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SHORT COMMUNICATION

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Reverse genetic screen for loss-of-function mutations uncovers a frameshifting deletion in the *melanophilin* gene accountable for a distinctive coat color in Belgian Blue cattle

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Summary

In the course of a reverse genetic screen in the Belgian Blue cattle breed, we uncovered a 10-bp deletion (c.87_96del) in the first coding exon of the *melanophilin* gene (*MLPH*), which introduces a premature stop codon (p.Glu32Aspfs*1) in the same exon, truncating 94% of the protein. Recessive damaging mutations in the *MLPH* gene are well known to cause skin, hair, coat or plumage color dilution phenotypes in numerous species, including human, mice, dog, cat, mink, rabbit, chicken and quail. Large-scale array genotyping undertaken to identify p.Glu32Aspfs*1 homozygous mutant animals revealed a mutation frequency of 5% in the breed and allowed for the identification of 10 homozygous mutants. As expression of a colored coat requires at least one wild-type allele at the co-dominant Roan locus encoded by the *KIT ligand* gene (*KITLG*), homozygous mutants for p.Ala227Asp corresponding with the missense mutation were excluded. The six remaining colored calves displayed a distinctive dilution phenotype as anticipated. This new coat color was named 'cool gray'. It is the first damaging mutation in the *MLPH* gene described in cattle and extends the already long list of species with diluted color due to recessive mutations in *MLPH* and broadens the color palette of gray in this breed.

Keywords bovine, cool gray, disruptive mutation, *KITLG*, *MLPH*, OMIA 00206-9913, whole-genome/whole-exome sequence

In an attempt to evaluate the fraction of disruptive mutations that could cause embryonic lethality and therefore affect fertility, we embarked on a next-generation-sequencing-based reverse genetic screen in modern cattle populations. As part of this study, we sequenced the whole genome of 50 and the whole exome of 30 sires from the Belgian Blue cattle breed. Sequence data were mined for highly disruptive loss-of-function variants corresponding to frameshift (FS), stop gain (SG) and essential donor/acceptor splice-site (SS) mutations. In this breed, we found 109 loss-of-function variants, including 56 FS, 42 SG and 11 SS. We have demonstrated that only a small fraction of these (~10%) likely causes embryonic lethality in homozygotes, highlighting the importance of molecular redundancy and the high proportion of non-essential genes (Charlier *et al.* 2014).

As a by-product of this study, we are currently searching for distinctive phenotypic features in animals that are apparently normal despite being homozygous for loss-of-function mutations in genes that are evolutionary highly conserved. In this process, we highlighted a 10-bp deletion in the first coding exon of the *melanophilin* gene (*MLPH*) at genomic position 117, 591, 518–117, 591, 527 bp on bovine chromosome 3 (BosTau6/UMD3.1 reference genome assembly) (Fig. 1). It is likely private to the Belgian Blue breed, as it was not listed in dbSNP (http://www.ncbi.nlm.nih.gov/snp/), not found in the 1000 bull genomes project (http://www.1000bullgenomes.com) (run 3: 429 sequenced key ancestors from 15 different breeds) and not found in an additional panel of 10 breeds, including two *bos indicus* breeds. This FS mutation (c.87_96del) is

