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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 doi:10.3324/haematol.2018.215426

Received: December 26, 2018

Accepted: March 6, 2019.

Pre-published: March 7, 2019.

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Supplementary Information

Supplementary Methods

Coagulation assays and FIX activity measurement

APTT (activated partial thromboplastin time) was measured coagulometrically using different commercially available activating reagents according to the manufacturer's test instructions. To standardize measurement results performed in different laboratories, ratio values (aPTT patient/median aPTT of healthy dogs) were calculated and reported. FIX activity was measured coagulometrically using human FIX deficient plasma and a commercial human aPTT reagent for activation. Canine pooled plasmas were used as reference (activity defined as 100 %).

Next generation sequencing (NGS) and genotyping

A 450 bp library was prepared from genomic DNA with the NEBNext Ultra DNA Library Prep Kit for Illumina (New England Biolabs GmbH, Frankfurt, Germany) following the manufacturer's instructions. Library quality was evaluated with Agilent2100 Bioanalyzer. The following assembly options were used: mer-size 31 nt, min. match percentage 98, high layout stringency, min. aligned length 120 nt, min. layout length 50 nt, max. gap size 5 nt. Duplicate reads were combined and clonal reads removed. For #4 4,590 and for #6 4,982 consistent paired reads were assembled. The sample wise insert size metrics for high quality aligned reads was median pair distance 383.1 bp (SD 117.46 bp, min. distance 151 bp, max. distance 894 bp) for #4 and 383.9 bp (SD 103.55 bp, min. distance 153 bp, max. distance 882 bp) for #6.

Electrophoretic mobility shift assay (EMSA)

Binding reactions included 2 µL 10 x binding buffer (100 mM Tris, 10 mM EDTA, 1 M KCl, 60% v/v Glycerol (86% solution), 0.1 mg/ml BSA, 0.5% Triton X-100, 1mM DTT; pH 7.5), 2 µg poly(dI-dC) and 1.2 µg human HNF4α or 4 µg poly(dI-dC) and 5 µg human AR lysate. As negative controls 1 pmol duplex DNA oligos were incubated without protein or with 1 µg BSA. Binding reactions were pre-incubated for 20 min on ice followed by 1 hour at room temperature after adding 1 pmol biotin-end labelled double-stranded oligonucleotide probes. The mixtures were loaded onto 12% Tris-Glycine gels (Invitrogen, USA). After electrophoresis at 80 V for 90 min (HNF4α) or 2 hours (AR), gels were blotted onto PVDF membranes (GE Healthcare Life Sciences, Germany) using a wet blotter for 30 min at 100 V. Membranes were crosslinked at 120 mJ/cm^2 using a commercial UV-light crosslinking instrument equipped with 254 nm bulbs for 1 minute.

Luciferase-Assay

Promoter fragment design was geared to an equivalence of the canine genomic situation choosing a respective distance between NC_006621.3:g.109,501,492delC and

the luciferase start codon. Recombinant pGL3 vectors were used for transformation of *E. coli* XL1-Blue according to the manufacturer´s protocol. Plasmid DNA of 17 colonies of pGL3Basic+970bpinsertF9_MT and 37 colonies of pGL3Basic+971bpinsertF9_WT were isolated using Promega PureYield Plasmid Miniprep Kit (Promega, Mannheim, Germany) and sequenced for validation. A validated clone of each construct was incubated in LB-medium and plasmid DNA was isolated using Qiagen Plasmid Maxi Kit (Qiagen, Hilden, Germany).

