Structure and evolution of the Fam20 kinases

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The Fam20 proteins are novel kinases that phosphorylate secreted proteins and proteoglycans. Fam20C phosphorylates hundreds of secreted proteins and is activated by the pseudokinase Fam20A. Fam20B phosphorylates a xylose residue to regulate proteoglycan synthesis. Despite these wide-ranging and important functions, the molecular and structural basis for the regulation and substrate specificity of these kinases are unknown. Here we report molecular characterizations of all three Fam20 kinases, and show that Fam20C is activated by the formation of an evolutionarily conserved homodimer or heterodimer with Fam20A. Fam20B has a unique active site for recognizing Galβ1-4Xylβ1, the initiator disaccharide within the tetrasaccharide linker region of proteoglycans. We further show that in animals the monomeric Fam20B preceded the appearance of the dimeric Fam20C, and the dimerization trait of Fam20C emerged concomitantly with a change in substrate specificity. Our results provide comprehensive structural, biochemical, and evolutionary insights into the function of the Fam20 kinases.
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inases are molecular activators and signal transducers, and regulate many essential cellular processes by phosphorylating target molecules. The human kinome, for example, encompasses more than 500 kinases that mediate signaling pathways, defects of which are frequently associated with diseases. However, most of the currently characterized kinase members function in the cytoplasm and nucleus to phosphorylate intracellular proteins, despite the fact that phosphorylation of secretory molecules, such as milk casein, had been acknowledged for many years. Recent studies have identified a group of kinases collectively referred to as the "secretory pathway kinases," which are specifically localized to the lumen of the endoplasmic reticulum (ER), Golgi apparatus, and extracellular space. Some of these kinases phosphorylate Ser, Thr, or Tyr residues in protein substrates, whereas others possess glycan kinase activities. For example, Drosophila four-jointed phosphorylates Ser/Thr residues in the extracellular domains of two atypical cadherins Fat and Dachsous. Vertebrate lonsome kinase (VLK; aka PKDC/Sgk-193) phosphorylates a variety of ER resident and extracellular proteins on their Tyr residues. In contrast, protein O-mannose kinase (POMK/Sgk-196), once considered an inactive kinase, phosphorylates a mannose residue to regulate the biosynthesis of alpha-dystroglycan, an essential extracellular matrix glycoprotein required for proper muscle function. Some of these kinases are secreted, although it remains unclear whether they can phosphorylate molecules in the extracellular space in a physiological context.

Besides the proteins mentioned above, the "family with sequence similarity 20" (Fam20) proteins also function in the secretory pathway to phosphorylate proteins and proteoglycans. The human genome encodes three Fam20 paralogs: Fam20A, Fam20B, and Fam20C. Remarkably, each has a distinct biochemical activity. Fam20C is the long-sought-after physiological casein kinase that mainly phosphorylates proteins within Ser-x-Glu/pSer motifs. More than 100 proteins are Fam20C substrates, which function broadly in biological processes such as biomineralization, phosphate metabolism, cell adhesion and migration, and cardiac function. Diminished Fam20C activity causes Raine syndrome, an incurable malady characterized by generalized osteodysplasia and ectopic calcifications. Both Fam20A and Fam20B are highly similar to Fam20C in amino acid sequence, but Fam20A lacks an active site residue critical for kinase activity, binds ATP in a catalytically incompetent manner, and is therefore a pseudokinase. Fam20A nonetheless promotes the phosphorylation of enamel matrix proteins by forming a complex with Fam20C and stimulating Fam20C activity. Mutations in fam20a lead to the dental and renal diseases known as Amelogenesis Imperfecta and Enamel Renal Syndrome. Despite these important functions of Fam20C and the Fam20A–Fam20C complex, the structural and molecular basis for regulation of Fam20C by Fam20A are unknown.

