

Structural and functional insights into γ -glutamyl carboxylase-factor IX interaction: implications for vitamin K-dependent bleeding disorders

Gamma-carboxylation, catalyzed by γ -glutamyl carboxylase (GGCX), is a critical post-translational modification essential for the biological activity of vitamin K-dependent proteins (VKDP).¹ Mutations in GGCX, depending on their specific location, result in vitamin K-dependent coagulation factor deficiency type 1, which encompasses a broad spectrum of clinical manifestations ranging from mild to severe.² Over 40 pathogenic GGCX mutations have been identified, most causing impaired substrate binding or abolishing enzymatic activity (*Online Supplementary Table S1*).^{2,3} Limited understanding of the structure and functional regions of GGCX hinders insights into the impact of its mutations. This study identified key functional regions of GGCX crucial for substrate binding and enzymatic activity, shedding light on the pathogenesis of vitamin K-dependent coagulation factor deficiency type 1 and advancing the development of targeted therapy.

We utilized molecular dynamics simulations to analyze the binding strength of GGCX with its substrates, factor IX (FIX) with retained propeptide (proFIX) and the cofactor reduced vitamin K (KH₂) (Figure 1A, *Online Supplementary Figure S1A, B*). The results indicated that GGCX-substrate binding involves multiple regions and sites (Figure 1A), potentially explaining the dispersed distribution of pathogenic GGCX mutations (Figure 1A, B).² Detailed binding interfaces are shown in Figure 1C-E, *Online Supplementary Figure S1C*, highlighting key structural elements such as loops (L), α -helices (α), and β -sheets (β). The transmembrane domains TM7 and TM8 of GGCX closely interact with the Gla domain of FIX and KH₂ (Figure 1D, E), indicating their critical role in enzymatic activity. Pathogenic mutations (F299S and S300F) in TM8, which abolish enzymatic activity,² further validate our structural model. These findings are consistent with the GGCX-VKDP binding model proposed in recent studies.⁴⁻⁶

Based on the binding mode, 15 binding hotspots exhibiting significant interaction probabilities were identified and mapped onto the GGCX structure for enhanced visualization (Figure 1B, *Online Supplementary Figure S1C*). To validate the binding model experimentally, we introduced missense mutations (obtained from the NCBI SNP database), alanine substitution mutations, and site-directed mutagenesis⁷ at selected sites within the 15 identified regions of GGCX. The effects of these mutations on carboxylation activity were systematically evaluated using cell-based assays.⁸ Some mutations markedly reduced enzymatic activity, while others enhanced it, confirming their critical roles in

substrate interactions (Figure 1F).

GGCX tightly binds VKDP via propeptides,^{9,10} which play a critical role in substrate recognition. We therefore comprehensively analyzed the GGCX-FIX propeptide interaction and identified key binding sites using MM/PBSA calculations (Figure 2A). Critical residues of GGCX (Y231, H410, K412, N427, Y458, E528, F543, Y586, and E608) were identified as essential for propeptide interactions. To investigate these key residues, we conducted site-directed mutagenesis and subsequently analyzed the impact of mutations on FIX binding. Bimolecular fluorescence complementation assays² (Figure 2B) revealed that nearly all GGCX mutations resulted in a decrease in the relative binding strength between GGCX and proFIX, with the H410P mutation exhibiting the most pronounced reduction.

To substantiate the significance of critical GGCX residues in propeptide binding, we assessed the effects of specific GGCX mutations on their interactions with different propeptides. *In vitro* analysis showed that FIX, protein C (PC), and matrix Gla protein (MGP) propeptides bind to GGCX with varying affinities, with dissociation constant (K_d) values of 4.97, 18.6, and 0.98 nM,¹⁰ respectively. The bimolecular fluorescence complementation results illustrated in Figure 2C indicate that most mutations of GGCX decrease its binding to the reporter proteins (directed by the propeptides of PC and MGP), similarly to the reductions seen with the FIX propeptide (Figure 2B), except for the Y586F mutation.

