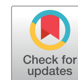

Proteolytic processing of secretory pathway kinase Fam20C by site-1 protease promotes biomineralization

Xinxin Chen[a,b,1], Jianchao Zhang[a,b,1], Pulan Liu[c], Yangyang Wei[c], Xi'e Wang[a], Junyu Xiao[c], Chih-chen Wang[a,b], and Lei Wang[a,b,2]

[a]National Laboratory of Biomacromolecules, Chinese Academy of Sciences (CAS) Center for Excellence in Biomacromolecules, Institute of Biophysics, CAS, Beijing 100101, China; [b]College of Life Sciences, University of CAS, Beijing 100049, China; and [c]The State Key Laboratory of Protein and Plant Gene Research, School of Life Sciences, Peking-Tsinghua Center for Life Sciences, Peking University, Beijing 100871, China

Edited by Kim Orth, The University of Texas Southwestern Medical Center, Dallas, TX, and approved July 8, 2021 (received for review January 5, 2021)

Family with sequence similarity 20C (Fam20C), the major protein kinase in the secretory pathway, generates the vast majority of the secreted phosphoproteome. However, the regulatory mechanisms of Fam20C transport, secretion, and function remain largely unexplored. Here, we show that Fam20C exists as a type II transmembrane protein within the secretory compartments, with its N-terminal signal peptide-like region serving as a membrane anchor for Golgi retention. The secretion and kinase activity of Fam20C are governed by site-1 protease (S1P), a key regulator of cholesterol homeostasis. We find that only mature Fam20C processed by S1P functions in osteoblast differentiation and mineralization. Together, our findings reveal a unique mechanism for Fam20C secretion and activation via proteolytic regulation, providing a molecular link between biomineralization and lipid metabolism.

Fam20C | Golgi | phosphorylation | proteolysis | site-1 protease

Extracellular and secretory pathway phosphorylation regulates numerous physiological processes, such as bone mineralization, blood coagulation, extracellular matrix remodeling (1, 2), and neuronal development (3). During the past decade, Fam20 proteins were identified as secretory pathway kinases. They are synthesized in the lumen of the endoplasmic reticulum (ER) and specifically localize in the Golgi apparatus and extracellular space where they phosphorylate secreted proteins (4, 5) or proteoglycans (6). Fam20C, the most-studied member of the Fam20 family, is the bona fide "Golgi casein kinase," which phosphorylates secretory proteins within the S-x-E/pS motif (4). Loss-of-function *FAM20C* mutations cause a rare and often lethal osteosclerotic bone dysplasia called Raine syndrome (7). Indeed, Fam20C has been found to phosphorylate the small integrin-binding ligand N-linked glycoproteins (SIBLINGs), consisting of bone sialoprotein (BSP), osteopontin (OPN), dentin matrix protein-1 (DMP1), dentin sialophosphoprotein (DSPP), and matrix extracellular phosphoglycoprotein (MEPE), the major extracellular components regulating biomineralization (4, 5). Moreover, the broad substrate spectrum of Fam20C suggests that this protein kinase participates in a wide range of biological processes besides biomineralization, such as cell adhesion and migration (8), prohormone processing (9–11), lipid homeostasis (12), protein trafficking (13), and ER homeostasis (14–17), underscoring the biological importance of Fam20C kinase within the secretory pathway.

Protein kinases can be regulated in multiple ways, such as through interactions with regulatory subunits, oligomerization (18), autoinhibition (19), and phosphorylation either by other kinases or by itself (20). Different from other protein kinases, Fam20C preferentially uses Mn^{2+} over Mg^{2+} as a metal ion cofactor and is insensitive to the broad-spectrum protein kinase inhibitor staurosporine (4). The crystal structure of the Fam20C ortholog from *Caenorhabditis elegans* revealed an atypical kinase architecture, and the position of the regulatory α-C helix and the lack of an activation loop indicated an architecture primed for efficient catalysis (21). Fam20C can be allosterically activated through the formation of a homodimer or a heterodimer with its paralog Fam20A (22–24), a pseudokinase that plays a critical role in dental development (25, 26). In addition, small molecules such as sphingosine and several sphingolipids were also reported to activate Fam20C, but the underlying molecular mechanisms are poorly understood (27, 28). Furthermore, little is known about the regulatory mechanisms of Fam20C transport and secretion.

In *C. elegans*, retention of Fam20C in the early secretory pathway impairs fertility, embryogenesis, and development of worms (29), suggesting that transit into the late secretory pathway and/or extracellular space is necessary for Fam20C to execute its functions. Moreover, some Raine syndrome mutants of Fam20C display mislocalization in the ER and secretion deficiency (4, 5). These results imply that proper transport of Fam20C in the secretory pathway is important for its function. The N terminus of the extracellular mature Fam20C has been characterized as Asp93 (8), but the details of Fam20C processing and its biological roles are currently unknown.

Limited proteolysis of proteins is a common irreversible post-translational modification for releasing active products. The major processing enzymes of the secretory pathway are subtilisin-like proprotein convertases, a family of nine serine proteases (PCSK1 to 9) (30). The Golgi-resident site-1 protease (S1P, also named PCSK8, SKI-1), encoded by the *MBTPS1* (membrane-bound transcription factor peptidase, site-1) gene, cleaves membrane-bound transcription factors at luminal sites, such as sterol regulatory element-binding proteins (SREBPs) (31, 32), activating transcription factor 6 (ATF6) (33), and cAMP response element-binding proteins (CREBs) (34), to regulate cholesterol homeostasis and

BIOCHEMISTRY

Significance

Fam20C is the bona fide "Golgi casein kinase" and generates the majority of the secreted phosphoproteome. Loss-of-function *FAM20C* mutations cause a type of lethal osteosclerotic bone dysplasia called Raine syndrome. In this study, we find that Fam20C resides in the Golgi apparatus as a transmembrane protein. Site-1 protease, a key regulator of cholesterol homeostasis, cleaves the Fam20C propeptide and promotes its secretion and activation. This proteolytic processing of Fam20C is critical for osteoblast differentiation and mineralization. Our results have important implications for both secretory pathway kinase regulation and biomineralization regulation.

Author contributions: X.C., J.Z., J.X., C.-c.W., and L.W. designed research; X.C., J.Z., P.L., Y.W., and X.W. performed research; X.C., J.Z., J.X., and L.W. analyzed data; and X.C. and L.W. wrote the paper.

The authors declare no competing interest.

This article is a PNAS Direct Submission.

Published under the PNAS license.

[1]X.C. and J.Z. contributed equally to the work.

[2]To whom correspondence may be addressed. Email: wanglei@ibp.ac.cn.

This article contains supporting information online at https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2100133118/-/DCSupplemental.

Published August 4, 2021.

ER stress responses. S1P also functions in the biogenesis of lysosomes via the proteolytic activation of the *N*-acetylglucosamine-1-phosphotransferase α-/β-subunit precursor (35). In addition, the processing of viral envelope glycoprotein precursors by S1P is crucial for arenavirus synthesis and spread (36). Interestingly, S1P is also implicated in biomineralization, as S1P deficiency causes human skeletal dysplasia (37). S1P has been reported to regulate osteoblastic mineralization (38, 39) and bone development (40, 41). However, it is currently unclear how S1P functions to regulate biomineralization.

In this study, we first identified Fam20C as a type II transmembrane protein with its N-terminal transmembrane (TM) domain integrated into the membrane but not a luminal protein as previously considered. We further demonstrated that the ER-to-Golgi anterograde transport of Fam20C is mediated by COPII vesicles and that the TM domain acts as a Golgi retention signal.