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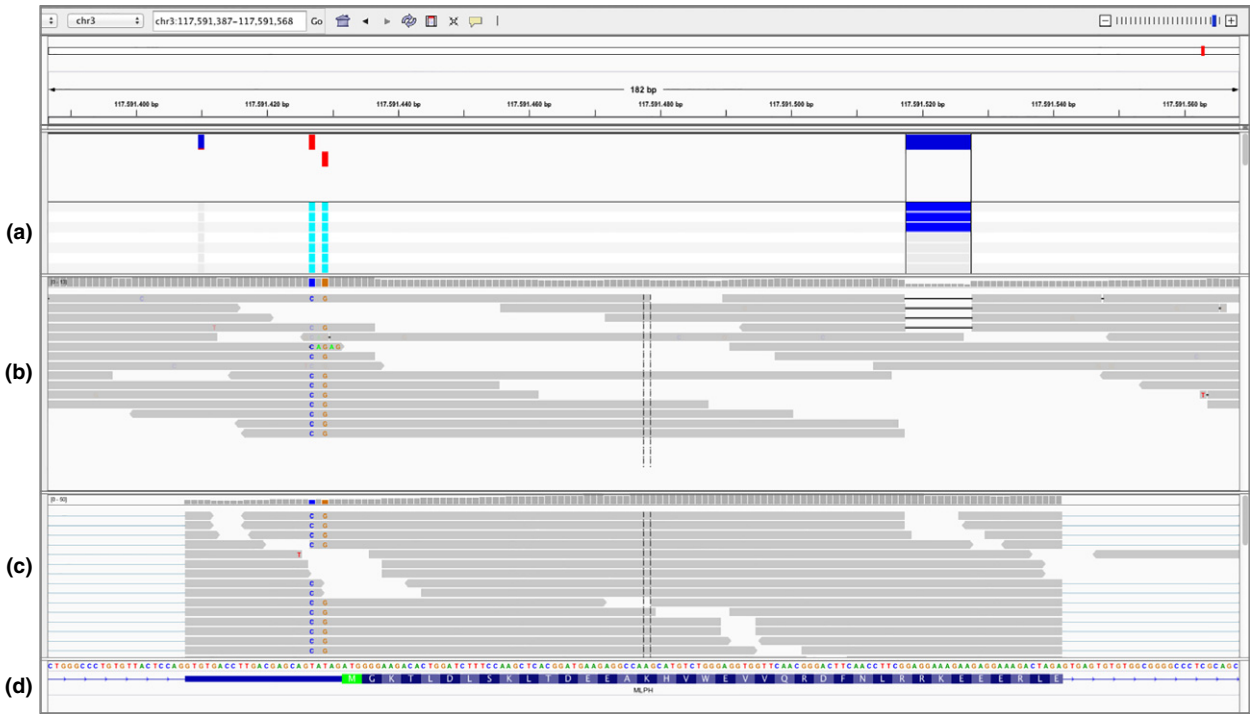

Figure 1 A 10-bp deletion (c.87_96del) in the first coding exon of the *melanophilin* gene. Screen capture of an INTEGRATIVE GENOMICS VIEWER (Robinson *et al.* 2011) output for a 182-bp genomic region encompassing the *MLPH* coding exon 1 with, from top to bottom (a) a 'VCF' file of 50 Belgian Blue sires (Druet *et al.* 2014) displaying three heterozygous animals for the c.87_96del mutation, (b) a 'BAM' file from a whole-genome sequence of a carrier animal, (c) fetal skin cDNA sequence reads from a wild-type animal defining the corresponding exon junctions and (d) a track of Ref-seq gene annotation.

predicted to introduce a premature stop codon in the same exon (p.Glu32Aspfs*1) and a resulting variant protein that is 94% shorter. Furthermore, the nonsense-mediated decay pathway is expected to degrade the variant mRNA.

The *melanophilin* gene encodes a protein expressed primarily in melanocytes, where it plays a central and active role in a tripartite complex (RAB27A-MLPH-MYO5A) (e.g. Strom *et al.*, 2002; Sckolnick *et al.* 2013). This complex is indispensable for active intracellular mature melanosome trafficking. In human, mutations in any of the three subunits of the complex cause Griscelli syndrome, a recessive disorder characterized by variable immune and neurological defects systematically accompanied by skin and hair hypopigmentation. Mutations in *MLPH* cause Griscelli syndrome type 3, which is strictly restricted to pigment dilution without any reported additional phenotypic change (Ménasché *et al.* 2003; Westbroek *et al.* 2012). This dilution is not generated by a defect in melanosome biosynthesis but, rather, caused by an impaired transport of mature melanosomes toward melanocyte dendritic tips where they are transferred to nearby keratinocytes (reviewed by Huizing *et al.* 2008). Similar hypopigmentation phenotypes, explained by recessive mutations in *MLPH*, have been described in numerous species including mouse (Matesic *et al.* 2001), cat (Ishida *et al.* 2006), dog (Philipp *et al.* 2005; Drögemüller *et al.* 2007), mink (Cirera *et al.* 2013), rabbit (Lehner *et al.* 2013; Fontanesi *et al.* 2014), chicken (Vaez *et al.* 2008) and quail (Bed'hom *et al.* 2012)–but not yet cattle. The identified causative mutation(s) and associated dilution phenotype for each species are summarized in Table S1. Therefore, the c.87_96del variant in the bovine *MLPH* gene appeared to be a strong candidate for a yet-to-be-described novel coat color in the Belgian Blue cattle.

