Supporting Information

Tagliabracci et al. 10.1073/pnas.1402218111

SI Methods

Molecular Biology, Cell Culture, and Transfection. Human cDNAs for FGF23, PCSK1, PCSK3 (furin), and the family with sequence similarity 20, member C (Fam20C) were from Open Biosystems. Human PCSK2 and *N*-acetylgalactosaminyltransferase 3 (GalNAc-T3) cDNAs were from DNASU. The ORFs were amplified by PCR and cloned into mammalian expression plasmids containing a C-terminal V5/His tag (pcDNA4.0; Invitrogen) or a C-terminal Flag tag (pCCF). QuikChange site-directed mutagenesis (Agilent Technologies) was performed to introduce mutations. U2OS and HEK293T cells were grown in DMEM containing 10% (vol/vol) FBS with 100 μg/mL penicillin/streptomycin (GIBCO) at 37 °C with 5% CO_2. For coexpression experiments, 5×10^5 cells were seeded in 2 mL in a six-well plate format. Approximately 24 h later, cells were transfected with 0.5 μg pCCF-PCSK1/2/3 or pCCF-Fam20C (or mutants) and 2 μg pcDNA4.0-FGF23 (or mutants) with 7 μL FuGENE 6 (Roche) as recommended by the manufacturer. Forty to 48 h after transfection, the conditioned medium and cell extracts were harvested and analyzed.

Protein Immunoblotting and Immunoprecipitations. V5- and Flag-tagged proteins were analyzed by immunoprecipitation and immunoblotting as described (1). Furin protein was analyzed by immunoblotting cell extracts with an anti-furin polyclonal antibody (1:1,000 dilution; GeneTex). To detect Fam20C protein in conditioned medium, cells were extensively washed with PBS and serum-free DMEM and then incubated for a minimum of 16 h in serum-free DMEM. The medium was centrifuged at 750 × *g* for 5 min to remove cell debris. The supernatant was further centrifuged at 10,000 × *g* for 10 min, and 100% trichloroacetic acid (one-quarter the volume of the medium) was added to the resulting supernatant. The proteins were precipitated overnight at 4 °C and washed two or three times with −20 °C acetone. The samples were dried in a SpeedVac and resuspended in 1× SDS loading buffer. Fam20C protein was analyzed by immunoblotting with a rabbit polyclonal anti-Fam20C antibody.

Generation of Anti-Fam20C Antibody. Anti-Fam20C antibodies were affinity-purified by coupling maltose-binding protein (MBP) fusion peptides to HiTrap NHS-activated HP columns (GE Healthcare). The N-terminal MBP-fusion peptides (residues 20–361, 381–496, 423–584, and 381–584 of human Fam20C) were produced in *Escherichia coli* and affinity-purified using amylose resin (New England Biolabs).

Metabolic Radiolabeling of U2OS Cells. For metabolic radiolabeling experiments, 5×10^5 cells were seeded in 2 mL in a six-well plate format. Approximately 24 h later, cells were transfected with 5 μg of pcDNA4.0-FGF23 or pcDNA4.0-osteopontin with 10 μL FuGENE 6. Forty to 48 h after transfection, the medium was replaced with phosphate-free DMEM containing 10% (vol/vol) dialyzed FBS and 1 mCi/mL [^{32}P]orthophosphate (PerkinElmer). The cells were incubated for an additional 6–8 h at which time the conditioned medium was centrifuged at 750 × *g* for 5 min to remove cell debris. The medium was further centrifuged at 10,000 × *g* for 10 min and V5-tagged proteins were immunoprecipitated from the supernatant, washed five times with PBS containing 0.4 mM EDTA and 1% Nonidet P-40, and then analyzed for protein and incorporated ^{32}P by immunoblotting and autoradiography.

Clustered Regularly Interspaced Short Palindromic Repeats/Cas9 Genome Editing. The 20-nt guide sequences targeting human *FAM20C* and *FURIN* were designed using the clustered regularly interspaced short palindromic repeats (CRISPR) design tool at www.genome-engineering.org/crispr (2) and cloned into a bicistronic expression vector (pX330) containing human codon-optimized Cas9 and the RNA components (2) (Addgene).

The guide sequences targeting exon 1 of human *FAM20C* and exon 7 of human *FURIN* are shown below.

FAM20C.

5′-GGGCTGCGCGCACGAACAGC-3′ (clone 5)

5′-CCGCCCCCGCAAGGCGCGCT-3′ (clone 9)

FURIN.

5′-TACACCACAGACACCGTTGT-3′ (clones 17 and 82)

The single-guide RNAs (sgRNAs) in the pX330 vector (4 μg) were mixed with EGFP (1 μg; Clontech) and cotransfected into U2OS cells using FuGENE 6. Twenty-four hours posttransfection, the cells were trypsinized, washed with PBS, and resuspended in fluorescence-activated cell sorting (FACS) buffer (PBS, 5 mM EDTA, 2% FBS, and 100 μg/mL penicillin/streptomycin). GFP-positive cells were single-cell–sorted by FACS (Human Embryonic Stem Cell Core, University of California, San Diego; BD Influx) into a 96-well plate format into DMEM containing 20% FBS and 100 μg/mL penicillin/streptomycin. Single clones were expanded and screened for Fam20C and furin by protein immunoblotting. Genomic DNA (gDNA) was purified from clones using the Quick-gDNA Prep Kit (Zymo Research), and the region surrounding the protospacer adjacent motif (PAM) was amplified with Q5 polymerase (New England Biolabs) using the following primers (linkers containing EcoRI and HindIII restriction sites are underlined).

