

# Christmas disease in a Hovawart family resembling human hemophilia B Leyden is caused by a single nucleotide deletion in a highly conserved transcription factor binding site of the *F9* gene promoter

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## ABSTRACT

Hemophilia B is a classical monogenic, X-chromosomal, recessively transmitted bleeding disorder caused by genetic variants within the coagulation factor IX gene (*F9*). Although hemophilia B has been described in dogs, it has not yet been reported in the Hovawart breed. Here we describe the identification of a Hovawart family transmitting typical signs of an X-linked bleeding disorder. Five males were reported to suffer from recurrent hemorrhagic episodes. A blood sample from one of these males with only 2% of the normal concentration of plasma factor IX together with samples from seven relatives were provided. Next-generation sequencing of the mother and grandmother revealed a single nucleotide deletion in the *F9* promoter. Genotyping of the deletion in 1,298 dog specimens including 720 Hovawarts revealed that the mutant allele was only present in the aforementioned Hovawart family. The deletion is located 73 bp upstream of the *F9* start codon in the conserved overlapping DNA binding sites of hepatocyte nuclear factor 4 $\alpha$  (HNF-4 $\alpha$ ) and androgen receptor (AR). The deletion only abolished binding of HNF-4 $\alpha$ , while AR binding was unaffected as demonstrated by electrophoretic mobility shift assay using human HNF-4 $\alpha$  and AR with double-stranded DNA probes encompassing the mutant promoter region. Luciferase reporter assays using wild-type and mutated promoter fragment constructs transfected into Hep G2 cells showed a significant reduction in expression from the mutant promoter. The data provide evidence that the deletion in the Hovawart family caused a rare type of hemophilia B resembling human hemophilia B Leyden.

## Introduction

Hemophilia B (Christmas disease) is a recessive, X-linked bleeding disorder caused by genetic variants within the clotting factor IX gene (*F9*) resulting in the absence or insufficient levels of factor IX (FIX) in the blood.<sup>1</sup> In humans hemophilia B is also known as the “royal disease” as it was transmitted into several European royal dynasties by Queen Victoria.<sup>2,3</sup> As of present, 1,113 unique *F9* variants have been described in humans.<sup>4</sup> The majority of the pathogenic variants are located within exons (n=923) and intronic regions (n=137) of *F9*. Only 33 variants (2.96%) have been described in the 5'-UTR (n=28) and 3'-UTR (n=5) accounting for 2.52% and 0.45% of human pathogenic hemophilia B variants, respectively.<sup>4</sup>

Although the first reports about canine hemophilia B date back to the early 1960s

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and this was the first disorder in dogs characterized on the DNA level, data on hemophilia B cases in dogs remain rather scarce compared to data from humans.<sup>5-8</sup> For instance, in the Cairn Terrier colony of the Francis Owen Blood Research Laboratory (University of North Carolina, Chapel Hill, NC, USA) a G>A transition (NC\_006621.3:g.109,532,018G>A) in exon 8 causing an amino acid exchange (NP\_001003323.1:p.Gly418Glu) that resulted in a complete lack of circulating FIX was detected in affected dogs.<sup>9</sup> Due to a complete deletion of *F9* in a Labrador Retriever, FIX inhibitors were produced after transfusion of canine blood products.<sup>10</sup> In a study of Pit Bull Terrier mixed breed dogs and Airedale Terrier dogs a large deletion of the entire 5' region of *F9* extending to exon 6 was found in the former and a 5 kb insertion disrupting exon 8 was described in the latter.<sup>11</sup> As in the Labrador Retriever with hemophilia B, FIX inhibitors were produced in both breeds. A mild form of hemophilia B in German Wirehaired Pointers was caused by a 1.5 kb Line-1 insertion in intron 5 of *F9* at position NC\_006621.3:g.109,521,130.<sup>12</sup> Until today, hemophilia B has been described in four mixed-breed dogs and nine dog breeds, i.e. German Shepherd, Lhasa Apso, Labrador Retriever, Rhodesian Ridgeback, Airedale Terrier, Cairn Terrier, Maltese, Mongrel and German Wirehaired Pointer.<sup>9-17</sup>

