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Frameshift variations in the *RHD* coding sequence: Molecular mechanisms permitting protein expression

Willy A. Flegel, **Kshitij Srivastava**

Department of Transfusion Medicine, NIH Clinical Center, National Institutes of Health, Bethesda, Maryland

The *RHD* gene, located on the short arm of Chromosome 1, encodes RhD, a highly immunogenic blood group protein.[1] A D-negative phenotype is caused by a complete loss of the RhD protein on the red blood cell (RBC),[2] whereas individuals with a DEL phenotype express minuscule amounts of the RhD protein.[2–4] Individuals lacking the D antigen can produce anti-D when exposed to D-positive RBCs through transfusion (causing hemolytic transfusion reaction), pregnancy (causing hemolytic disease of the fetus and newborn), or transplantation (causing graft rejection).[5]

Several molecular mechanisms can lead to a D-negative phenotype. The Human RhesusBase[6] lists 90 D-negative alleles with a variety of genetic causes, including 31 deletions, 19 nonsense variants, 15 splice site variants, 15 *RHD-CE-D* hybrids,[7] five missense variants, three insertions, and two duplications. Of these, the *RHD* gene deletion is the most frequent D-negative allele worldwide.[8] Frameshift variations are deletions or insertions of, for example, 1, 2, or 4 nucleotides, which alter the ribosome reading frame, resulting in premature termination of translation.[9,10] Ordinarily, frameshift variants induce the degradation of the mRNA transcript by nonsense-mediated decay and thus obliterate the expression of protein.[11]

Previous studies explained the conversion of a D-positive phenotype to a D-negative phenotype[12] through genetic rearrangement at Chromosome 1[13–18] and subsequent clonal expansion.[12,14,19–23] However, the conversion of a D-negative to a D-positive phenotype involving clonal expansion has never been reported.

Many patients and donors harboring distinct frameshift variants in the *RHD* gene have been described.[6] Most such alleles encode a D-negative phenotype, as expected, but some unexpectedly express traces of the RhD protein and their carriers present with weak D or DEL phenotypes. Mechanisms such as transcriptional frameshifting, translational frameshifting, and alternate translation start site may explain these enigmatic observations.

Correspondence: Willy A. Flegel, Laboratory Services Section, Department of Transfusion Medicine, NIH Clinical Center, National Institutes of Health; Bethesda, MD 20892, USA. waf@nih.gov.

AUTHOR CONTRIBUTION

Willy Albert Flegel and Kshitij Srivastava wrote the paper.

CONFLICT OF INTEREST

The authors declare no conflict of interest relevant.

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We review the known *RHD* alleles with frameshift variants and discuss molecular mechanisms that can explain those with D-positive phenotypes.

1 | METHODS

We searched the Human RhesusBase[6] and GenBank[24] and catalogued all known frameshift variants. Accompanying the online search, we checked the reference lists on the Web page of each *RHD* variant in the Human RhesusBase Web site for potentially pertinent original reports or additional variants.

2 | RESULTS

Among the 51 published frameshift variants in the *RHD* gene, the phenotype for 44 alleles has been reported (Table 1),[25–34] of which 36 alleles present a D-negative phenotype, as expected. The remaining eight alleles express, however, some RhD protein; six were reported as DEL and two as weak D phenotype.[6,35]

3 | DISCUSSION

The total number of frameshift variants in the *RHD* gene currently stands at 51 (Table 1). The canonical mechanism for the D-negative phenotype of *RHD* frameshift variants is a premature stop codon.[2,36] However, several noncanonical mechanisms permit expression of a cell-surface protein from genes with distinct frameshift variants. We summarized the accumulated evidence and outlined the underlying principle for hitherto inexplicable D-positive phenotypes[30,35,37–39] that were observed associated with some *RHD* frameshift variants, including premature stop codons or elongated proteins.

In-frame deletion,[40–43] insertion,[44] or duplication[45,46] of 3N nucleotides (three nucleotides or a multiple thereof), without affecting the *RHD* open reading frame, causes either a weak D,[41,45,46] partial D,[40,42,44] or D-negative phenotype.[43,47] Insertion, deletion, or duplication of 3N ± 1 nucleotides (any number of nucleotides that is not a multiple of 3) in the coding sequence of a gene alters the reading frame: These frameshift variants are expected to cause the lack of full-length proteins.[27] However, molecular mechanisms, known to overcome the effects of a frameshift variation, can lead to the synthesis of full-length, truncated, or elongated, yet functional, proteins. These mechanisms include “transcriptional frameshifting or slippage,”[48,49] “translational or ribosomal frameshifting,”[29,50] “alternate translation start site,”[51] and “ribosomal termination-reinitiation.”[52,53] “Addition of amino acids” due to frameshift variants at the carboxy-terminal end is a mechanism that leads to elongated proteins, which may or may not be functional. We examined the 51 known frameshift variants (Table 1) and detailed the most plausible reasons why most of the frameshift variants cause a D-negative phenotype while some cause a DEL or weak D phenotype.