Our results indicate that those GGCX residues are involved in VKDP propeptide binding, consistent with recent studies⁴⁻⁶ showing that the propeptide binding region spans S405 to V604. This supports our concept that multiple GGCX regions (e.g., amino acids 228-255, 451-461, 468-478, 540-553, 581-588, as shown in *Online Supplementary Figure S1A*) converge to form a binding interface, with critical residues essential for propeptide interaction. This integrated structural arrangement underscores the cooperative role of these regions in substrate recognition and subsequent enzymatic catalysis. Consequently, dispersed pathogenic mutations, including those outside the designated critical regions of GGCX, such as R485P³ (*Online Supplementary Table S1*), may disrupt binding interface integrity, affecting substrate recognition and binding. Previous experimental results¹¹ further support this, showing that multiple GGCX regions exhibit indirect cooperative structural dependency in substrate binding without directly contributing to propeptide recognition.

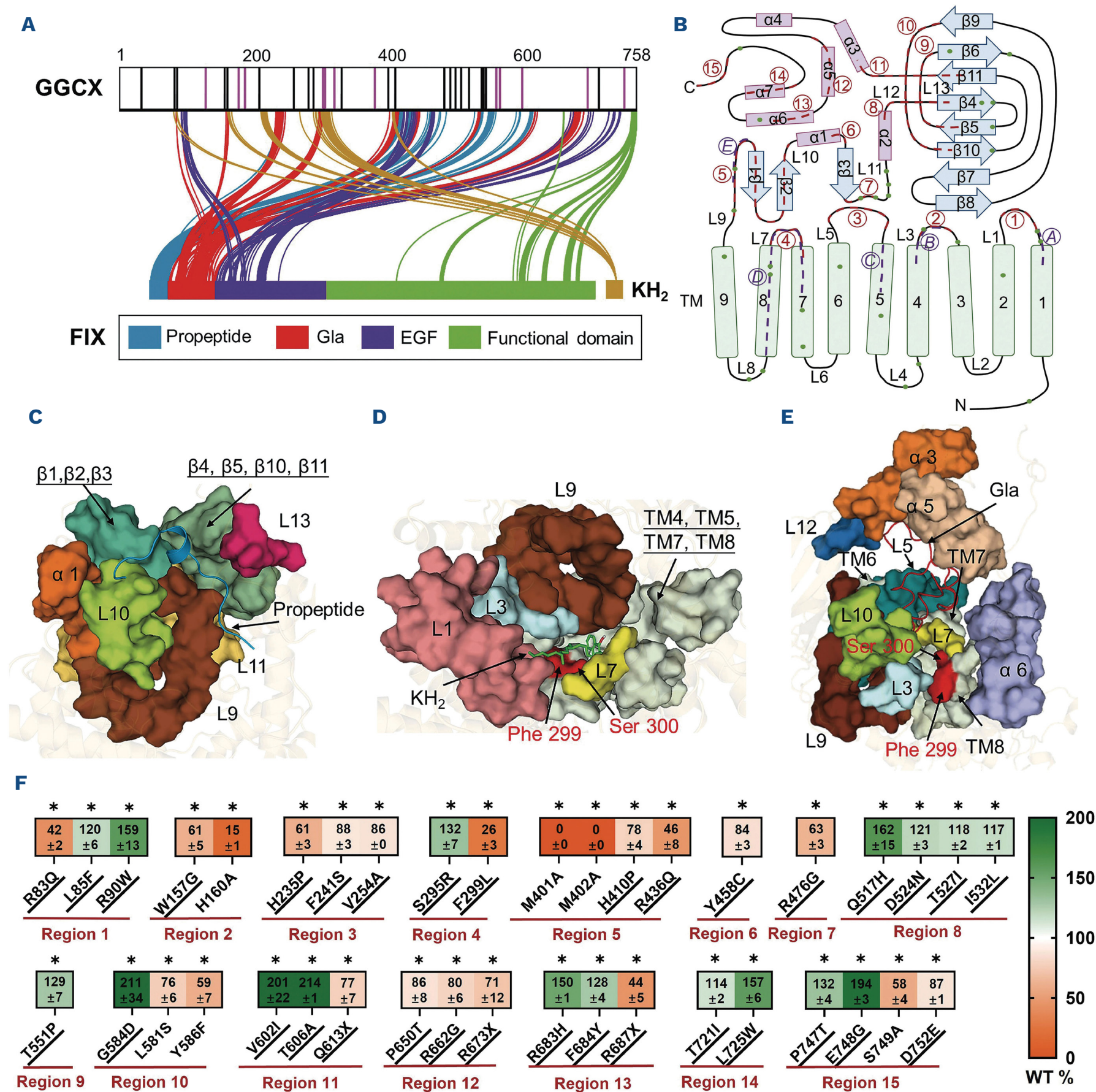


Figure 1. Model of γ -glutamyl carboxylase binding to factor IX propeptide and reduced vitamin K. (A) Contact model (with known pathogenic γ -glutamyl carboxylase [GGCX] mutations) depicting the interactions between residues of GGCX and its substrates, propeptide of factor IX (proFIX) and reduced vitamin K (KH_2 , colored orange). The GGCX–proFIX complex was predicted using AlphaFold3, while the GGCX– KH_2 interaction was modeled via molecular docking with AutoDock Vina (version 1.1.2), followed by a 100 ns molecular dynamics simulation using GROMACS (version 2020.6). The colored regions represent different domains of factor IX (FIX): the propeptide (blue), Gla domain (red), EGF domain (purple), and functional domain (green). Pathogenic GGCX mutations are indicated by lines, and mutations with carboxylation activity below 30% are marked in pink. Data are representative of three independent experiments. (B) A topological map based on molecular dynamics simulations, illustrating the