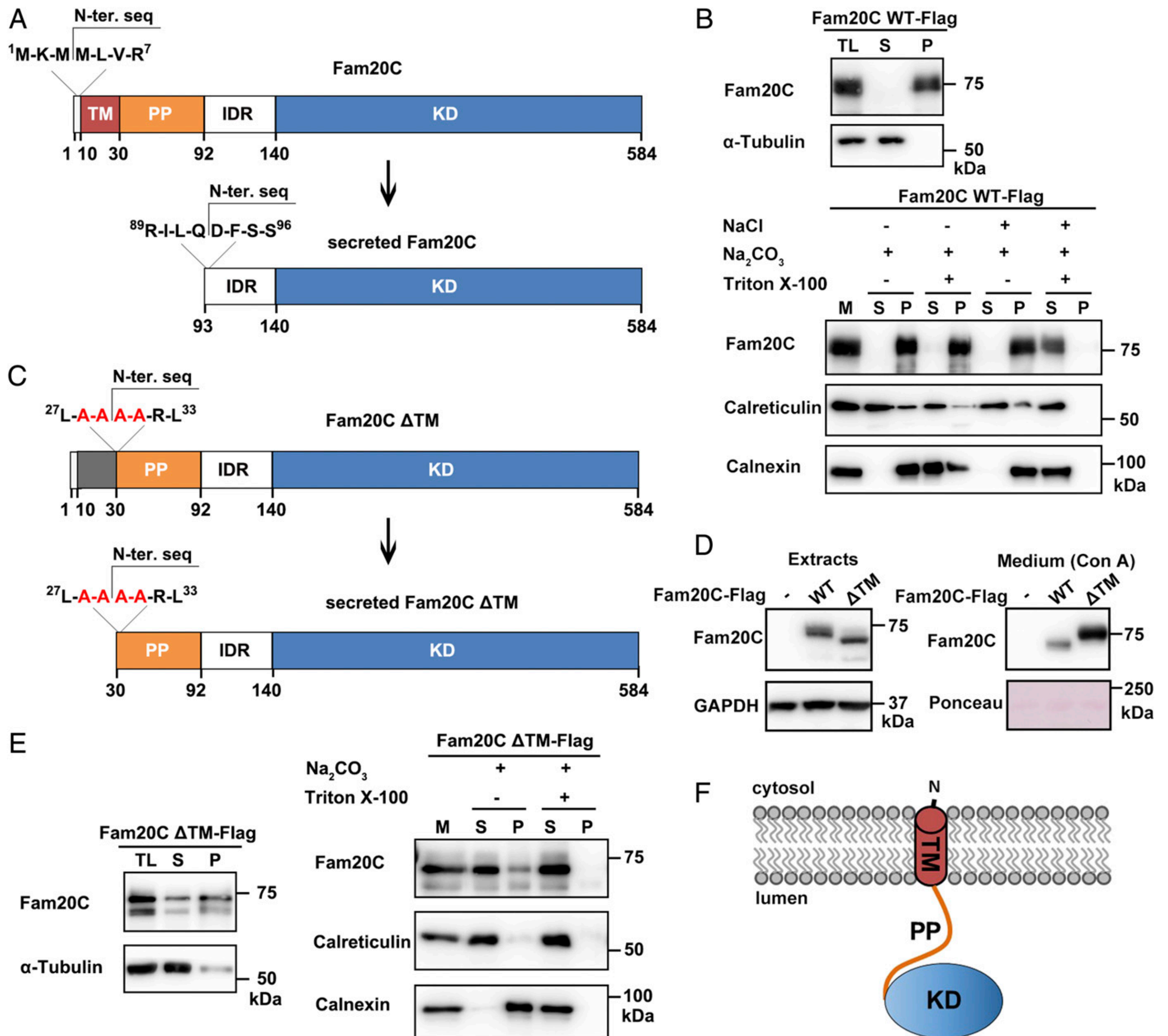

Fig. 1. Identification of Fam20C as a type II transmembrane protein. (*A*) Schematic representation of full-length human Fam20C (*Upper*) and secreted mature Fam20C (*Lower*), depicting the TM domain, the propeptide (PP), the intrinsic disorder region (IDR), and the kinase domain (KD). The C-terminally Flag-tagged Fam20C expressed in HeLa cells was purified from cell extracts or conditioned medium, and the N-terminal amino acid sequence was determined by Edman degradation. The amino acid residues surrounding the sequenced region were shown, and the domain boundaries were numbered from the C terminus. (*B*, *Upper*) The total lysates (TL) of HeLa cells expressing Flag-tagged Fam20C WT were separated into soluble (S) and pelleted membrane (P) fractions. Fam20C and the cytosolic protein α-tubulin were immunoblotted. (*Lower*) The P fraction in *Upper* was taken as total membrane (M), extracted with Na_2CO_3 in the absence or presence of NaCl and Triton X-100, and then separated into soluble (S) and pelleted membrane (P) fractions. Fam20C, the ER lumen protein calreticulin, and the ER membrane protein calnexin were immunoblotted. (*C*) Schematic representation of Fam20C ΔTM (*Upper*) and its secreted form (*Lower*), depicting the amino acid mutations (^{28}DLLP^{31} to ^{28}AAAA^{31}) in red. Note that the original TM of Fam20C is changed to a cleavable signal peptide (gray). The C-terminally Flag-tagged Fam20C ΔTM was ectopically expressed in *FAM20C* KO HeLa cells, purified from cell extracts or conditioned medium, and sequenced by Edman degradation. The amino acid residues surrounding the sequenced region were shown. (*D*) Immunoblotting of Fam20C in cell extracts and Concanavalin A (Con A) precipitates from conditioned medium of *FAM20C* KO HeLa cells expressing Flag-tagged Fam20C WT or ΔTM. (*E*) The membrane fractionation of *FAM20C* KO HeLa cells expressing Flag-tagged Fam20C ΔTM was performed as in *B*. (*F*) Topological diagram of Fam20C in cells.

We provided evidence that cleavage of Fam20C propeptide is catalyzed by the Golgi-resident protease S1P, and this processing increases the secretion and kinase activity of Fam20C. Importantly, we established a functional link between S1P and Fam20C in terms of osteoblast differentiation and mineralization. Overall, we have unraveled the mechanism for proteolysis-mediated maturation of the secretory pathway kinase Fam20C, which plays a critical role in biomineralization.

Results

Fam20C Is a Type II Transmembrane Protein in the Secretory Pathway. Fam20C is a secreted kinase that traverses the secretory pathway, and its N-terminal 22 amino acid sequence was defined as a canonical signal peptide targeting the kinase domain into the ER lumen (4). However, the cleavage probability between residues 22 and 23 is very low, as predicted by SignalP-5.0 Server (*SI Appendix*, Fig. S1*A*). N-terminal sequencing analysis of the C-terminally Flag-tagged Fam20C protein immunoprecipitated from HeLa cells revealed that it begins with Met4 (Fig. 1*A*), suggesting that the signal peptide of Fam20C is not cleaved. In addition, the Fam20C secreted from HeLa cells begins with Asp93 (Fig. 1*A*), in line with previous results from HEK293T cells that mature Fam20C is truncated by 92 residues (8). Indeed, topology predictions using multiple methods have suggested that 11 to 30 amino acid residues form an N-terminal TM domain (*SI Appendix*, Fig. S1*C*). Sedimentation and alkaline extraction were performed to investigate whether Fam20C is integrated into the membrane. Flag-tagged Fam20C that precipitated with membrane fractions was not extracted into the supernatant by alkaline and high salt washes unless detergent was added (Fig. 1*B*). Similar results were obtained with endogenous Fam20C (*SI Appendix*, Fig. S1*D*), indicating that Fam20C is a membrane protein but not a luminal protein.

Furthermore, we mutated four residues at the end of the TM region (^{28}DLLP^{31} to ^{28}AAAA^{31}). This mutant was predicted to be cleaved by signal peptidase during cotranslational translocation into the ER, and the generated protein lacked the TM domain, hereafter referred to as Fam20C ΔTM (*SI Appendix*, Fig. S1*B*). In *FAM20C* knockout (KO) HeLa cells (15), intracellular Fam20C ΔTM begins with Ala30, as determined by N-terminal sequencing analysis (Fig. 1*C*), and its molecular weight is less than that of Fam20C wild type (WT) (Fig. 1*D*). Fam20C ΔTM can be extracted into the supernatant by alkaline washes alone, suggesting that the N-terminal TM domain anchors Fam20C to the membrane (Fig. 1*E*). To determine the intramembrane orientation of Fam20C, we performed a fluorescence protease protection assay by measuring the restricted proteolytic digestibility of Fam20C tagged with mApple at the C terminus. We confirmed the Golgi localization of Fam20C and demonstrated that the C terminus of Fam20C resides in the Golgi lumen, distinct from the cytoplasmic peripheral Golgi protein GM130 (*SI Appendix*, Fig. S1*E*). Taken together, these results indicated that intracellular Fam20C is a type II membrane protein in the Golgi apparatus and is composed of a short cytoplasmic amino terminus, a single-pass TM domain, and a large luminal domain (Fig. 1*F*).

The TM Domain Retains Fam20C in the Golgi Apparatus. Notably, Fam20C ΔTM secreted much more efficiently than WT, and its propeptide remained uncleaved (Fig. 1 *C* and *D*). We employed the retention using selective hooks (RUSH) system to investigate the possible functions of the TM domain for Golgi retention and secretion. Fam20C was tagged with streptavidin binding protein (SBP) and EGFP at the C terminus and was initially trapped in the ER by luminal streptavidin fused with the ER retention signal KDEL (Fig. 2*A*). Biotin competed with streptavidin for SBP and induced a synchronized release of Fam20C from the ER to the downstream secretory pathway, with a peak in the Golgi approximately at 60 min postbiotin, in line with the idea that Fam20C is secreted via the Golgi, a canonical pathway (Fig. 2 *B* and *C*). Compared with Fam20C WT, Fam20C ΔTM displayed a faster transport rate from the ER to the Golgi, with a peak approximately at 30 min postbiotin. Moreover, the EGFP signal from Fam20C ΔTM was lost within 90 min but was maintained up to 6 h for Fam20C WT (Fig. 2 *B* and *C*), suggesting that the TM domain mediated the Golgi retention of Fam20C. To further confirm this, we fused Fam20C amino acid sequences 1 to 28 and 1 to 31 to the N terminus of mEmerald, named SP-mEmerald and TM-mEmerald, respectively (*SI Appendix*, Fig. S2*A*). SignalP-5.0 Server prediction showed that the signal peptide of SP-mEmerald has a high cleavage probability, while TM-mEmerald is less cleavable (*SI Appendix*, Fig. S2 *B* and *C*). Fluorescence imaging showed that SP-mEmerald exists throughout the secretory pathway, while TM-mEmerald displayed dominant Golgi localization and little secretion (*SI Appendix*, Fig. S2 *D* and *E*). These results further demonstrated that the TM domain of Fam20C is a Golgi retention signal. Previous studies have suggested that oligomerization is a potential mechanism for Golgi protein retention (42). We found that high salt was required for the extraction of Fam20C WT (Fig. 1*B*) but not for ΔTM (Fig. 1*E*), implying that the TM domain may contribute to the oligomerization of Fam20C in the Golgi membrane via electrostatic interactions. To validate this, we analyzed the oligomerization of Fam20C WT and ΔTM by a discontinuous sucrose gradient. Fam20C WT tended to form higher molecular weight complexes than Fam20C ΔTM, underlining an important role of the TM domain in Fam20C oligomerization (*SI Appendix*, Fig. S2*F*).

COPII vesicles are crucial for ER export of a large majority of secretory proteins. COPII vesicle formation involves recruitment of the Sec23–Sec24 complex triggered by Sar1 GTPase, which is followed by polymerization of the Sec13–Sec31 complex on the outer layer of the vesicle (43). To explore whether the transport of Fam20C from the ER to the Golgi depends on COPII, we used the dominant-negative mutant Sar1A H79G to detect its effect on the subcellular localization and secretion of Fam20C. The overexpression of Sar1A WT promoted the secretion of Fam20C without changing the Golgi localization of Fam20C (Fig. 2 *D* and *E*). However, Sar1A H79G dramatically inhibited the secretion of Fam20C and trapped Fam20C in the ER (Fig. 2 *D* and *E*). Furthermore, coimmunoprecipitation (co-IP) showed that Fam20C physically interacted with the COPII components Sec23A and Sec24A (Fig. 2*F*). We also generated RUSH-Fam20C tagged with mApple to visualize its colocalization with COPII during transport by using GFP-tagged Sec23A as a COPII marker. In the initial stage after biotin addition, Fam20C and Sec23A colocalized at ER exit sites, but their colocalization disappeared 60 min postbiotin, by which time most Fam20C had transited to the Golgi (Fig. 2*G*). Thus, the transport of Fam20C from the ER to the Golgi is mediated by COPII vesicles.

