GGCX mutations show different responses to vitamin K thereby determining the severity of the hemorrhagic phenotype in VKCFD1 patients

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Abstract
Background: Vitamin K dependent coagulation factor deficiency type 1 (VKCFD1) is a rare hereditary bleeding disorder caused by mutations in γ-glutamyl carboxylase (GGCX). VKCFD1 patients are treated lifelong with high doses of vitamin K in order to correct the bleeding phenotype. However, normalization of clotting factor activities cannot be achieved for all VKCFD1 patients.

Objective: The current study aims to investigate the responsiveness to vitamin K for all reported GGCX mutations with respect to clotting factors in order to optimize treatment.

Methods: This study developed an assay using genetically engineered GGCX−/− cells, in which GGCX mutations were analyzed with respect to their ability to γ-carboxylate vitamin K dependent pro-coagulatory and anti-coagulatory clotting factors by ELISA. Additionally, factor VII activity was measured in order to proof protein functionality. For specific GGCX mutations immunofluorescent staining was performed to assess the intracellular localization of clotting factors with respect to GGCX wild-type and mutations.

Results: All GGCX mutations were categorized into responder and low responder mutations, thereby determining the efficiency of vitamin K supplementation. Most VKCFD1 patients have at least one vitamin K responsive GGCX allele that is able to γ-carboxylate clotting factors. In few patients, the hemorrhagic phenotype cannot be reversed by vitamin K administration because GGCX mutations on both alleles affect either structural or catalytically important sites thereby resulting in residual ability to γ-carboxylate clotting factors.

Conclusion: With these new functional data we can predict the hemorrhagic outcome of each VKCFD1 genotype, thus recommending treatments with either vitamin K or prothrombin complex concentrate.
INTRODUCTION

γ-glutamyl carboxylase (GGCX) is an enzyme that converts specific glutamic acid residues (Glu) into γ-carboxyglutamic acid residues (Gla). This post-translational modification is a vitamin K-dependent process, in which vitamin K hydroquinone (KH$_2$) is oxidized to vitamin K 2,3-epoxide by GGCX. γ-carboxylation takes place in vitamin K-dependent (VKD) proteins, which is required for their function. VKD proteins include hemostatic proteins as the pro-coagulatory factor II (FII), VII (FVII), IX (FIX), X (FX), and the anti-coagulatory proteins C (PC), S, and Z. There are also non-hemostatic proteins known that are γ-carboxylated, such as osteocalcin (BGLAP), matrix Gla protein (MGP), upper zone of growth plate and cartilage matrix associated protein (UCMA/GRP), growth arrest specific 6 (GAS6), proline-rich Gla proteins (PRGPs) 1 and 2, and transmembrane Gla proteins (TMGs) 3 and 4. Almost all VKD proteins have similar structural organization in which the N-terminal propeptide initially binds to GGCX and specific Glu residues in the Gla domain are γ-carboxylated. However, propeptides of clotting factors have different affinities for GGCX. FX has the highest affinity followed by FVII and FIX, whereas PC and FII have a weak affinity for GGCX. After this modification in the endoplasmic reticulum, the propeptide is cleaved and the VKD protein is transported to its destined location.

Mutations in GGCX lead to a rare bleeding disorder called vitamin K–dependent coagulation factor deficiency type 1 (VKCFD1). VKCFD1 patients are characterized by decreased activity of VKD clotting factors that cause spontaneous hemorrhages as intracranial or nose bleeds. In addition, some VKCFD1 patients show additional non-bleeding phenotypes as skin hyperlaxity, and skeletal and cardiac abnormalities. Oral administration of vitamin K$_2$ (K$_4$) increases clotting factor activities to normal ranges in most cases. However, there are patients reported for whom normalization or the increase of clotting factor activities cannot be achieved with vitamin K supplementation.