structural distribution of known pathogenic GGCX mutations. The map delineates 15 critical regions mediating GGCX binding to proFIX and five key regions involved in the interaction of GGCX with KH_2 . Two sets of demarcation systems are used: numeric sectors 1–15 (indicated by red dashed lines and numbered circles) correspond to proFIX-binding regions, while alphabetic sectors A–E (denoted by purple dashed lines and lettered circles) represent KH_2 -binding regions. Pathogenic GGCX mutations are marked with green dots. (C–E) Detailed binding interfaces between GGCX, FIX's propeptide and Gla domain, and KH_2 . The loops are col-

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or-coded as follows: L1 (salmon), L3 (light cyan), L5 (dark cyan), L7 (yellow), L9 (brown), L10 (lemon yellow), L11 (yellow-orange), L12 (sky blue), and L13 (hot pink). The α -helices are colored as follows: α 1 and α 3 (orange), α 5 (wheat), and α 6 (light blue). β -sheets are color-coded: β 1-3 (aquamarine), β 4, β 5, β 10, and β 11 (pale green). Transmembrane domains (TM) are shown in light green. (F) Carboxylation activity validation of GGCX mutations within binding regions of GGCX. The heatmap summarizes the evaluation of missense mutations (underlined) and site-directed mutations across 15 distinct GGCX binding regions, highlighting their effects on carboxylation activity. In designing mutations, we prioritized naturally occurring missense or previously reported pathogenic variants. When such variants were unavailable, we selected mutations predicted by AlphaFold3 to cause the most significant structural perturbation, as indicated by the highest effective strain values.⁷ GGCX or its mutants were transiently transfected into a GGCX-knockout cell line stably expressing a FIX reporter, and the carboxylation of the reporter proteins was subsequently assessed using enzyme linked immunosorbent assay. Data are presented as the mean $\pm$ standard deviation of three independent experiments (N=3). Statistical significance between GGCX mutants and wild-type GGCX was determined as $*P<0.05$.