In the canine cases analyzed so far on the DNA level, mutations have been observed only in exons and introns of *F9*, whereas alterations of the *F9* promoter have not yet been reported. In humans promoter variants have been detected and result in the so-called hemophilia B Leyden characterized by low levels of FIX until puberty, whereas after puberty FIX concentrations rise to almost normal levels.<sup>18-20</sup> Since its first description, the genetic background of human hemophilia B Leyden was elucidated by various studies identifying variants in different transcription factor binding sites in the *F9* promoter including the androgen-responsive element (ARE), hepatocyte nuclear factor 4 $\alpha$  (HNF4 $\alpha$ ), one cut homeobox (ONECUT1/2) and CCAAT/enhancing-binding protein  $\alpha$  (C/EBP $\alpha$ ) binding sites.<sup>21,22</sup> HNF4 $\alpha$  is a liver-enriched member of the nuclear receptor superfamily of ligand-dependent transcription factors and has been associated with several disorders, including diabetes, atherosclerosis, hepatitis, cancer, and hemophilia.<sup>23</sup> Promoter analyses have identified at least 140 genes with HNF4 $\alpha$  binding sites. A recent, more detailed analysis using protein binding microarrays identified an additional 1,400 potential binding sites.<sup>24,25</sup> Hence, HNF4 $\alpha$  plays an important role in the regulation of numerous genes especially in the maintenance of many liver-specific functions. Liver-specific HNF4 $\alpha$ -null mice have been used to study the involvement of hepatic HNF4 $\alpha$  in blood coagulation. In the murine model it was shown that expression of factors V, XI, XII, and XIII B depends directly on hepatic HNF4 $\alpha$  and FIX expression was decreased with significantly prolonged activated partial thromboplastin time (aPTT).<sup>26</sup> Ten of the so far identified 28 5'-UTR variants (35.7%) are located within the overlapping binding sites of the androgen receptor (AR) and HNF4 $\alpha$  in the human *F9* promoter.<sup>4,21</sup> Four variants at positions -21, -20 and -19 only affect HNF4 $\alpha$  binding and all of them have been shown to cause hemophilia B Leyden.<sup>19,27-30</sup> The remaining six variants at positions -26, -24 and -23, located in the overlapping region, cause the so-called hemophilia B

Brandenburg.<sup>31,32</sup> Unlike the classical hemophilia B Leyden, FIX levels in patients with these variants cannot be restored by testosterone-driven AR activity and remain low after puberty with no clinical recovery.<sup>21,32</sup>

## Methods

### Animals and genomic DNA isolation

Canine blood and/or hair samples were collected by local veterinarians. The collection of samples was approved by the Lower Saxony State Office for Consumer Protection and Food Safety (33.19-42502-05-15A506) according to §8a Abs. 1 Nr. 2 of the German Animal Protection Law (TierSchG). Blood collected into EDTA and/or hair samples were provided by different Hovawart and dog breeders with written consent from the dogs' owners. DNA was extracted from 30-50 hair roots using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.<sup>33</sup> A salting out procedure was used to obtain DNA from the EDTA blood samples.<sup>34</sup> Additional DNA samples deposited with the Institute of Veterinary Medicine were used as controls. All samples were pseudonymized using internal identities.

### Next-generation sequencing and genotyping

DNA from animals #4 and #6 was used for next-generation sequencing on an Illumina HiSeq2500. The quality of the fastq-files was analyzed using FastQC 0.11.7.<sup>35</sup> Total reads of 1,029,601,630 (#4; sequencing depth 51x) and 1,000,503,256 (#6; sequencing depth 50x) were obtained and mapped to the reference canine *F9* gene (NC\_006621.3, region 109,501,341 to 109,533,798; CanFam3.1) using DNASTAR Lasergene Genomics Suite SeqMan NGen 15.2.0 (130).<sup>36-40</sup>

Targeted genotyping of the promoter deletion was done by polymerase chain reaction (PCR) amplification with primers *cfa\_F9\_Ex1\_F* (5'-CCACTGAGGGAGATGGACAC-3') and *cfa\_F9\_Ex1\_R* (5'-CCCACATGCTGACGACTAGA-3') resulting in a fragment of 328 bp (wildtype) or 327 bp (deletion) spanning the variant position. The resulting PCR products were either directly sequenced on an ABI 3730 Genetic Analyzer (Thermo Fisher Scientific, Basel, Switzerland) or genotypes were determined by restriction fragment length polymorphism analysis after cleavage with *RsaI*. The wildtype allele generated two fragments of 52 bp and 276 bp while the allele with the deletion remained uncut.