To understand the pathological mechanism of the hemorrhagic phenotype of VKCFD1 patients, we have characterized 22 reported missense mutations with respect to γ-carboxylation of VKD pro-coagulatory and anti-coagulatory clotting factors in a CRISPR/Cas9 genome-engineered GGCX$^{-/-}$ HEK293 T cell line. Our analyses will help to predict and improve the treatment of the hemorrhagic phenotype of each GGCX genotype.

METHODS

Construction of human GGCX/VKD clotting factor expression vectors

The wildtype (wt) human GGCX (hGGCX) cDNA was cloned into MCSB of the bicistronic vector pIRES (Clontech). cDNAs of VKD proteins (F2, F7, F9, F10, PC) were cloned into MCSA of the same vector together with a c-myc tag at the C-terminus. GGCX mutations (mut) were introduced by site-directed mutagenesis.

Generation of GGCX$^{-/-}$ HEK293 T cell line

A GGCX$^{-/-}$ HEK293 T cell line was generated by CRISPR/Cas9 gene editing. The target crRNA sequence was CCTACGTACATGCTGGCCAGCAGC, the vector used pRZ-Cas9-mCherry. Genetic profile of single cell colonies were validated twice by MiSeq next generation deep sequencing (Illumina). Absence of GGCX protein was verified by western blot using anti-GGCX antibody (Abcam, ab170921).

Expression of GGCX and VKD clotting factors in GGCX$^{-/-}$ cells

GGCX$^{-/-}$ HEK293 T were seeded in Dulbecco’s modified Eagle medium containing 1% non-essential amino acids and 10% heat-inactivated fetal bovine serum (FBS), which does not contain vitamin K (Gibco/ThermoFisher). After 24 h, cells were transfected with pIRES vectors containing cDNAs of GGCX wt or mut together with one of the clotting factors by lipofectamin 2000 (ThermoFisher). Different K$_i$ concentrations prepared in ethanol (0.1, 0.316, 1, 3.16, 6.32, 10, 31.6, and 100 µM; Sigma Aldrich)
were added 4 h post-transfection. After 48 h, supernatants were collected to measure γ-carboxylation of secretory VKD clotting factors (FII, FVII, FIX, FX, PC). Cell lysates were collected in 80 µl Nonidet P-40 buffer including protease inhibitors to detect GGCX antigen.

2.4 | Measurement of γ-carboxylation of VKD clotting factors

γ-carboxylation of VKD clotting factors was measured by ELISA in which the VKD protein was captured by anti-myc antibody followed by γ-carboxylation detection by a monoclonal antibody directed against human Gla residues (Sekisui, 3570). Medium of untransfected cells as well as of transfected cells that were not treated with K1 served as negative control. Ninety-six-well plates were coated overnight at 4°C with the anti-myc antibody (Applied Biological Materials, G077) in coating buffer (30 mM Na2CO3, 200 mM NaHCO3, pH9). After blocking (2% bovine serum albumin [BSA] in 1x phosphate buffered saline [PBS], 0.05% Tween20), cell supernatants were incubated overnight at 4°C (1:6 dilution in 1x PBS, 0.1% BSA). Wells were then incubated for 3 h with anti-Gla antibody prepared in buffer (1x PBS, 0.1% BSA, 0.05% Tween20). After incubation with a horseradish peroxidase (HRP)-conjugated antibody (Dako, PO260) for 1 h at room temperature (RT) an HRP-dependent chemiluminescence substrate (Roche, 11582950001) was added and luminescence was detected by a plate reader (Synergy 2, Biotek). Wells were washed 3x between each step with washing-buffer (1x PBS, pH 7.4, 0.05% Tween 20, 3 mM MgCl2).

2.5 | Measurement of GGCX antigen levels

GGCX antigen levels were measured by ELISA. Coating, washing, blocking, and detection were performed as described above. For coating an anti-GGCX antibody detecting aa 684–733 was used (Antibodiesonline, ABIN2781826). The detection antibody against GGCX (746–757 aa; ThermoFisher, PA5-19155) was incubated for 3.5 h followed by HRP-conjugated antibody (Dako, PO160). The immunogenic regions for coating and detection anti-GGCX antibodies exclude any analyzed mutation. The antigen levels were quantified by GGCX wt standard curves.

