralogs, suggesting that it plays an important role in FZD function. In addition, genotyping data revealed that the mutant allele is restricted to the Montbéliarde breed, at a 3.7% frequency. All homozygous cows for the mutant allele exhibited the milca phenotype whereas all heterozygotes had no coat color defects. In conclusion, this study strongly suggests that, in cattle, a mutation of *FZD7* alone is sufficient to cause a coat color phenotype without any strong other adverse effect.

Keywords cattle, coat color, frizzled, polymorphism

Recently, the Montbéliarde breed has seen the emergence of a new recessive condition characterized by dilution of the coat color. A total of 106 affected animals were reported to the French National Observatory for Bovine Abnormalities (ONAB, https://www.onab.fr/, Grohs *et al.* 2016). The first case reported to the ONAB was a cow born in 2008. At birth, the affected animals, which are otherwise healthy, present a dilution of the pigmented, normally red, areas of the coat (Fig. 1). This phenotype, denominated 'milca' by the breeders, is considered undesirable because it deviates from the red pied standard of the breed (https://www.montbeliarde.org/discovering-the-montbeliarde.html). Pedigree analysis of the 106 cases identified 'EZOZO' as a common ancestor of all of the parents of all of the cases at three to eight generations back and, therefore, as the most probable carrier ancestor (Fig. S1). This pedigree analysis, with inbred cases from the same ancestor, spread in many sire families and herds, strongly suggests a recessive determinism.

To search for the causative genetic factors responsible for this genetic condition, a genome-wide homozygosity scan followed by whole-genome sequence data analysis was performed. The genome-wide homozygosity scan was carried out using genotyping data from 44 initially identified affected animals and from 40 150 control Montbéliarde animals using several Illumina SNP chips including the BOVINESNP50 beadchip v1 and v2 (Matukumalli *et al.* 2009), the LD chip (Boichard *et al.* 2012) and the EUROGENOMICS EUROG10K (Boichard *et al.* 2018; Illumina Inc.). First, genotypes were processed using the standard French genomic selection pipeline, as previously described (Boichard *et al.* 2012; Hozé *et al.* 2013). This process includes an imputation step with FIMPUTE software (Sargolzaei *et al.* 2014) in order to recover all BOVINESNP50 marker information. The resulting genotypes were subsequently analysed using an in-house implementation of the HOMAP (homozygosity mapping) tool (Charlier *et al.* 2008). A single 1.68 Mb long homozygous region shared by all of the cases was found in the [89.95–91.63 Mb] interval of BTA2

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Figure 1 Example of normal and 'milca' Montbéliarde animals. Photos of healthy (upper photos) and 'milca' (lower photos) Montbéliarde adult (right photos) and calf (left photos) animals showing the dilution of the coat color.

(Fig. S2) on the ARS-UCD1.2 reference genome (Rosen *et al.* 2020). Analysis of gene content using the bovine Ensembl gene database (release 93) retrieved with the BioMart data mining tool (http://www.ensembl.org/biomart/) revealed that our candidate region contained or overlapped 19 known protein-encoding genes (Table S1).

The whole genomes of four affected animals were determined by 2 × 150 bp paired-end sequencing using HiSeq 3000 technology (Illumina). The average whole-genome sequencing coverage was 9×, ranging from 8× (for three samples) to 12× (for the fourth sample). Firstly, sequences were aligned against the cattle ARS-UCD1.2 reference genome (Rosen *et al.* 2020). Secondly, small genomic variants and structural variants were searched using GATK-HaplotypeCaller and pindel software respectively, as described by Boussaha *et al.* (2015, 2016), Daetwyler *et al.* (2014) and Letaief *et al.* (2017). Thirdly these variants were compared with those present in the genomic variant databases available in Run 7 of the 1000 Genomes Project for a total of 2333 animals (Daetwyler *et al.* 2014) and in our laboratory database for a total of 394 animals from several different cattle breeds, including the common ancestor EZOZO and 27 other Montbéliarde animals (Boussaha *et al.* 2015, 2016; Letaief *et al.* 2017). Small genomic variants were also compared with the most recent 1000 Genomes Project Run 8 database that included 4109 samples, out of which there are 63 animals from the Montbéliarde breed.