For analysis of promoter activity human hepatoma derived cell line Hep G2 (ATCC HB-8065) was cultivated in Roti-CELL DMEM High Glucose (Carl Roth GmbH, Karlsruhe, Germany) [1]. Constructs were transfected using Effectene Transfection Reagent (Qiagen, Hilden, Germany). Firefly and *Renilla* luciferase luminescence was measured using the Dual-Glo Luciferase Assay System (Promega, Mannheim, Germany) on a Tecan GENios Pro 96/384 Multifunction Microplate Reader (Tecan GmbH, Crailsheim, Germany) with the analysis software XFlour v4.64 after cell lysis with Passive Lysis 5X Buffer (Promega, Mannheim, Germany). For normalization *Renilla* luciferase activity was measured by co-transfecting phRL-TK(Int^-) (Promega, Mannheim, Germany). Experiments were repeated 5-times with two measurements each. Background luminescence values were subtracted from raw luminescence values. *Renilla* luciferase activities were used for normalization [2].

Supplementary Figures

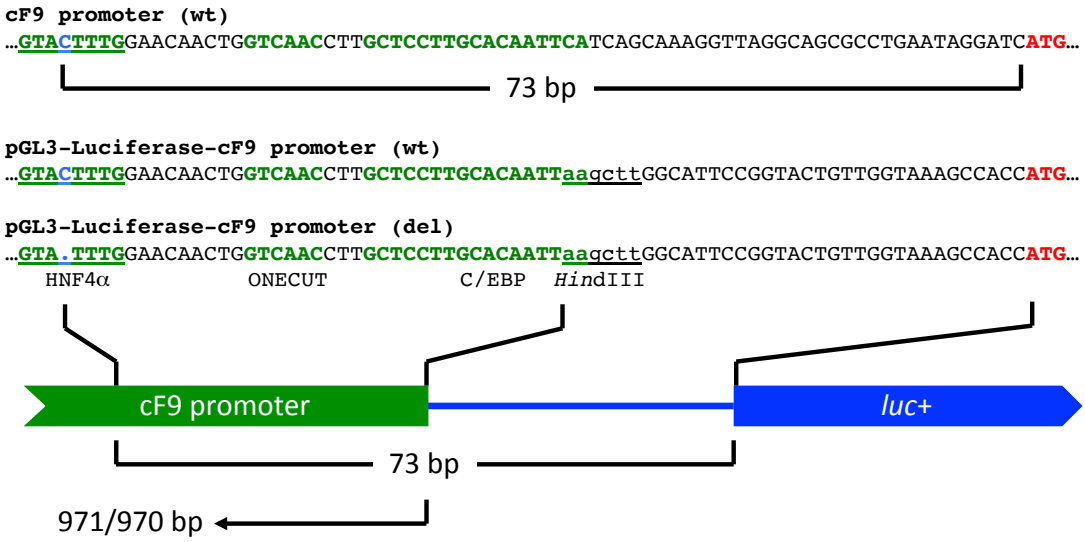

Figure S1. Structure of the pGL3 Luciferase Reporter Vector constructs

Fragments of the canine *F9* gene wild type (971 bp) and mutant (970 bp) promoter were amplified and cloned into the *Hin*dIII restriction site of the pGL3-Basic Vector multiple cloning site upstream of the *luc*+ coding region. The distance of the C-deletion within the overlapping AR/HNF4α binding sites 73 bp upstream the start codons (indicated in bold red) and the potential binding sites of ONECUT and C/EBP were cloned so that the distances of the positions in the promoter inserts to the luciferase reporter gene exactly mimic the *in vivo* genomic distances.

