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ORIGINAL ARTICLE

Six novel mutations including triple heterozygosity for Phe31Ser, 514delT and 516T → G factor X gene mutations are responsible for congenital factor X deficiency in patients of Nepali and Indian origin

G. JAYANDHARAN,* A. VISWABANDYA,* S. BAIDYA,† S. C. NAIR,† R. V. SHAJI,* B. GEORGE,* M. CHANDY* and A. SRIVASTAVA*

*Departments of *Hematology and †Clinical Pathology, Christian Medical College, Vellore, India*

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Summary. Factor X (FX) deficiency is a rare (1 : 100000) autosomal recessive disorder caused by heterogeneous mutations in FX gene. We have studied the molecular basis this disease in six Indian and one Nepali patients. Diagnosis was confirmed by measuring the FX coagulant activity (FX: C) using a PT based assay. Six of them had a FX: C of < 1% and one patient had 24% coagulant activity. Mutations were identified in all the seven patients. These included eight (88.8%) missense and one frame-shift (11.2%) mutations of which six were novel. Three of the novel mutations, a Phe31Ser affecting 'Gla' domain and 514delT and 516T → G mutations affecting Cys132 in 'connecting region' were identified in a triple compound heterozygous state in a Nepali patient presenting with a severe phenotype. Two other novel mutations, Gly133Arg, may affect the disulphide bridge between Cys132-Cys302 in the connecting region while Gly223Arg may perturb the catalytic triad (His236, Asp282 and Ser379). The other novel mutation, Ser354Arg, involves the replacement of a small-buried residue by a large basic aminoacid and is likely to have steric or electrostatic effects in the pocket involving Lys351-Arg347-Lys414 that contributes to the core epitope of FXa for binding to FVa. Three previously reported mutations, Thr318Met; Gly323Ser; Gly366Ser were also identified. This is the first report of the molecular basis of FX deficiency in patients from the Indian subcontinent.

Keywords: conformation sensitive gel electrophoresis, factor X, India.

Correspondence: Alok Srivastava, Department of Hematology, Christian Medical College, Vellore, 632 004, India.
Tel.: +91 416 222 2102 Extn 2353; fax: +91 416 223 2035; e-mail: aloks@cmcvellore.ac.in

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Introduction

Human coagulation factor X (FX) is a vitamin K dependent serine protease of central importance in blood coagulation [1]. FX gene is composed of eight exons, spanning 27 kb on chromosome 13q34 [2]. Mutations in all these exons have been shown to cause FX deficiency, a rare autosomal recessive bleeding disorder affecting 1 : 1000000 in general population [3–5]. It is more common with a eight- to 10-fold increased frequency in populations (Iran, south India) practicing consanguineous marriages [6]. The clinical features of this disorder are among the most severe among patients with rare coagulation defects [7] and typically include hemarthroses, muscle hematomas, umbilical cord bleeding, gastrointestinal and central nervous system (CNS) bleeds [8]. The molecular basis of this disease is highly heterogeneous with a variety (approximately 65) of deletions, missense, frame shift and splice site mutations reported [3,9,10] to date. In this report, we describe for the first time, the molecular abnormalities found in the FX gene of Indian patients.

Patients and methods

Patients

Six unrelated Indian patients and one from Nepal were evaluated. Their diagnosis was based on prolonged prothrombin time and activated partial thromboplastin time and their FX coagulant activity (FX: C).

Factor X gene mutation analysis

Factor X gene was amplified by nine pairs of primers (Table 1) designed by Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) (Date of last access 25 March 05). Mutations were screened by conformation sensitive gel electrophoresis (CSGE) as previously described

Table 1 Primers used for amplification of factor X gene. PCR for exonic fragments 3, 7, 8A, 8B was performed in a 25 μL volume containing 500 ng of genomic DNA, 7.5–15 pmoles of each primer, 250 μM of each dNTPs, 0.87 units of Expand High Fidelity PCR system in supplied buffer-2 (Roche Diagnostics, GmbH, Mannheim, Germany). For PCR fragments 1, 2, 4–6 amplification was performed in a 25 μl reaction volume containing 7.5–15 pmoles of each primer in a 1X concentration of a ready reaction mix (ABgene®, Epsom, UK) containing 75 mM Tris HCl (pH 8.8), 20 mM $(NH)_2SO_4$, 0.2 mM of each of dNTP, 0.01% (v/v) Tween 20 and 1.25 units of Thermoprime plus DNA polymerase. PCR conditions for each fragment are detailed below. Following an initial denaturation at 94°C for 5 min, 30 cycles of amplification was performed with denaturation at 94°C for 40 s, annealing at temperatures as mentioned below for 40 s and extension at 72°C for 40 s. The final extension was at 72°C for 5 min