FAM20C.

Forward: 5′-AAAAGAATTCTGGAGAGGAGCGCGCTGAGGATC-3′

Reverse: 5′-AAAAAAGCTTTCCGGGTTCTCCGCCGCTTTG-3′

FURIN.

Forward: 5′-AAAAGAATTCACTCAGGGGATGATGGGTGTC-3′

Reverse: 5′-AAAAAAGCTTAGAGAAGGAAAAAGAGAACACCTCC-3′

PCR products were purified using the DNA Clean & Concentrator Kit (Zymo Research) and cloned into pBluescript II KS+. To determine the indels of individual alleles, ∼10 bacterial colonies were expanded and the plasmid DNA was purified and sequenced.

Protein Purification. Flag-tagged Fam20C and FGF23 R176Q were immunopurified from conditioned medium of HEK293T cells as previously described (1). FGF23 R176Q was further purified by Superdex 200 size-exclusion chromatography. To generate a secreted form of GalNAc-T3 (sGalNAc-T3), the nucleotides encoding residues 38–633 of human GalNAc-T3 were cloned into a modified retroviral (pQCXIP; Clontech) vector containing an N-terminal interleukin 2 signal peptide and a C-terminal Flag

tag. Stable expression and purification of sGalNAc-T3 from conditioned medium were performed as described (1).

Enzyme Assays. ***Kinase assays.*** In vitro kinase assays were performed essentially as described (1). The reaction mixture contained 32.5 mM Tris·HCl (pH 7.5), 100 mM NaCl, 12.5% glycerol, 10 mM $MnCl_2$, 0.5 mM [γ-^{32}P]ATP (specific activity 100–500 cpm/pmol), 0.5 mg/mL FGF23 R176Q, and 20 μg/mL Fam20C-Flag or Fam20C (D478A)-Flag.

Glycosylation assays. In vitro O-glycosylation assays were performed essentially as described (3). Reactions were performed in a 20-μL mixture containing 25 mM sodium cacodylate (pH 7.4), 10 mM $MnCl_2$, 1.5 mM UDP-GalNAc (Sigma), 0.5 mg/mL peptide substrate: PIPRRHTRSAEDDSERDPL; FGF23(172–190) or PIPRRHTRpSAEDDSERDPL; pFGF23(172–190), and 50 μg/mL sGalNAc-T3. Reactions were incubated at 37 °C and terminated at the indicated time points by the addition of an equal volume of Sigma-Aldrich universal MALDI matrix resuspended in 78% acetonitrile and 0.1% trifluoroacetic acid.

Protease assays. Furin cleavage assays were performed as previously described (3). Reactions were performed in a 20-μL mixture containing 50 mM Hepes (pH 7.5), 1 mM $CaCl_2$, 0.5 mg/mL FGF23(172–190) or pFGF23(172–190), and 0.02 units per μL furin (New England Biolabs). Reactions were incubated at 37 °C and terminated at the indicated time points by the addition of an equal volume of Sigma-Aldrich universal MALDI matrix resuspended in 78% acetonitrile and 0.1% trifluoroacetic acid.

Deglycosylation and λ-phosphatase assays. V5-immunoprecipitates from conditioned medium of U2OS cells transiently expressing V5-tagged FGF23 were treated with O-glycosidase and α-(2→3,6,8,9)-neuraminidase to remove O-linked glycosylation or PNGase F to remove N-linked glycosylation according to the manufacturer's instructions (Sigma-Aldrich; Enzymatic Protein Deglycosylation Kit). λ-Phosphatase assays were performed as described (1).

Matrix-Assisted Laser Desorption/Ionization Analysis. Glycosylation and protease reaction products (1 μL) were plated on an Applied Biosciences (ABI) MALDI target plate for analysis. The ABI 4800 MALDI-TOF/TOF was calibrated at 25 ppm mass error with a peptide mix standard. After calibration, each spot was analyzed using reflectron positive ionization and a laser power of 30%. Masses that corresponded to truncated forms of peptide PIPRRHTRSAEDDSERDPL and the phosphorylated version PIPRRHTRpSAEDDSERDPL were sequenced de novo for validation. In addition, b- and y-ion masses were used to localize phosphorylated residues on fragment ions.

Peptide Synthesis. Peptides and phosphopeptides were synthesized by standard O-(Benzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium hexafluorophosphate/1-Hydroxybenzotriazole (HBTU/HOBt) Fmoc solid-phase chemistry on Wang resin using an ABI 431A synthesizer and incorporating pSer as its Fmoc-*O*-benzyl ester. Peptide–resin was cleaved with reagent K (2-Ethyl-5-phenylisoxazolium-3′-sulfonate). Identity and purity were assessed by electrospray ionization-Fourier transform ion cyclotron resonance mass spectrometry; the purity of crude peptide was at least 90%. Peptides were dissolved in 10 mM Hepes and the pH was adjusted to 7 with NaOH before use.