### Electrophoretic mobility shift assay

For the electrophoretic mobility shift assay, biotin-labeled, double-stranded wildtype (*cfa\_F9n\_wt\_Biotin*: 5'-CAGAAGTAAAT-ACAGCTCAACTTGTACTTTGGAACAACTGGTCAACC-3') and mutated (*cfa\_F9n\_mut\_Biotin*: 5'-CCAGAAGTAAAT-ACAGCTCAACTTGTATTTGGAACAACTGGTCAACC-3') oligonucleotides were synthesized (Integrated DNA Technologies IDT, Leuven, Belgium) harboring the overlapping HNF4 $\alpha$  and AR binding sites (underlined). The position of the deleted C-nucleotide is indicated in bold and italics. Recombinant human HNF4 $\alpha$  and human AR overexpression lysate were purchased from Origene Technologies Inc. (Rockville, MD, USA).

DNA was detected using the Chemiluminescent Nucleic Acid Detection Module Kit (Thermo Scientific, USA) with minor modifications, i.e. membranes were incubated for 1 min in the substrate working solution.

### Luciferase assay

pGL3 Luciferase Reporter Vectors (pGL3-Basic, pGL3-Control)

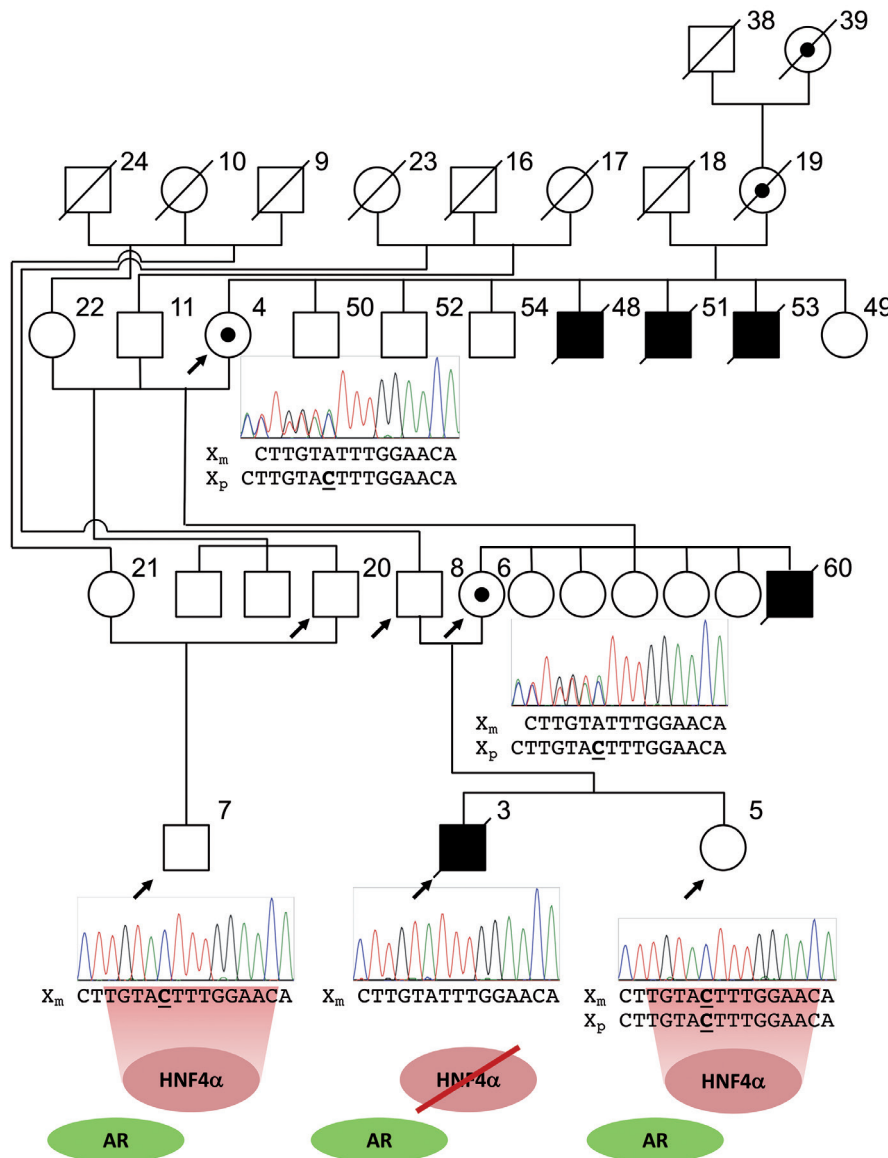
were used for the luciferase assay (Promega, Mannheim, Germany). The wildtype *F9* promoter fragment (971 bp wildtype) was generated by PCR using primers *cfa9\_HindIII\_F\_neu* (5'-CGTAGACTTAGCACTGTTCAAAGCTTTCACACACACAGTTCTTAAAT-3') and *cfa9\_HindIII\_R\_neu* (5'-ATGGCTAGCAACCGTCTAAGAAGCTTAATTGTGCAAGGAGCAAGG-3'). The mutated *F9* promoter fragment (970 bp) was generated by PCR using primers *cfa9\_HindIII\_F* (5'-ATCGTCAAGCTTTCACACACACAGTTCTTAAAT-3') and *cfa9\_HindIII\_R* (5'-CGTACGAAGCTTAATTGTGCAAGGAGCAAGG-3'). For cloning into the *HindIII* restriction site of pGL3, primers