Transcriptional frameshifting occurs when the RNA polymerase encounters a template with homopolymeric sequences and adds or deletes one or more nucleotides to the run of repeat bases.[54] Variants in the repeat region, such as *RHD*93_94insT*,[37] may produce full-length protein, explaining the reported DEL phenotype.[37] Reading-frame restoration by this mechanism was first shown in vivo for the human apolipoprotein B gene.[55] The

*RHD*93_94insT* may also express DEL by other mechanisms (Table 1), which are not mutually exclusive and described in the next two paragraphs.

Translational frameshifting occurs in the presence of a heptanucleotide site X-XXY-YYZ, which allows slippage of ribosome-bound tRNAs.[29] The XXX can be any homopolymeric sequence; YYY can be either AAA or TTT; and Z can be A, T, or C. The presence of such a "slippery" heptanucleotide at position c.87 to c.93 in *RHD* Exon 1 (T-TTT-TTT) may allow translational frameshifting. Variants around this region, such as *RHD*93_94insT*,[37] may translate some *RHD* mRNAs to produce a full-length protein, as previously observed.[56]

Alternate translation start site requires a modified initiation mechanism, such as the leaky scanning mechanism.[57] Ribosomal termination-reinitiation can also produce proteins truncated at their amino-terminal end. Previous studies have shown mRNAs containing premature termination codons due to nonsense, frameshift, and splice site variants near the translation start site can resist nonsense-mediated decay.[58–60] These mRNAs produce truncated proteins when the ribosome resumes scanning and reinitiate translation at downstream methionine codons. We have postulated RhD protein expression from alternate translation start sites (ATG) in *RHD* Exon 2 for the *RHD* Exon 1 deletion (*RHD*ex1del type 1 allele).[28] The D-positive phenotypes of *RHD*29delGGCGCTGCCTGCCC*,[35] *RHD*93_94insT*,[37] and *RHD*147delA*[30] alleles may be explained by the same mechanism. However, an alternate translation start site requires additional initiation sequences in its vicinity.[61–64] Some *RHD* Exon 1 variant alleles express a D-positive phenotype and others do not, which may depend on the presence or absence of a working copy of a translational initiation sequence nearby.

Addition of amino acids at the carboxy-terminal end is known to permit a D-positive phenotye.[38,39] Frameshift variants in Exons 9 and 10 may result in such elongated proteins. The carboxy-terminal amino acids encoded by part of *RHD* Exons 9 and 10 are involved in the interaction of the RhD protein with the RBC cytoskeleton.[28] Genetic variants in this region often lead to a weakened D expression in the RBC membrane,[6] as observed for the frameshift variants *RHD*1248_1249insG*[39] and *RHD*1252_1253insT*[38] (Table 1).

A complete loss of *RHD* Exon 10 can lead either to a DEL phenotype (*RHD*ex10del type 1[28,65]) or to a D-negative phenotype (*RHD*1228–1_1248delTTTCCTCATTTGGCTGTTGGA*[5] and *RHD*ex10del type 2[28]). Molecular variants affecting the carboxy-terminal cytoplasmic amino acid positions 391 to 417[66] warrant systematic screening. Sensitive serologic methods may discover DEL or weak D phenotypes.

Neither of the four noncanonical mechanisms suggested for *RHD* frameshift alleles with D-positive phenotypes (Table 1) applies to *RHD*510insG*,[67] and *RHD*822delG*,[32] and *RHD*941_942delGG*.[68] Other possible mechanisms include "alternate mRNA isoforms" and "stop codon readthrough." Either mechanism may apply to any of the listed 51 *RHD* frameshift alleles, although no functional data have supported these two mechanisms in *RHD* so far. To prove or disprove these, a detailed molecular and serologic workup of samples will be required.

Alternate mRNA isoforms are a likely explanation in at least one known *RHD* allele: the deletion of a whole *RHD* exon such as *RHD* Exon 8 (*RHD*ex8del type 1)[69] has been shown to permit expression of an RhD protein in RBC membranes. Although functional data are lacking, alternate *RHD* mRNA isoforms[64,65] skipping an exon[70,71] that contains a frameshift variant could in principle lead to the expression of a truncated RhD protein, particularly if the reading frame is not altered.

Stop codon readthrough, where the ribosome miscodes at a premature termination codon, can produce full-length proteins.[62,72,73] Factors such as identity of the stop codon and surrounding sequence contexts,[72,74–78] proximal RNA structures,[79,80] RNA modifications,[81] and presence of RNA binding proteins[82] influence the likelihood of a readthrough.