The ^{89}RILQ^{92} Sequence of Fam20C Conforms to the Consensus Recognition Motif of S1P. The secreted Fam20C begins with Asp93, and its propeptide is cleaved (Fig. 1*A*). We generated a constitutively cleaved form of Fam20C by mutating Asp28 to Ala and deleting amino acid residues 29 to 92, hereafter referred to as Fam20C ΔPP (Fig. 3*A* and *SI Appendix*, Fig. S3*A*). N-terminal sequencing confirmed that Fam20C ΔPP begins with Asp93, both in cells and in conditioned medium (Fig. 3*A*). Fam20C ΔPP secreted more efficiently than WT with a similar molecular size (Fig. 3*B*) and showed faster transport through the secretory pathway (Fig. 3 *C* and *D*), suggesting that propeptide cleavage promotes Fam20C secretion. Further evidence showing that the Fam20C propeptide is cleaved came from the redox analysis of Fam20C protein. Interestingly, while the secreted Fam20C migrated at a similar position (~70 kDa) under either reducing or nonreducing conditions, the intracellular Fam20C presented an apparent molecular weight of 150 kDa on a nonreducing gel and

BIOCHEMISTRY

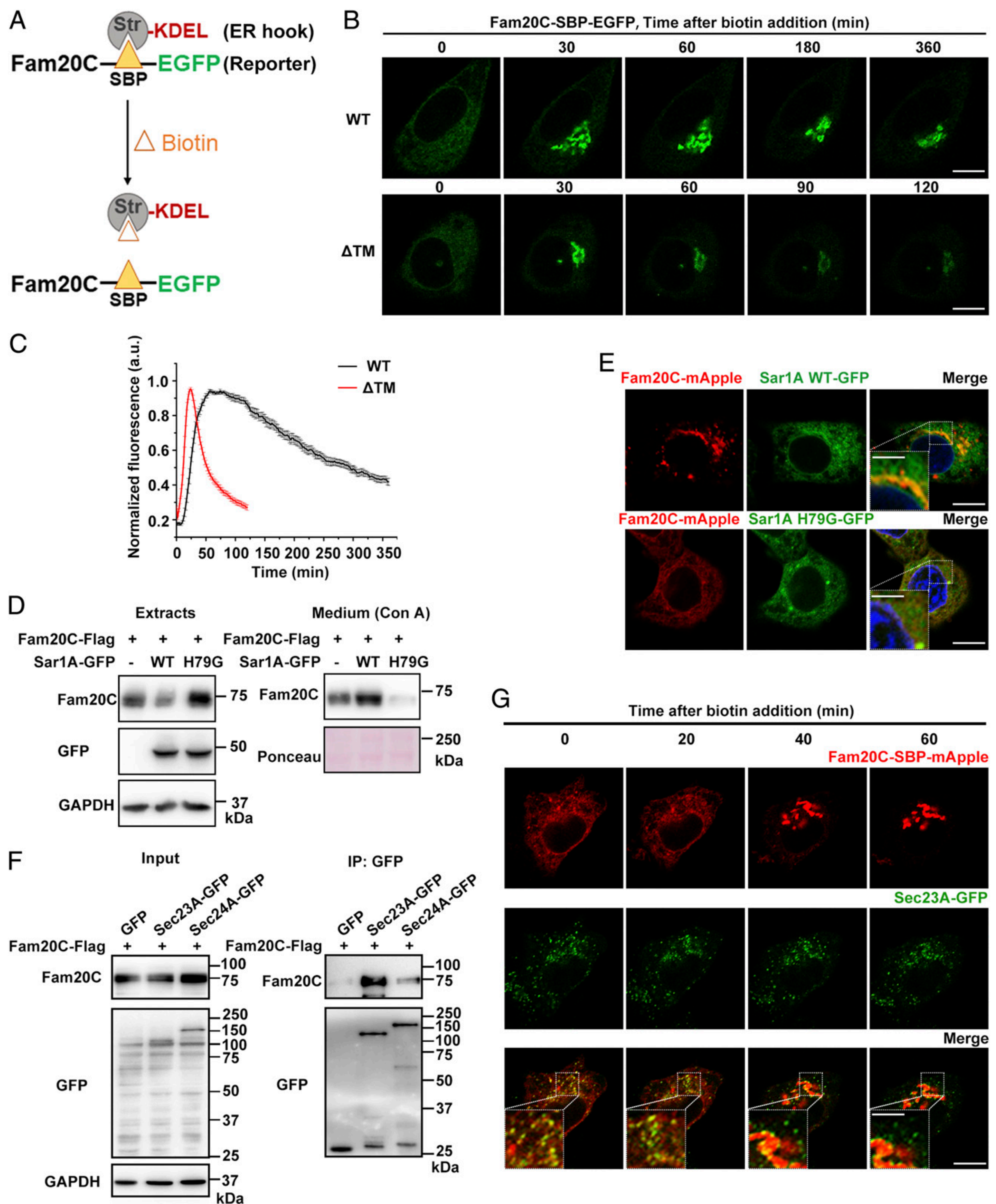

Fig. 2. The COPII vesicle mediates ER-to-Golgi anterograde transport of Fam20C. (*A*) Schematic depicts that Fam20C fused to the SBP, and EGFP (Fam20C-SBP-EGFP) is retained in the ER via its interaction with the ER hook, KDEL fused to streptavidin (Str-KDEL). This interaction is mediated by the core streptavidin and the SBP. The release is induced by the addition of biotin to allow trafficking of the Fam20C to the downstream secretory pathway. (*B*) Representative confocal live-cell imaging of *FAM20C* KO HeLa cells expressing Str-KDEL with Fam20C WT-SBP-EGFP or ΔTM-SBP-EGFP at the indicated time points after the addition of biotin. (Scale bar, 10 μm.) (*C*) The plot showing fluorescence intensity in the Golgi region at each time point in *B*, normalized to the maximum value. Data were shown as mean ± SEM of at least 20 cells. (*D*) Immunoblotting of Fam20C in cell extracts and Concanavalin A (Con A) precipitates from conditioned medium of HeLa cells coexpressing Fam20C-Flag and Sar1A WT-GFP or Sar1A H79G-GFP. (*E*) Representative confocal imaging of HeLa cells coexpressing Fam20C-mApple and Sar1A WT-GFP or Sar1A H79G-GFP. (Scale bar, 10 μm.) (*F*) Co-IP of Flag-tagged Fam20C and GFP-fused Sec23A or Sec24A in HeLa cells. (*G*) Representative confocal live cell imaging of HeLa cells coexpressing Fam20C-SBP-mApple, Str-KDEL, and Sec23A-GFP at the indicated time points after the addition of biotin. (Scale bar, 10 μm; Zoom scale bar, 4 μm.)

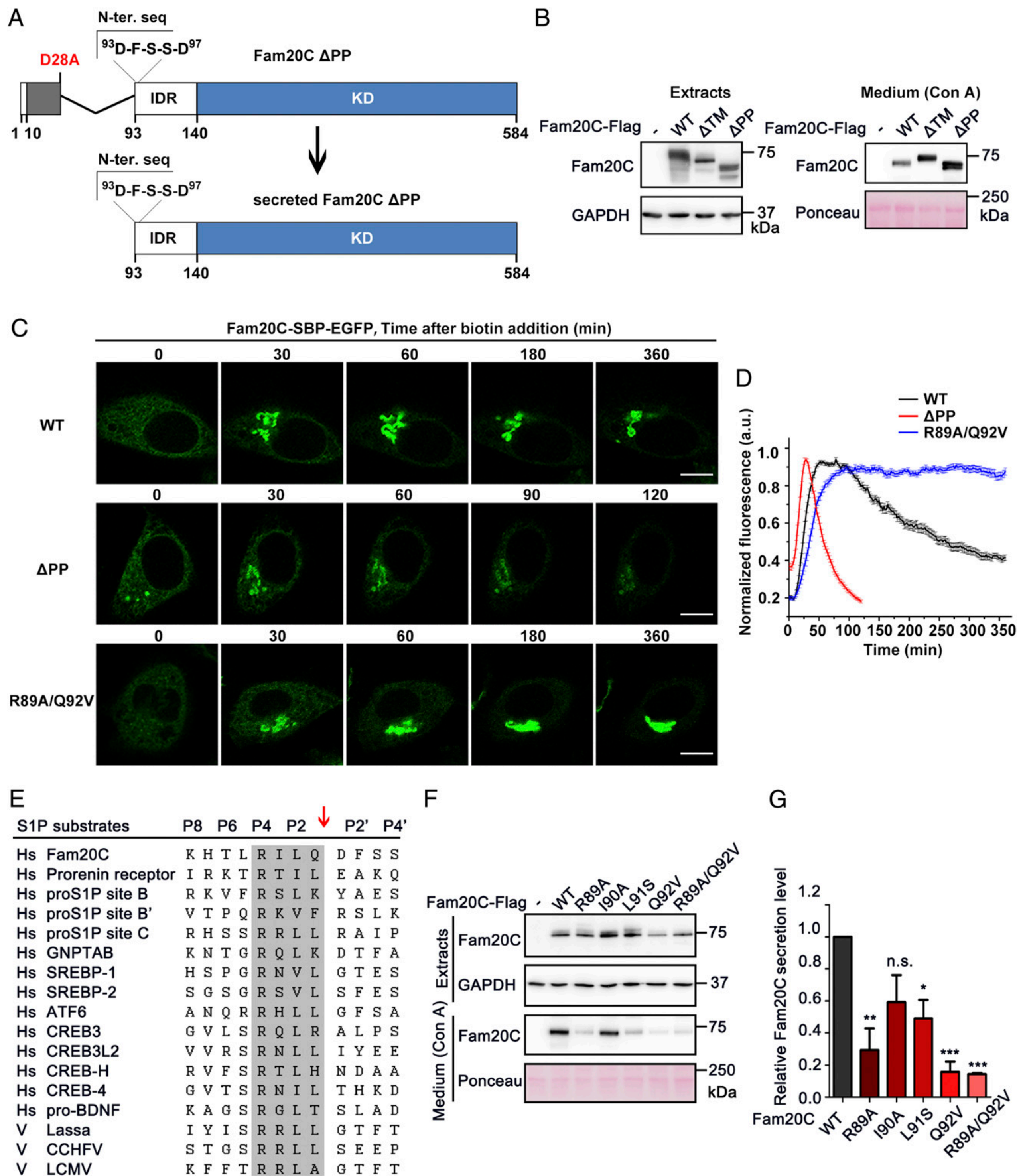

Fig. 3. Structural requirements for cleavage of the propeptide of Fam20C. (*A*) Schematic representation of Fam20C ΔPP (*Upper*) and its secreted form (*Lower*) depicting the amino acid mutation (D28A) and truncation from 29 to 92. Note that the N-terminal 28 residues in this mutant are a cleavable signal peptide (gray). Fam20C ΔPP purified from cell extracts or conditioned medium of HeLa cells was sequenced by Edman degradation. The amino acid residues surrounding the sequenced region were shown. (*B*) Immunoblotting of Fam20C in cell extracts and Concanavalin A (Con A) precipitates from conditioned medium of *FAM20C* KO HeLa cells expressing Flag-tagged Fam20C WT, ΔTM, or ΔPP. (*C*) Representative confocal live-cell imaging of *FAM20C* KO HeLa cells expressing Str-KDEL with Fam20C WT-SBP-EGFP, R89A/Q92V-SBP-EGFP, or ΔPP-SBP-EGFP at the indicated time points after the addition of biotin. (Scale bar, 10 μm.) (*D*) The plot showing fluorescence intensity in the Golgi region at each time point in *C*, normalized to the maximum value. Data were shown as mean ± SEM of at least 20 cells. (*E*) Cleavage site sequence alignment of Fam20C and known S1P substrates. The red arrow indicates the cleavage site, and the conserved cleavage motif in gray shading is critical for S1P recognition. Hs, *Homo sapiens*; V, virus. (*F*) Immunoblotting of Fam20C in cell extracts and Con A precipitates from conditioned medium of *FAM20C* KO HeLa cells expressing Flag-tagged Fam20C WT or its mutants. (*G*) Quantification of the relative Fam20C secretion level by calculating the medium/extracts ratio of Fam20C in *F*. Data were shown as mean ± SEM of three independent experiments; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; n.s., no significance (one-way ANOVA, Tukey's multiple comparison test).