Exon	Forward primer (5′–3′)	Reverse primer (5′–3′)	Amplicon size (bp)	Forward and reverse primers (pmoles)	Final concentration of Mgcl2 (mM)	Annealing temperature (°C)
1	GGGCCAGAGTGGATGTGTT	ATGAGAGGGTTTCCCCAACT	329	7.5	1.5	58
2	CTGGATATGGCAAGGGACAT	CCTGGATGGACGGAGCAG	343	7.5	1.5	58
3	TTCACAGATGCAGAAGATCTGA	GAAACACCCTGAGGGAAAAA	350	15	1.5	48
4	CCAAAGAGGGGGAGTTGTTT	TTAGGCGTTTTCACTTCTTCC	341	15	3.5	54.5
5	GATGTAGCTGGCACCCTTG	GGCTCAGTCCTGTCCTCTTG	353	10	1.5	62
6	GCTATGGGGAGCCTCTCTCT	GACAGGTGGTCTCTCCAGCA	345	7.5	1.5	58
7	CAGGAGACCGAAGAGGACAG	TCAACCAGTGTTCCCAACAA	346	10	1.5	51
8A	GATGTGCGAGAGCATGTCC	CTTGAAGCGGGTGACGTG	476	7.5	1.5	57
8B	GCTACGACACCAAGCAGGAG	GTATCTGGGGAAAGGAATGC	475	15	1.5	52

[11]. Abnormal CSGE patterns were sequenced by the Big Dye Terminator cycle sequencing kit (Applied Biosystems, Warrington, UK) on an ABI 310 genetic analyzer (*PE* Applied Biosystems, Foster City, CA, USA). Mutation nomenclature in the FX gene was used as previously described [12]. PCR products from exon 2 and exon 6, were digested with restriction enzymes (PCR-REA) Dde*I* (New England BioLabs, Hitchin, UK) and Sma*I* (MBI Fermentas, St. Leon-Rot, Germany). All the mutations identified were analyzed by the splice site prediction program (http://www.fruitfly.org/seq_tools/splice.html) (Date of last access 25 March 05) to predict changes in RNA splicing. FX aminoacid sequences from 12 different species and five related serine proteases were obtained from SwissProt and Trembl databases (http://us.expasy.org/sprot/) (Date of last access 25 March 05) using PSI-BLAST to study the conservation of an aminoacid mutated by missense change. Multiple sequence alignment was performed with CLUSTALW (http://www.ebi.ac.uk/clustalw/). Missense mutations were studied based on the normal type FXa three-dimensional structure (PDB: 1hcg) [13]. Their potential effects were modeled by SwissPdb Viewer [14]. The final images were created by ViewerPro 4.2 software (http://www.accelrys.com).

Results

Patients

The clinical and hematological data of the patients evaluated in this study is given in Table 2. The clinical features consisted of easy bruisability (71%), epistaxis, hemarthroses, gum bleeding, gastro-intestinal bleeding, CNS bleeding (28% each), ecchymoses, hematuria and umbilical cord bleeding (14% each).

Mutation analysis

The CSGE analysis followed by DNA sequencing identified five polymorphisms and eight of nine disease causing mutations (Table 2). CSGE failed to detect a T → C transition predicting a Phe31Ser mutation in PCR fragment/exon 2. This mutation was identified by a complete sequencing of the FX gene. Of the five different polymorphisms identified, four [IVS 1 (−52) ins16 bp, IVS 2 (−17) T → C, IVS 3 (+98) C → A, IVS 7 (+33) A → G] were novel while a C → T transition for Thr224Thr has been described [15]. Of the nine mutations, eight (88.8%) were missense while one was a frame-shift (11.2%). Six of these were novel mutations (Fig. 1).