RBCs with rescued RhD expression could be expanded through clonal hematopoiesis,[13,83,84] giving rise to either a weak D or a DEL phenotype. The presence of predisposing genetic[85–88] and environmental factors[89,90] and stochastic processes may influence the probability of clonal emergence, explaining why some *RHD* frameshift alleles express RhD protein while others do not.[91]

Various mechanisms exist that can lead to the expression of miniscule amounts of protein. We listed potential mechanisms for 8 of the 51 known frameshift variants in the *RHD* gene that lead to the expression of the RhD protein (Table 1). Due to the variability across samples as well as across reagents or methods for adsorption-elution testing,[30,43] the rest of the 36 *RHD* frameshift alleles, currently listed with a D-negative phenotype (Table 1), might still express a DEL phenotype. Therefore, researchers should investigate those and newly identified frameshift variants by serology, even if predicted to encode truncated RhD proteins.

In summary, we propose that most frameshift variants identified in *RHD* Exon 1[30,35,37] will likely lead to a weak D or DEL phenotype. Most frameshift variants in *RHD* Exons 2 to 7 will lead to a D-negative phenotype; these variants require serologic workup because exceptions are known. Frameshift variants identified in *RHD* Exons 8 to 10[69,92] will lead to a weak D or DEL phenotype. These predictions are based on the observations collated in Table 1 and our derived possible mechanisms for rescued RhD protein expression.

Since 2004,[93] observations of frameshift variants encoding D-positive samples, otherwise predicted to be D-negative, had remained unexplained. The wealth of data accumulated for the *RHD* gene allowed us to outline principles that can explain these enigmatic observations. This review summarized published data accumulated in 15 years' worth of literature that shared no systematic study design. Red cell genotyping and the *RHD* gene as a study subject still allowed the collation of representative examples for a host of mechanisms described by molecular biology researchers in disparate model systems before. We propose *RHD* frameshift variants, readily found in our donor and patient cohorts, and their RBC samples, readily accessible by a simple blood draw, are worth a systematic study.

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TABLE 1

Frameshift variants identified in the *RHD* gene

Molecular variation[a]		Phenotype[b]		Possible mechanism[c]						Allele designation	
					Noncanonical						
Name	Exon	Negative	Positive	Premature stop	Transcriptional frameshifting	Translational frameshifting	Alternate translation start site	Addition of amino acids	Other	GenBank number	ISBT allele
*RHD*4T, 5C, 7insG*	1	X		X						MT318152	*RHD*01N.85*
*RHD*29delGGCGCTGCCTGCCC*	1		X				X			JN696683	*NA*
*RHD*8G, 49delG*	1	X		X						MN366001	*NA*
*RHD*53delT*	1	X		X						LT596613	*RHD*01N.81*
*RHD*78delC*	1	X		X						GQ477180	*RHD*01N.32*
*RHD*93dup (RHD*93_94insT)* [d]	1		X		X	X	X			AM998541	*RHD*01EL.18*
*RHD*124_125delAA*	1	X		X						NA	*RHD*01N.65*
*RHD*147delA*	1		X				X			AM998539	*RHD*01EL.04*
*RHD*208delinsTG*	2	X		X						MF488716	*NA*
*RHD*216_217dup (RHD*216_217dupCA), 1195A*	2	X		X						JX193764	*RHD*01N.45*
*RHD*297_319delCCAGTTCCCTTCT GGGAAGGTGG*	2	X		X						KC290447	*RHD*01N.37*
*RHD*325delA*	2	X		X						DQ309581	*RHD*01N.11*
*RHD*330_331delGT*	2	X		X						EF105440	*RHD*01N.35*
*RHD*343delC*	3	X		X						AM998542	*RHD*01N.23*
*RHD*361_371delTTGTCGGTGCT*	3	X		X						JN696684	*RHD*01N.41*
*RHD*395_396dup (RHD*396insGG)*	3	-			-					KU859401	*NA*
*RHD*400delG*	3	X		X						MN624143	*NA*
*RHD*421delG*	3	X		X						MN365995	*NA*
*RHD*449delT*	3	X		X						NA	*RHD*01N.12*
*RHD*487_490delACAG*	4	X		X						AF037626	*RHD*01N.13*
*RHD*510dup (RHD*510insG)*	4		X						X	KR611039	*NA*
*RHD*545_548delCTGT*	4	X		X						HE613975	*RHD*01N.46*
*RHD*581dup (RHD*581_582insG)*	4	X		X						KU899995	*RHD*01N.75*
*RHD*615_616delCA*	4	X		X						GQ289585	*RHD*01N.34*