Mass Spectrometry. Fifty picomoles of Flag-tagged FGF23 R176Q was oxidized with 4 mM DTT and alkylated with 8 mM iodoacetamide and digested with either trypsin, endoproteinase GluC, or chymotrypsin. The resulting peptide extract was diluted into a solution of 2% acetonitrile, 0.1% formic acid (buffer A) for analysis. Two picomoles of each digest was analyzed by automated microcapillary liquid chromatography-tandem mass spectrometry. Fused-silica capillaries (100-μm inner diameter; i.d.) were pulled using a P-2000 CO_2 laser puller (Sutter Instruments) to a 5-μm i.d. tip and packed with 10 cm of 5-μm Magic C18 material (Agilent) using a pressure bomb. This column was then installed in-line with a Dionex 3000 HPLC pump running at 300 nL/min. Peptides were loaded with an autosampler directly onto the column and were eluted from the column by applying a 30-min gradient from 5% buffer B to 40% buffer B (98% acetonitrile, 0.1% formic acid). The gradient was switched from 40% to 80% buffer B over 5 min and held constant for 3 min. Finally, the gradient was changed from 80% buffer B to 100% buffer A over 0.1 min, and then held constant at 100% buffer A for 15 more minutes. The application of a 1.8-kV distal voltage electrosprayed the eluting peptides directly into an LTQ XL ion trap mass spectrometer equipped with a nano-liquid chromatography electrospray ionization source. Full mass spectra were recorded on the peptides over 400–2,000 *m/z*, followed by five tandem mass (MS/MS) events on the five most intense ions. Mass spectrometer scan functions and HPLC solvent gradients were controlled by the Xcalibur data system (Thermo Finnigan). MS/MS spectra were extracted with ReAdW.exe (http://sourceforge.net/projects/sashimi). The resulting mzXML file contains all of the data for all MS/MS spectra and can be read by the subsequent analysis software. The MS/MS data were searched with Inspect (4) against a database containing protein sequences for all *E. coli* proteins, common contaminants, and the sequence for FGF23 R176Q (2,786 proteins) with modifications: +16 on methionine (oxidation), +57 on cysteine (carbamidomethyl), and + 80 on threonine, serine, or tyrosine (phosphorylation). Only peptides with at least a *P* value of 0.01 were analyzed further. The MS/MS data of putative phosphorylated peptides were manually verified. In addition, further verification was performed by analyzing putative phosphorylated peptides in targeted MS/MS mode.

1. Tagliabracci VS, et al. (2012) Secreted kinase phosphorylates extracellular proteins that regulate biomineralization. *Science* 336(6085):1150–1153.
2. Hsu PD, et al. (2013) DNA targeting specificity of RNA-guided Cas9 nucleases. *Nat Biotechnol* 31(9):827–832.
3. Kato K, et al. (2006) Polypeptide GalNAc-transferase T3 and familial tumoral calcinosis. Secretion of fibroblast growth factor 23 requires O-glycosylation. *J Biol Chem* 281(27): 18370–18377.
4. Tanner S, et al. (2005) InsPecT: Identification of posttranslationally modified peptides from tandem mass spectra. *Anal Chem* 77(14):4626–4639.

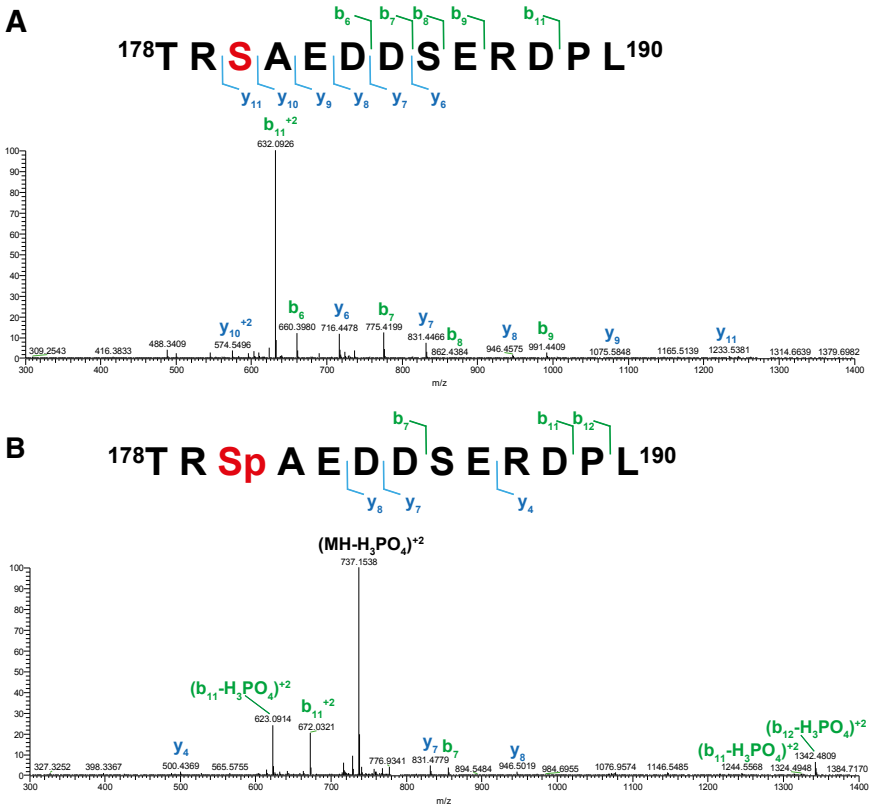

Fig. S1. FGF23 is phosphorylated on Ser[180]. Representative MS/MS fragmentation spectra of a chymotryptic peptide (FGF23 178–190) depicting Ser[180] phosphorylation of FGF23 R176Q purified from conditioned medium of HEK293T cells. The nonphosphopeptide and the phosphopeptide are shown in *A* and *B*, respectively.