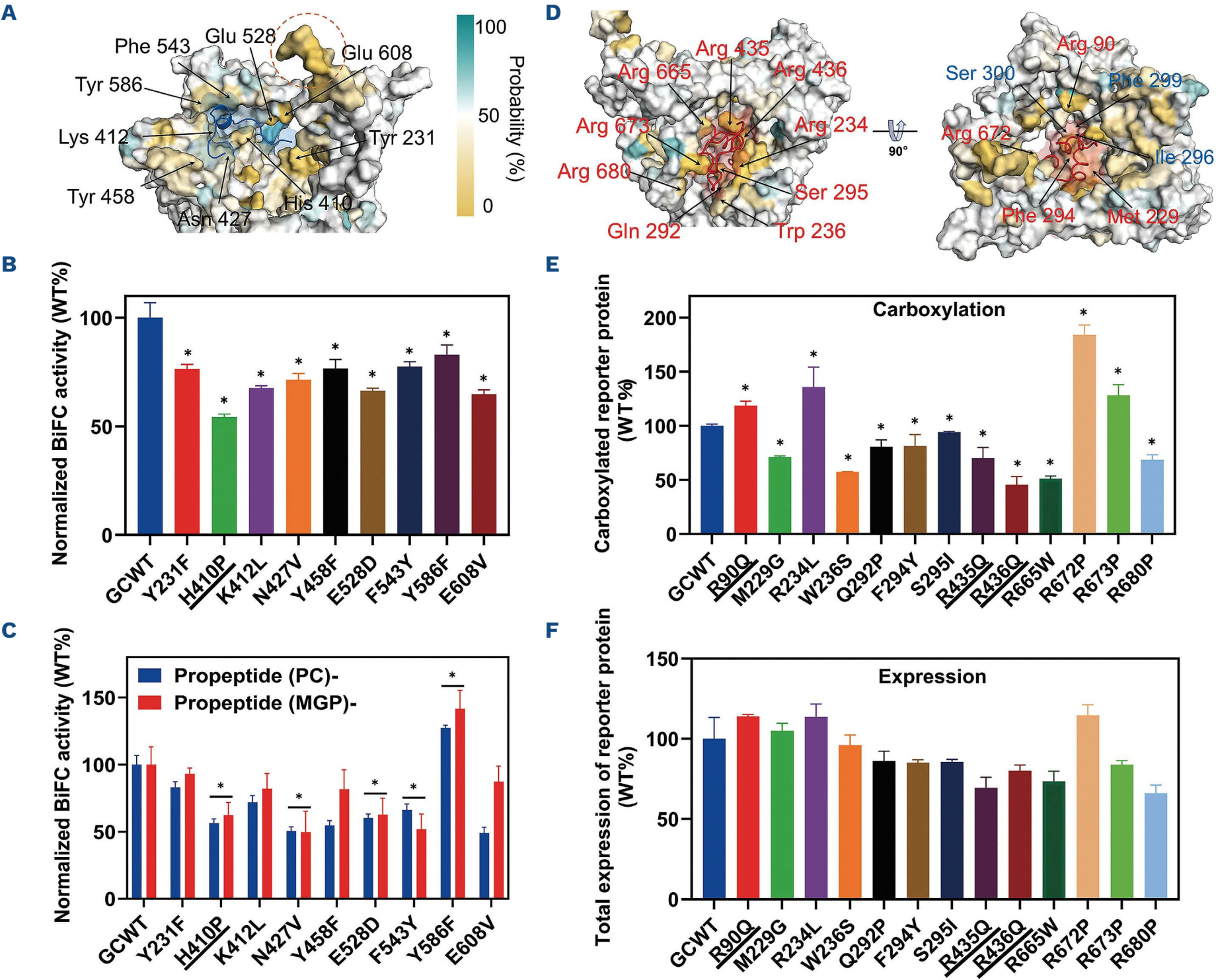


Figure 2. Factor IX propeptide and Gla domain binding to γ -glutamyl carboxylase is mediated by multi-site interactions. Missense mutations are underlined. (A) Molecular visualization of binding sites between γ -glutamyl carboxylase (GGCX) and factor IX (FIX) propeptide (in blue) derived from MM/PBSA analysis. Teal indicates weaker binding, while yellow represents stronger binding interactions. The red dashed circle highlights the C-terminal region of GGCX. The scale bar reflects normalized probabilities for residue contact. (B, C) Assessment of interactions between wild-type or mutant GGCX and reporter proteins guided by the propeptides of FIX (B), protein C, or matrix Gla protein (MGP) (C) using the Venus-based bimolecular fluorescence complementation assay. Wild-type or mutant GGCX linked to one of two complementary fragments of Venus, with the reporter protein linked to the other. Bimolecular fluorescence complementation analysis was performed on these constructs transiently transfected into

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HEK293 cells without endogenous GGCX. After 48 hours of incubation, fluorescence intensity within the transfected live cells was measured to assess the interaction between GGCX and reporter proteins. (D) Molecular visualization of the key binding residues of GGCX with the Gla domain (in red) of FIX. Residues colored in blue are implicated in GGCX's enzymatic activity. Binding strengths are visualized as a gradient from yellow (strong) to teal (weak). (E, F) The carboxylation activity and expression levels of chimeric reporter protein FIXglaPC under various GGCX mutations. GGCX and specific mutations with the reporter protein were co-expressed in a GGCX-knockout cell line. After 48 hours in complete medium with 10 μ M vitamin K1, carboxylation and expression levels were measured by enzyme-linked immunosorbent assay. Data are presented as the mean $\pm$ standard deviation of three independent experiments (N=3). Statistical significance between GGCX mutants and wild-type GGCX was determined as $*P<0.05$.