Molecular variation[a]		Phenotype[b]		Possible mechanism[c]						Allele designation	
					Noncanonical						
Name	Exon	Negative	Positive	Premature stop	Transcriptional frameshifting	Translational frameshifting	Alternate translation start site	Addition of amino acids	Other	GenBank number	ISBT allele
*RHD*652delA,653G*	5	X		X						EF010986	*RHD*01N.17*
*RHD*660delG*[d]	5	X		X						AM998547	*RHD*01N.29*
*RHD*679delCT*	5	-			-					33	*NA*
*RHD*683delTGAGAAGTCCAATCGA*	5	-			-					HG326209	*NA*
*RHD*697delG*	5	X		X						MG496271	*RHD*01N.82*
*RHD*702delG*[d]	5	X		X						KY229721	*RHD*01N.83*
*RHD*711delC*	5	X		X						AF390112	*RHD*01N.16*
*RHD*712delG*	5	X		X						AM998548	*RHD*01N.33*
*RHD*745_759delinsAG*[d]	5	X		X						EU499361	*RHD*01N.47*
*RHD*784delC*	5	X		X						MN365997	*NA*
*RHD*785deLA*[d]	5	X		X						AM998549	*RHD*01EL.13*
*RHD*822delG*[d]	6		X						X	HG779212	*RHD*01N.48*
*RHD*908_909insTGGCT, 939+2_939+5delTAAG*	6	X		X						AF390113	*RHD*01N.27*
*RHD*915delC*	6	X		X						KF861933	*RHD*01N.49*
*RHD*941_942delGG*	7		X						X	NA	*NA*
*RHD*950delA*	7	X		X						JN644481	*RHD*01N.51*
*RHD*970_972delCAC, 976_991delTCCATCATGGGCTACA*	7	X		X						EF195359	*RHD*01N.28*
*RHD*993delC*[d]	7	X								LN680540	*RHD*01EL.28*
*RHD*1026insAC*	7	-			-					KJ145903	*NA*
*RHD*1067dup (RHD*1067insA)*	7	-			-					KY659317	*NA*
*RHD*1080_1089delCTTCCAGGTC*	8	X		X						GU362076	*RHD*01N.36*
*RHD*1166delA*	9	-			-					KC292216	*NA*
*RHD*1174delA*	9	X		X						NA	*RHD*01N.66*
*RHD*1209delT*	9	-			-					LN680544	*NA*

Molecular variation[a]		Phenotype[b]		Possible mechanism[c]						Allele designation	
					Noncanonical						
Name	Exon	Negative	Positive	Premature stop	Transcriptional frameshifting	Translational frameshifting	Alternate translation start site	Addition of amino acids	Other	GenBank number	ISBT allele
*RHD*1228–1_1248delTTTCCTCATTTGGCTGTTGGA*	10	X		X						LN680543	*RHD*01N.44*
*RHD*1248_1249insG*	10		X					X		KJ145906	*RHD*01EL.26*
*RHD*1252dup (RHD*1252_1253insT)* [d]	10		X					X		AJ630384	*RHD*01EL.11*

Note: -, "phenotype not known" or "mechanism not applicable."

[a] Nomenclature as used by ISBT.[25] As recommended by the Human Genome Variation Society,[26] we labeled six alleles as duplications, which were listed by ISBT as insertions. Among 11 alleles with "insertions," four alleles harbor de novo insertions and seven duplications.

[b] "Positive" phenotype includes weak D and DEL alleles; "Negative" phenotype includes D-negative alleles with or without adsorption-elution testing.

[c] Premature stop, as the canonical mechanism, leads to a premature stop codon with truncated protein[27]; Alternate translation: alternate translation start site in *RHD* Exon 2 may permit traces of cell-surface protein.[28] Ribosomal frameshifting: ribosomal frameshifting may permit traces of cell-surface protein.[29] Addition of amino acids: additional amino acids at the carboxy-terminal end may permit integration of some protein in the red cell membrane.[3]

[d] *RHD*93_94insT* is also listed as *RHD*93dupT* (*RHD*01N.50*) by ISBT; *RHD*660delG* is also listed as *RHD*659delG* (*RHD*01N.78*) by ISBT; *RHD*785delA* listed as RhD-negative in the original publication by Flegel et al,[30] DEL by Ma et al,[31] and both as DEL and RhD-negative by ISBT; *RHD*822delG* incorrectly labelled as *RHD*882delG* in the original publication[32] and listed incorrectly as RhD-negative by ISBT; *RHD*993delC* listed as DEL by ISBT may refer to the nucleotide deletion rather than the phenotype; *RHD*1252_1253insT* is also associated with AJ630375 in GenBank; *RHD*702delG* with *RHD-CE(2)-D* allele listed as MG496272 in GenBank; and *RHD*745_759delinsAG* is also listed as *RHD*01N.30* by ISBT.[25] *RHD*93delC* listed as *RHD*01N.31* by ISBT has been declared obsolete.[6]