2.6 | Measurement of FVII clotting factor activity

GGCX−/− cells were seeded on 24-wells and transfected with pIRES vectors using Lipofectamine. After 4 h medium was changed to OptiMEM (ThermoFisher) containing 5% BSA (Sigma-Aldrich, protease-free) including 10 µM K1 in order to express FVII under serum-free conditions. After 72 h medium was collected and FVII clotting factor activity was assessed using Atellica coagulometer (Siemens). Sample aliquots were added to FVII-depleted plasma (Siemens) and activities were normalized to a calibration curve of standard pooled plasma.

2.7 | Immunofluorescence staining

GGCX−/− cells were seeded on gelatin-coated coverslips and transfected with vectors containing GGCX wt/mut. After 4 h, 10 µM K1 was added. 24 h post-transfection cells were washed 3x with PBS and fixed in 4% paraformaldehyde followed by blocking with 10% FBS for 30 min. Cells were incubated overnight with an anti-GGCX antibody (Abcam, ab170921). FII and FX were stained with antibodies from Affinity Biologicals (SAFII-AP, GAFX-AP). Secondary Alexa Fluor 488 or 594-conjugated antibodies incubated for 1 h at RT in dark. Mounting was performed with ProLong Glass (ThermoFisher) including NucBlue. Images were taken by Apotome2 (Zeiss) and Pearson’s correlation coefficient was calculated using Zen software.

3 | RESULTS

3.1 | Evaluation of γ-carboxylation of VKD clotting factors

In the current study, we developed an assay to measure the effect of GGCX mutations on γ-carboxylation of VKD clotting factors by ELISA (Figure 1A). Therefore, a GGCX−/− cell line was generated, which has a deletion of 46 nucleotides in exon 8 that leads to absence of GGCX protein (Figure S2 in supporting information). These GGCX−/− cells were transiently transfected with bicistronic vectors harboring cDNAs of GGCX wt or mut and a VKD pro-coagulatory or anti-coagulatory clotting factor. VKD clotting factors were captured equally by a myc-tag and Gla residues were

FIGURE 1  Method to study γ-carboxylation of vitamin K--dependent (VKD) clotting factors. A, A Myc tag was introduced at the C-terminus of VKD clotting factors and cloned into the multiple cloning site A (MCSA) of a bicistronic vector (pIRES). The cDNA of γ-glutamyl carboxylase (GGCX) wild type/mutation (wt/mut) was cloned into multiple cloning site B (MCSB). These vectors were transfected into a HEK293T GGCX−/− cell line. γ-carboxylation was measured by an ELISA in which VKD clotting factors were captured by an anti-Myc antibody and detected by a Gla-specific antibody. GGCX antigen level was measured by another sandwich ELISA in which two GGCX antibodies were used. B, In our study, γ-carboxylation level of 4 pro-coagulatory factors (factors II, VII, IX, and X), and one anti-coagulatory factor (protein C) were measured. C, γ-Carboxylation dose-response curves of GGCX wt with respect to VKD clotting factors. The y-axis represents normalized γ-carboxylation (%). The x-axis display K1 concentrations (0.1–100 µM). Error bars depict SD of triplicate measurements of n = 3 experiments.
detected by a Gla-specific antibody (Figure 1A, B). Medium of untransfected cells served as negative control. With respect to GGCX wt transfected cells, levels of γ-carboxylated VKD clotting factors increased in a K_1-dependent manner (Figure 1C). GGCX mutations exhibited different dose responses as described in the following sections.

### 3.2 The hemorrhagic phenotype: GGCX mutations show variable responses to vitamin K

The direct influence of GGCX mutations on clotting factor’s γ-carboxylation status was assessed by ELISA. FII and FX were chosen as pro-coagulatory factors with highest and lowest affinity to GGCX, respectively. PC was selected as representative anti-coagulatory clotting factor. To validate γ-carboxylation values, FVII activity was measured by a clotting assay, which detects functional, secreted protein only. GGCX antigen levels were assessed to confirm expression of the analyzed mutations.

