PART 14 Molecular Genetic Analysis

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TOPICS COVERED

- ✓ Molecular Characteristics of Hemophilia-Related Genes and Proteins
- ✓ Spectrum of Causative Variants in Hemophilia
- ✓ Other Phenotypes Associated with the F8 Gene
- ✓ Other Phenotypes Associated with the F9 Gene
- ✓ Deep Vein Thrombosis (DVT) Protection
- ✓ Spectrum of Practical Approaches Applied in Genetic Testing
- ✓ Standardized Nomenclature and Pathogenic Potential of Variants

Genetic analysis of hemophilia is important in defining the underlying cause of the bleeding disorder in affected individuals and their family members. Understanding of the genetic variant associated with the phenotype can help to predict the severity of the disorder, including the risk of inhibitor development. It also aids in the identification of female carriers of hemophilia, who may be offered prenatal diagnosis. It is important that individuals who are proceeding to genetic investigation undergo appropriate genetic counselling prior to any testing. The recent WFH Guidelines for the Management of Hemophilia (3rd edition) (Srivastava et al, 2019) contain a chapter dedicated to the genetic assessment of individuals with hemophilia A and hemophilia B. This laboratory manual chapter describes the genetic basis of hemophilia A and B and highlights the heterogeneity of practical approaches that are currently available throughout the world that may be used to investigate the underlying genetic variants. It also describes the use of standardized nomenclature for the description of genetic variants, and their classification of pathogenicity, as well as highlighting the importance of a clear, concise interpretive report which outlines the genetic result and the implications for the individual and their family.

Molecular Characteristics of Hemophilia-Related Genes and Proteins: The molecular features of hemophilia genes, coagulation FVIII or F8, and coagulation FIX or F9, are shown in Table 41. Table 41 shows the genomic coordinates on GRCh38 (hg38), gene size and cytogenetic location of F8 and F9, their exon complexity (exon number), updated curated versions of RefSeq files (i.e., NG_..., NM_..., NP_...), and the relevant molecular size of the main gene transcripts and their derived polypeptide isoforms.

Table 41. Molecular features of F8 and F9 genes

HGNC official symbol	Gene name	Cytogenetic location	GRCh38 (hg38) (coordinates) NC_000023.11 (length [bp])	Genomic RefSeq (coordinate range)	Main transcript* RefSeq (length [nts]) (exons)	Main protein* RefSeq (length [aa])	OMIM #
F8	coagulation factor VIII	Xq28	complement (154,835,792-155,022,723) (186,931)	NG_011403.2 (5,001-191,932)	NM_000132.4 (9032) (26)	NP_000123.1 (2351)	300841
F9	coagulation factor IX	Xq27.1	(139,530,739-139,563,459) (32,720)	NG_007994.1 (5,001-37,723)	NM_000133.4 (2800) (8)	NP_000124.1 (461)	300746

HGNC: HUGO Gene Nomenclature Committee. Length [Units]: [bp], base pairs; [nts], nucleotides; [aa]; amino acids. *Only the longest (most significant) transcript variant and their derived main isoform are

indicated. OMIM: Online Mendelian Inheritance in Man (https://omim.org/); #, accession number. Data were collected from NCBI (National Center from Biotechnology Information) accessed Jan/18/2024 (https://www.ncbi.nlm.nih.gov/). RefSeq indicates Reference Sequence files from the NCBI browser platform.

Additionally, Table 42 shows the most relevant characteristics of the coagulation FVIII and FIX proteins. Table 42 compiles data from the main protein isoforms, the amino-acid (aa) cartography of recognizable protein domains (specified by UNIPROT database), and protein database (PDB) files associated with 3D-structure models (FVIIIa and FIXa atom 3-dimensional coordinates).