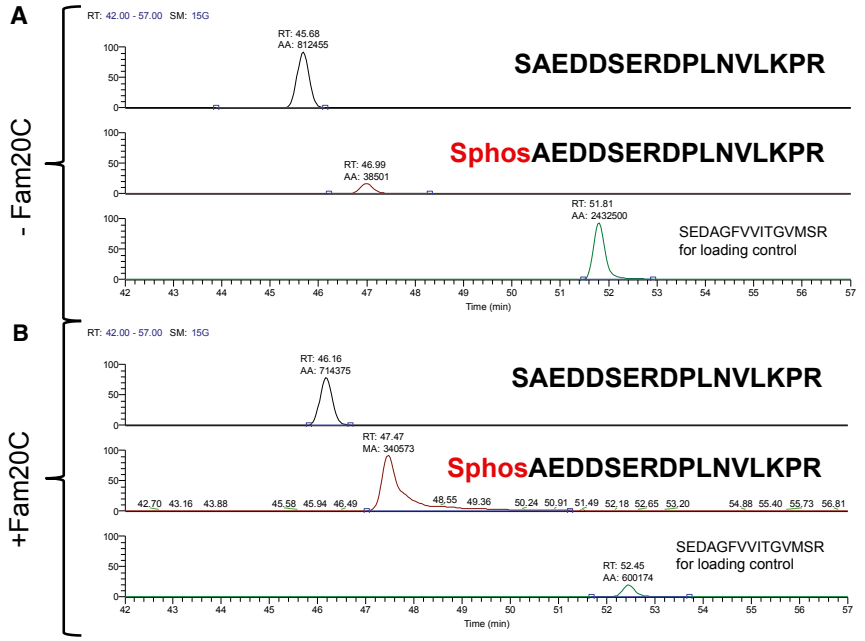

Fig. S2. Fam20C phosphorylates FGF23 on Ser[180]. Selected ion chromatograms of tryptic peptides (FGF23 180–196) from FGF23 R176Q (*A*) or FGF23 R176Q that had been treated with recombinant Fam20C (*B*). Note the relative 10-fold increase in the relative abundance of the phosphopeptide upon treatment with Fam20C. Calculations are estimated based on the areas under the curve for the selected ion species (AA phospho/nonphospho; 38,501/812,455 = 0.05; 340,573/714,375 = 0.5). AA and MA, area; RT, retention time.