Analysis of sequence-derived small genomic variations within our candidate region in the four affected animals revealed 2025 homozygous alternate variants. Of these, 2011 were filtered out because the same genotype was also found in the control population and 14 remained as potential candidates. Out of them, 12 were located in genes and two were intergenic (Table S2). Among these variants, two of them were in coding sequence at positions 90 572 567 (NC_037329.1:g.90572567G>T) and 90 572 569 (NC_037329.1:g.90572569G>C) respectively, both within the *FZD7* (*Frizzled-7*) gene. The common ancestor EZOZO was heterozygous for both variants. In addition, no structural variant (deletions, duplications, inversions and translations) was identified in affected animals within our candidate region.

According to the Variant Effect Predictor pipeline v96 (McLaren *et al.* 2010) annotation, the NC_037329.1:g.90572567G>T substitution is synonymous. However, the NC_037329.1:g.90572569G>C substitution is a missense mutation that changes the glycine residue at position 414 of the protein into an alanine (p.Gly414Ala).

The monoexonic bovine *FZD7* gene-coding sequence is 1725 Nt in length, encoding a 574 amino acid protein. The FZD7 protein belongs to the frizzled family that contains 10 G-protein-coupled receptor proteins. The frizzled family members serve as receptors in several processes, including melanogenesis, and signaling pathways, e.g. Wnt signaling (Huang & Klein 2004; MacDonald & He 2012).

Cross-species alignments showed that the reference guanine allele at the mutated position is evolutionarily conserved between FZD proteins, with the exception of FZD9 (Fig. S3). The high degree of conservation of FZD7 orthologs across more mammals and distantly related species (such as chicken and *Xenopus tropicalis*) is indicative of its potential structural and functional importance. Indeed this modification occurs within the functionally important and evolutionarily highly conserved second extracellular loop domain of the protein (Carroll *et al.* 2012). Accordingly, the identified mutation was classified as pathogenic by the UMD-Predictor software (http://umd-predictor.eu/registration.php) and the SIFT score derived by vep was 0.02.

Previous studies showed that the *FZD7* gene is expressed, among others, in developing feather follicles in chick embryos (Kawakami *et al.* 2000; Chodankar *et al.* 2003)

as well as during mouse hair follicle morphogenesis and postnatal hair growth (Reddy *et al.* 2004).

So far, no human patient with mutated FZD7 has been reported. Knockout of the mouse *Fzd7* gene alone did not induce any coat color phenotype, but was instead associated with tail truncation and kinking and with a low penetrance of ventricular septal defects (Yu *et al.* 2012). The penetrance of this latter phenotype increases with the co-knockout of *Fzd2*, revealing partial genetic redundancy of these two genes. Knockout of both mouse *Fzd1* and *Fzd7* was reported to induce a gray coat color, suggesting that these two genes have a biological redundancy in some aspect of melanocyte development (Yu *et al.* 2012). However, no deleterious mutation could be detected in the Montbéliarde genome at the *FZD1* locus (data not shown), suggesting that, as for *Fzd4* in mouse (Wang *et al.* 2001), knockdown of *FZD7* alone can affect bovine coat color.

PCR sequencing of the 106 affected Montbéliarde animals (including the four sequenced samples) reported to the ONAB and for which good-quality genomic DNA was available, using primers surrounding the mutation (forward primer, 5′-GTTCCATCTGGTGGGTCATC-3′ and reverse primer, 5′-ACGTGCCGATGAAGAGGTAG-3′), confirmed their homozygous mutated status.