Table 42. Molecular features of coagulation factor VIII and IX proteins

Protein symbol	Protein Name main isoform	Isoform RefSeq (length [aa])	UNIPROT* #	Isoform domains: UNIPROT (aa coordinates)	3D-structure PDB id (aa coordinates)
FVIII	coagulation factor VIII isoform a preproprotein	NP_000123.1 (2351)	P00451	**Signal peptide**: (1-19) **A1**: F5/8 type A 1 (20-348) **A2**: F5/8 type A 2 (399-730) **B:** B-region (760-1667) **A3**: F5/8 type A 3 (1713-2040) **C1**: F5/8 type C 1 (2040-2188) **C2**: F5/8 type C 2 (2193-2345)	**Mature FVIIIa 2R7E.pdb**** A: Heavy chain A1-A2 (Legacy 1-725) B: Light chain A3-C1-C2 (Legacy 1689-2332)
FIX	coagulation factor IX isoform 1 preproprotein	NP_000124.1 (461)	P00740	**Signal peptide**: (1-28) – **Pro-peptide**: (29-46) **Gla**: γ-carboxyglutamate-rich (47-92) **EGF1:** Ca++-binding EGF-like 1 (93-129) **EGF2:** EGF-like 2 (130-171) **Act_peptide**: Activation peptide (192-226) **Tryp_SPc**: Trypsin-like serine protease (227-457)	**Homology model FIXa.pdb***** **L**: Light chain Gla-EGF1-EGF2 (47-171) **H**: Heavy chain Tryp_SPc (227-461)

*UNIPROT protein browser (URL: https://www.uniprot.org/). **Shen et al, 2008. ***Curators from the EAHAD Variant Databases Project (Rallapalli et al, 2013; McVey et al, 2020). Most data were collected from the EAHAD (European Association for Haemophilia and Allied Disorders) Coagulation Factor Variant Databases (URL: https://dbs.eahad.org/) accessed Jan/18/2024. Codons and amino acids (aa) are numbered following HGVS rules (i.e., codon +1 coding for the first residue (Met) of the primary polypeptide in FVIII and FIX). In Legacy numbering, codon/amino-acid +1 refers to that coding for the first amino-acid of the mature FVIII protein (excluding 19 aa of the signal-peptide) and FIX protein (excluding 46 aa of the signal-peptide and the pro-peptide). Although HGVS numbering is recommended, Legacy numbering has been extensively used in former publications.

Spectrum of Causative Variants in Hemophilia: Most pathogenic variants affecting the F8 gene cause hemophilia A, whilst most pathogenic F9 variants cause hemophilia B. The Online Mendelian Inheritance in Man (OMIM) database (https://www.omim.org) compiles a wide set of comprehensive information about human genes, indicates variants affecting their function, and describes and classifies their associated phenotypes. The OMIM database indicates that F8 variants are associated with two different phenotypes: hemophilia A (#306700) and thrombophilia 13 (X-linked, due to FVIII defect) (#301071) ((THPH13); while F9, with four phenotypes: hemophilia B (#306900), thrombophilia 8 (X-linked, due to FIX defect) (#300807) (THPH8), protection against deep venous thrombosis (DPV) (#300807), and warfarin sensitivity (#301052). Table 43 and Table 44, respectively, show the spectrum of F8 mutations causing hemophilia A according

to the coagulation FVIII activity levels (FVIII:C) and F9 mutations causing hemophilia B, associated with the FIX levels (FIX:C). Most relative prevalences of hemophilia A and hemophilia B causative variants, listed in Tables 43 and 44, were extracted from the European Association for Hemophilia and Allied Disorders (EAHAD) databases. The EAHAD F8 variant database excludes prevalent inversions associated with severe hemophilia A involving almost half of patients. In order to present an unbiased prevalence of severe hemophilia A causative mutations, their relative frequencies were merged taking into account the worldwide averages of the F8 intron 22 inversions (Inv22) reported by Antonarakis et al (1995), and those estimated for the F8 intron 1 inversion (Inv1) from an international hemophilia A patient series (Rossetti et al, 2004) (Table 43).

Table 43. Most typical hemophilia A causative F8 variants in hemizygous patients from international sources.