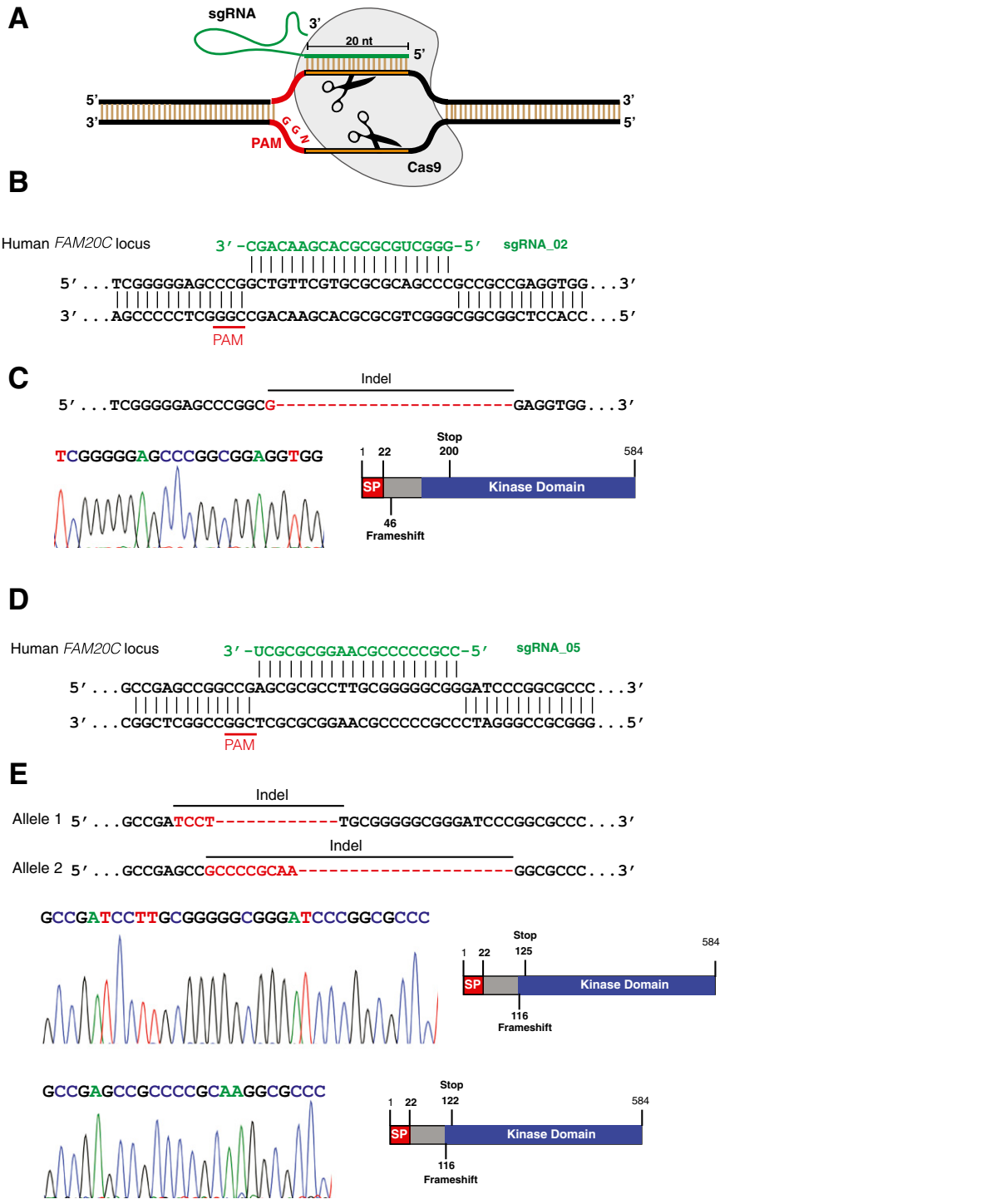

Fig. S3. Generation of Fam20C knockout cells using CRISPR/Cas9 genome editing. (*A*) The type II prokaryotic CRISPR/Cas9 from *Streptococcus pyogenes* has been shown to facilitate RNA-guided site-specific DNA cleavage and can be harnessed to target the destruction of specific genes in mammalian cells (1–3). Any genomic locus followed by a 5′-NGG PAM (red) can be targeted by a chimeric single guide RNA (sgRNA) (green) consisting of a 20-nt guide sequence and a scaffold. The sgRNA directs the Cas9 nuclease (gray) to the genomic target, resulting in a double-strand break. The double-strand break is repaired by error-prone nonhomologous end joining or by homologous recombination. (*B* and *D*) Schematic representations of the base pairing between guide RNAs and the targeting locus of exon 1 in the human *FAM20C* gene. (*C* and *E*) The sequences of the mutated alleles in *FAM20C* clone 5 (*C*) and clone 9 (*E*) and representative chromatograms depicting the indels (red) are shown. The indels are predicted to cause frameshift mutations producing inactive copies of the protein. SP, signal peptide.

1. Cong L, et al. (2013) Multiplex genome engineering using CRISPR/Cas systems. *Science* 339(6121):819–823.
2. Jinek M, et al. (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* 337(6096):816–821.
3. Jinek M, et al. (2013) RNA-programmed genome editing in human cells. *Elife* 2:e00471.

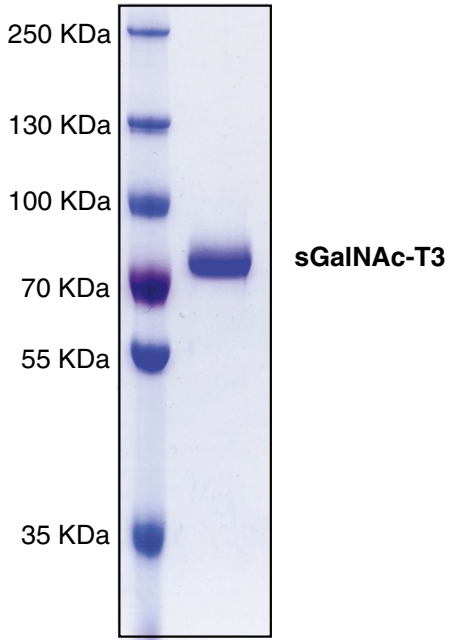

Fig. S4. Expression and purification of sGalNAc-T3. SDS/PAGE and Coomassie staining of Flag-tagged sGalNAc-T3 immunopurified from the conditioned medium of HEK293T cells.