### 3.3 GGCX mutations that can be corrected with vitamin K

We observed for most GGCX mutations markedly reduced levels of γ-carboxylated FII, FX, and PC at low K_1 concentrations (1 µM K_1) compared to wt at the same condition (Table 1). Interestingly, at high K_1 doses, γ-carboxylation of clotting factors increased to substantial levels for mutations GGCX:p.(W157R), GGCX:p.(R204C), GGCX:p.(V255M), GGCX:p.(S284P), GGCX:p.(R325Q), GGCX:p.(R476C),
| Abbreviations: | CH, compound heterozygous; FII, factor II; FVII, factor VII, FX, factor X; GGCX, homozygous; HTZ, heterozygous; LOF, loss-of-function; NA, not available; PC, protein C; WT, wild type. |
|----------------|------------------------------------------------------------------------------------------------|

| GGCX mutation | Genotype | Reported patient’s data |
|---------------|----------|-------------------------|
| **GGCX:p.(W493C)** | Unusually high levels of \(\gamma\)-carboxylated clotting factors (Figure 2C). | |
| **GGCX:p.(R204C)** | Three responder mutations decreased antigen levels were detected. | |
| **GGCX:p.(R83W)** | There are some mutations that did not respond with markedly increased levels of \(\gamma\)-carboxylated clotting factors and FVII activities at high \(K_1\) concentrations. | |
| **GGCX:p.(D153G)** | Moreover, we observed for four more responding mutations (GGCX:p.(I532T), GGCX:p.(G537A), GGCX:p.(W501S), and GGCX:p.(W493C)) unusually high levels of \(\gamma\)-carboxylated clotting factors at low and high \(K_1\) conditions compared to wt (Figure 2D, Table 1). | |

3.4 | **GGCX mutations that are difficult to treat with vitamin K** |

There are some mutations that did not respond with markedly increased levels of \(\gamma\)-carboxylated clotting factors and FVII activities at high \(K_1\) concentrations. We categorized them as low responders, which will be difficult to treat in patients harboring these mutations. Low responder mutations are GGCX:p.(R83W), GGCX:p.(R83P), GGCX:p.(D153G), GGCX:p.(L394R), GGCX:p.(S300F), and GGCX:p.(H404P) (Figure 3A,B). GGCX antigen levels were reduced for GGCX:p.(R83W) and GGCX:p.(D153G), whereas the other mutations showed similar expression compared to wt (Figure 3C).