Variant type by effect	Severe non inversions # cases (%)	Severe Global # cases (%)	Moderate # cases (%)	Mild # cases (%)	References
Missense	1418 (30.2)	(16.2)	1340 (79.9)	3048 (95.8)	F8_var_db EAHAD*
In-frame-indel	70 (1.5)	(0.8)	19 (1.1)	32 (1.0)	F8_var_db EAHAD*
Frameshift-indel	1487 (31.7)	(17.0)	142 (8.5)	27 (0.9)	F8_var_db EAHAD*
Splicing defect	320 (6.8)	(3.7)	98 (5.8)	68 (2.1)	F8_var_db EAHAD*
Nonsense	968 (20.6)	(11.1)	59 (3.5)	4 (0.1)	F8_var_db EAHAD*
Large deletion (SV)	426 (9.1)	(4.9)	19 (1.1)	3 (0.1)	F8_var_db EAHAD*
Total non-inversions	4689 (100)	(53.7)	1677 (100)	3182 (100)	F8_var_db EAHAD*
Inv22 (SV) type 1 **Inv22 (SV) type 2** **Inv22 (SV) other types**		740 (35.4) 140 (6.7) 25 (1.1)			Antonarakis et al, 1995
Total Inv22		2093 (43.2)			
Inv1 (SV)		19 (3.1)			Rossetti et al, 2004
Total Inv1		622 (3.1)			

SV indicates structural variants including copy number variants (CNVs) as large F8 deletions and large F8 inversions (non-CNV) as the intron 22 inversion (Inv22) and intron 1 inversion (Inv1). Data from F8 inversions' uninformative patients were obtained from EAHAD (European Association for Haemophilia and Allied Disorders) databases registering individual patients. *F8 variant database (http://f8-db.eahad.org/).

The most characteristic and recurrent variant causing severe hemophilia A worldwide, is the F8 intron 22 inversion, a large perfect inversion of 600 kb mediated by recombination between inverted repeats of 10 kb (int22h or h) disrupting the F8 structure impeding the normal RNA splicing between exons 22 and 23 (Lakich et al, 1993; Naylor et al, 1993). There is a F8 intragenic copy of int22h within intron 22 (h1) and two extragenic copies (h2 and h3). Depending on which extragenic copy recombines with the intragenic one, the Inv22 shows a pattern type 1 (h1/h3) or a pattern type 2 (h1/h2). The Inv22 originates almost exclusively from male germ cells (Rossiter et al, 1995) and, consequently, the majority of mothers of patients with the Inv22 are carriers (Tizzano et al, 1995). The molecular mechanism of non-allelic homologous recombination between large inverted repeats in male meiosis supports the Inv22 recurrence as the most prevalent cause for severe hemophilia A worldwide (Table 43). Similarly, the F8 intron 1 inversion (Inv1) is a large perfect DNA inversion caused by recombination between 1 kb inverted repeats (int1h) disrupting the F8 structure at intron 1 (Bagnall et al, 2001) and involves an estimated average of 3% of severe hemophilia A patients worldwide (Table 43).

The remaining group of patients with severe, moderate, or mild hemophilia A (Table 43), uninformative for the F8 inversions, and all patients with hemophilia B (Table 44) show a typical spectrum of deleterious variants, including single nucleotide substitutions (SNV) predicting missense, nonsense, or splicing defects; small insertions/deletions (INDEL) predicting frameshifts or in-frame changes; or, less frequently, large copy number variations (CNVs), mostly large deletions.

Table 44. Most typical hemophilia B causative F9 variants in hemizygous patients from international sources

Variant type by effect	Severe # cases (%)	Moderate # cases (%)	Mild # cases (%)	References
Missense	999 (52.3)	1039 (85.1)	719 (95.0)	F9_var_db EAHAD*
In-frame-indel	27 (1.4)	9 (0.7)	1 (0.1)	F9_var_db EAHAD*
Frameshift-indel	185 (9.7)	42 (3.4)	2 (0.3)	F9_var_db EAHAD*
Splicing defect	135 (7.1)	66 (5.8)	30 (4.0)	F9_var_db EAHAD*
Nonsense	459 (24.0)	62 (5.1)	5 (0.7)	F9_var_db EAHAD*
Large deletion (SV)	107 (5.6)	3 (0.3)		F9_var_db EAHAD*
Total	1912 (100)	1221 (100)	757 (100)	F9_var_db EAHAD*

SV indicates structural variants as large deletions affecting partially or totally the F9 gene. Data from HB patients were obtained from EAHAD (European Association for Haemophilia and Allied Disorders) databases registering individual patients. *F9 variant database (https://f9-db.eahad.org/).