The 109 loss-of-function variants detected in Belgian Blue cattle, including the *MPLH:*c.87_96del mutation, were added to the Illumina low-density custom array and used to genotype 5201 Belgian Blue animals as part of a genomic selection program (Charlier *et al.* 2014). The *MLPH:* c.87_96del mutation was shown to segregate at a frequency of 5% in Belgian Blue cattle and to be in Hardy–Weinberg equilibrium ($P = 0.37$; 10 del/del, 501 del/+, 4690 +/+). We identified 10 homozygous mutants. It is worth mentioning that the Belgian 'Blue' cattle breed received its name from the segregation of a co-dominant mutation, known as the Roan mutation, originating from the Shorthorn breed (Jones 1947). Homozygous wild-types, heterozygotes and homozygotes for the Roan mutation are black, roan blue (mixture of black and white hairs) and white, respectively (Charlier *et al.* 1996). These phenotypes are fully explained by a non-synonymous mutation (p.Ala227Asp; inferred annotation after gene model correction) in the *KIT ligand* (*KITLG*) gene (Seitz *et al.* 1999). This missense mutation was also present on our custom

array and was shown to have a frequency of 56% in the breed. Among the 10 *MLPH* homozygous mutants identified, two were black (*KITLG:*Ala227/Ala227), four roan blue (*KITLG*:p.Ala227Asp) and four white *(KITLG:*Asp227/Asp227*)*. As the phenotypic effect of the *MLPH*:c.87_96del mutation would be epistatically masked in white animals, we traced the six non-white animals back to their farms to examine their coat color. As expected, all six displayed a distinctive dilution phenotype, which we called 'cool gray' (Fig. 2, Appendix S1). Non-white animals, heterozygous for the *MLPH*:c.87_96del mutation, were indistinguishable from homozygote wild-type relatives (fully recessive mutation).

Figure 2 *MLPH* knockout dilution phenotypes in Belgian Blue animals and epistasis with the *KITLG* alleles. (a) Two animal homozygotes (del/del) for the c.87_96del mutation at the *MLPH* locus: homozygote wild-type (Ala/Ala, left) and heterozygote (Ala/Asp, right) for the p.Ala227Asp non-synonymous mutation from the *KITLG* locus; the animal on the left displays a black dilute coat color (dark 'cool gray') and the one on the right shows the epistatic 'cool gray' coat color; however, distinguishing a phenotypic difference between these combinations of genotypes remains challenging when animals are not side by side. (b) Two animals heterozygotes (Ala/Asp) for the p.Ala227Asp non-synonymous mutation at the *KITLG* locus: homozygote mutant and wild type (del/del, left; +/+, right) for the c.87_96del mutation at the *MLPH* locus; the animal on the right is under the classical 'roan blue' coat color.

In all the species with loss-of-function mutations in *MLPH*, the reported phenotype appeared to be strictly restricted to skin and hairs. However, it is noteworthy that, in addition to melanocytes, *MLPH* is also highly expressed in mast cells, which are key cellular players mediating allergic and inflammatory reactions. In mice, a recent study by Singh *et al.* (2013) has shown that the Rab27a/Mlph/MyoVa complex seemed to regulate the docking of mast cell granules to the mast cell plasma membrane by modulating its cytoskeleton integrity. It is thus tempting to speculate that loss-of-function mutations in *MLPH* could affect mast cell degranulation and have a pleiotropic effect on an allergic and/or inflammatory reaction's time-course. Moreover, in human, it has been reported that the *MLPH* locus exhibits a strong signal of recent positive selection in non-African populations (Pickrell *et al.* 2009). A link between this sweep in human, underlying alleles and a putative advantageous phenotype–correlated or not with pigmentation–remains to be established.

Up until now, there have been characterized mutations segregating in the Belgian Blue breed at four coat-color loci (*MC1R*, *KITLG*, *KIT* and *MLPH*) (Klungland *et al.* 1995; Charlier *et al.* 1996; Seitz *et al.* 1999; Durkin *et al.* 2012; this study). All known mutations, their modes of inheritance, associated phenotypic effects and respective frequencies within this breed are listed in Table S2. Collectively, they are responsible for the observed variety of colored patterns and subtle blue or gray shades in both Belgian Blue purebred and crossbred animals.

This study stands as proof of the concept that a population-based next-generation-sequencing reverse screen can uncover segregating variations underlying novel phenotypes of biological interest.

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Authors' contributions

WL performed WGS/WES analysis; AS was in charge of cases collection and phenotyping; NT genotyped the samples on the Illumina custom array; WC supervised the

GIGA-genomics platform; and MG and CC designed the study, analyzed the data and wrote the manuscript with the help of all co-authors.

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Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Known recessive mutations in the *MLPH* gene and associated effect at the molecular level in various vertebrate species.

Table S2 Known mutations and their associated phenotypic effect in the four coat color genes molecularly characterized in the Belgian Blue breed.

Appendix S1 Photographs of Belgian Blue animals and genotype/phenotype table.