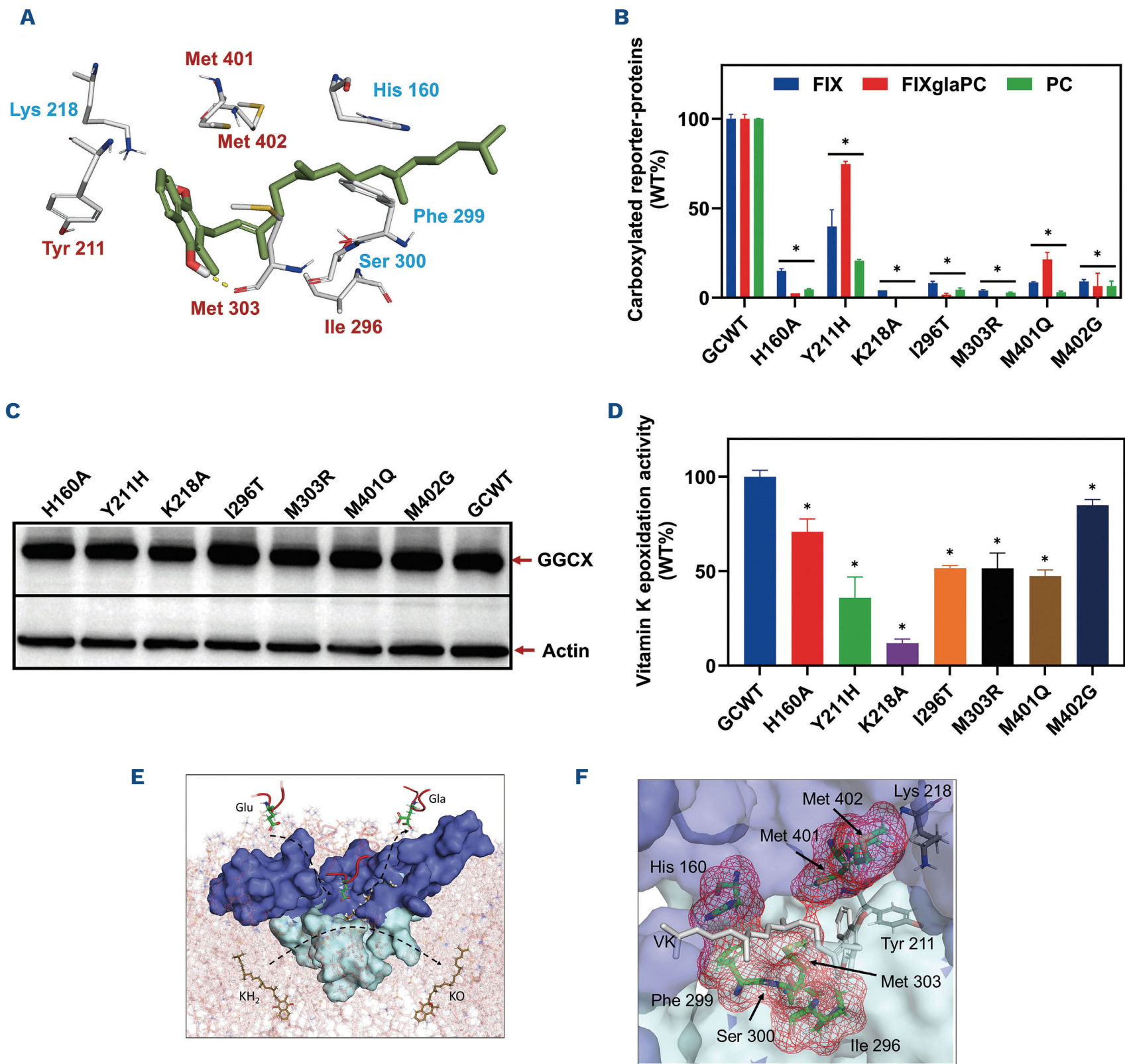


Figure 3. Spatial connection between the carboxylation and vitamin K epoxidation centers of γ -glutamyl carboxylase. (A) Key residues in the binding of γ -glutamyl carboxylase (GGCX) to reduced vitamin K (KH₂). Reported residues are colored in blue. (B) Carboxylation activity of three reporter proteins under different GGCX mutations. The three reporter proteins are factor IX (FIX), protein C (PC), and the chimeric reporter protein FIXglaPC (PC with its Gla domain replaced by that of FIX). GGCX or specific mutations were co-transfected with reporter proteins into a GGCX-knockout cell line. After 48 hours in complete medium with 10 μ M vitamin K1, carboxylation levels were quantified by enzyme-linked immunosorbent assay. (C) Expression of GGCX mutants

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as assessed by western blot. Wild-type (WT) GGCX and its mutants were transiently expressed in an HEK293 cell line without endogenous GGCX. After 48 hours of incubation, cell lysate was analyzed with western blot using a rabbit anti-GGCX polyclonal antibody. (D) Effects of GGCX mutations on vitamin K epoxidation activity. Wild-type GGCX and its mutants were transiently expressed in GGCX-deficient HEK293 cells. Transfected cells were incubated with vitamin K1, and the conversion to vitamin K epoxide by GGCX was measured using reversed-phase high-performance liquid chromatography. (E) Molecular visualization illustrates the coupling between vitamin K-dependent protein carboxylation and vitamin K epoxidation. (F) Molecular visualization displays partially overlapping active sites of carboxylation and vitamin K epoxidation. The Gla binding regions are colored in slate and the residues related to vitamin K epoxidation are colored in pale cyan. Data are presented as the mean $\pm$ standard deviation of three independent experiments (N=3). Statistical significance between GGCX mutants and WT GGCX was determined as $*P<0.05$.

In addition to the propeptide, we also analyzed simulation results of GGCX interacting with the Gla domain of FIX (Figure 2D). We identified critical residues (R90, M229, R234, W236, Q292, F294, S295, I296, F299, S300, R435, R436, R665, R672, R673, and R680) essential for VKDP Gla domain binding. Site-directed mutagenesis and cell-based assays showed mutations at R90, R234, R672, and R673 enhanced carboxylation activity, whereas others reduced it to varying degrees (Figure 2E), without significantly altering reporter protein expression (Figure 2F). These results further support the hypothesis that multiple regions of GGCX work in coordination to facilitate VKDP substrate binding and carboxylation (Figure 1A).