In contrast to Fam20A, Fam20B is a bona fide kinase. However, unlike Fam20C, which phosphorylates protein substrates, Fam20B is a glycan kinase like POMK, and its activity is a critical switch during the biosynthesis of chondroitin sulfate (CS) and heparan sulfate (HS) proteoglycans. In fact, Fam20B and POMK represent the only two known glycan kinases in animal cells. Proteoglycans are unique macromolecules of the cell surface and major constituents of the extracellular matrix. They are fundamental to a wide spectrum of physiological processes such as adhesion, growth and differentiation, receptor-ligand interactions, and microbial infections. The biosynthesis of CS and HS proteoglycans requires the formation of a tetrasaccharide linker, established by the sequential actions of xylosyltransferase (XylT), galactosyltransferase I (GalT-I), galactosyltransferase II (GalT-II), and glucuronyltransferase I (GlcAT-I) (Supplementary Fig. 1). During this process, Fam20B recognizes the initiator Galβ1-4Xylβ1 disaccharide and phosphorylates the xylose residue at the C2 hydroxyl position, and this phosphorylation event is important for priming the activity of GalT-II. Without the priming phosphorylation GalT-II activity is greatly reduced, and formation of the tetrasaccharide linker and subsequent elongation of the CS and HS glycosaminoglycan chains are abolished. Ablation of the fam20b gene in mice results in embryonic lethality at E13.5, and is associated with severe developmental defects. Tissue-specific deletions of fam20b in joint cartilage and dental epithelium cause chondrosarcoma and biomineralization abnormalities such as supernumerary incisors. Zebras fish deficient in Fam20B display malformation of cartilage matrix and bone. These phenotypes highlight the importance of Fam20B function in proteoglycan synthesis and related developmental processes. Despite high sequence similarity, the mechanisms by which Fam20B and Fam20C achieve substrate specificity are unknown.

Here, we use a combination of structural biology, biochemistry, and phylogenetics to elucidate the molecular functions and evolutionary relationships of the three Fam20 kinases. First, we show that Fam20C activation requires the formation of an evolutionarily conserved homodimer, or heterodimer with Fam20A. Compared to Fam20C, Fam20B is a more efficient Fam20C-binding surface and is a specialized Fam20C-allosteric activator. We also reveal the mechanism by which Fam20B recognizes its substrate by solving the crystal structure of a Fam20B ortholog in complex with the Galβ1-4Xylβ1 disaccharide. Our phylogenetic analyses suggest that the monomeric Fam20B xylosylkinase activity likely emerged first in evolution, and the evolutionary change in Fam20C substrate specificity correlated with dimer formation. Collectively, these results provide comprehensive insights into the function of this unique and biomedically important family of kinases and shed light on their evolutionary history.

Results
Structure of the human Fam20A–Fam20C complex. To elucidate the molecular basis of how Fam20A regulates Fam20C activity, we determined the crystal structure of the human Fam20A–Fam20C complex (Table 1). The structure reveals that Fam20A and Fam20C form a reversed face-to-face heterodimer (Fig. 1a). The Fam20A–Fam20C interface buries ~1000 Å², or ~5% of the solvent-accessible surfaces from each molecule. At the heart of the interface are interactions between the Kβ3-βKα3 loop (we use “K” to denote the kinase core, and “N” to denote the N-terminal segment) and the N-lobe insertion domain (Kβ5-Kβ7) of each molecule (Supplementary Fig. 2). In addition, residues from the Kβ8-κα6 loop in the C-lobe of each protein (Leu365A, Lys413C; superscripts A and C indicate human Fam20A and Fam20C, respectively), as well as from the Kβ1-Kβ2 insertion of Fam20A (Ile214A) also contribute to the interaction. Specifically, Ile214A, Phe251A, Phe252A, Ile255A, and Leu365A form a continuous hydrophobic surface patch on Fam20A that docks onto Fam20C residues including Phe354A, Pro357C, Tyr364C, and Thr373C (Fig. 1b). Phe360A and Pro309A form hydrophobic interactions with Phe300C, and Phe306A also forms a cation–π interaction with Lys413C. Lys324A uses its alphatic side chain to pack against Tyr369C, and its main chain carbonyl group to form a hydrogen bond with His375C. Tyr327A forms hydrophobic contacts with Phe299C, and a hydrogen bond with Tyr305C. Other hydrogen bonds present at the Fam20A–Fam20C interface include Asp250A-Ser356C, Asp250A-Asn360C, Ser308A-Asp298C,
mutants to activate endogenous Fam20C in cells, we analyzed enamelin (ENAM 173-277) (Fig. 2b). To test the function of these Fam20C in vitro when assayed against the Fam20C substrate, we also examined the ability of multiple WT Fam20A and Fam20C. 