Novel mutations

Triple compound heterozygous mutations were observed in-patient BL-58 of Nepali origin with a severely reduced FX activity. DNA sequencing revealed a heterozygous T → C transition in exon 2 predicting a Phe31Ser substitution in 'Gla' domain. Additionally, two different heterozygous (514delT, 514T → G) mutations in exon 6 were found within a single codon (Cys132) in the 'connecting region' (Fig. 2). PCR-REA confirmed the paternal origin of Phe31Ser substitution, while 514delT and 516T → G mutations affecting Cys132 was derived from a single allele from the mother.

A G → A transition in a homozygous state was responsible for a Gly133Arg substitution in exon 6 to cause severe FX deficiency (BL-17). Patient BL- 40 (FX: C < 1%) had a homozygous G → A mutation, predicting Gly223Arg in the catalytic region. An A → C homozygous transversion in exon 8 of FX gene resulted in Gly354Arg in the catalytic region (BL-22).

Previously reported mutations

All the three previously reported mutations, namely Thr318Met [16], Gly323Ser [10] and Gly366Ser [17] missense mutations were present in exon 8 and affected the catalytic region.

Table 2 Clinical features and mutation data on patients with hereditary factor X deficiency

UPN	AOI*/sex	Con†	Family history	PT‡ (S), control (10–12)	APTT§ (S), control (28.3–30)	FX:C¶	Nucleotide change	Mutation/ codon**	Domain	Restriction site altered ††	Comments	Clinical features
17	11 months/ male	No	Sporadic	> 60	96.5	< 1%	GGG → AGG‡‡	Gly133Arg	Connecting region	SfcI+	Homozygous	Umblical stump bleeds, epistaxis,
							IVS 1 (−52) insCCTCTT CACCCAGGCT‡‡				Homozygous	hemarthroses, hematoma, hemetemesis, CNS§§ bleeds
22	24/male	Yes	Sporadic	> 60	> 180	< 1%	AGC → CGC‡‡	Ser354Arg	Catalytic	AciI+	Homozygous	Easy bruisability, epistaxis,
							ACC → ACT	Thr224Thr			Homozygous	hematuria, hemarthroses,
							IVS 1 (−52) insCCTCTT CACCCAGGCT‡‡				Homozygous	hematomas
							IVS 7 (+33) A → G‡‡				Homozygous	
40	2/male	Yes	Sporadic	> 60	115.2	< 1%	GGA → AGA‡‡	Gly223Arg	Catalytic	NA	Homozygous	Gastro-intestinal
							ACC → ACT	Thr224Thr			Homozygous	CNS bleeds
							IVS 3 (+98) C → A‡‡				Homozygous	
							IVS 7 (+33) A → G‡‡				Homozygous	
58	13/male	Yes	Sporadic	> 60	118	< 1%	TTT → TCT‡‡	Phe31Ser	Gla	DdeI+	Heterozygous	Ecchymoses,
							514delT‡‡	Cys132FS	Connecting region	SmaI+	Heterozygous	hematoma
							516T → G‡‡		Connecting region	SmaI+	Heterozygous	
							IVS 2 (−17) T → C‡‡				Homozygous	
							IVS 3 (+98) C → A‡‡				Homozygous	
64	18/male	Yes	Sporadic	> 60	178.8	< 1%	GGC → AGC	Gly323Ser	Catalytic	AluI+	Homozygous	Gum bleeds, hematoma
119	1/female	Yes	Sporadic	> 180	> 180	< 1%	GGC → AGC	Gly366Ser	Catalytic	MspI−	Homozygous	Gum bleeds
134	3/female	Yes	Sporadic	13.7	70.4	24%	ACG → ATG	Thr318Met	Catalytic	BbsI−	Homozygous	Easy bruisability
							IVS 1 (−52) insCCTCTT CACCCAGGCT‡‡				Homozygous	Subcutaneous hematomas on trivial injury
							IVS 3 (+98) C → A‡‡				Homozygous	

*Age at first investigation.
†Consanguinity status.
‡Prothrombin time.
§Activated partial thromboplastin time.
¶Factor X coagulant (FX: C) activity.
**Nucleotide numbering according to Messier *et al*, 1991 [12].
††(+) sign indicates that a mutation creates a restriction site and a (−) indicates that the mutation abolishes a restriction site for the restriction enzyme; NA- restriction enzyme not available.
‡‡Novel mutations/polymorphisms, not reported previously in Peyvandi *et al*, 2002 [3] or in http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=Details&DB=PubMed.
§§Central nervous system (CNS) bleeds.