Information about F8 and F9 variants is compiled in publicly accessible databases, such as those developed by the CDC (Centers for Disease Control and Prevention) named CHAMP and CHBMP for hemophilia A and hemophilia B, respectively (https://www.cdc.gov/ncbddd/hemophilia/champs.html), and by EAHAD (European Association for Haemophilia and Allied Disorders) for F8 (http://f8-db.eahad.org/) and F9 (http://f9-db.eahad.org/) (Tables 43 and 44). Accessed on January 18, 2024, EAHAD databases contain information from 3052 unique F8 variants corresponding to 10144 individual cases, and 1244 unique F9 variants corresponding to 4713 individual cases. In Tables 43 and 44, genetic variants are classified by their predicted effect from the observed DNA nucleotide sequence evidence (i.e. missense, in-frame-indel, frameshift-indel, splicing defect, nonsense, large deletion). F8 and F9 variants, respectively, listed in Tables 43 and 44 represent those hemophilia causative variants with significant frequencies worldwide in contrast with those prevalent variants found in particular populations typically associated with non-severe phenotypes (e.g. F8 exon 13 duplication prevalent in the Italian population of mild hemophilia A (Acquila et al, 2004).

Other Phenotypes Associated with the F8 Gene

Thrombophilia 13 (X-linked, due to FVIII defect): Shen et al (2013) evaluated FVIII:C activity levels and F8 gene copy number in patients with venous thromboembolism (VTE) versus healthy controls. VTE patients showed significantly higher FVIII:C and greater number of copies of the F8 gene. Simioni et al (2021) reported two Italian families with thrombophilia 13 and identified a partial F8 tandem duplication, which is consistent with X-linked dominant inheritance pattern as hemizygous male patients are more severely affected than female carriers.

Other Phenotypes Associated with the F9 Gene

Thrombophilia 8 (due to FIX defect) is an X-linked recessive inherited phenotype associated with early onset VTE caused by a F9 missense defect, R338L or variant Padua, reported by Simioni et al (2009). FIX-Padua was reported to enhance the fibrinolytic resistance of plasma clots (Ammollo et al, 2014).

Deep Vein Thrombosis (DVT) Protection:

The common polymorphic variant FIX-Mälmo (minor allele frequency of 0.32), p.(Thr148Ala) due to a G>A single nucleotide substitution (SNV), associates with a DVT risk protection with odds ratios (OR) of 0.8 in male patients and 0.89 in female patients (Bezemer et al, 2008). However, the molecular mechanisms for DVT protection conferred by the FIX-Mälmo polymorphism remained unknown.

Warfarin is a widely prescribed anticoagulant for the prevention of thromboembolic events in 'at risk' patients. Warfarin sensitivity (X-linked, due to FIX variants) refers to a bleeding phenotype complication during anticoagulation therapy with vitamin K antagonists. Pezeshkpoor et al (2018) reported an association between F9 missense variants affecting the pro-peptide, such as p.(Ala37Thr) and p.(Ala37Val), and warfarin sensitivity characterized by a disproportionate reduction of FIX:C levels during anticoagulation therapy.

The causal relationship between a given genetic variant (e.g. F8 Inv22) and a particular phenotype (e.g. severe hemophilia A in a hemizygous patient) can be modified in rare cases by the involvement of a genetic mosaicism (GM), which is defined as a coexistence of at least two genetically different clones in an individual (e.g. Inv22-positive and -negative cells). A GM may involve partially or totally, some or all organs/tissues from an affected individual, resulting for example, in milder phenotypes if it affects somatic cells (e.g. hepatic endothelium-derived FVIII/FIX producing cells) and the gene variant heritability if it affects germ cells (e.g. a germinal mosaic male hemophilia patient may be the father of non-carrier daughters) (Abelleyro et al, 2018).