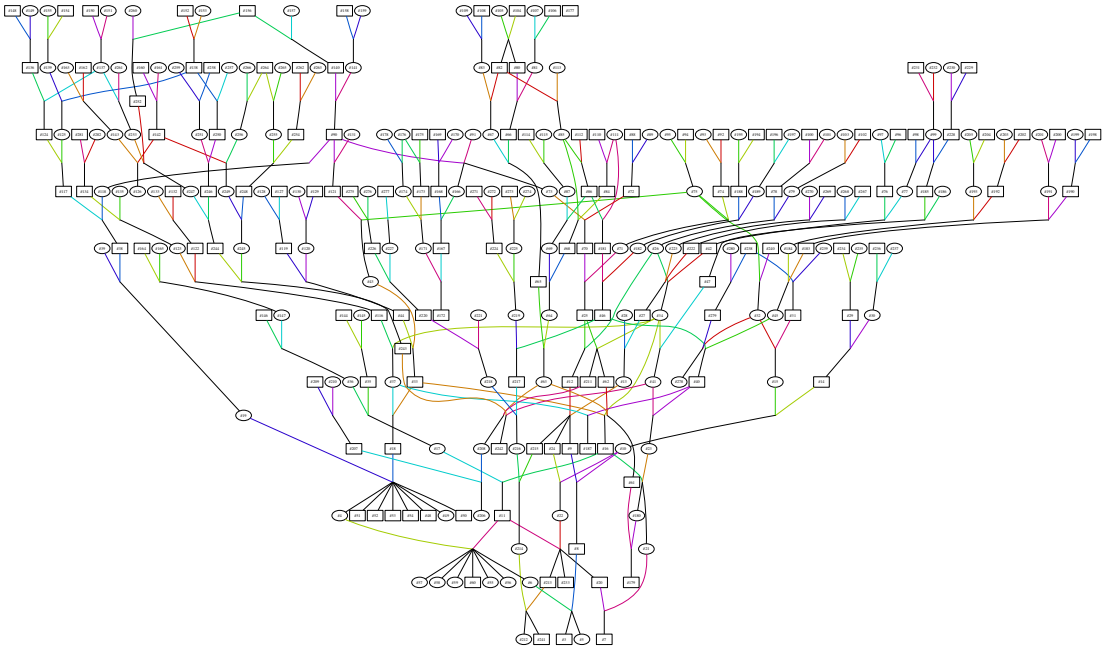

Figure S2. Detailed pedigree of the affected Hovawart family

The ancestry of the affected Hovawart #3 was reconstructed using the online dog breed database "Working dog" and pedigree data of individual dogs provided by the owners [3]. The pedigree was drawn using Pedigraph [4]. Males are indicted with squares, females with circles. Pseudonymized individual dog numbers are given within the pedigree symbols.