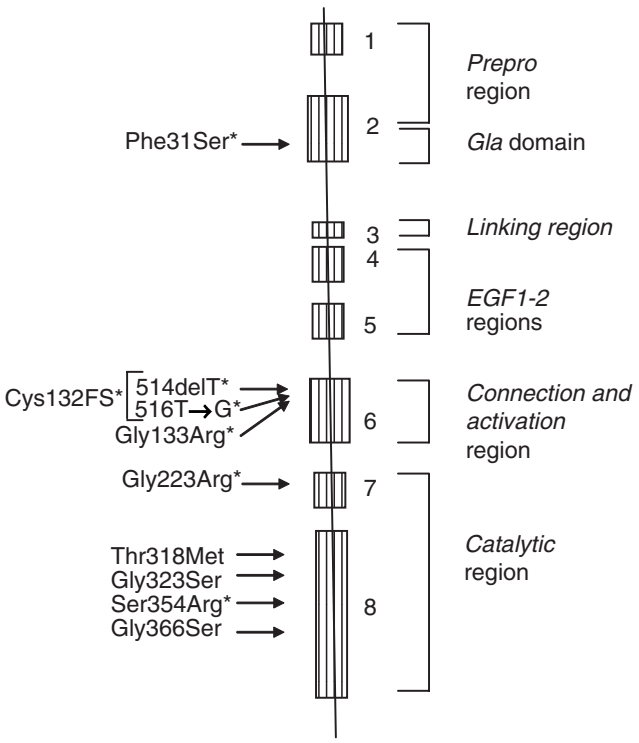

Fig. 1. Schematic representation of factor X gene mutations identified in this study. *Novel mutations, not reported previously in [3,9].

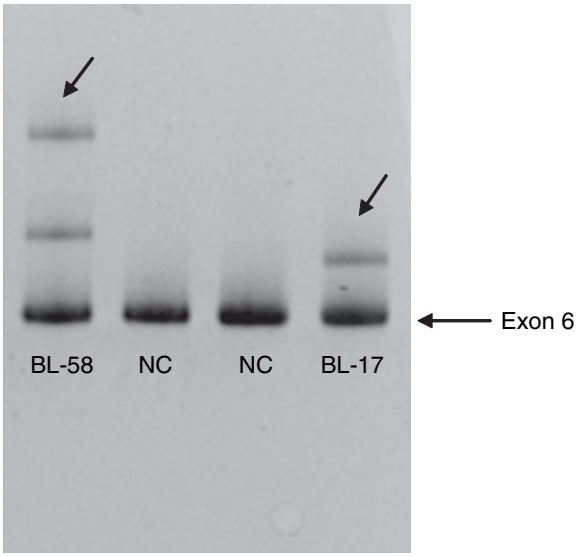

Fig. 2. Representative gel photograph of CSGE analysis for exon 6 of factor X gene: patient PCR products were mixed with equal volume of normal control (NC) PCR product and analyzed in a 12.5% CSGE gel. The abnormal mobility of patient PCR product relative to a normal control is shown by an arrow. Shown here are 514delT, 516T → G transversion mutations in patient BL-58 and a G → A transition predicting Gly133Arg aminoacid change in patient BL-17.

Discussion

We have identified the causative molecular defects in seven unrelated patients with FX deficiency in India. Triple heterozygosity (Phe31Ser, 514delT and 516T → G) for novel mutations was the cause for FX deficiency in one patient. Phe31 is located in light chain of FX at the amino-terminal 'Gla' domain. 'Gla' domain is involved in cell membrane and calcium binding through its 11 γ-carboxylated glutamic acid residues [18]. Phe31 is an aromatic hydrophobic residue conserved in 16 of 17 FX protein species examined (Table 3). The substitution of this aromatic side chain by the hydroxylic group of serine may open a cavity inside the hydrophobic core to change the secondary structure or result in partial misfolding of 'Gla' domain. This could probably affect conformation-dependent 'Gla' domain binding to phospholipid membrane [19] and normal activation [20]. Several other mutations flanking Phe31 namely Glu26Asp [21] and Glu32Gln (Factor X Tokyo) [22] have been shown to be pathognomic. The presence of a topologically equivalent mutation in the FIX gene [23] – Phe32Ser causing hemophilia B shows the importance of this codon in 'Gla' domain.