Finally, two GGCX mutations, GGCX:p.(F299S) and GGCX:p.(M174R), exhibited loss-of-function for all clotting factors by showing no \(\gamma\)-carboxylation at any \(K_1\) concentration (Figure 3D). In addition,
FIGURE 2  The hemorrhagic phenotype: γ-glutamyl carboxylase (GGCX) mutations that respond well to vitamin K. A, D, γ-carboxylation values of hemostatic proteins measured by ELISA that were expressed along with GGCX wild type (wt) or mutation (mut) in GGCX−/− HEK293T cells. The y-axis represents normalized γ-carboxylation (%) to wt for factor II, factor X, and protein C. The x-axis represents K1 concentrations (0.1–100 µM). In each figure dose-response curve for GGCX wt is shown in black. Error bars depict SD of n = 3 technical replicates of n = 3 experiments. B, E, Factor VII activity measured in media from GGCX−/− HEK293T cells that were transfected with bicistronic vectors harboring cDNAs of GGCX wt/mut together with F7. Cells were treated with 1 µM or 10 µM K1. Error bars depict SD of n = 3 experiments. C, F, Antigen levels of GGCX mutations. Error bars depict SD of n = 3 technical replicates of n = 3 experiments. A, Dose-response curves of mutations that respond to vitamin K with increasing values of γ-carboxylation. B, Factor VII activity for GGCX mutations depicted in (A). C, Antigen levels of GGCX mutations depicted in (A). D, Dose-response curves of mutations that responded with greater γ-carboxylation levels compared to wt. E, Factor VII activity measured for GGCX mutations depicted in (D) under 1 µM or 10 µM K1 treatment. F, Antigen levels of GGCX mutations depicted in (D). AG, antigen levels
The hemorrhagic phenotype: difficult to treat $\gamma$-glutamyl carboxylase (GGCX) mutations. A, D, $\gamma$-carboxylation values of hemostatic proteins measured by ELISA that were expressed along with GGCX wild type (wt) or mutation (mut) in GGCX$^{-/-}$ HEK293T cells. The $y$-axis represents normalized $\gamma$-carboxylation (%) to wt for factor II, factor X, and protein C. The $x$-axis represents $K_1$ concentrations (0.1–100 µM). In each figure dose-response curve for GGCX wt is shown in black. Error bars depict SD of $n=3$ technical replicates of $n=3$ experiments. B, E, Factor VII activity measured in media from GGCX$^{-/-}$ HEK293T cells that were transfected with bicistronic vectors harboring cDNAs of GGCX wt/mut together with F7. Cells were treated with 10 µM $K_1$. Error bars depict SD of $n=3$ experiments. C, F, Antigen levels of GGCX mutations. Error bars depict SD of $n=3$ technical replicates of $n=3$ experiments. A, Dose-response curves of GGCX mutations that did not respond well to $K_1$ administration. B, Factor VII activity measured for GGCX mutations depicted in (A). C, Antigen levels of GGCX mutations depicted in (A). D, Dose-response curves of mutations that showed loss-of-function. E, Factor VII activity measured for GGCX mutations depicted in (D). F, Antigen levels of GGCX mutations depicted in (D). AG, antigen levels.
Differentially responding mutations

A

DAX

FX

PC

Normalized γ-Carboxylation (%)

Vitamin K₁ (μM)

B

FVII activity at 10 μM K₁

C

GGCX Ag (μM)

WT

R485P

G555R

T591K

D

DFFI

FX

GGCX

Merged

E

Correlation coefficient

FX

Correlation coefficient

FII
Figure 4: Mutations affecting the propeptide binding site. A, \(\gamma\)-Carboxylation dose-response curves of vitamin K-dependent (VKD) clotting proteins with respect to mutations showing different responses to vitamin K measured by ELISA. The \(\gamma\)-axis represents normalized \(\gamma\)-carboxylation (%). The x-axis represents \(K_1\) concentrations (0.1–100 \(\mu\)M). In each figure dose-response curve for \(\gamma\)-glutamyl carboxylase (GGCX) wild type (wt) is depicted in black. Error bars depict SD of triplicate measurements of \(n = 3\) experiments. B, Factor VII activity measured for GGCX mutations depicted in (A). C, Antigen levels of GGCX mutations depicted in (A). AG, antigen levels. D, \(\gamma\)-carboxylase (GGCX) localized FX and FVII even at high \(K_1\) (G558R) likely indirect effects on the known propeptide binding site in GGCX.

Mutations affecting the propeptide binding site. A, \(-\)Carboxylation dose-response curves of vitamin K-dependent clotting proteins with respect to mutations showing different responses to vitamin K measured by ELISA. The \(-\)axis represents normalized \(-\)carboxylation (%). The x-axis represents \(K_1\) concentrations (0.1–100 \(\mu\)M). In each figure dose-response curve for \(-\)glutamyl carboxylase (GGCX) wild type (wt) is depicted in black. Error bars depict SD of triplicate measurements of \(n = 3\) experiments. B, Factor VII activity measured for GGCX mutations depicted in (A). C, Antigen levels of GGCX mutations depicted in (A). AG, antigen levels. D, \(-\)carboxylase (GGCX) localized FX and FVII even at high \(K_1\) (G558R) likely indirect effects on the known propeptide binding site in GGCX.