75 kDa on a reducing gel (*SI Appendix*, Fig. S3*B*). These results suggested that Fam20C mainly exists as a disulfide-linked homodimer in cells and that this covalent dimer is disrupted by propeptide cleavage. Two cysteine residues, Cys46 and Cys48, are present in the Fam20C propeptide. The single mutation of either Cys46 or Cys48 to Ser had no obvious effect on disulfide-linked dimer formation. However, double mutation of Cys46 and Cys48 resulted in a complete disruption of the Fam20C covalent dimer (*SI Appendix*, Fig. S3*C*). Therefore, intracellular Fam20C exists as a *cis*-disulfide-linked homodimer via Cys46 and Cys48 (*SI Appendix*, Fig. S3*D*). Nevertheless, double mutation of Cys46 and Cys48 did not affect Fam20C secretion (*SI Appendix*, Fig. S3*E*), suggesting that these intermolecular disulfide bonds are not involved in the regulation of Fam20C secretion.

Compared with WT Fam20C, secreted Fam20C ΔTM reserved the propeptide (Fig. 1 *C* and *D*) and showed a shorter retention time in the Golgi (Fig. 2 *B* and *C*), suggesting that Golgi localization is required for the cleavage of the Fam20C propeptide. Therefore, we searched Golgi-resident proteases and found that the sequence ^{89}RILQ^{92} near the cleavage site of the Fam20C propeptide was in accordance with the consensus recognition motif of S1P. S1P recognizes and cleaves the (R/K) X(hydrophobic)Z motif, where X represents any amino acid residue, and Z preferentially represents Leu or Thr but excludes Val, Pro, Glu, Asp, and Cys (44) (Fig. 3*E*). According to the S1P recognition code, we generated Fam20C mutants in the ^{89}RILQ^{92} motif and expressed them in *FAM20C* KO HeLa cells. The R89A, L91S, Q92V, and R89A/Q92V (but not I90A) mutants exhibited drastically decreased secretion compared with Fam20C WT (Fig. 3 *F* and *G*), albeit all these mutants showed normal Golgi distribution (*SI Appendix*, Fig. S3*F*). In line with this, the RUSH assays showed that the R89A/Q92V mutant normally transited from the ER to the Golgi but was unable to leave the Golgi (Fig. 3 *C* and *D*). Altogether, the above results suggested that cleavage of the propeptide after the ^{89}RILQ^{92} motif in the Golgi is critical for the efficient secretion of Fam20C.

S1P Catalyzes Fam20C Propeptide Cleavage and Promotes Fam20C Secretion. To identify whether S1P is responsible for Fam20C propeptide cleavage, we first detected the interaction between Fam20C and S1P. Immunofluorescence analysis showed that S1P and Fam20C colocalized in the *cis*-Golgi (Fig. 4*A*), and co-IP experiments supported the physical interaction between Fam20C and S1P (Fig. 4*B* and *SI Appendix*, Fig.S4*A*). We generated S1P-encoding gene (*MBTPS1*) KO HeLa cells by CRISPR/Cas9 technology (*SI Appendix*, Fig. S4*B*). Successful KO of endogenous S1P was also supported by the observation that the ectopically expressed S1P catalytically inactive mutant S414A migrated as a single band, while S1P WT was further cleaved (Fig. 4*C*), as it has been reported to undergo autoprocessing (45). *MBTPS1* KO largely suppressed the secretion of endogenous Fam20C, which could be restored by replenishment of S1P WT but not the inactivated mutant S414A (Fig. 4*C*). Consistently, knockdown of *MBTPS1* by RNA interference also significantly reduced Fam20C secretion (*SI Appendix*, Fig. S4 *C* and *D*). In addition, compared with S1P S414A, overexpression of S1P WT further promoted the secretion of Fam20C WT but did not affect the secretion of the cleavage-resistant mutant Fam20C R89A/Q92V (Fig. 4*D*). We also checked the effect of PF-429242, a specific S1P inhibitor (46), on the secretion of Fam20C. PF-429242 clearly inhibited the secretion of Fam20C WT but not Fam20C ΔPP (Fig. 4*E*). PF-429242 did not inhibit the secretion of Fam20C ΔTM either (Fig. 4*E*), which is in line with the observation that Fam20C ΔTM quickly transits the Golgi and bypasses S1P. Taken together, these results indicated that the Golgi-resident protease S1P cleaves the Fam20C propeptide and promotes Fam20C secretion.

Removal of the Fam20C Propeptide Enhances Its Kinase Activity. To explore whether the propeptide cleavage of Fam20C regulates its kinase activity, we purified recombinant Fam20C (23 to 584) and Fam20C (141 to 578), with or without a disordered region containing the propeptide, respectively. In vitro kinase assays showed that Fam20C (141 to 578) exhibited higher activity to phosphorylate OPN than Fam20C (23 to 584), as phosphorylated OPN migrated slower on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 5*A*). Similar results were observed when using ER sulfhydryl oxidase Ero1α as a substrate, analyzed by the slowly migrating bands and by p-Ero1α blotting (*SI Appendix*, Fig. S5 *A* and *B*). These results suggested that the propeptide-containing region hinders Fam20C from being fully active. To further confirm this concept, we engineered recombinant Fam20C^{3C} with the human rhinovirus (HRV) 3C protease recognition motif inserted after the propeptide cleavage site of Fam20C (Fig. 5*B*). In vitro proteolysis assays showed that the Fam20C propeptide can be efficiently cleaved by 3C protease (Fig. 5*C*). As shown in Fig. 5*D*, Fam20C^{3C} with propeptide cleavage was able to catalyze OPN phosphorylation much faster than the uncleaved protein. Similar results were observed for Ero1α phosphorylation (*SI Appendix*, Fig. S5*C*).

At the cellular level, although Fam20C ΔTM displayed more secretion than WT, its kinase activity for the substrate Ero1α was lower than that of WT (*SI Appendix*, Fig. S5 *D* and *E*) because the propeptide was retained in Fam20C ΔTM (Fig. 1*C*). By contrast, Fam20C ΔPP displayed increased activity to phosphorylate Ero1α than Fam20C WT and ΔTM (*SI Appendix*, Fig. S5 *D* and *E*). Interestingly, secreted Fam20C ΔPP displayed higher phosphorylation levels than Fam20C WT and ΔTM (Fig. 5*E*). We found that the phosphorylation of Fam20C is strictly dependent on its kinase activity, as the inactive mutant D478A is not autophosphorylated (Fig. 5*F*). Mass spectrum analysis revealed that Ser106 in an S-x-E motif is heavily phosphorylated in Fam20C ΔPP, and mutation of Ser106 largely abolished its autophosphorylation level (Fig. 5*F*). The above data showed that removal of the propeptide enhances the kinase activity of Fam20C.

We performed a radiolabeling experiment to further confirm the physiological role of S1P in Fam20C kinase activity regulation. The S1P inhibitor PF-429242 suppressed OPN phosphorylation in a dose-dependent manner, as assessed by the incorporation of ^{32}P into V5 immunoprecipitates when the cells were radiolabeled with ^{32}P orthophosphate; moreover, the secretion of Fam20C was also inhibited (Fig. 5 *G* and *H*). Similar results were also observed when Ero1α was used as the substrate (*SI Appendix*, Fig. S5*F*). Consistently, knockdown of *MBTPS1* by RNA interference decreased the phosphorylation levels of both OPN (Fig. 5 *I* and *J*) and Ero1α (*SI Appendix*, Fig. S5*G*). In addition, compared with inactive S1P S414A, overexpression of S1P WT in *MBTPS1* KO cells promoted the phosphorylation of both OPN (Fig. 5 *K* and *L*) and Ero1α (*SI Appendix*, Fig. S5*H*). Altogether, these results indicated that S1P catalyzes the propeptide cleavage and enhances the kinase activity of Fam20C.