The 514delT and 516T → G mutations occur in a single codon Cys132. Cys132 is disulphide bonded to Cys302. This links residues Ile125 to Arg139 of light chain of both FX and FXa to their heavy chain [4]. The combinatorial effect of these two mutations at codon 132 creates a glycine at this position and a frame shift leading to 'TGA' at residue 226 in catalytic region. As a consequence, there could be destabilization of heavy and light chain disulphide-bridge with a truncated protein of 226 aminoacids, formed.

Gly133Arg mutation was detected in a patient with FX: C of < 1%. Gly133 is a highly conserved (Table 3) and small aminoacid present in a hydrogen bonded turn in the connecting region. This aminoacid is present in a hydrophobic environment (solvent accessibility – 0) [5]. The replacement of this residue by Arg133 may place the larger side chain and polar basic chemical groups of arginine to make additional hydrogen bonds with Gln98, Ala112 and Phe392 (Fig. 3). This mutant may probably destabilize disulphide linkage between the adjoining residues Cys132 with Cys302. An Arg139Ser (Factor X Kurayoshi) mutation around Gly133 has been postulated to functionally affect FX interaction with FVIIIa and FVa [24]. The identification of two corresponding mutations in FIX [23], namely a Gly133Arg and Gly133Glu to cause hemophilia B suggests its importance in the connecting region of FX. Interestingly, in our splice prediction analysis for this mutation (517G → A), a cryptic acceptor splice site between nucleotides 516t***a***g-gaa521 is created (0.84, score for mutant FX) and the actual splice site at nucleotide 503 (500cag-ggc506) getting a concurrent score of 0.91. In the wild type DNA sequence the efficiency of splicing at the physiological acceptor splice site is 0.95. This effect could not be confirmed at the RNA level because of the non-availability of patient sample.

Gly223Arg corresponds to severe FX deficiency. Gly223 is conserved in 14 of 17 FX or related proteases studied. Gly223 is a very small aminoacid present in beta-strand C in serine protease subdomain 1 [5], and in a hydrophobic environment between Gly219 to Leu226 (solvent accessibility – 0). Its replacement by Arg223 may cause the positive charge and the

Table 3 Alignment of aminoacid sequence derived from factor X from different species and its related serine proteases. The conservation of aminoacids in humans is shown in bold letters and their missense mutations are italicized. Aminoacid sequences were obtained from the SwissProt database [14] with the accession numbers mentioned below. Multiple sequence alignment was performed with CLUSTALW (http://www.ebi.ac.uk/clustalw/)

		Codon							
Accession No.	Protein	31	132	133	223	318	323	354	366
P00742	Human coagulation factor X precursor	F	C	G	G	T	G	S	G
Q28511	Rhesus macaque coagulation factor X	F	C	–	–	T	G	S	G
O88947	Mouse coagulation factor X precursor	F	C	G	G	T	G	T	G
Q63207	Rat factor X	F	C	G	G	T	G	T	G
O19045	Rabbit coagulation factor X precursor	F	C	G	G	T	G	S	G
P00743	Bovine coagulation factor X precursor	F	C	G	G	T	G	S	G
Q9GMD9	Duckbill platypus coagulation factor X	F	C	G	G	A	G	S	G
P81428	Troca coagulation factor X	F	C	G	G	S	G	S	G
P83370	Hopst coagulation factor X	F	C	G	G	S	G	S	G
P25155	Chick coagulation factor X precursor	F	C	G	G	S	G	T	G
AAH56804	Zebrafish coagulation factor X	F	C	G	G	D	G	S	G
Q804W9	Fugu coagulation factor X precursor	F	C	G	G	D	G	T	G
P00740	Human coagulation factor IX precursor	F	C	G	G	S	G	T	G
P08709	Human coagulation factor VII precursor	F	C	G	G	F	G	S	G
P22891	Human protein Z precursor	F	C	G	G	S	G	L	–
P04070	Human protein C precursor	F	C	G	A	T	G	M	G
P00734	Human Prothrombin precursor	L	T	G	A	T	G	T	G
Mutation		S	FS	R	R	M	S	R	S