were designed with an unspecific random 5'-tag (italics) followed by a *HindIII* restriction site (underlined) (*Online Supplementary Figure S1*). DNA from female carrier #6 served as a template for amplification. Low expression levels of C/EBP in Hep G2 cells were complemented by co-transfection of a C/EBP $\alpha$  expression vector.<sup>22</sup> Data are presented as relative response ratios.<sup>41</sup> A Mann-Whitney U test was used to determine statistical significance. Values were considered statistically significant when  $P < 0.05$  (weakly significant),  $P < 0.01$  (medium-strength significance) and  $P < 0.001$  (strongly significant).

**Other methods**

Further details of the study methods are given in the *Online Supplementary Appendix*.

**Results**

Hemophilias are rare diseases in dogs and hence it was rather coincidental that a case in a Hovawart (#3, Figure 1) was reported to us. With the reconstruction of the pedigree using the online dog breed database and pedigree data of individual dogs provided by the owners it was possible to trace the disease back to the female carrier #39 (Figure 1, *Online Supplementary Figure S2*).<sup>42</sup> In the studied family the hemophilia was transmitted to animals #19, #4 and #6. Bitch #19 had one litter with three hemophilic males (#48, #51, #53). Bitches #4 and #6 had litters with one affected male each, #60 and #3, respectively. Although DNA samples from animals #48, #51, #53 and #60 were not available, blood parameters and medical reports about recurrent hemorrhagic episodes were provided (*Online*