3.5 | GGCX mutations that \(\gamma\)-carboxylate hemostatic proteins differentially

Mutations GGCX:p.(R485P), GGCX:p.(G558R), and GGCX:p.(T591K) exhibited different dose responses for FII, FX, and PC. Therefore, measurement of \(\gamma\)-carboxylation of FVII and FIX were included for these mutations. Mutation GGCX:p.(R485P) exhibited low values of \(\gamma\)-carboxylated FII, but was responding well with increasing levels of \(\gamma\)-carboxylated FVII, FIX, FX, and PC similar to wt (Figure 4A). FVII activity was within normal range when treated with 10 \(\mu\)M \(K_1\) (Figure 4B). GGCX:p.(G558R) and GGCX:p.(T591K) did not \(\gamma\)-carboxylate FIX and FVII even at high \(K_1\) concentrations and exhibited markedly reduced or zero FVII activity (Figure 4A,B). GGCX:p.(G558R) \(\gamma\)-carboxylated FII as wt but did not \(\gamma\)-carboxylate FIX and PC. GGCX:p.(T591K) is slightly responding for \(\gamma\)-carboxylation of FII, FIX, and PC (Figure 4A). GGCX antigen levels were similar to wt for all differentially responding mutations (Figure 4C).

3.6 | Mutations affecting the propeptide and glutamate binding site

Specific mutations that were found to be within or close to the propeptide or glutamate binding site or that showed different dose-responses among the VKD clotting factors were additionally analyzed with respect to their location to know functional binding sites and by immunofluorescent staining (IF).

Two mutations, GGCX:p.(H404P) and GGCX:p.(L394R), are located within the reported glutamate binding site. We observed for both mutations that VKD clotting factors are \(\gamma\)-carboxylated only at high concentrations of \(K_1\), indicating that the catalytic efficiency is affected (Figure 3A). The amino acid exchange to a positively charged arginine or a bulky proline seems to alter the nature of the glutamate binding site thereby affecting GGCX catalysis.

Moreover, there are some mutations that showed direct or most likely indirect effects on the known propeptide binding site in GGCX (aa 495–513).

4 | DISCUSSION

Here, we show how GGCX mutations led to a differential \(\gamma\)-carboxylation of VKD clotting factors, thereby resulting in variable hemostatic VKCFD1 phenotypes. Our data can be used to predict the clinical course of VKCFD1 patients and facilitate decisions on treatment with either vitamin K or prothrombin complex concentrate (PCC), in order to correct the hemorrhagic phenotype.
This study demonstrates that most GGCX mutations found in VKCFD1 patients exhibited markedly reduced levels of γ-carboxylated clotting factors at low vitamin K1 conditions (e.g., 1 μM K1, Table 1). This explains why VKCFD1 patients have markedly reduced clotting factor activities under normal diet. However, most GGCX mutations responded to high K1 administration (e.g., 10 μM K1) with increasing levels of γ-carboxylated FII, FX, and PC and increased FVII activities in our assays. This is in concordance with the available patient data showing increased clotting factor activities under K treatment for responder mutations (Table 1). For example, one patient harboring GGCX:p.(R204C);p.(R204C) exhibited clotting factor activities close to normal range under high K1 supplementation (FIIc:69%, FXc:71%; 13 mg K1 per day). Administration of only 3 mg K1 per day for another patient with the same genotype demonstrates that this low dose of K1 is insufficient to correct for the bleeding phenotype. Thus, supplementation with increased doses of K1 should lead to clotting factor activities within normal range. Patients harboring the responder mutations GGCX:p.(S284P) or GGCX:p.(W493C) in a compound heterozygous state with a nonsense mutation, exhibited markedly increased clotting factor activities under K1 administration, even though the second allele is not functional (Table 1). This demonstrates that only one allele has to respond to K1 treatment in order to correct the bleeding phenotype.

