Supplemental Materials

Supplemental Methods

Research subject

The subject was the first reported clinical case of VKCFD. Written informed consent for genomic analysis and blood sample collection was obtained from the patient at the time of sample collection. Genetic studies were conducted in accordance with the protocols approved by the Office of Human Research Ethics, University of North Carolina at Chapel Hill.

Genetic analysis

Genomic DNA was isolated from the patient’s peripheral blood or lymphoblastoid cells using a Blood and Cell Culture DNA kit (QIAGEN, Valencia, CA). Exome sequencing was performed by GENEWIZ NGS laboratory (New Jersey, USA). The GGCX mutations identified by exome sequencing were further confirmed by sequencing the PCR-amplified fragments of the corresponding regions in the genomic DNA. For mutation zygosity analysis, PCR fragments were cloned into TOPO TA cloning vector (Invitrogen, Carlsbad, CA) and single colonies were picked up for sequencing.

Minigene splicing assay of the GGCX intronic mutation

The effects of the GGCX intronic mutation (c.1889-6G>A) on GGCX pre-mRNA splicing were assessed using a minigene splicing assay based on the pSPL3 exon trapping vector\textsuperscript{1,2} (vector kindly provided by Dr. Karen E. Heath, Hospital Universitario La Paz, Spain). Briefly, exon 14 was amplified, along with its upstream and downstream intronic sequences (166 bp and 195 bp, respectively), using Advantage 2 proofreading DNA polymerase (Clontech Laboratories, Inc., Mountain View, CA). The PCR product was subcloned into the NotI and BamHI sites of the pSPL3
vector. Single colonies were picked and sequenced to obtain the wild-type GGCX and the c.1889-6G>A mutant constructs. The minigene constructs and the empty pSPL3 vector were transiently transfected into HEK293 cells. The transfected cells were harvested 48 hours post-transfection, and total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA). The first-strand cDNA was synthesized by reverse transcription using M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA) and oligo(dT)18 primer. Spliced exon 14 of GGCX was amplified by PCR, purified by electrophoresis, and directly sequenced to characterize the effect of the mutation on pre-mRNA splicing.

**Functional characterization of GGCX and its mutational variants using a GGCX-deficient cell-based assay**

The effects of the GGCX mutations were examined using a GGCX-deficient cell-based assay as previously described. Briefly, the wild-type or mutant GGCX was transiently expressed in the GGCX-deficient reporter cells. The transfected cells were cultured under different conditions and the cell culture medium was used directly for sandwich-based ELISA to quantitate vitamin K-dependent carboxylation activity. Wild-type GGCX activity was normalized to 100%.

**GGCX subcellular localization and immunoblotting analysis**

Subcellular localization of GGCX and its mutants was examined by fluorescence confocal microscopy as previously described. The EGFP (enhanced green fluorescent protein) tagged GGCX or its variants was transiently co-expressed with the ER marker mCherry-ER-3 (a gift from Dr. Michael Davidson, Addgene plasmid # 55041) in GGCX-deficient HEK293 cells. Forty-eight hours post-transfection, the nuclei of the cells were stained with 2 μM Hoechst 33342 (Life Technologies, Carlsbad, CA) at 37°C for 20 minutes. The cells were gently washed twice with
growth medium without phenol red and used directly for fluorescence confocal microscope imaging.

To assess GGCX expression and stability, HPC4-tagged GGCX and its truncation mutants were transiently expressed in GGCX-deficient HEK293 cells. Forty-eight hours post-transfection, cells were lysed with 1% Triton X-100 in the presence of protease inhibitors. The cell lysates were directly used for SDS-PAGE and western blot detection. Protein bands were probed using anti-HPC4 as the primary antibody. To detect GGCX glycosylation mutants, protein bands were probed using anti-GGCX (Proteintech Group, Inc., Rosemont, IL) as the primary antibody.

**Supplemental References**


Supplemental Table 1 – *GGCX* and *VKOR* mutations found in the VKCFD patient by whole exome sequencing

<table>
<thead>
<tr>
<th>Gene</th>
<th>Reference position</th>
<th>db SNP rs#</th>
<th>Nucleotide change</th>
<th>Region</th>
<th>aa-position-codon</th>
<th>Zygosity</th>
<th>Allele frequency</th>
<th>aa change</th>
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<td>E630*</td>
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Supplemental Figure 1  
**N-linked glycosylation affects GGCX activity**  
(A) Immunoblotting of GGCX (lane 1) and the N605/627Q mutant (lane 2).  
(B) Carboxylation activity of GGCX and the N605/627Q mutant measured by GGCX-deficient cell-based assay with 5 µg/mL vitamin K.  
(C) Carboxylation of the reporter protein by GGCX and the N605/627Q mutant in GGCX-deficient HEK293 reporter cells in response to increasing concentrations of vitamin K.
Supplemental Figure 2  Localization of GGCX and the identified truncation mutants in HEK293 cells by fluorescence confocal microscopy imaging EGFP-tagged GGCX and its truncation mutants (green) were co-expressed with the ER marker mCherry-ER-3 (red) in GGCX-deficient HEK293 cells. Forty-eight hours post-transfection, cell nuclei were stained with Hoechst 33342 (blue) before imaging.
Supplemental Figure 3  C-terminal truncation mutations affect GGCX folding and stability

HPC4-tagged GGCX and the identified truncation mutations were transiently expressed in GGCX-deficient HEK293 cells. Forty-eight hours post-transfection, cell lysates were directly used for immunoblotting analysis. Lane 1: GGCX; lane 2: the I553* mutant; lane 3: the E630* mutant. Full-length GGCX band is indicated by an arrow; expected GGCX truncation mutation bands are indicated by open circles.