The S1P–Fam20C Axis Is Responsible for Efficient Osteoblast Differentiation. S1P and Fam20C are both implicated in biomineralization. To explore whether the S1P–Fam20C axis regulates biomineralization, we first investigated the role of S1P in osteoblast differentiation and mineralization. In mouse MC3T3-E1 preosteoblasts treated with the S1P inhibitor PF-429242, Fam20C secretion decreased (*SI Appendix*, Fig. S6*A*), which was similar to the results from HeLa cells (Fig. 4*E*). Moreover, PF-429242 also inhibited the phosphorylation of endogenous OPN in MC3T3-E1 cells (*SI Appendix*, Fig. S6 *A* and *B*), which is a well-characterized Fam20C substrate and one of the major extracellular components regulating biomineralization. Importantly, the S1P inhibitor largely suppressed osteoblast differentiation and mineralization (*SI Appendix*, Fig. S6 *C* and *D*). Consistently, the transcription

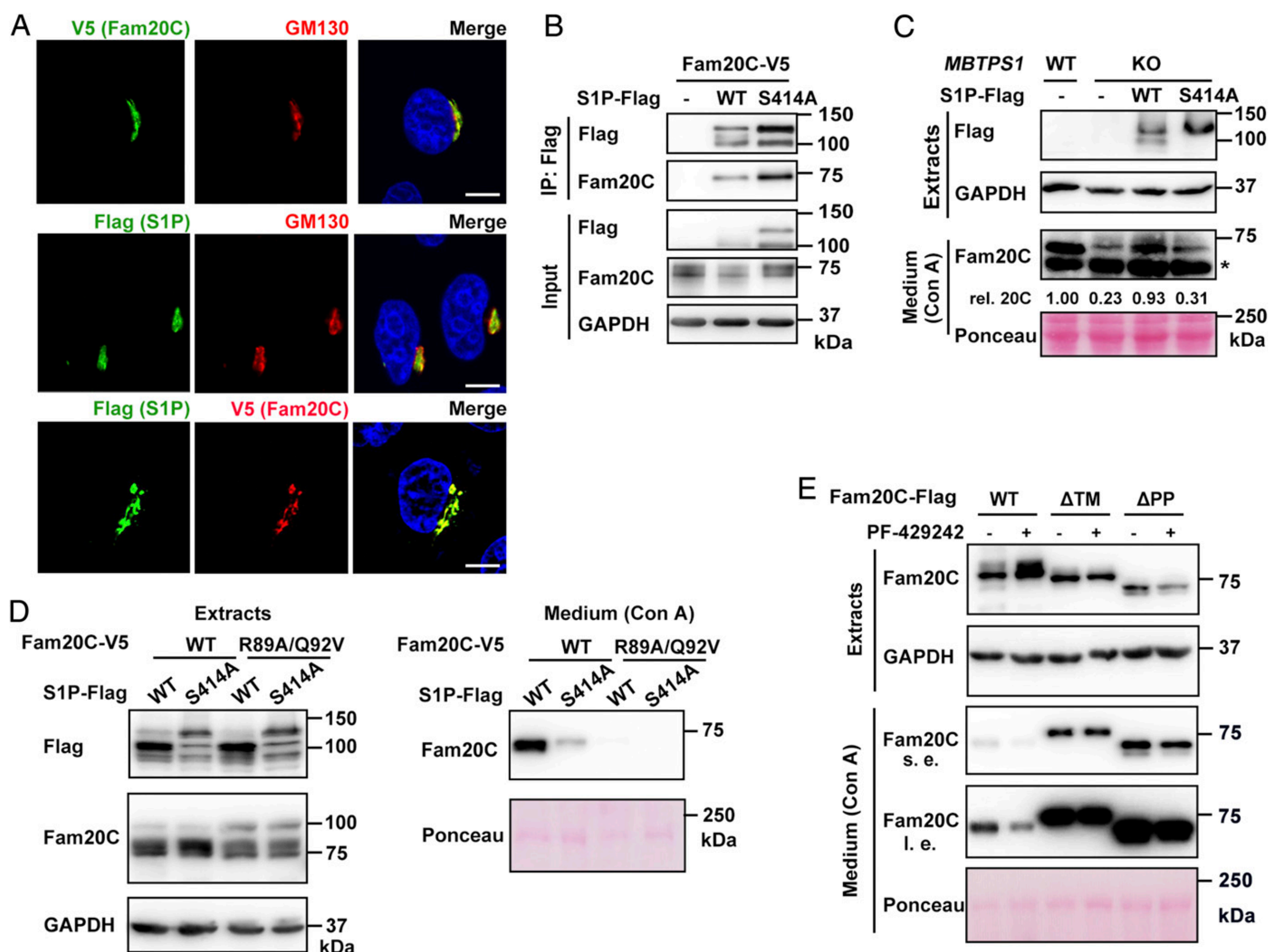

Fig. 4. The secretion of Fam20C is modulated by S1P. (*A*) Immunofluorescence analysis of HeLa cells coexpressing Flag-tagged S1P and V5-tagged Fam20C. GM130 was used as a *cis*-Golgi marker. (Scale bars, 10 μm.) (*B*) Co-IP of V5-tagged Fam20C and Flag-tagged S1P WT or its catalytic inactive mutant S414A in HeLa cells. (*C*) Immunoblotting of endogenous Fam20C in Concanavalin A (Con A) precipitates from conditioned medium of WT HeLa cells or *MBTPS1* KO cells expressing Flag-tagged S1P WT or its catalytic inactive mutant S414A. Asterisk indicated a nonspecific protein band. The relative amounts of secreted Fam20C (rel. 20C) were quantified. (*D*) Immunoblotting of Fam20C in cell extracts and Con A precipitates from conditioned medium of *FAM20C* KO HeLa cells coexpressing Flag-tagged S1P WT or S414A and V5-tagged Fam20C WT or its cleavage-resistant mutant R89A/Q92V. Please note that S1P S414A was processed by endogenous S1P and showed a triplet band, and the cleavage efficiency of S1P WT was much higher than S414A. (*E*) Immunoblotting of Fam20C in cell extracts and Con A precipitates from conditioned medium of *FAM20C* KO HeLa cells expressing Flag-tagged Fam20C WT, ΔTM, or ΔPP treated with or without 100 μM PF-429242, an S1P inhibitor for 18 h. s.e., short exposure; l.e., long exposure.

levels of genes associated with osteogenic differentiation, such as *Dmp1*, *Mepe*, *Bsp*, and *Opn*, were all dramatically down-regulated by PF-429242 treatment (*SI Appendix*, Fig. S6*E*). These results indicated that S1P is crucial for osteoblast differentiation and mineralization.

Next, we investigated whether S1P-catalyzed cleavage of the Fam20C propeptide is involved in biomineralization. We generated MC3T3-E1 stable cell lines expressing different Fam20C mutants by lentivirus infection (Fig. 6*A*). Compared with mock cells, the overexpression of Fam20C WT promoted osteoblast differentiation and mineralized nodule formation (Fig. 6 *B* and *C*). By contrast, the S1P cleavage-resistant mutant Fam20C R89A/Q92V was unable to promote osteoblast differentiation (Fig. 6 *B* and *C*) and showed little secretion (Fig. 6*A*). Notably, the MC3T3-E1 cells expressing the constitutively active mutant Fam20C ΔPP displayed enhanced differentiation and mineralization compared with Fam20C WT cells (Fig. 6 *B* and *C*), consistent with its increased secretion (Fig. 6*A*). As a negative control, the catalytically inactive Fam20C ΔPP D478A had little effect on osteoblast differentiation and mineralization (Fig. 6 *B* and *C*), though its secretion was as efficient as Fam20C ΔPP (Fig. 6*A*). We also detected the transcription of osteogenic differentiation markers in these transduced cells. The overexpression of Fam20C WT and ΔPP led to a similar up-regulation of the messenger RNA (mRNA) levels of *Dmp1* and *Mepe*, though it had less effect on the transcription of *Bsp* and *Opn*. The uncleaved R89A/Q92V mutant had little effect on any of the four osteogenic differentiation markers (Fig. 6*D*). Thus, these results indicated that propeptide cleavage is indispensable for Fam20C to regulate osteoblast differentiation and mineralization.

Discussion

Fam20C was identified as the Golgi casein kinase (G-CK) in 2012 (4, 5), but the molecular mechanism by which Fam20C is retained in the Golgi apparatus and subsequently secreted is not yet understood. In this study, we demonstrated that Fam20C is a type II transmembrane protein, and its N-terminal signal peptide-like region is not cleaved after targeting the nascent polypeptide