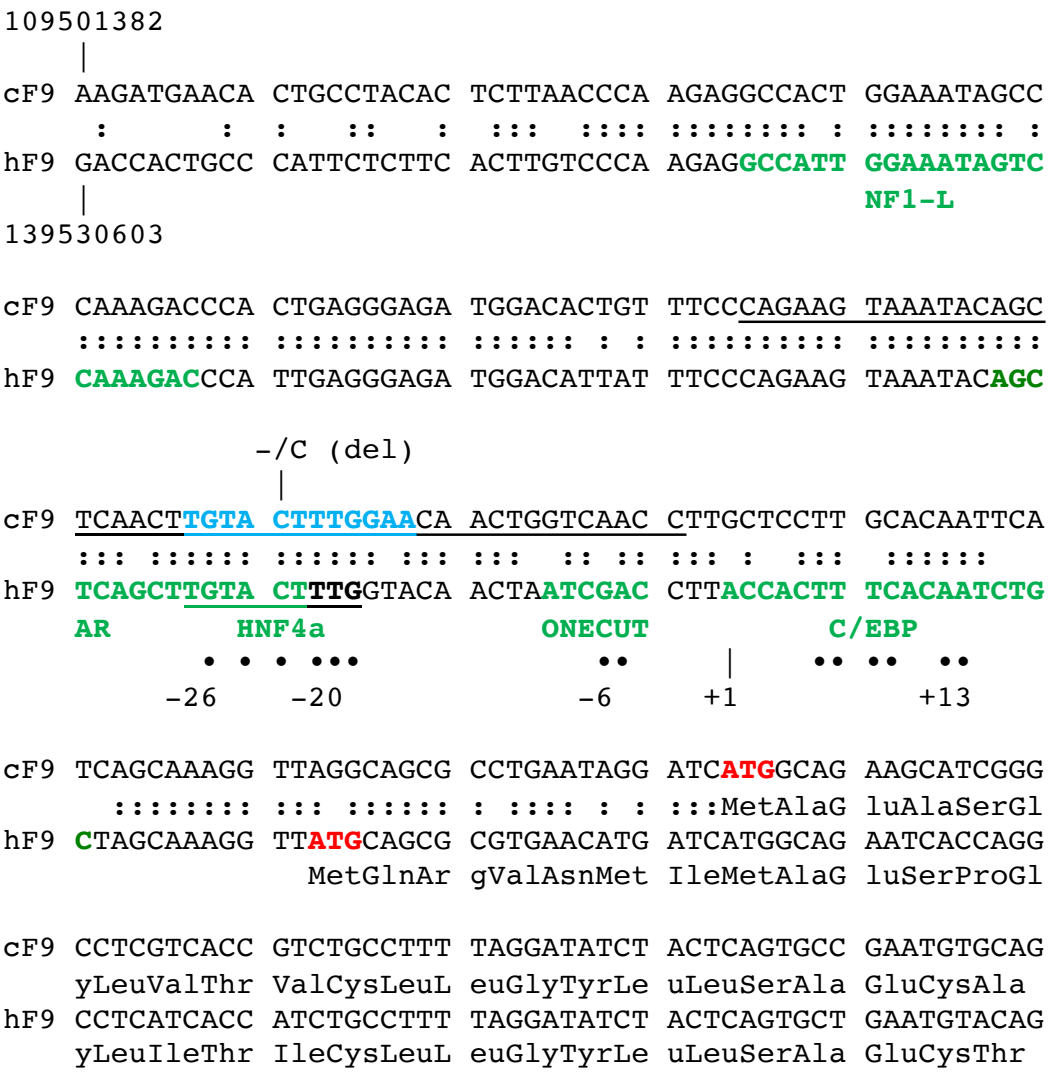

Figure S3. Sequence comparison of the human and canine *F9* promoter region including exon 1

Position 109501382 of the canine *F9* promoter (cF9) corresponds to accession number ENSCAFG00000018998 (CM000039.3, CanFam3.1) [5]. Position 139530603 of the human *F9* promoter (hF9) corresponds to accession number NC_000023.11 (GRCh38.p12) [5, 6]. The start codons of exon 1 are indicated in red [5]. Amino acids of the first exons are shown in 3-letter code below the corresponding DNA sequence. Identical nucleotide positions are depicted with colons. Consensus sequences of transcription factor binding sites identified in the human *F9* promoter are shown in bold and/or green: NF1-L (Nuclear factor-1 liver) [5, 7], AR (Androgen receptor) [8, 9], HNF4α (Hepatocyte nuclear factor 4α) [9-11], ONECUT (ONECUT homeobox transcription factor 1 and 2) [5, 9, 11, 12], C/EBP (CCAAT/enhancer-binding protein alpha) [5, 7, 9, 11]. In the overlapping binding sites of AR and HNF4α, the binding site of HNF4α is also underlined. The position (73 bp upstream of the ATG) of the C-deletion (-/C (del)) causative for Hemophilia B Leyden in the Hovawart family is indicated. The predicted HNF4α binding site in the canine *F9* promoter using the online database PROMO 3.0 is shown in bold blue [13, 14]. The oligo

used for EMSA is underlined in cF9. The transcription start site of the human *F9* gene is indicated with +1 [5, 7]. Bullet points and numbers below the human *F9* promoter DNA sequence mark nucleotide positions that have been shown to associated hemophilia B (+6: T>A [9], +7: T>C [9], +8: T>C [9], +9: T>G [9], +12: A>G [9], +13: A>G, A>C or delA [7, 9]), hemophilia B Leyden (-5: A>T or A>G [12], -6: G>A or G>C [12], -19: G>C [12], -20: T>A or T>C [8, 11, 12], -21: T>G [10, 12], -23: C>T or C>G [12], -24: A>G [12]) and hemophilia B Brandenburg (-26: G>C, G>T or G>A [8, 9, 11, 12]).