As a dual-function enzyme, GGCX oxidizes KH_2 to vitamin K epoxide, generating the energy required for γ -glutamate deprotonation during substrate carboxylation.¹² To explore the active site of vitamin K epoxidation, we analyzed the interaction of GGCX with KH_2 , identifying five key regions (A-E) critical for KH_2 binding (*Online Supplementary Figure S2*). The binding mode, including specific residues, is illustrated in Figure 3A. The functional impact of these residues on carboxylation was evaluated using cell-based assays² with three reporter proteins: FIX, PC, and the chimeric reporter FIXglaPC.² Mutations at residues H160, K218, I296, M303, M401, and M402 abolished carboxylation activity across all reporter proteins, while the Y211 mutation had a milder impact (around 50% reduction for FIX's carboxylation) (Figure 3B). These mutations did not affect levels of GGCX expression (Figure 3C), suggesting that these residues primarily affect enzymatic function rather than protein stability.

We also evaluated the impact of GGCX mutations on vitamin K epoxidation activity. The conversion of KH_2 to vitamin K epoxide was assessed in GGCX-deficient cells expressing wild-type GGCX or its mutants.¹³ The K218A mutation nearly eliminated epoxidase activity, while mutations at H160, I296, M303, and M401 retained approximately 50% of epoxidase activity (Figure 3D) but showed no carboxylation activity (Figure 3B). This differentiated effect of GGCX mutations on carboxylation and epoxidation activity could be due to the effect on the efficiency of the coupling of these two activities, as previously observed.^{4,14}

The visualized simulation structure reveals that regions binding the VKDP Gla domain (e.g., amino acids 82-98, 155-160, 288-301, and 395-443 in *Online Supplementary Figure S1A*) form a circular cavity essential for carboxylation (Fig-

ures 1E and 2D). This Gla domain binding cavity is spatially connected to GGCX's vitamin K epoxidation center (KH_2 binding regions at amino acids 78-84, 155-164, 283-310, and 395-402, shown in *Online Supplementary Figure S2*) (Figure 3E), as evidenced by overlapping residues (H160, I296, F299, S300, M303, M401, and M402) (Figure 3F) critical for enzymatic activity.