BIOCHEMISTRY

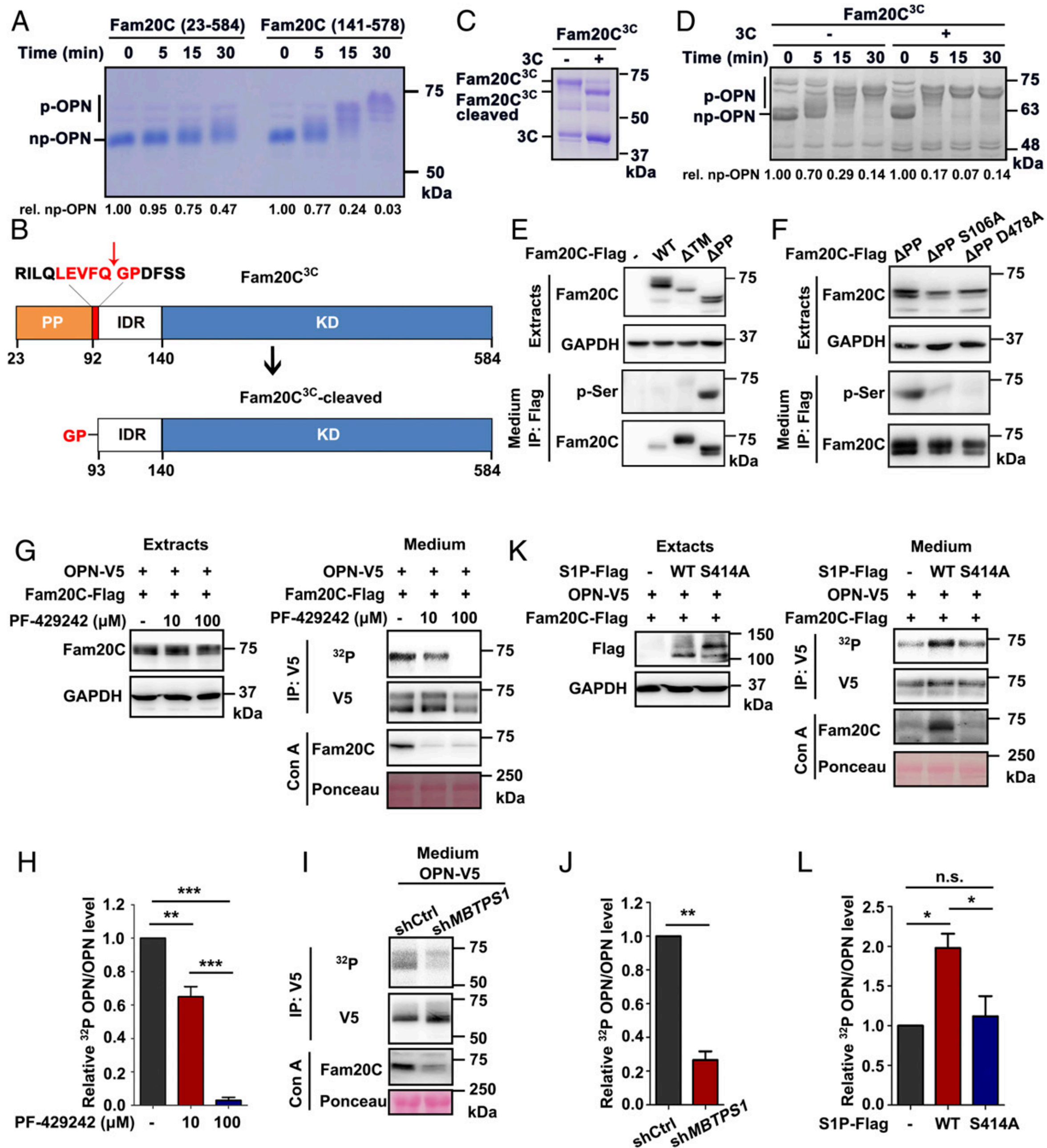

Fig. 5. S1P-dependent propeptide cleavage enhances the kinase activity of Fam20C. (*A*) Phosphorylation of OPN catalyzed by recombinant Fam20C (23 to 584) or Fam20C (141 to 578) protein was measured by an in vitro kinase assay. Reaction products were separated by SDS-PAGE and visualized by Coomassie blue staining. The relative amounts of nonphosphorylated OPN (rel. np-OPN) in different time points were quantified. (*B*) Schematic representation of Fam20C inserted with HRV 3C protease cleavage site (Fam20C^{3C}) and its cleaved form. The inserted motif was colored in red, and the 3C protease cleavage site was indicated by a red arrow. (*C*) In vitro proteolytic processing of Fam20C^{3C} protein by 3C protease. Reaction products were analyzed by Coomassie blue staining. (*D*) Phosphorylation of OPN catalyzed by recombinant Fam20C^{3C} or Fam20C^{3C} preincubated with 3C protease was measured by an in vitro kinase assay. Reaction products were separated by SDS-PAGE and visualized by Coomassie blue staining. The relative amounts of nonphosphorylated OPN (rel. np-OPN) in different time points were quantified. (*E*) Detection of phosphoserine (p-Ser) of Fam20C in Flag immunoprecipitates from conditioned medium of *FAM20C* KO HeLa cells expressing Flag-tagged Fam20C WT, ΔTM, and ΔPP. (*F*) Detection of p-Ser of Fam20C in Flag immunoprecipitates from conditioned medium of *FAM20C* KO HeLa cells expressing Flag-tagged Fam20C ΔPP, ΔPP S106A and ΔPP D478A. (*G*) Immunoblotting and autoradiography of V5 immunoprecipitates from conditioned medium of HeLa cells coexpressing Flag-tagged Fam20C and V5-tagged OPN treated with different concentrations of PF-429242 and radiolabeled with ^{32}P-orthophosphate. (*H*) Quantification of relative ^{32}P OPN/OPN ratio in *G*. Data were shown as mean ± SEM of three independent experiments; $^{**}P < 0.01$, $^{***}P < 0.001$ (one-way ANOVA, Tukey's multiple comparison test). (*I*) Immunoblotting and autoradiography of V5 immunoprecipitates from conditioned medium of HeLa cells transfected with control short hairpin RNA (shCtrl) or sh*MBTPS1* and V5-tagged OPN radiolabeled with ^{32}P-orthophosphate. (*J*) Quantification of relative ^{32}P OPN/OPN ratio in *I*. Data were shown as mean ± SEM of three independent experiments, $^{**}P < 0.01$ (two-tailed Student's *t* test). (*K*) Immunoblotting and autoradiography of V5 immunoprecipitates from conditioned medium of *MBTPS1* KO HeLa cells coexpressing V5-tagged OPN, Flag-tagged S1P WT, or S414A and Fam20C radiolabeled with ^{32}P-orthophosphate. (*L*) Quantification of relative ^{32}P OPN/OPN ratio in *K*. Data were shown as mean ± SEM of three independent experiments, $^{*}P < 0.05$; n.s., no significance (one-way ANOVA, Tukey's multiple comparison test).

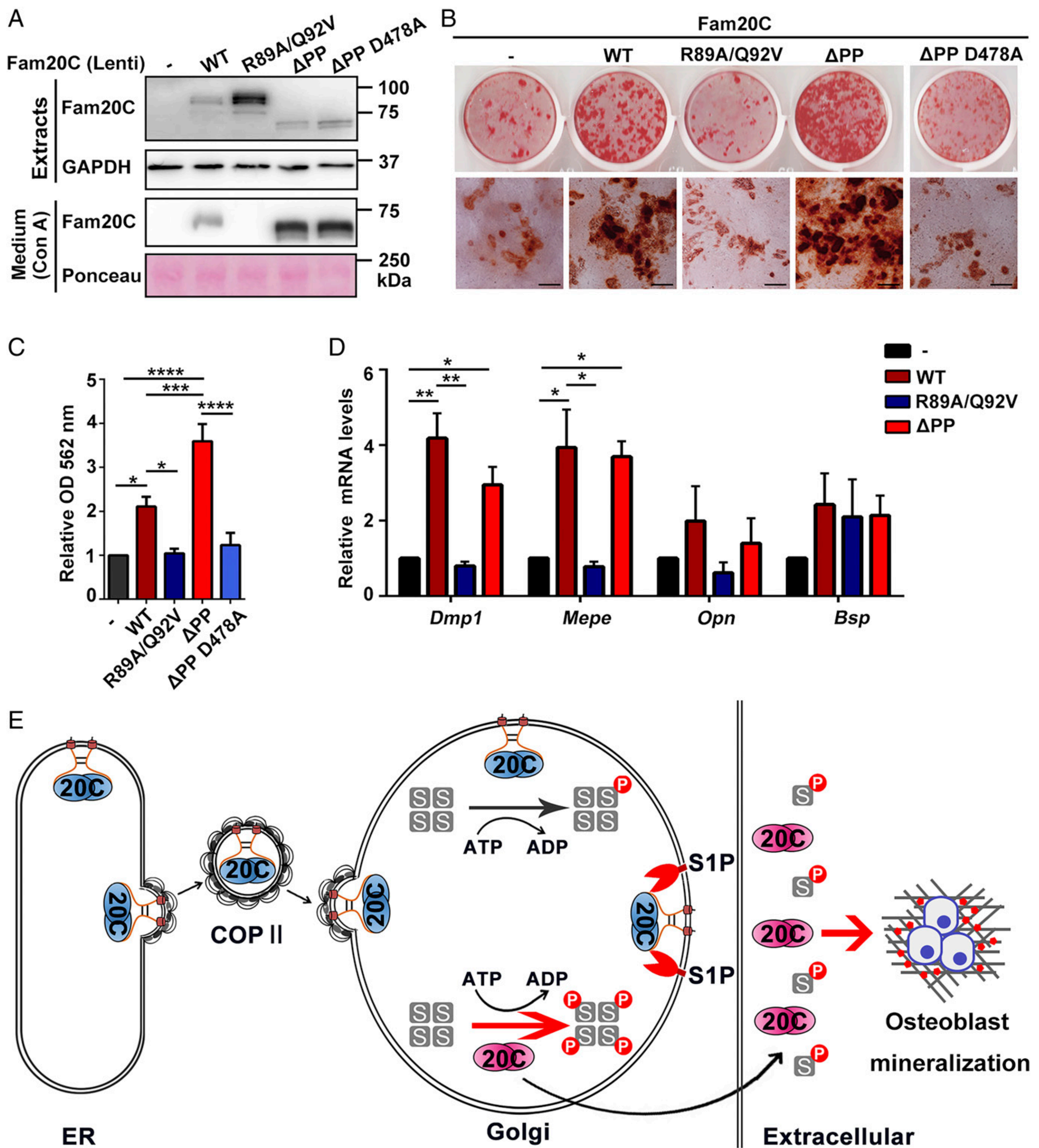

Fig. 6. Cleavage of Fam20C propeptide is indispensable for osteoblast differentiation. (*A*) Immunoblotting of Fam20C in cell extracts and Concanavalin A (Con A) precipitates from conditioned medium of MC3T3-E1 cells stably transfected with Flag-tagged Fam20C WT, R89A/Q92V, ΔPP, or ΔPP D478A. (*B*) Plate view (*Upper*) and microscopic view (*Lower*) of Alizarin Red S staining of MC3T3-E1 cells transfected with Flag-tagged Fam20C WT, R89A/Q92V, ΔPP, or ΔPP D478A after osteogenic induction. (Scale bars, 400 μm.) (*C*) Quantification of Alizarin Red S Staining in *B*. Data were shown as mean ± SEM of at least four independent experiments; $*P < 0.05$, $***P < 0.001$, $****P < 0.0001$ (one-way ANOVA, Tukey's multiple comparison test). (*D*) Detection of *Dmp1*, *Mepe*, *Opn*, and *Bsp* mRNA levels by real-time qPCR in MC3T3-E1 cells transfected with Flag-tagged Fam20C WT, R89A/Q92V, or ΔPP after osteogenic induction. Data were shown as mean ± SEM of three independent experiments; $*P < 0.05$, $**P < 0.01$ (one-way ANOVA, Tukey's multiple comparison test). (*E*) Proposed model for the proteolytic regulation of Fam20C. Fam20C is a type II transmembrane protein with an N-terminal TM region. In the lumen of the secretory pathway, Fam20C exists as a *cis*-disulfide-linked homodimer and may form higher molecular weight oligomers mediated by the transmembrane region. The ER to Golgi anterograde transport of Fam20C is mediated by COPII vesicle, and the TM region acts as a retention motif to enrich Fam20C in the Golgi. S1P, a Golgi-resident proprotein convertase, cleaves the propeptide of Fam20C, therefore increasing both the secretion and kinase activity of Fam20C. Importantly, the cleavage of Fam20C propeptide is necessary for efficient osteoblast differentiation and mineralization. 20C, Fam20C; S, substrate protein; P, phosphate group.

chain into the ER lumen, rather, it serves as a Golgi-resident membrane anchor (Fig. 6*E*). Furthermore, we reveal that the Golgi-resident protease S1P catalyzes the propeptide cleavage of Fam20C, and the processed kinase can then be secreted and activated. The link between Fam20C and S1P has been established to function in osteoblast differentiation and mineralization (Fig. 6*E*).