**Figure 1.** Pedigree section of the hemophilia B Leyden Hovawart family and DNA sequence comparison of the mutant hepatocyte nuclear factor 4 $\alpha$ /androgen receptor binding site in the promoter of canine *F9* in the hemophilic male (#3) and relatives (#4 grandmother, #5 sister, #6 mother, #7 cousin). Pedigree symbols are according to the standardized human pedigree nomenclature.<sup>61</sup> Individuals are pseudonymized using internal identities. DNA samples were available from individuals indicated with an arrow. DNA sequences of heterozygous bitches #4 and #6 (female carriers) show overlapping peaks with similar heights 5' of the deletion position. For males #48, #51, #53 and #60, signs of hemophilia (*Online Supplementary Table S1*) were reported and the dogs had to be euthanized after recurrent hemorrhages. X<sub>m</sub>: maternal X chromosome; X<sub>p</sub>: paternal X chromosome; HNF4 $\alpha$ : hepatocyte nuclear factor 4 $\alpha$  binding site (consensus sequence: 5'-TGNACTTTG-3');<sup>21,48</sup> AR: 3'-part of the androgen receptor binding site (consensus sequence: 5'-AGNACANNNTGNTCT-3').<sup>21,48</sup>

Supplementary Table S1). These males had increased aPTT ratios of 2.93 (#53) to 4.76 (#51) indicative of defects in the intrinsic coagulation pathway and also reduced FIX concentrations in the blood as is normally the case in hemophilia B. The affected dog #3 presented only 2% of the standard FIX concentration. The female carriers #4 and #6 showed aPTT ratios within the reference range. FIX concentrations, however, were slightly below the reference range (#6). This was not surprising as it has been shown that minute reductions in FIX concentrations might not always be reflected in an aPTT increase because of the sensitivity of commercial reagents.<sup>43</sup> The clinical signs together with the blood coagulation parameters and X-linked transmission supported the diagnosis of hemophilia B. The definite clinical diagnosis prompted us to search for the molecular cause initially on the DNA level. The canine *F9* gene is located on chromosome X (CFAX) between positions 109,501,341 (transcription start site) and 109,533,798 and has a length of 32,458 bp (NC\_006621.3, CanFam3.1). The canine *F9* gene, similarly to that of other mammals, has eight exons with an open reading frame of 1,356 bp coding for 452 amino acids.<sup>44</sup> DNA of female carriers #4 and #6 was subjected to whole genome sequencing and aligned to the canine reference *F9* gene sequence. Surprisingly, only six sequence variants outside the coding regions of *F9* were identified (Table 1). Five variants were located in introns and were excluded as the cause of the hemophilia B in the Hovawarts because these variants were also detected in unaffected controls. The remaining variant (deletion) was located in the promoter of *F9* 73 bp upstream of the start codon (Online Supplementary Figures S1 and S3). As this deletion was located within a putative transcription factor binding site of HNF4 $\alpha$  and AR, which had been shown in humans to be important for *F9* expression and mutated in hemophilia B Leyden and Brandenburg,<sup>31,32</sup> this position was analyzed in more detail.

Figure 1 shows the segregation of the nucleotide deletion in the affected Hovawart family. The female carriers #4 and #6 were heterozygous, as evidenced by the overlap-

ping peaks with similar heights 5' of the deletion position. The affected male #3 was hemizygous for the deleted allele whereas his sister #5 and cousin #7 were homozygous wildtype. Genotyping of 1,298 dogs (including 83 different breeds, 720 unrelated Hovawarts, and 12 Hovawart family members) demonstrated the occurrence of the deletion only among members of the affected Hovawart family (Table 2, Online Supplementary Table S2). To provide proof that the deletion represented the causative genetic variant and resulted in the low expression of *F9*, functional analyses using electrophoretic mobility shift and luciferase reporter assays were performed.

As shown in Figure 2, no binding of recombinant HNF4 $\alpha$  to the mutated promoter region was detected. On the other hand, the AR lysate clearly showed binding to both fragments and hence the deletion seems not to influence AR binding to the androgen-responsive element in the canine *F9* promoter. To analyze the effect of the promoter variant on *F9* expression, wildtype and mutated promoter fragment luciferase constructs were transfected into Hep G2 cells. As shown in Figure 3 the mutated promoter fragment resulted in a statistically highly significant ( $P=2.2 \times 10^{-9}$ ) reduction of the relative response ratio to approximately 34.6% of the wildtype promoter in the presence of C/EBP (+ C/EBP). C/EBP is clearly also an important transcription factor in the regulation of the canine *F9* promoter as shown when C/EBP was not co-transfected (- C/EBP). In the absence of C/EBP the relative response ratio of the wildtype promoter was 29.8%. On the other hand there were no significant differences between the mutated promoter fragment (+/- C/EBP) and the wildtype promoter fragment (- C/EBP). As for variants of the HNF4 $\alpha$  site, disruption of the C/EBP binding site has also been shown to be causative for hemophilia B in humans.<sup>45,46</sup>

## Discussion

As in humans, hemophilia A and B are also rare diseases in dogs caused by sequence variants in the coagulation

**Table 1.** DNA sequence variants in the canine *F9* gene determined by next-generation sequencing of DNA of animals #4 and #6.

