Supplementary Methods

DNA manipulation and plasmid constructions: For the cell-based GGCX activity assay, mammalian expression vector pBudCE4.1 (Life Technologies, Carlsbad, CA), that independently expresses two genes from a single vector, was used for expressing GGCX or its variants in HEK293 cells. *Metridia* luciferase cDNA was cloned into one of the multi-cloning sites of pBudCE4.1 (pBudCE4.1-*Met*.Luc) as an internal control for transfection efficiency; GGCX or its mutant was cloned into the other multi-cloning site, as previously described (Tie *et al.*, 2014). The GGCX Arg485Pro mutant was created by QuickChange site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) according to the manufacturer's instructions. The GGCX-Δex3 mutant was created by deleting amino acid residues Phe73 to Gly125 using two-step PCR based on the cDNA sequence from the patients as reported by Kariminejad *et al*. (2014) (Kariminejad *et al.*, 2014). For the fluorescence confocal study, EGFP was fused to the C-terminus of GGCX or its mutant. For the immunoblotting study, an HPC_4 (EDQVDPRLIDGK) epitope tag was introduced to the C-terminus of GGCX or its mutant.

Cell-based GGCX function study: Most of our knowledge concerning GGCX's function has been obtained from an *in vitro* activity assay under artificial conditions using the pentapeptide FLEEL as the substrate (Rishavy and Berkner, 2012; Suttie *et al.*, 1979). Thus, it has limited usefulness in understanding carboxylation of vitamin K-dependent (VKD) proteins in their native milieu. In the present study, to better clarify the functional consequences of the GGCX mutations, we determined GGCX activity in human embryonic kidney cells (HEK293) using two structure distinct VKD proteins as the

reporters (Tie *et al.*, 2016). The first reporter protein is a chimeric prothrombin (PT), FIXgla-PT (prothrombin with its gla domain exchanged with that of factor IX), which is used to evaluate the carboxylation efficiency of coagulation factors. This gla domain replacement allowed us to use a monoclonal antibody specific for the carboxylated gla domain of FIX for quantitative detection. The second reporter protein is MGP whose carboxylation status has been linked to non-bleeding disorders, such as PXE-like syndrome. To eliminate the background signal, the endogenous GGCX gene of the reporter cells was knocked out by CRISPR-Cas9-mediated genome-editing (Cong *et al.*, 2013; Mali *et al.*, 2013).

For GGCX function study, plasmid DNA (pBudCE4.1-*Met*.Luc) carrying the cDNA of either the wild-type or the mutant GGCX was transiently transfected into the GGCX-deficient dual-reporter cells in a 48-well plate using Xfect transfection reagent according to the manufacturer's instructions (Clontech, Mountain View, CA). Five hours post-transfection, the transfection medium was replaced by a complete cell culture medium containing either 5μg/ml vitamin K or with increasing concentrations of vitamin K for activity titration. The cell culture medium was collected after 48 hours incubation and used directly for sandwich-based ELISA to quantitate carboxylated FIXgla-PT and MGP, as previously described (Tie *et al.*, 2016). Wild-type GGCX activity was normalized to 100%.

Fluorescence confocal microscopy and immunoblotting: For subcellular localization of the wild-type GGCX and the GGCX-Δex3 mutant, C-terminal tagged EGFP fusion of these proteins were transiently co-expressed with the ER marker mCherry-ER-3 (a gift from Dr. Michael Davidson, Addgene plasmid # 55041) in HEK293 cells on glass coverslips. Forty-eight hours post-transfection, the nuclei of the cells were stained with

Hoechst 33342 (Life Technologies, Carlsbad, CA) by replacing the growth medium with staining solution (2 μM Hoechst 33342 in fresh growth medium) and incubated at 37ºC for 20 minutes. Cells were washed once with growth medium, and used directly for fluorescence confocal microscope imaging. Confocal microscopy was performed on a Zeiss LSM710 confocal laser-scanning microscope (Carl Zeiss Microimaging, Thornwood, NY). Images were collected using a 40×/1.2NA C-Apochromat objective lens. Visualization of the Hoechst 33342 was achieved by using a 405nm diode laser for excitation with the detector set to collect emission at 406-485nm. Visualization of the EGFP was achieved by using a 488nm argon laser line for excitation with the detector set to collect emission at 505–544nm. Visualization of the mCherry was achieved by using a 561nm diode laser for excitation with the detector set to collect emission at 565-701nm.

For protein expression and stability study, HPC_4-tagged GGCX and GGCX-Δex3 were transiently expressed in HEK293 cells. Forty-eight hours post-transfection, cells were washed twice with PBS (pH 6.8) and lysed with 1% Triton X-100 in the presence of protease inhibitor cocktail III (RPI Corp, Mount Prospect, IL) and kept on ice for 20 minutes. Cell lysate was used directly for SDS-PAGE and western blot detection. Protein bands were probed by anti-HPC_4 mouse monoclonal antibody as the primary antibody, horseradish peroxidase conjugated goat anti-mouse antibody as the secondary antibody, and visualized by ECL western blotting detection reagents (GE Healthcare Bio-Sciences, Pittsburgh, PA).

Supplementary references

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