As historical paradigms of X-linked recessive disorders, hemophilia A (OMIM #306700) and hemophilia B (OMIM #306900) are typically expressed in hemizygous male patients (46,XY) and heterozygous females (46,XX) are usually asymptomatic. According to a consensual point of view among hematologists, a new classification of female hemophilia considers the factor coagulation activity levels, indicating severe disease when <1 IU/dl, moderate 1-5 IU/dl, and mild hemophilia 5-40 IU/dl; and when factor levels are >40 IU/dl, individuals are classified as symptomatic and non-symptomatic carriers (van Galen et al, 2021). The molecular basis of female hemophilia involves the impaired expression or silencing of F8 or F9 alleles mediated by the phenomenon of X-chromosome inactivation (XCI), which silences the gene expression in cis from one X in each cell to compensate doses with males. XCI takes place early in embryogenesis normally at random in each cell and this state is inherited clonally in the adult life of women. A homozygous female carrier and a compound heterozygote are expected to express hemophilia as well as heterozygous carriers with skewed XCI preferentially silencing the normal allele (Radic et al, 2015). Moreover, Garagiola et al (2021) proved a significant association between FVIII/FIX clotting activity levels and the pattern of XCI measured in peripheral blood leukocytes from heterozygous hemophilia A carriers with ≤50 IU/dl.

Spectrum of Practical Approaches Applied in Genetic Testing: Depending on the availability of resources and expertise, there are a variety of techniques that may be employed for the investigation of genetic variants associated with hemophilia A and hemophilia B. This chapter provides examples of these practical approaches and references, where available. A number of different techniques are available for the investigation of the F8 intron 22 inversion, including Southern blot, long range polymerase chain reaction (PCR), and inverse-shifting PCR (Lakich et al, 1993; Liu et al, 1998; Bagnall et al, 2006; Rossetti et al, 2008; Abelleyro et al, 2016; Ding et al, 2016; Hudecova et al, 2017; Pan et al, 2014; Kumar et al, 2015; Edison et al, 2016). The F8 intron 1 inversion can be detected by techniques such as double PCR or inverse shifting PCR (Bagnall et al, 2002; Rossetti et al, 2008). Analysis of SNVs in F8 and F9 can be performed by a range of techniques, including PCR and Sanger sequencing, or high throughput sequencing technologies, such as next generation sequencing (NGS) (Al-Allaf et al, 2019; Li et al, 2014; Lyu et al, 2016; Manderstedt et al, 2019; Edison et al, 2016). Where resources are limited, a screening approach prior to Sanger sequencing could be employed (Salviato et al, 2019), such as heteroduplex analysis using conformation sensitive gel electrophoresis (CSGE). For analysis of CNVs in F8 and F9, there are a number of techniques such as gap-PCR, multiplex ligation-dependent probe amplification (MLPA), quantitative real-time PCR, and NGS (Rossetti et al, 2004; Payne et al, 2012; Costa et al, 2004; Belvini et al, 2017; Kinkle et al, 2017; You et al,

2013; Wu et al, 2014; Fernandez-Lopez et al, 2007; Tizzano et al, 2005; Johnsen et al, 2017). In cases of hemophilia A and hemophilia B where an underlying genetic variant is not found in the essential regions of the F8 or F9 genes using the techniques described above, analysis of deep intronic regions for potential splicing defects may be available by targeted massive parallel sequencing (MPS) or whole genome sequencing (WGS) (Jourdy et al, 2018; Jourdy et al, 2020; Bach et al, 2015; Inaba et al, 2017; Castaman et al, (2011; Chang et al, 2019). Linkage analysis may also be considered for family studies where there is no identifiable F8 or F9 variant (Sun et al, 2015). The investigation of individuals with atypical phenotypes which may be due to complex genomic rearrangements may be by cytogenetic microarray analysis (Jourdy et al, 2016; Jourdy et al, 2017; Janczar et al, 2016; Lannoy et al, 2018). Analysis of X-chromosome inactivation may be performed by a number of techniques such as methylation specific restriction enzyme, PCR and fragment analysis, or other quantitative techniques (Nisen et al, 1989; Coleman et al, 1993; Johansson et al, 2023; Machado et al, 2014).