The membrane-bound property of G-CK was initially detected in the Golgi-enriched membrane fraction isolated from lactating guinea pig mammary gland (47), although the molecular identity of G-CK was unknown. The mechanisms for Golgi protein retention are complex and varied. TM domain-mediated retention is one of the major determinants of protein localization to the Golgi apparatus. The membrane thickness model states that TM domains of Golgi proteins are typically shorter than those of plasma membrane proteins to prevent their entry into the sterol-enriched and thicker bilayers of Golgi-derived transport vesicles destined to the cell surface (42). In vertebrates, the mean lengths of the TM domains of Golgi and plasma membrane proteins are 20.6 and 24.4 amino acids, respectively (48). The TM domain of Fam20C consists of 20 amino acids (Fig. 1*A*), consistent with typical Golgi membrane proteins. Protein aggregation/oligomerization is another general mechanism for TM domain-mediated Golgi retention by enlarging the protein size and excluding it from transport vesicles (42). Consistent with this model, our results show that the Fam20C TM domain mediates its oligomerization (*SI Appendix*, Fig. S2*F*), which is further stabilized by Cys46-Cys46 and Cys48-Cys48 interchain disulfides (*SI Appendix*, Fig. S3*D*) and noncovalent interactions between the kinase domains (24). Our findings, that Fam20C is a type II transmembrane protein, support and enrich the view that the majority of Golgi enzymes such as glycosyltransferases and glycosidases are type II transmembrane proteins (49). We analyzed the membrane-bound properties of all known kinases in the secretory pathway by bioinformatics approaches. Many secretory pathway kinases show no or low cleavage probability by signal peptidase and are predicted to contain an N-terminal TM domain, including Fam20A, Fam20B, Fam20C, Fam198B, Fam69A, Fam69B, Fam69C, vertebrate lonesome kinase (VLK), and Sgk196 (*SI Appendix*, Table S1). This information implies that membrane spanning might be a general property for secretory pathway kinases, but this needs to be verified in future studies. Moreover, it will be interesting to investigate the relationship between the oligomeric regulation (22, 24) and proteolytic regulation of Fam20C.

Proteolysis is a general mechanism for the maturation and activation of many proproteins. However, proteolysis-induced activation of secretory pathway protein kinases has been less reported. We identified S1P, a Golgi-resident protease, as responsible for Fam20C propeptide cleavage. Our results show that the propeptide cleavage of Fam20C is crucial for its secretion and maximum kinase activity. It has been reported that mutation of ^{91}LQD^{93} to ^{91}AAA^{93} near the cleavage site does not affect Fam20C secretion (8). This result can be explained because the mutated ^{89}RIAA^{92} sequence still conforms to the recognition motif of S1P. Instead, we generated cleavage-resistant Fam20C mutants with changes in the ^{89}RILQ^{92} motif, which does not adhere to the S1P recognition rule. The R89A, L91S, Q92V, and R89A/Q92V mutants exhibited significantly decreased secretion compared with Fam20C WT (Fig. 3 *F* and *G*). In line with the observation that S1P prefers to cleave membrane proteins (30, 36, 50), the propeptide of Fam20C ΔTM was not cleaved by S1P (Fig. 1 *C* and *D*). One possibility for this is that Golgi retention by the TM domain provides a sufficiently long duration for efficient cleavage of Fam20C by S1P. Another possibility could be that Fam20C undergoes conformational changes at the Golgi membrane that are essential for S1P recognition and cleavage. In cultured cells, Fam20C-catalyzed protein phosphorylation was believed to occur inside the cells (15) and mostly in the Golgi lumen and trans-Golgi network–derived vesicles (13). However, Fam20C can also be partially retained in the ER and phosphorylates ER-localized proteins under ER stress (16). Since S1P-catalyzed propeptide cleavage of Fam20C enhances its kinase activity, we believe that immature full-length Fam20C with basal activity can phosphorylate specific substrates under certain conditions and that fully active mature Fam20C phosphorylates substrates more efficiently in the late secretory pathway after S1P cleavage.

Loss-of-function *FAM20C* mutations are associated with Raine syndrome, a human skeletal disorder. Notably, some Raine syndrome mutants of Fam20C display mislocalization in the ER and secretion deficiency (4, 5). In mice, *Fam20c* deletion results in hypophosphatemic rickets (51, 52), as well as severe enamel (53) and dentin defects (54, 55). The overexpression of Fam20C accelerates the odontoblast mineralization process (56), and transgenic expression of Fam20C in osteoblasts increases cortical bone formation and osteoclastic bone resorption in mice (57). Our data clearly demonstrated that only the mature active form of Fam20C can execute its function to promote osteoblast differentiation and mineralization (Fig. 6 *B* and *C*). Moreover, the inhibition of Fam20C cleavage decreased the phosphorylation of endogenous OPN (*SI Appendix*, Fig. S6 *A* and *B*), one of the major extracellular components regulating biomineralization (4), which partially explains how the Fam20C processing defect leads to the failure of osteoblast differentiation and mineralization. Thus, it is likely that mature fully active Fam20C generated by S1P cleavage efficiently phosphorylates extracellular matrix proteins (such as SIBLINGs, including OPN) in the late secretory pathway to promote biomineralization. Previous studies showed that the addition of recombinant Fam20C protein to culture medium promotes MC3T3-E1 osteoblast differentiation (51). In addition, Fam20A-mediated Fam20C secretion into the extracellular space seemingly regulates Fam20C function in biomineralization (58). Thus, it is also possible that as a calcium-binding protein (56), autophosphorylated Fam20C may function to promote the mineral deposition process itself. Interestingly, in *C. elegans*, Fam20C must also transit into the late secretory pathway for worm fertility and embryonic development (29), further underlining that proper transport of Fam20C in the secretory pathway is important for its physiological function.

Previous studies have shown that S1P is essential for mammalian skeletal development, possibly because S1P cleaves TM transcription factor precursors that are required for the expression and secretion of bone matrix proteins (37, 39, 59). Our results reveal that S1P mediates the maturation of the secretory pathway kinase Fam20C, which can directly phosphorylate SIBLINGs that are crucial for biomineralization, providing mechanistic insight into S1P-regulated bone development at the posttranslational level. It should be noted that S1P is a pleiotropic protease in the secretory pathway and participates in the processing of many substrates, therefore we cannot exclude the possibility that PF-429242 inhibits osteoblast differentiation through other unidentified mechanisms. S1P is also known as a key regulator of lipid metabolism and cholesterol homeostasis by converting the SREBP transcription factors, which regulate cholesterol and fatty acid synthesis and uptake. Interestingly, Fam20C has also been reported to be involved in lipid transport (8) and uptake (12). Thus, it would be interesting to investigate whether the cellular cholesterol content and membrane lipid composition can regulate the transport and processing of Fam20C, which shall shed new light on the crosstalk between lipid metabolism and biomineralization.

Materials and Methods

Protein Expression and Purification. Recombinant full-length human Ero1α and OPN proteins were expressed in *Escherichia coli* BL21 (DE3) cells and purified as before (4, 60). Fam20C^{3C} was generated by inserting a HRV 3C protease cleavage site (LEVLFQGP) between Q92 and D93. For expression in insect cells, Fam20C protein (141 to 578), Fam20C (23 to 584), and Fam20C^{3C} were cloned into the psMBP2 vector and expressed as His6-MBP fusion

proteins (61). More details on protein purification are presented in *SI Appendix, Materials and Methods*.

Cell Culture and Transfection. HeLa and HEK293T cells were cultured in Dulbecco's modified Eagle's medium (Gibco; C11995500BT) respectively supplemented with 5% and 10% fetal bovine serum (FBS) (Gibco; 10091-148). Mouse preosteoblast MC3T3-E1 subclone 4 (ATCC; CRL-2593) were cultured in Minimum Essential Medium-α (Gibco; 12571-063) supplemented with 10% FBS. All media were supplemented with 100 units/mL penicillin and 100 μg/mL streptomycin (Invitrogen; 15140-122), and the cells were cultured at 37 °C with 5% CO_2. More details on cell culture and transfection are presented in *SI Appendix, Materials and Methods*.

Detailed descriptions of all materials and methods are available in *SI Appendix, Materials and Methods*.

Data Availability. All study data are included in the article and/or *SI Appendix*.

ACKNOWLEDGMENTS. We thank Hong Zhang, Guangyan Miao, Junjie Hu, Yanfang Wu, and Likun Wang (Institute of Biophysics, CAS) for providing materials and helpful suggestions and Hongjie Zhang (Institute of Biophysics, CAS) for technical assistance. This work was supported by the Strategic Priority Research Program of CAS (XDB37020303), the National Natural Science Foundation of China (32022033, 31771261), the National Key R&D Program of China (2017YFA0504000, 2016YFA0500200), and the Youth Innovation Promotion Association, CAS to L.W.