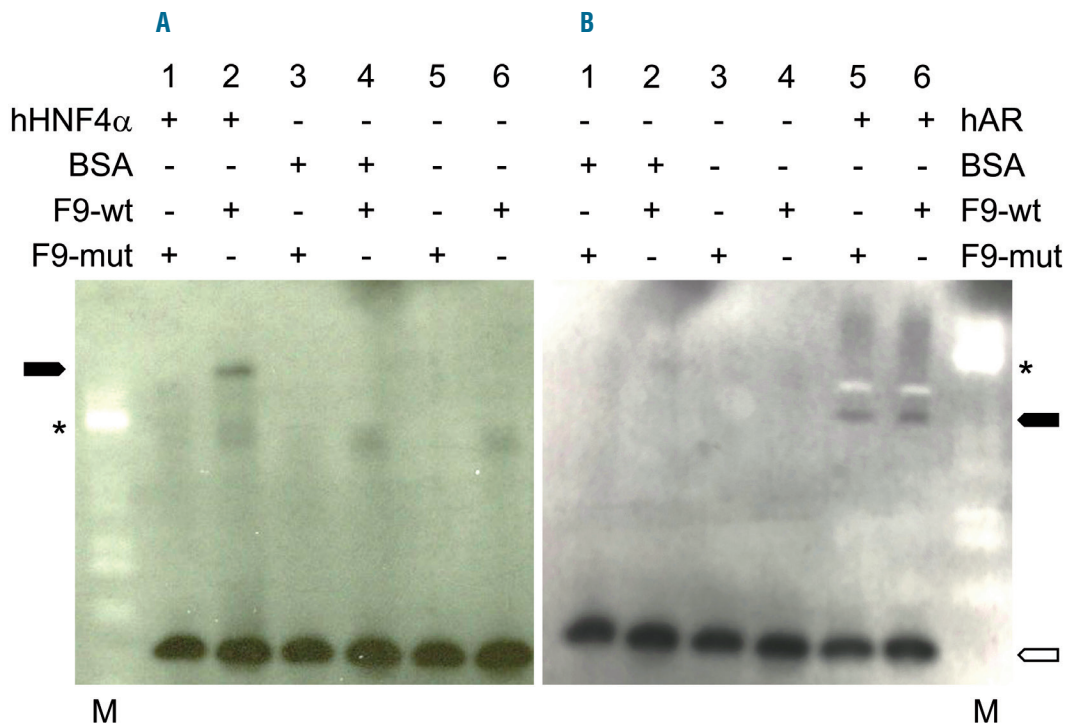
Position	Ref/Alt <sup>a</sup>	Gene region	HGVS <sup>b</sup> g.
X:109501492	C/-	5'-flanking region	NC_006621.3:g.109501492delC
X:109504229	C/-	intron 1	NC_006621.3:g.109504229delC
X:109505462	-/AG	intron 1	NC_006621.3:109505462_109505463insAG
X:109507446	-/A	intron 2	NC_006621.3:109507446_109507446insA
X:109510986	G/A	intron 3	NC_006621.3:g.109510986G>A
X:109524055	A/G	intron 6	NC_006621.3:g.109524055A>G

<sup>a</sup>Ref/Alt: reference nucleotide/alternate nucleotide; <sup>b</sup>HGVS: Human Genome Variation Society (<http://www.hgvs.org>).