The active residue K218¹⁵ catalyzes the oxidation of KH_2 to vitamin K epoxide, a process essential for glutamate carboxylation on VKDP, which is consistent with our findings. The residues F299, S300, M303, M401, and M402, situated at the interface of GGCX's dual enzymatic active centers (Figure 3F), are associated with the coupling of its dual enzymatic activities. Mutations at these sites may disrupt enzymatic coupling, leading to the symptoms of vitamin K-dependent coagulation factor deficiency type 1 resistant to high concentrations of vitamin K, as exemplified by pathogenic mutations such as F299S and S300F² (*Online Supplementary Table S1*). These findings are consistent with recent cryo-electronmicrography structural studies of GGCX.⁴⁻⁶

In summary, we propose a GGCX-proFIX binding and carboxylation model, identifying critical regions for substrate binding and enzymatic activity. Mutations in enzymatic regions, particularly at the newly identified residues I296, M303, M401, and M402, result in irreversible loss of activity, while high-dose vitamin K partially rescues function in substrate-binding region mutations (*Online Supplementary Table S1*). This study offers the first comprehensive analysis correlating the structural locations of GGCX mutations with their pathogenic mechanisms, significantly advancing the understanding of vitamin K-dependent coagulation disorders. The present study fully adhered to all applicable ethical standards and national regulations. No procedures or experiments involving human participants or animals were conducted.

Authors

Kang Liu,^{1*} Shixin Li,^{1*} Guomin Shen,^{2*} Jiangbo Tong,¹ Nan Jiang,¹ Minwen Hong,¹ Yi Gu,¹ Luju Chen,¹ Yuan Zhao,¹ Jinlin Huang,^{1,3} Jian-Ke Tie⁴ and Zhenyu Hao^{1,5}

¹College of Bioscience and Biotechnology, Yangzhou University, Yangzhou, China; ²Department of Cell Biology, School of Basic

Medical Sciences, Harbin Medical University, Harbin, China; ³Joint International Research Laboratory of Agriculture and Agri-Product Safety, Ministry of Education of China, Yangzhou, Jiangsu, China; ⁴Department of Biology, the University of North Carolina at Chapel Hill, Chapel Hill, NC, USA and ⁵Affiliated Hospital, Yangzhou University, Yangzhou, Jiangsu, China

**KL, SL and GS contributed equally as first authors.*

Correspondence:

J. HUANG - jinlin@yzu.edu.cn
J.-K. TIE - jktie@email.unc.edu
Z. HAO - zhyuhao@hotmail.com

<https://doi.org/10.3324/haematol.2025.287736>

Received: March 20, 2025.

Accepted: August 1, 2025.

Early view: August 21, 2025.

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Disclosures

No conflicts of interest to disclose.

Contributions

ZH, J-KT and JH conceived the studies. KL, GS and MH performed the cell-based functional studies. KL and LC performed the bimolecular fluorescence complementation assay. SL, J-KT and JT performed molecular dynamics simulations and molecular docking studies. KL and GS constructed plasmids and created GGCX mutations. KL and YG performed the chemical cross-linking. KL and YZ performed epoxidase activity assays. ZH, J-KT, JH and SL analyzed the data and wrote the manuscript. All authors reviewed and contributed to the manuscript.

Funding

This work was supported by grants 82370136 (to ZH) and 82170133 (to GS) from the National Natural Science Foundation of China; grant HL131690 (to J-KT) from the National Institutes of Health, USA; grants BK20231333 (to ZH) and BK20210828 (to SL) from the Natural Science Foundation of Jiangsu Province; Jiangsu Specially-Appointed Professor Start-up Funds grant (to ZH); grants 2023M732983 (to SL), and 2022M712691 (to NJ) from the China Postdoctoral Science Foundation; and grant YZ2023245 (to ZH) from the Yangzhou Innovation Capability Enhancement Fund/Program.

Data-sharing statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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