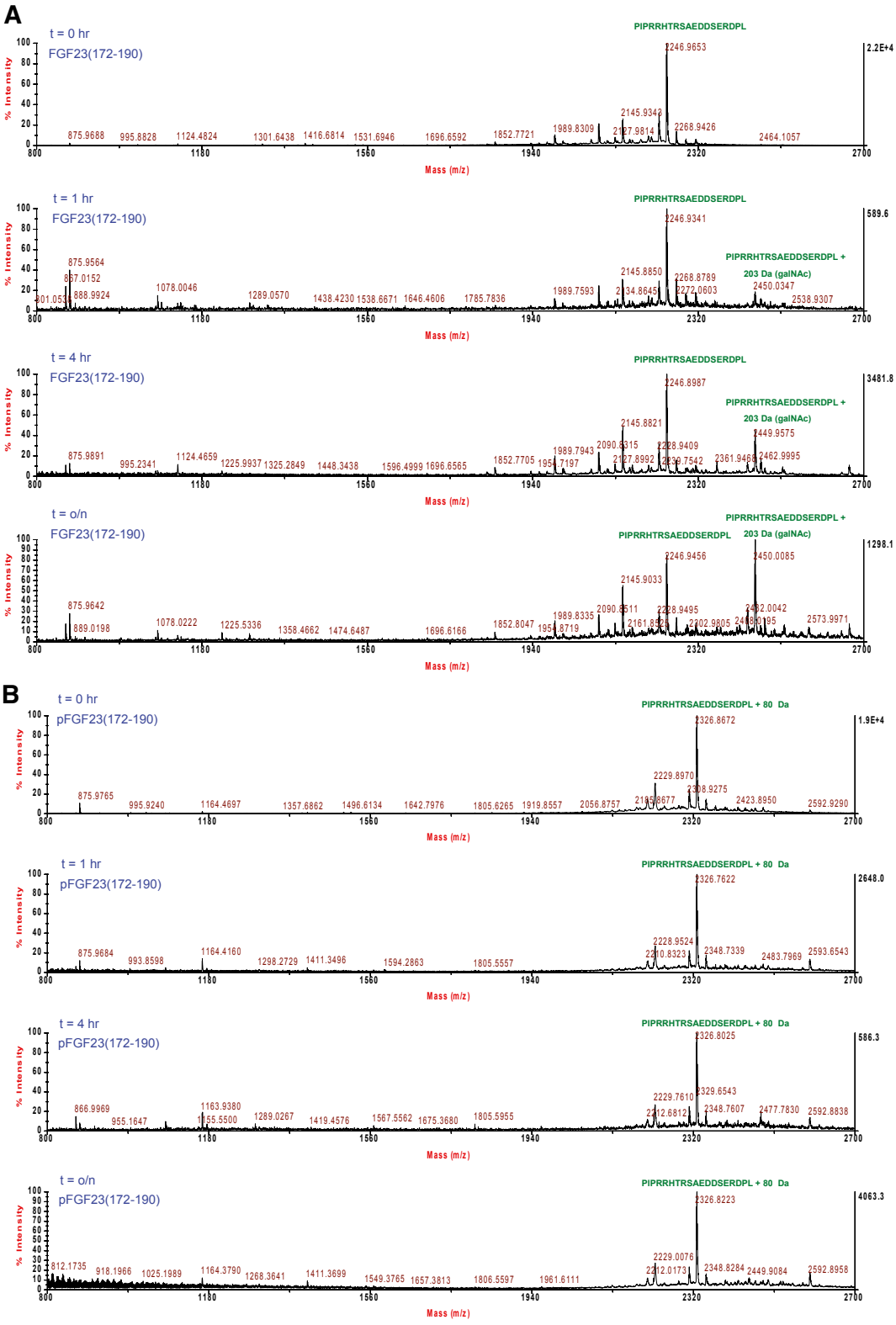

Fig. S5. Ser^{180}-phosphorylated FGF23(172–190) is not a substrate for sGalNAc-T3. Full MALDI-TOF MS spectra depicting the time-dependent incorporation of GalNAc from UDP-GalNAc into FGF23(172–190) (*A*) or Ser^{180}-phosphorylated FGF23(172–190) (*B*).

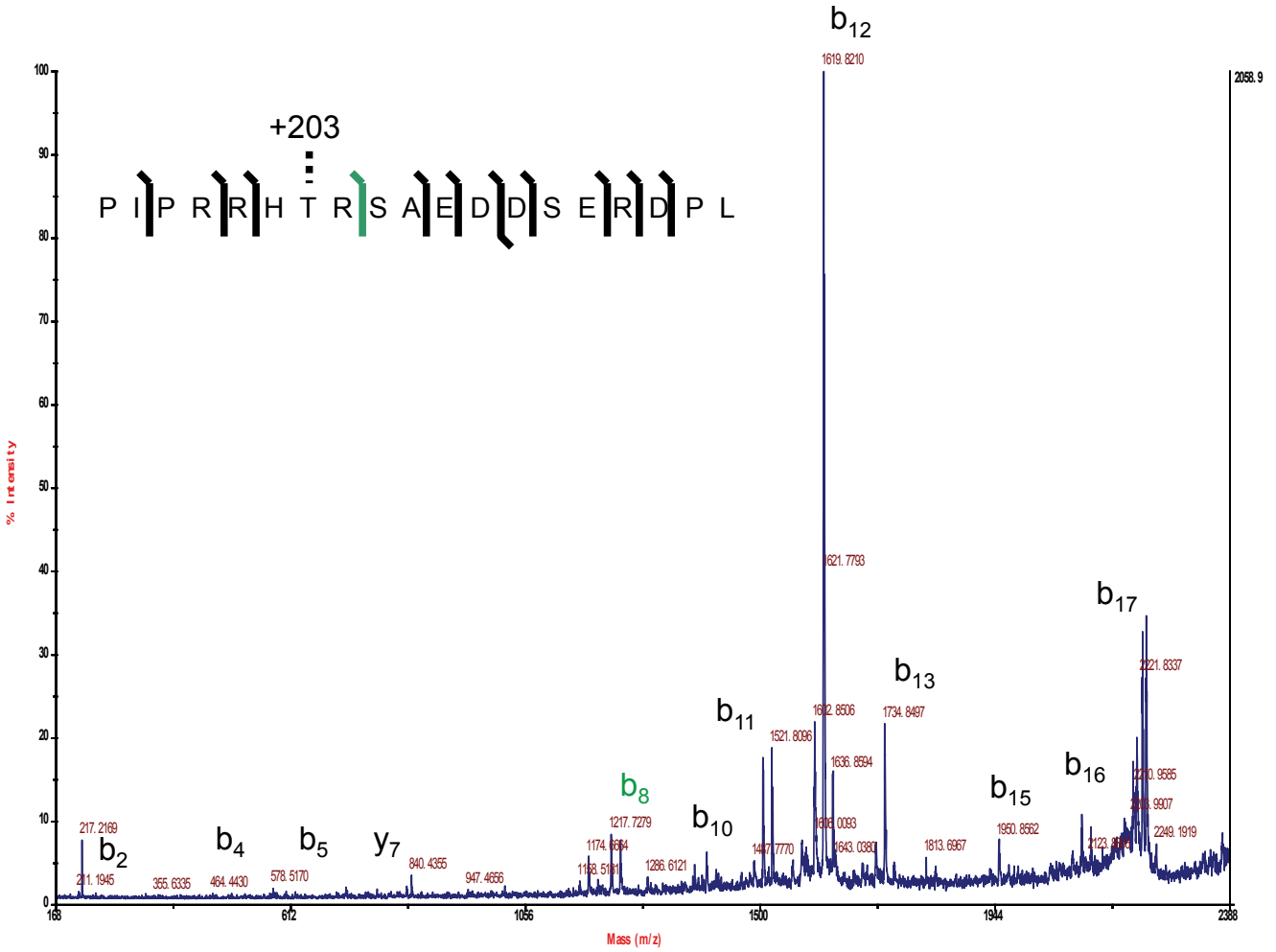

Fig. S6. Thr^{178} is O-glycosylated by sGalNAc-T3. Representative MS/MS fragmentation spectrum of the selected ion peak at *m/z* 2450 in Fig. S5*A*, depicting the addition of GalNAc to Thr^{178}.