1. G. Yalak, V. Vogel, Extracellular phosphorylation and phosphorylated proteins: Not just curiosities but physiologically important. *Sci. Signal.* **5**, re7 (2012).
2. E. Klement, K. F. Medzihradszky, Extracellular protein phosphorylation, the neglected side of the modification. *Mol. Cell. Proteomics* **16**, 1–7 (2017).
3. H. Harada *et al*., Extracellular phosphorylation drives the formation of neuronal circuitry. *Nat. Chem. Biol.* **15**, 1035–1042 (2019).
4. V. S. Tagliabracci *et al*., Secreted kinase phosphorylates extracellular proteins that regulate biomineralization. *Science* **336**, 1150–1153 (2012).
5. H. O. Ishikawa, A. Xu, E. Ogura, G. Manning, K. D. Irvine, The Raine syndrome protein FAM20C is a Golgi kinase that phosphorylates bio-mineralization proteins. *PLoS One* **7**, e42988 (2012).
6. T. Koike, T. Izumikawa, J. Tamura, H. Kitagawa, FAM20B is a kinase that phosphorylates xylose in the glycosaminoglycan-protein linkage region. *Biochem. J.* **421**, 157–162 (2009).
7. M. A. Simpson *et al*., Mutations in FAM20C are associated with lethal osteosclerotic bone dysplasia (Raine syndrome), highlighting a crucial molecule in bone development. *Am. J. Hum. Genet.* **81**, 906–912 (2007).
8. V. S. Tagliabracci *et al*., A single kinase generates the majority of the secreted phosphoproteome. *Cell* **161**, 1619–1632 (2015).
9. V. S. Tagliabracci *et al*., Dynamic regulation of FGF23 by Fam20C phosphorylation, GalNAc-T3 glycosylation, and furin proteolysis. *Proc. Natl. Acad. Sci. U.S.A.* **111**, 5520–5525 (2014).
10. B. Ramos-Molina, I. Lindberg, Phosphorylation and alternative splicing of 7B2 reduce prohormone convertase 2 activation. *Mol. Endocrinol.* **29**, 756–764 (2015).
11. I. Lindberg *et al*., FGF23 is endogenously phosphorylated in bone cells. *J. Bone Miner. Res.* **30**, 449–454 (2015).
12. A. Ben Djoudi Ouadda *et al*., Ser-phosphorylation of PCSK9 (proprotein convertase subtilisin-kexin 9) by Fam20C (family with sequence similarity 20, member C) kinase enhances its ability to degrade the LDLR (low-density lipoprotein receptor). *Arterioscler. Thromb. Vasc. Biol.* **39**, 1996–2013 (2019).
13. T. K. Hecht *et al*., Fam20C regulates protein secretion by Cab45 phosphorylation. *J. Cell Biol.* **219**, e201910089 (2020).
14. A. J. Pollak *et al*., Phosphorylation of serine96 of histidine-rich calcium-binding protein by the Fam20C kinase functions to prevent cardiac arrhythmia. *Proc. Natl. Acad. Sci. U.S.A.* **114**, 9098–9103 (2017).
15. J. Zhang *et al*., Secretory kinase Fam20C tunes endoplasmic reticulum redox state via phosphorylation of Ero1α. *EMBO J.* **37**, e98699 (2018).
16. J. Yu *et al*., Phosphorylation switches protein disulfide isomerase activity to maintain proteostasis and attenuate ER stress. *EMBO J.* **39**, e103841 (2020).
17. A. J. Pollak *et al*., A secretory pathway kinase regulates sarcoplasmic reticulum Ca^{2+} homeostasis and protects against heart failure. *eLife* **7**, e41378 (2018).
18. J. A. Endicott, M. E. Noble, L. N. Johnson, The structural basis for control of eukaryotic protein kinases. *Annu. Rev. Biochem.* **81**, 587–613 (2012).
19. R. Bayliss, T. Haq, S. Yeoh, The Ys and wherefores of protein kinase autoinhibition. *Biochim. Biophys. Acta* **1854** (10 Pt B), 1586–1594 (2015).
20. J. Beenstock, N. Mooshayef, D. Engelberg, How do protein kinases take a selfie (autophosphorylate)? *Trends Biochem. Sci.* **41**, 938–953 (2016).
21. J. Xiao, V. S. Tagliabracci, J. Wen, S. A. Kim, J. E. Dixon, Crystal structure of the Golgi casein kinase. *Proc. Natl. Acad. Sci. U.S.A.* **110**, 10574–10579 (2013).
22. J. Cui *et al*., A secretory kinase complex regulates extracellular protein phosphorylation. *eLife* **4**, e06120 (2015).
23. J. Cui *et al*., Structure of Fam20A reveals a pseudokinase featuring a unique disulfide pattern and inverted ATP-binding. *eLife* **6**, e23990 (2017).
24. H. Zhang *et al*., Structure and evolution of the Fam20 kinases. *Nat. Commun.* **9**, 1218 (2018).
25. P. Vogel *et al*., Amelogenesis imperfecta and other biomineralization defects in Fam20a and Fam20c null mice. *Vet. Pathol.* **49**, 998–1017 (2012).
26. J. O'Sullivan *et al*., Whole-Exome sequencing identifies FAM20A mutations as a cause of amelogenesis imperfecta and gingival hyperplasia syndrome. *Am. J. Hum. Genet.* **88**, 616–620 (2011).
27. G. Cozza *et al*., A new role for sphingosine: Up-regulation of Fam20C, the genuine casein kinase that phosphorylates secreted proteins. *Biochim. Biophys. Acta* **1854** (10 Pt B), 1718–1726 (2015).
28. G. Cozza, M. Salvi, V. S. Tagliabracci, L. A. Pinna, Fam20C is under the control of sphingolipid signaling in human cell lines. *FEBS J.* **284**, 1246–1257 (2017).
29. A. Gerson-Gurwitz *et al*., Ancestral roles of the Fam20C family of secreted protein kinases revealed in *C. elegans*. *J. Cell Biol.* **218**, 3795–3811 (2019).
30. N. G. Seidah, A. Prat, The biology and therapeutic targeting of the proprotein convertases. *Nat. Rev. Drug Discov.* **11**, 367–383 (2012).
31. J. Sakai *et al*., Molecular identification of the sterol-regulated luminal protease that cleaves SREBPs and controls lipid composition of animal cells. *Mol. Cell* **2**, 505–514 (1998).
32. M. S. Brown, J. L. Goldstein, A proteolytic pathway that controls the cholesterol content of membranes, cells, and blood. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 11041–11048 (1999).
33. J. Ye *et al*., ER stress induces cleavage of membrane-bound ATF6 by the same proteases that process SREBPs. *Mol. Cell* **6**, 1355–1364 (2000).
34. M. Llarena, D. Bailey, H. Curtis, P. O'Hare, Different mechanisms of recognition and ER retention by transmembrane transcription factors CREB-H and ATF6. *Traffic* **11**, 48–69 (2010).
35. K. Marschner, K. Kollmann, M. Schweizer, T. Braulke, S. Pohl, A key enzyme in the biogenesis of lysosomes is a protease that regulates cholesterol metabolism. *Science* **333**, 87–90 (2011).
36. D. J. Burri, J. R. da Palma, S. Kunz, A. Pasquato, Envelope glycoprotein of arenaviruses. *Viruses* **4**, 2162–2181 (2012).
37. Y. Kondo *et al*., Site-1 protease deficiency causes human skeletal dysplasia due to defective inter-organelle protein trafficking. *JCI Insight* **3**, e121596 (2018).
38. J. P. Gorski *et al*., Potential role of proprotein convertase SKI-1 in the mineralization of primary bone. *Cells Tissues Organs* **189**, 25–32 (2009).
39. J. P. Gorski *et al*., Inhibition of proprotein convertase SKI-1 blocks transcription of key extracellular matrix genes regulating osteoblastic mineralization. *J. Biol. Chem.* **286**, 1836–1849 (2011).
40. D. Patra *et al*., Site-1 protease is essential for endochondral bone formation in mice. *J. Cell Biol.* **179**, 687–700 (2007).
41. D. Patra, E. DeLassus, J. Mueller, G. Abou-Ezzi, L. J. Sandell, Site-1 protease regulates skeletal stem cell population and osteogenic differentiation in mice. *Biol. Open* **7**, bio032094 (2018).
42. D. K. Banfield, Mechanisms of protein retention in the Golgi. *Cold Spring Harb. Perspect. Biol.* **3**, a005264 (2011).
43. F. Brandizzi, C. Barlowe, Organization of the ER-Golgi interface for membrane traffic control. *Nat. Rev. Mol. Cell Biol.* **14**, 382–392 (2013).
44. A. Elagoz, S. Benjannet, A. Mammarbassi, L. Wickham, N. G. Seidah, Biosynthesis and cellular trafficking of the convertase SKI-1/S1P: Ectodomain shedding requires SKI-1 activity. *J. Biol. Chem.* **277**, 11265–11275 (2002).
45. J. R. da Palma, L. Cendron, N. G. Seidah, A. Pasquato, S. Kunz, Mechanism of folding and activation of subtilisin kexin isozyme-1 (SKI-1)/site-1 protease (S1P). *J. Biol. Chem.* **291**, 2055–2066 (2016).
46. B. A. Hay *et al*., Aminopyrrolidineamide inhibitors of site-1 protease. *Bioorg. Med. Chem. Lett.* **17**, 4411–4414 (2007).
47. J. C. Pascall, A. P. Boulton, R. K. Craig, Characterisation of a membrane-bound serine-specific casein kinase isolated from lactating guinea-pig mammary gland. *Eur. J. Biochem.* **119**, 91–99 (1981).
48. H. J. Sharpe, T. J. Stevens, S. Munro, A comprehensive comparison of transmembrane domains reveals organelle-specific properties. *Cell* **142**, 158–169 (2010).
49. L. G. Welch, S. Munro, A tale of short tails, through thick and thin: Investigating the sorting mechanisms of Golgi enzymes. *FEBS Lett.* **593**, 2452–2465 (2019).
50. T. Nakagawa *et al*., Site-1 protease is required for the generation of soluble (pro) renin receptor. *J. Biochem.* **161**, 369–379 (2017).
51. X. Wang *et al*., Inactivation of a novel FGF23 regulator, FAM20C, leads to hypophosphatemic rickets in mice. *PLoS Genet.* **8**, e1002708 (2012).
52. P. Liu *et al*., Specific ablation of mouse Fam20C in cells expressing type I collagen leads to skeletal defects and hypophosphatemia. *Sci. Rep.* **7**, 3590 (2017).
53. X. Wang *et al*., The specific role of FAM20C in amelogenesis. *J. Dent. Res.* **92**, 995–999 (2013).
54. X. Wang *et al*., The specific role of FAM20C in dentinogenesis. *J. Dent. Res.* **94**, 330–336 (2015).
55. X. Wang *et al*., FAM20C plays an essential role in the formation of murine teeth. *J. Biol. Chem.* **287**, 35934–35942 (2012).
56. J. Hao, K. Narayanan, T. Muni, A. Ramachandran, A. George, Dentin matrix protein 4, a novel secretory calcium-binding protein that modulates odontoblast differentiation. *J. Biol. Chem.* **282**, 15357–15365 (2007).
57. K. Hirose *et al*., Overexpression of Fam20C in osteoblast in vivo leads to increased cortical bone formation and osteoclastic bone resorption. *Bone* **138**, 115414 (2020).
58. Y. Ohyama *et al*., FAM20A binds to and regulates FAM20C localization. *Sci. Rep.* **6**, 27784 (2016).
59. T. Murakami *et al*., Signalling mediated by the endoplasmic reticulum stress transducer OASIS is involved in bone formation. *Nat. Cell Biol.* **11**, 1205–1211 (2009).
60. L. Wang *et al*., Reconstitution of human Ero1-Lalpha/protein-disulfide isomerase oxidative folding pathway in vitro. Position-dependent differences in role between the a and a′ domains of protein-disulfide isomerase. *J. Biol. Chem.* **284**, 199–206 (2009).
61. V. S. Tagliabracci, J. Wen, J. Xiao, Methods to purify and assay secretory pathway kinases. *Methods Mol. Biol.* **1496**, 197–215 (2016).