**Table 2.** *F9* genotype frequencies

Genotype	HB <sup>a</sup> affected (n=1)	Hovawart		Control, unknown relationship (n= 720)	Other breeds <sup>b</sup> Controls (n= 567)
		HB carrier (n=2)	Control, related (n=12)		
C/C			12	720	567
C/-		2			
-/-	1				

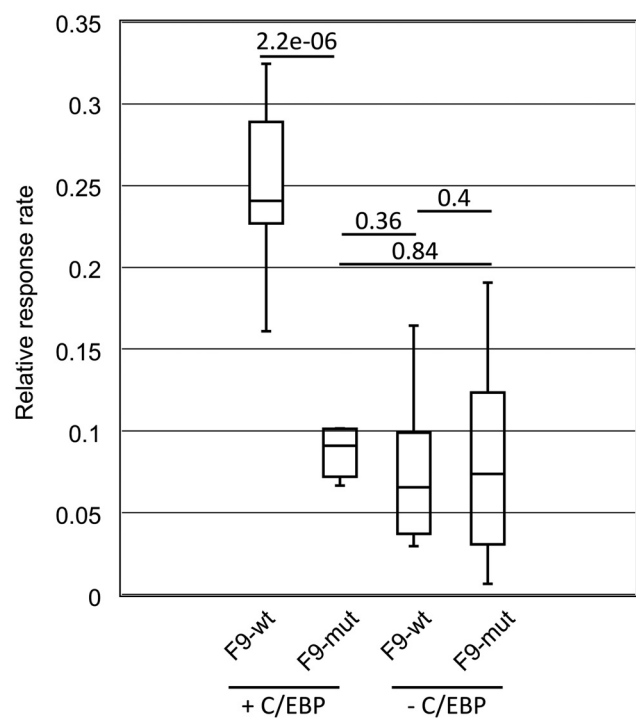
<sup>a</sup>HB: hemophilia B; <sup>b</sup>dog breeds used as controls are listed in Online Supplementary Table S2.



**Figure 2. Analysis of hepatocyte nuclear factor 4α and androgen receptor binding of wildtype and mutated F9 promoter regions using an electrophoretic mobility shift assay.** (A, B) Human hepatocyte nuclear factor 4α (HNF4α) and (B) androgen receptor (AR) were used to bind biotin-labeled wildtype and mutated F9 promoter fragments (F9-wt, F9-mut). Specific shifted bands (solid arrowheads) are detected in lane 2 (A) for HNF4α and lanes 5 and 6 (B) for AR. To test specificity, binding reactions were also performed using bovine serum albumin [BSA; lanes 3 and 4 (A), lanes 1 and 2 (B)]. In lanes 5 and 6 (A) and lanes 3 and 4 (B) no protein was added. Binding reactions were separated on 12% Tris-Glycine gels. X-ray films were cropped using GIMP 2.8.22. The 70 kDa protein marker band (PageRuler Prestained Protein Ladder, Fermentas) is indicated with an asterisk (lane M). The open arrowhead indicates unbound, free DNA.

factor VIII (*F8*) and IX (*F9*) gene, respectively.<sup>47</sup> Since the 1960s cases of canine hemophilia B have been reported and in 1989 the first description of the molecular cause in a Cairn Terrier population at the Francis Owen Blood Research Laboratory was published.<sup>5,8,9,44</sup>

Since then only six further types of variants, all of them affecting the coding region of the *F9* gene, *i.e.* deletions, insertions, missense variants, have been described as causative for canine hemophilia B.<sup>10-12,15,16</sup> The identification of a causative promoter variant in the Hovawart dogs described here is therefore unique in two respects: it is the first regulatory variant described in dogs and secondly this variant resembles a specific subtype of hemophilia B, known as hemophilia B Leyden, in humans.<sup>21</sup> Hitherto, 21 distinct variants in the human *F9* promoter have been determined in families affected by hemophilia B Leyden.<sup>48</sup> These variants cluster in the so-called Leyden-specific region (LSR) and interfere with the binding of different transcription factors, *e.g.* AR, HNF4α, ONECUT, and C/EBPα.<sup>49</sup> The deletion identified in the Hovawart dogs was located 73 bp upstream of the start codon of the canine *F9* gene corresponding to position -23 in the third human promoter cluster harboring the overlapping binding sites of AR and HNF4α.<sup>48</sup> Similar to analyses in humans, it was possible to show by electrophoretic mobility shift assay that the deletion in the canine promoter abolished HNF4α binding because it affects the highly conserved core sequence of the HNF4α binding motif.<sup>24</sup> On the other hand, binding of AR was not affected. This might be due to the fact that AR DNA-binding