Standardized Nomenclature and Pathogenic Potential of Variants: Accuracy in the univocal description of genetic variants is essential for research and clinical care. To address this requirement, the Human Genome Variation Society (HGVS) Variant Nomenclature Committee (HVNC) under the auspice of the Human Genome Organization (HUGO) developed a set of recommendations. These include that genetic variants should be described at the most basic level, the DNA level, and descriptions at the RNA and/or protein level, in general predicted from the DNA evidence, may be given in addition (https://hgvs-nomenclature.org/stable/) (den Dunnen et al, 2016). HGVS nomenclature recommends a specific numbering for gene positions indicating codon +1 coding for the first residue (Met) of the primary polypeptide and nucleotide +1 for the A of the initiation codon AUG. In some former hemophilia publications, legacy numbering of codon/amino-acid +1 refers to that coding for the first amino-acid of the mature protein (i.e. in HGVS numbering, FVIII codon 20 and FIX codon 47). To adjust and normalize the nomenclature of variants according to HGVS, the Mutalyzer website offers efficient algorithms to check and verify their correct description from HGVS recommendations (https://mutalyzer.nl/) (Lefter et al, 2021). All detected variants should be classified according to their potential to cause the observed phenotype according to the guidelines produced by the American College of Medical Genetics and Genomics/Association for Molecular Pathology (ACMG/AMP) (Richards et al, 2015). ACMG recommendations can be applied to conventional or next-generation sequencing-based genetic tests used in clinical laboratories and comprise a five-tier system of classification for variants relevant to Mendelian disorders: (1) pathogenic, (2) likely pathogenic, (3) uncertain significance, (4) likely benign, and (5) benign. To achieve this categorization, the ACMG/AMP recommends a thorough analysis of (a) population data, (b) computational data, (c) functional data, and (d) segregation data.

For example, the analysis of F8 and F9 variants involve:

(a) The study of the genotyped variant in the general population and its frequency in hemizygous, heterozygous individuals, etc. consulting gnomAD (https://gnomad.broadinstitute.org/), ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/), and in the F8/HA and F9/HB gene specific databases, such as EAHAD and CHAMP (referenced above).

(b) The application of in silico bioinformatic tools to analyze missense changes by predicting eventual structural or functional changes using, for example, PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/), MutationTaster (https://www.mutationtaster.org/) and Varsome (https://varsome.com/) among others; or to evaluate eventual splicing defects, such as NNSplice (https://www.fruitfly.org/seq_tools/splice.html), NetGene2 (https://services.healthtech.dtu.dk/services/NetGene2-2.42/) and ESEFinder for searching differences in exonic splicing enhancer sequences (esefinder.ahc.umn.edu); and many other computational tools to estimate the impact of variants on the promoter, 5'- or 3'-UTR, etc.

(c) Experimental data obtained from in vitro and in vivo functional studies of the mutated versus normal version to be tested, or a part of it, provide significant information to establish the impact of a genetic variant.

(d) Segregation data associated with an X-linked recessive mode of inheritance and co-segregation with hemophilia in multiple affected family members is indicative of pathogenicity. Taking into account this analysis, the ACMG/AMP indicate the criteria for classifying pathogenic variants scoring the evidence as very strong (PVS), strong (PS), moderate (PM) and supporting (PP); and the criteria for classifying benign or neutral impact of variants scoring the evidence as stand-alone (BA), strong (BS) and supporting (BP). Final classification in a category to estimate pathogenicity (1-5) results from the combination of P_ and B_ evidence scores (Richards et al, 2015).

Interpretive Reports: Interpretive reports should be clear and concise, and address the diagnosis of the person under investigation. Beyond its main focus stating its overall molecular conclusion in answering the genetic question, an interpretive report should include sufficient details to allow identification of the variant in other laboratories (i.e. indicate the practical approaches used, limitations of the techniques, genomic reference sequence used, and the classification of pathogenicity according to the ACMG guidelines, including the evidence applied for classification and references; ACGS reporting guidelines, 2020; Deans et al, 2022; Claustres et al, 2014; Gomez et al, 2019). Interpretive reports should include information explaining the actual extent of the molecular diagnosis in plain language with clear indication of, for example, specific risks for developing specific phenotypes in the family.

Quality assurance: In genetic testing, quality assurance covers all aspects of the diagnostic process, from nucleic acid extraction and analytical procedures, through to the classification and description of the variants detected and the production of an interpretive report. Internal quality control (IQC) of genetic tests should be routinely performed to ensure the validity of the results produced. Formal EQA schemes are available to ensure that diagnostic process and reporting procedures are in agreement with other laboratories (e.g. Genomics Quality Assessment [GenQA], and specifically for hemophilia genetic assessment by the U.K. National External Quality Assessment Service [UK NEQAS] for Blood Coagulation). Genetics laboratories should undergo periodic accreditation, if available, against internationally agreed standards, by an approved body. This ensures high quality provision of the genetic diagnostic service.

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