Table S1. Determination of hemophilia relevant blood parameters and medical reports

ID[a)]	Sex	aPTT ratio[b)]	FIX (%)[c)]	Medical report
#3	m	ND[d)]	2	severe bleeding after chipping, blood transfusion, death caused by blood loss
#4	f	1.08	92	
#5	f	1.1	64	
#6	f	1.1	58	
#7	m	1.03	83	
#48[e)]	m	4.2	3	slight bleeding during second dentition, lameness/joint problems (age 4 months), several blood transfusions
#50	m	1.22	ND[d)]	
#51[e)]	m	4.76	70	umbilical hernia with internal bleeding after surgery, blood transfusion, minor bleeding episodes
#52	m	3.19	110	
#53[e)]	m	2.93	ND[d)]	recurrent slight bleeding, prolonged bleeding during second dentition, lameness/joint problems
#54	m	1.09	55	
#60[e)]	m	4.47	5	severe bleeding after first vaccination (age 8 weeks)
Control 1	m	1.0	83	healthy unrelated Hovawart control
Control 2	f	0.98	>100	healthy unrelated Hovawart control

a) Animal IDs refer to Fig 1; b) aPTT ratio: reference range 0.88-1.14; c) FIX (%): FIX % of standard (reference range: 75-140%); d) ND: not determined; e) #48, #51, #53 and #60 had been euthanized.

Table S2. Dog breeds used for determination of *F9* genotype frequencies shown in Table 2

Airedale Terrier (n=1), Akita Inu (n=8), Alaskan Malamute (n=1), Appenzell Cattle Dog (n=8), Australian Cattle Dog (n=8), Australian Shepherd (n=8), Barbet (n=8), Barzoi (n=8), Bavarian Mountain Scent Hound (n=3), Beagle (n=8), Bearded Collie (n=8), Belgian Shepherd Dog (n=15), Bernese Mountain Dog (n=8), Border Collie (n=11), Boston Terrier (n=8), Boxer (n=2), Briard (n=8), Cairn Terrier (n=8), Canadian Sheepdog (n=1), Catalan Sheepdog (n=1), Chinese Crested Dog (n=8), Chihuahua (n=7), Cocker Spaniel (n=1), Dachshund (n=11), Dalmatian (n=1), German Hound (n=2), Doberman (n=8), Elo (n=8), Entlebuch Cattle Dog (n=8), Eurasier (n=1), Flat Coated Retriever (n=8), Fox Terrier (n=1), French Bulldog (n=9), German Shepherd (n=12), German Shorthaired Pointer (n=2), German Spaniel (n=10), Giant Schnauzer (n=6), Giant Spitz (n=8), Golden Retriever (n=15), Great Dane (n=8), Greyhound (n=8), Irish Terrier (n=8), Jack Russel Terrier (n=10), Keeshound (n=8), Kromfohrländer (n=8), Labrador Retriever (n=10), Lagotto Romagnolo (n=8), Landseer (n=8), Leonberger (n=8), Magyar Viszla (n=1), Maltese (n=2), Xoloitzcuintle (n=6), Miniature Spitz (n=8), Miniature Pinscher (n=8), Miniature Poodle (n=2), Mudi (n=7), Mongrel (n=34), Newfoundland (n=7), Norwich Terrier (n=8), Nova Scotia Duck Tolling Retriever (n=8), Peruvian Hairless Dog (n=6), Polish Lowland Sheepdog (n=8), Pomeranian (n=8), Poodle (n=10), Portuguese Sheepdog (n=8), Pudelpointer (n=1), Pyrenean Sheepdog smoothfaced (n=2), Rhodesian Ridgeback (n=8), Saluki (n=4), Schapendoes (n=10), Scottish Terrier (n=8), Sibirian Husky (n=9), Shi Tzu (n=1), Spanish Water Dog (n=8), St. Bernard (n=8), Tibet Terrier (n=8), Welsh Terrier (n=3), West Highland White Terrier (n=7), Whippet (n=8), Yorkshire Terrier (n=10)

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