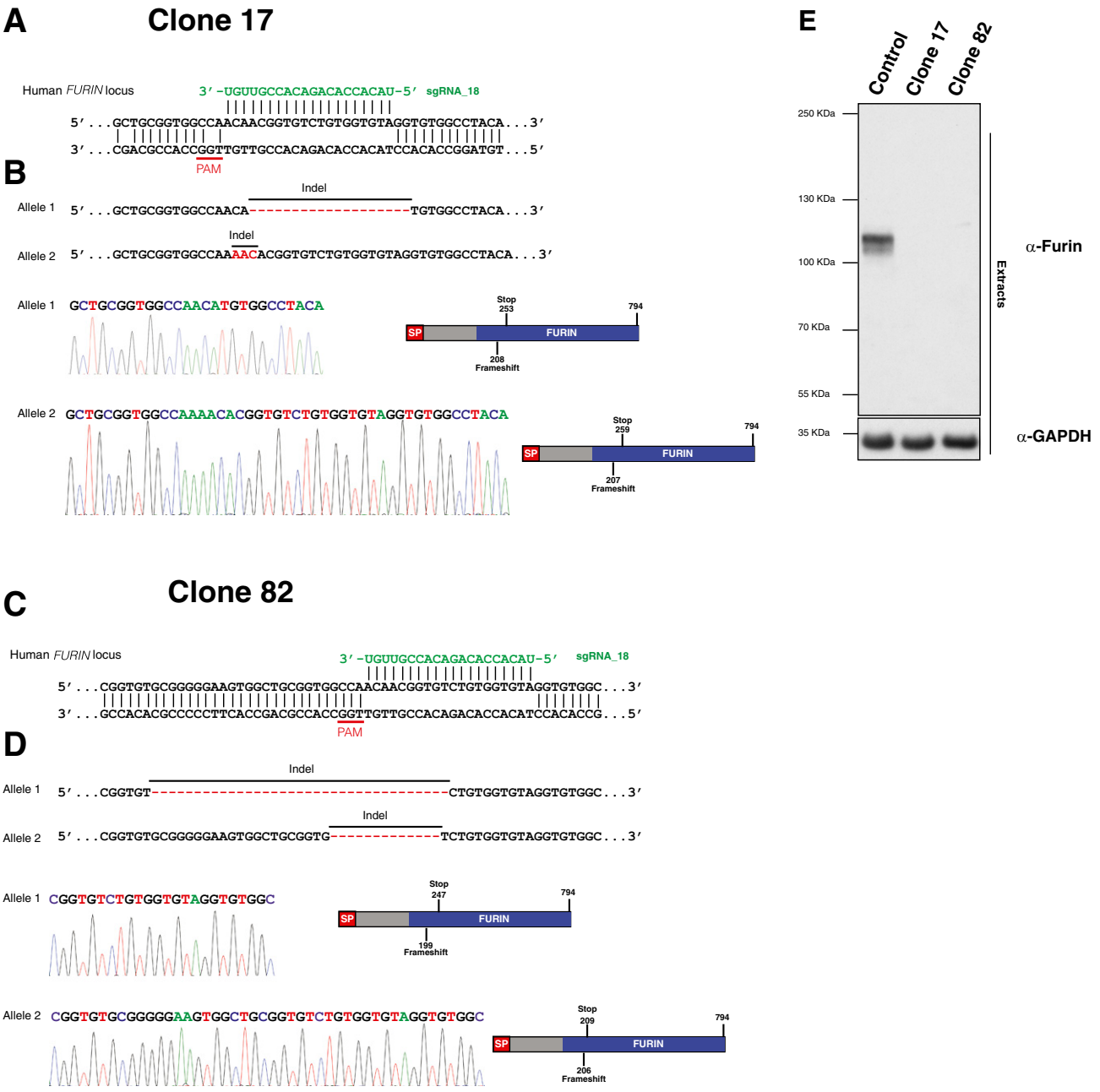

Fig. S7. Generation of *FURIN* knockout cells using CRISPR/Cas9 genome editing. (*A* and *C*) Schematic representations of the base pairing between a guide RNA (sgRNA_18) and the targeting locus of exon 7 in the human *FURIN* gene. (*B* and *D*) The sequences of the mutated alleles in *FURIN* clone 17 (*B*) and clone 82 (*D*) and representative chromatograms depicting the indels (red) are shown. The indels are predicted to cause frameshift mutations producing inactive copies of the protein. (*E*) Protein immunoblotting of cell extracts from control and furin KO cells.