**Figure 3. Dual-luciferase reporter analysis of F9 promoter activities in Hep G2 cells.** Box and whisker plot showing the change of relative response ratios (RRR) between the wildtype (F9-wt) and mutant promoter (F9-mut) gene constructs. The lines in the boxes represent the medians. Whiskers indicate minimum and maximum RRR values. Values were normalized as described in the *Online Supplementary Methods*. *P*-values are indicated.

sites display an exceptional amount of sequence variation.<sup>50</sup> Although the C-deletion is located in the consensus TGTNCT-motif of class I AR-binding sites several alternative motifs, e.g. TGT TTC in the stomatin-like protein 3 gene or TGTATC in the prostate-specific antigen gene enhancer III region, have been reported.<sup>50-52</sup> Therefore, it can be assumed that the affected males would have recovered from hemophilia during puberty. To what extent a sex- or possibly also age-dependent amelioration of the hemophilia B Leyden, as described in humans and also a mouse model, might be present in the affected Hovawarts remains to be determined.<sup>49,53</sup> In this respect the Hovawart family could also be of interest as a model in the comparative analysis of age-dependent normalization of *F9* expression in symptomatic carriers of hemophilia B Leyden in humans.

To analyze whether the deletion not only affected HNF4 $\alpha$  binding but also resulted in a downregulation of *F9* expression, we performed luciferase assays. Although relative response ratios do not directly reflect *F9* mRNA or FIX levels *in vivo*, silencing of the promoter activity due to the deletion was obvious from these experiments. Similar findings have been made using HNF4 $\alpha$ -null mice.<sup>26</sup> In the murine model it was shown that expression of factors V, XI, XII, and XIII B directly depends on hepatic HNF4 $\alpha$ . Northern blot analysis also demonstrated that *F9* expression was decreased with a significantly prolonged aPTT in the HNF4 $\alpha$ -null mice.<sup>26</sup> The finding that *F9* expression was not completely dependent on HNF4 $\alpha$  is in agreement with the observation that control of *F9* transcription in mice and humans is complex and depends on a plethora of factors.<sup>26</sup> For instance, in earlier studies it was shown, using DNaseI footprinting, that there are further binding

sites of HNF4 $\alpha$  and other factors, e.g. ARP1, COUP/Ear3, in the human *F9* promoter influencing *F9* expression.<sup>54</sup> However, the classical HNF4 $\alpha$  binding site at position -26 to -19 only binds HNF4 $\alpha$ . When analyzing the canine *F9* promoter using transcription factor binding site prediction algorithms, further potential binding motifs for HNF4 $\alpha$  and other transcription factors were predicted (*data not shown*).<sup>55-57</sup> As ectopic expression of *F9* *in vivo* can be excluded or at least ignored according to recent RNA-sequencing analyses,<sup>58</sup> a remaining reduced hepatic activity of the mutated promoter is in agreement with the clinical findings of residual FIX activity in the affected males (*Online Supplementary Table S1*) and the results of the electrophoretic mobility shift assay showing binding of AR in androgen-dependent promoter activation.

In summary, we have identified and elucidated the causative genetic variant for hemophilia B Leyden in Hovawarts. This is the first report on a single nucleotide deletion within the binding sites of HNF4 $\alpha$  and AR in the *F9* promoter causing hemophilia B Leyden in dogs. As the deletion only abolishes the binding of HNF4 $\alpha$ , it can be assumed that male dogs will most likely recover during puberty, as reported in humans.<sup>30,59,60</sup> However, to prevent any risk of a further propagation of the disorder, genotyping of females is recommended in further breeding.

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