The candidate variant was also added on the custom part of the EuroG10K SNP chip used for genomic selection and genotyped for 236 246 animals belonging to 19 different beef and dairy cattle breeds, out of which 74 216 were Montbéliarde (Table 1). The designed probe used in the genotyping processes contains the following source sequence surrounding the SNP: 5′-TCACCATCCTGGCTATG GGCCAGGTGGACGGGGACCTGCTCAGCGGGGTGTGCTATG G[G/C]CCTGTCCAGCGTGGACGCGCTGCGGGGCTTCGTGCT GGCGCCCCTGTTCGTCTACCTCTT-3′. This variant was only observed in the Montbéliarde breed out of the 19 breeds genotyped, indicating that the mutation is probably breed specific. In this population of 74 216 Montbéliarde animals genotyped, the allelic frequency was 3.7%, 5368 animals were heterozygous (7.2%) and 66 animals were homozygous (0.09%) for the mutant allele. The milca phenotype was confirmed for all 66 homozygous animals.

Table 1 Genotype frequencies in 19 cattle breeds.

Breed name	Number of genotyped animals	Genotype	Genotype frequency (%)
Abondance	2717	G/G	100
Aubrac	87	G/G	100
Jersey	1143	G/G	100
Brown Swiss	1922	G/G	100
Salers	524	G/G	100
Bazadaise	1	G/G	100
Tarentaise	1416	G/G	100
Limousine	761	G/G	100
Simmental	286	G/G	100
Charolaise	6911	G/G	100
Rouge des Prés	37	G/G	100
Montbéliarde	**66**	**C/C**	**0.1**
	5368	**C/G**	**7.2**
	68 782	**G/G**	**92.7**
Normande	15 881	G/G	100
Vosgienne	536	G/G	100
Holstein	124 725	G/G	100
Parthenaise	663	G/G	100
Gasconne	3	G/G	100
Blonde d'Aquitaine	4381	G/G	100
INRA95	36	G/G	100

Bold value are indicated only for the Montbéliarde breed.

In summary, we reported the identification of a candidate recessive mutation probably responsible for an emerging phenotype in the Montbéliarde breed that is characterized by a diluted coat color. Combination of homozygosity mapping and whole-genome sequencing approaches allowed for the identification of a missense deleterious mutation within the *FZD7* gene-coding region. This mutation changes the glycine residue at position 414 of the protein into an alanine. We cannot formally exclude the existence of another variant (either one of the 12 remaining small variants detected in the four cases or an unnoticed complex structural variant) in close LD with this missense mutation that either acts in concert with it or is responsible for the observed phenotype. However, (i) the nature and location of the identified missense mutation and (ii) the thorough analysis of the existing SNPs in the region make this hypothesis unlikely.

This is the first report of a coat-color-induced phenotype resulting from a mutation affecting the *FZD7* locus alone. It might suggest that the genes' redundancies observed within the *FZD* gene family differ between species. The underlying biological mechanism remains to be determined.

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Conflict of interests

The authors declare that they have no competing interests.

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Authors' contributions

MeB, SFL, JLV and DB designed the study and drafted the manuscript. AD, CG, MCD and GF collected data and samples from the affected animals and extracted the DNA. CG supervised the ONAB activities and the genotyping work. MeB, CH and SFR performed the mapping work and whole-genome sequencing data analysis. All of the authors read and approved the final manuscript.

Consent to participate

The present work complies with INRAE Animal Care Committee's rules and the requirements of Directive 86/609 of the European Community Council. No experiment was performed on animals.

Data availability statement

The Illumina short reads generated for the affected animals have been submitted to the European Nucleotide Archive with study accession no. PRJEB42790 and are available at https://www.ebi.ac.uk/ena/browser/view/PRJEB42790.
The Illumina short reads generated for the common ancestor 'EZOZO' and for 16 healthy Montbéliarde animals have been previously submitted (Boussaha *et al*. 2015) to the European Nucleotide Archive with study accession no. PRJEB9343 and are available at https://www.ebi.ac.uk/ena/browser/view/PRJEB9343.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Pedigree analysis.

Figure S2. Results of the homozygosity mapping on a portion of BTA2 for 106 'milca' animals.

Figure S3. Alignment of amino acid sequences for the different bovine, mouse, human, chicken and *Xenopus tropicalis* FZD proteins.

Table S1. Gene content of the candidate region.

Table S2. Genomic variations within the candidate region.