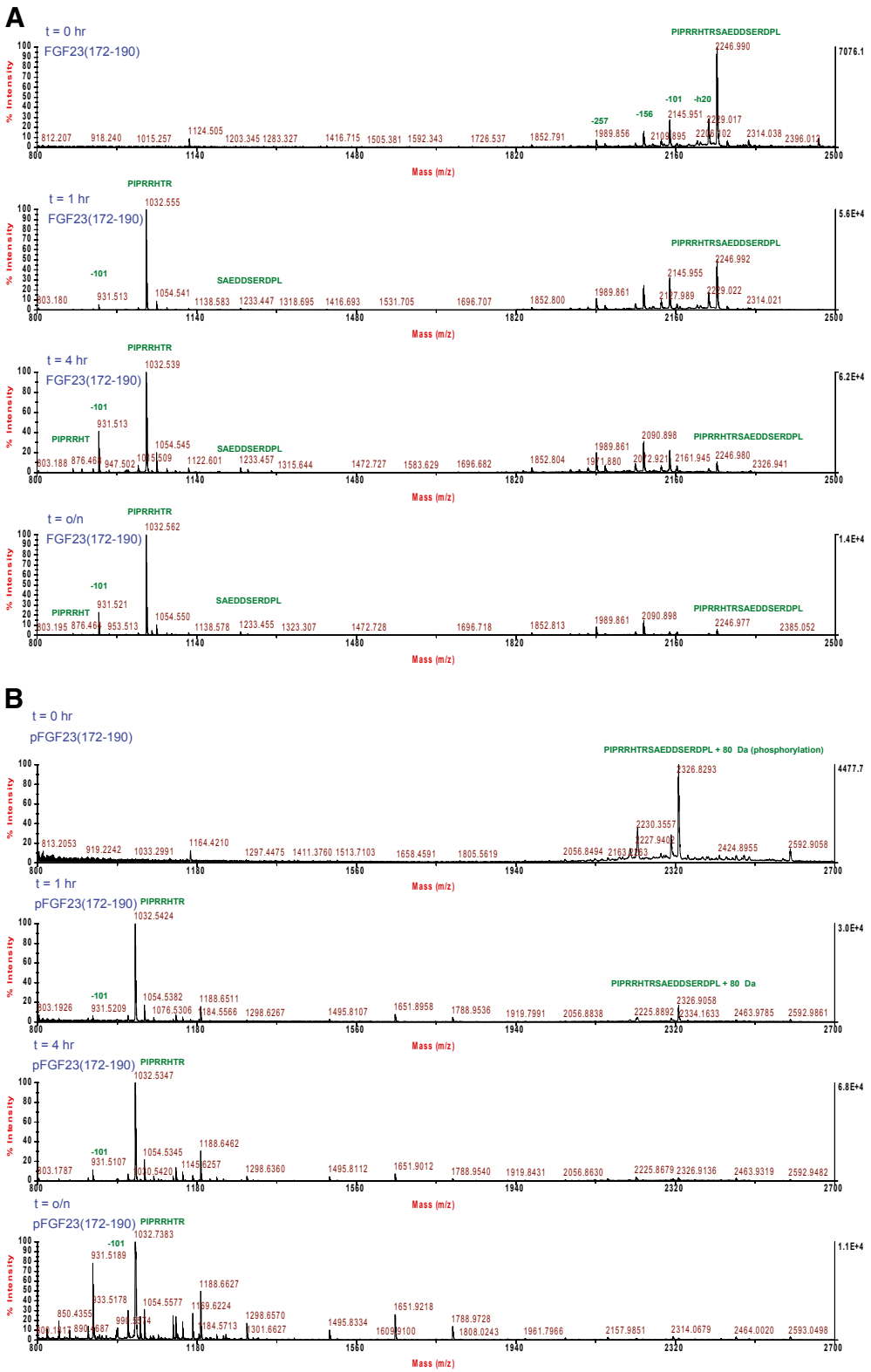

Fig. S8. Ser^{180}-phosphorylated FGF23(172–190) is cleaved by furin. Full MALDI-TOF MS spectra depicting the time-dependent cleavage of FGF23(172–190) (*A*) or Ser^{180}-phosphorylated FGF23(172–190) (*B*).

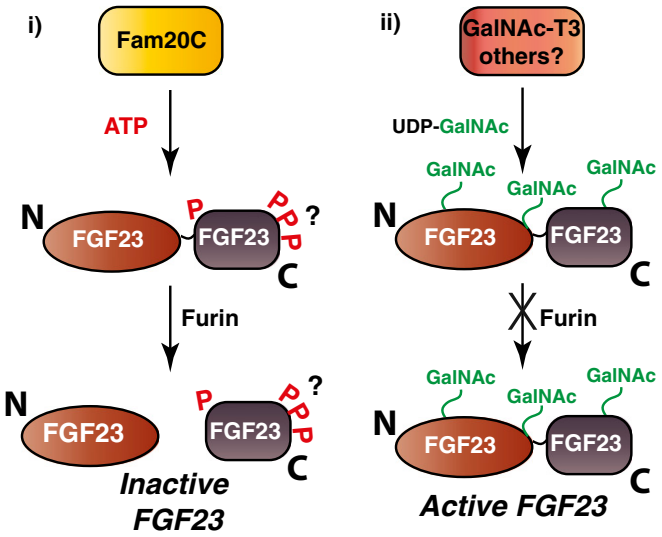

Fig. S9. Model for the regulation of FGF23 by Fam20C. We propose that interplay between phosphorylation by Fam20C (i) and O-glycosylation by GalNAc-T3 (ii) regulates the processing and activity of FGF23 by furin. Phosphorylation of FGF23 at Ser^{180} by Fam20C inhibits GalNAc-T3-mediated O-glycosylation at Thr^{178} and promotes inactivation by furin. Fam20C may also phosphorylate predicted Ser-x-Glu/pSer residues in the C-terminal region of FGF23.