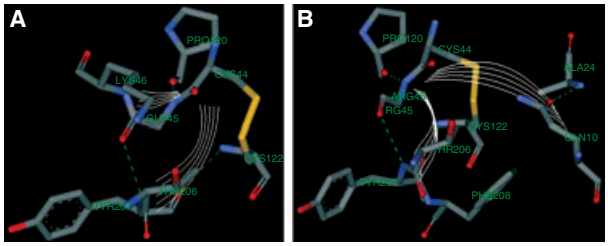

Fig. 3. Predicted effect of Gly133Arg (PDB: 1hcg numbering, B45) mutation. Glycine at codon 133 is a very small aminoacid and is present in a hydrogen bonded turn in the connecting region (A). This aminoacid is present in a hydrophobic environment (solvent accessibility – 0). The replacement of this residue by arginine at codon 133 may place the larger side chain and polar basic chemical groups of arginine to make additional hydrogen bonds with Gln98 (1hcg: B10), Ala112 (1hcg: B24), Phe392 (1hcg: A208) (B) thereby probably destabilizing the disulphide linkage (Cys132-Cys302) that connects the heavy and light chains of FX and FXa.

larger side chain of arginine to impair the correct folding of this protein or perturb the catalytic triad. The mutant also gains hydrogen bonds with aminoacids in adjacent hydrophobic (solvent accessibility – 0/1) beta strand B (Leu212), beta strand D (Ile231) and beta strand H (Leu286). Topologically equivalent missense mutations causing FVII deficiency, a Gly180Arg [25] and FIX deficiency -Gly208Asp, Gly208Val, Gly208Ser [23] suggest a critical role of Gly223 for FX activity. Apart from mutations at codons 133 and 223 identified in this series, mutations such as Gly20Arg (FX Santo Dominigo), Gly114Arg (FX Ockero), Gly204Arg, Gly249Arg, Gly380Arg [26] demonstrate the striking effect of Gly → Arg substitutions on FX protein stability.

Ser354Arg was detected in catalytic domain. Ser354 is inaccessible (solvent accessibility – 0) in between the alpha helix A2 (Arg347- Ser353) and a bend (Ser355-Phe356) in serine protease subdomain 2 [5]. Replacement of Ser354 by Arginine creates additional hydrogen bonds with Trp399, Tyr409 and Ile411. Ser354 is close to Lys351. Lysine at codon 351 with Arg347 and Lys414 is likely to contribute to the core epitope of FXa for binding to FVa [27]. The mutation of a small-buried residue by a large basic aminoacid is likely to have steric or electrostatic effects on this pocket. Identification of a Ser339Pro equivalent mutation in FIX [23] provides evidence that mutations in this codon are severe enough to cause FX deficiency.

Thr318Met (Factor X Roma) [16], Gly323Ser [10], Gly366-Ser (Factor X Nagoya 2) [17] mutations identified here have been reported previously in patients of Italian, British and Japanese descent, respectively. Thr318Met represents an example of a dysfunctional variant affecting FX activation. Gly323Ser replaces a small amino acid with a considerably larger one to yield a partially misfolded protein. Gly366Ser at the primary substrate-binding pocket is predicted to compromise substrate binding, consistent with loss of enzymatic activity.

Two other large series on mutations causing FX deficiency from Iran [5] and Europe [10] have been reported. Missense mutations are the most frequent (88%) in this study as in the earlier reports, 78% and 83%, respectively. The reported absence of nonsense mutations in homozygosity in any of these studies indicates that complete absence of FX is incompatible with survival, in agreement with experimental data from knockout mice [28]. None of the mutations in the present study involved a 'CpG' dinucleotide, the mutational hotspot found frequently (66.6%) [10] in other studies. In contrast to previous reports [10,29] most ($n = 6$ of 7) of our patients were homozygous for their mutations providing the clinical data

that will be a true reflection of their phenotype of some of these novel mutations. However, a functional characterization [27,30] of these mutants will be needed to demonstrate their effect on FX biosynthesis and secretion.

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