However, in a few patients, normalization of clotting factor activities with K1 supplementation will be difficult or impossible. These patients carry mutations on both alleles showing low ability or loss-of-function to γ-carboxylate clotting factors at high K1 concentrations (Figures 3 and 4, Table 1). These mutations affect structural or catalytically important sites as the glutamate binding site (GGCX:p.(L394R), GGCX:p.(H404P)), or the propeptide binding site (GGCX:p.(R485P), GGCX:p.(G558R), GGCX:p.(T591K)). Low-responding mutations also affect protein stability as do mutations GGCX:p.(R83P) and GGCX:p.(M174R). The loss-of-function mutation GGCX:p.(F299S) and the low responding mutation GGCX:p.(S300F) potentially affect vitamin K epoxidation. Patients harboring a low responding mutation in homozygous state or in compound heterozygous state with a loss-of-function (GGCX:p.(F299S), GGCX:p.(M174R)), or a nonsense mutation are difficult to treat with vitamin K and result in the most severe hemostatic phenotypes. For example, the patient carrying GGCX:p.(M174R+R325Q);p.(D153G) harbors the loss-of-function mutation GGCX:p.(M174R) in one allele that results in zero activities for all clotting factors. Mutation GGCX:p.(D153G) in the other allele is a low-responding mutation (Table 1). This is also reflected by the patients’ data, in which clotting factor activities increased to a maximum of 38% only, even though treated with high doses of K1 (20 mg/day). Thus, in the case of bleeding or surgery, this patient should be treated with PCC because K1 administration will not correct the bleeding phenotype. This is also our recommendation for all patients harboring low responder mutations in combinations with a loss-of-function or nonsense mutation.

With respect to the genotype, all VKCFD1 patients are homozygous or compound heterozygous for GGCX mutations except for two reported patients that were found to be heterozygous. The genotype of these patients is not correlating to the overall data because parents or siblings of other VKCFD1 patients, for whom even one allele is deficient due to nonsense or loss-of-function mutations, exhibit normal clotting factor activities under normal diet (Table S1 in supporting information). This demonstrates that both GGCX alleles have to be affected in order to exhibit a clinically relevant hemostatic phenotype, which is also supported by the phenotype of Ggcx−/− mice that exhibit clotting factor activities within normal range, whereas Ggcx−/− mice are intraterine lethal. Finally, this brings us to the conclusion that the genotype of the solely two reported heterozygous VKCFD1 patients should be re-evaluated.

Moreover, due to the lethal phenotype of Ggcx−/− mice, we suspect that there will never exist a genotype in which both GGCX alleles harbor a nonsense mutation or a loss-of-function mutation in humans. There will be at least always one allele, or both alleles in the case of homozygous mutations, that is partially responding with residual amounts of γ-carboxylated VKD clotting factors. These residual amounts of γ-carboxylated VKD clotting factors are sufficient for viability but result in different degrees of severity for the clotting phenotype.

Although VKD clotting factors are similar in their structure, they have different affinities for GGCX and share only five conserved residues within their propeptides (−18, −16, −10, −6, −1aa). In our present study, we report for the first time that GGCX mutations can also affect VKD clotting factors differently. Another study has previously shown that GGCX mutations can also affect the non-hemostatic VKD proteins MGP and BGLAP differentially. The selective deficiency to γ-carboxylate for example FX or FII for some mutations are new, important findings, which will help to treat respective patients with clotting factor concentrates more efficiently. Hence, our mutational analysis will help us further understand the mechanism of propeptide binding and processing of VKD clotting factors in more detail in the future.

In conclusion, our study highlights the physiological importance of GGCX to γ-carboxylate VKD clotting factors. Our data will help to predict the clinical course and treatment of VKCFD1 patients more efficiently.

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CONFLICTS OF INTEREST
The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS
Experimental design (KJC, JM, SG), generation of GGCX−/− cells (VH, KH, KJC), data collection (SG, KK, ALB), data analysis and figure production (SG, KJC), manuscript drafting and editing (SG, KJC, AB, FF, HS, VH, KH, MW, JO).
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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.

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