To verify the functional relevance of the Fam20A–Fam20C heterodimer, we generated several Fam20A mutants based on the structural observations above, including F251A/F252A, I255E, F300A, F354A/P357G, H375Y, and E374S/H375T (Fig. 1b). We also generated several mutants of Fam20C that we predicted would disrupt dimerization with Fam20A, including F299A/F300A, F354A/P357G, the mutant that displays the lowest kinase activity—drFam20C—F306A/P309G, and L365D. These residues were chosen because they are involved in making hydrophobic contacts with Fam20C (Fig. 1b), and we predicted that disrupting them would inhibit Fam20A–Fam20C interaction. We then tested the ability of these Fam20A mutants to bind and activate Fam20C. All of them exhibit greatly reduced interaction with Fam20C as shown by Maltose Binding Protein (MBP) pull-down experiments (Fig. 2a). They also exhibit a substantially diminished capacity to activate Fam20C in vitro when assayed against the Fam20C substrate, as judged by the change in electrophoretic mobility of V5-tagged OPN on SDS-PAGE (Fig. 2d). The F251A/F252A and F306A/P309G mutants of Fam20A failed to activate the Fam20C Raine mutants, whereas I255E and L365D showed only partial activation. The various defects of these Fam20A mutants confirm that the dimer interface observed in the crystal structure is critical for Fam20A to bind Fam20C and regulate its activity.

### Fam20C proteins function as evolutionarily conserved dimers

We also generated several mutants of Fam20C that we predicted would disrupt dimerization with Fam20A, including F299A/F300A, F354A/P357G, H375Y, and E374S/H375T (Fig. 1b). Interestingly, these mutants show substantially decreased basal kinase activity when assayed in the absence of Fam20A (Fig. 3a). All these mutant proteins were secreted from insect cells as efficiently as WT Fam20C, suggesting that the mutations do not severely disrupt protein folding. These observations raise the intriguing possibility that Fam20C may also be regulated by homodimerization. Indeed, purified Fam20C oligomerizes in a concentration-dependent manner in solution, and is mainly dimer at ~1 mg per ml, as shown by analytical ultracentrifugation (Supplementary Fig. 3A) and size exclusion chromatography experiments (Supplementary Fig. 3B). In contrast, Fam20C–F354A/P357G, the mutant that displays the lowest kinase activity (Fig. 3a), is largely monomeric (Supplementary Fig. 3B).

The human Fam20C protein crystallized but the crystals diffracted poorly. To gain structural insights into the Fam20C...
homodimer, we crystallized a Fam20C ortholog from zebrafish (Danio rerio, drFam20C), which is 86% identical (94% similar) to human Fam20C in the kinase domain, and determined its structure (Table 1). There are 12 molecules of drFam20C in the crystal asymmetric unit, which organizes into six pairs of homodimers similar in overall structure to the Fam20A–Fam20C heterodimer (Fig. 3b, Supplementary Fig. 4A). All the residues that participate in the drFam20C dimer interface are conserved in human Fam20C (Fig. 3c, Supplementary Fig. 2), including residues corresponding to Phe299C, Phe300C, Phe354C, Pro357C, Glu374C, and His375C (Phe275, Phe276, Phe330, Pro333, Glu350, and His351 in drFam20C), mutations of which reduce Fam20C activity (Fig. 3a). These analyses, together with the biochemical results described above, suggest that the human Fam20C protein probably forms a similar homodimer, and that dimer formation is critical for allosterically activating kinase activity.

Interestingly, a homologous homodimer is also present in the crystal lattices of ceFam20, formed by two symmetry-related molecules (Fig. 3d, Supplementary Fig. 4A). ceFam20 is a Fam20C ortholog in Caenorhabditis elegans34,35, which does not have Fam20A. Consistently, ceFam20 is a dimer in solution, and ceFam20-F260A/P263G (analogous to human Fam20C-F354A/P357G, Supplementary Fig. 2) is monomeric (Supplementary Fig. 3C). Importantly, the kinase activity of this mutant is also greatly reduced (Fig. 3e). Together, these results demonstrate that the Fam20C proteins function as evolutionarily conserved dimers.

A comparison of the drFam20C homodimer with the Fam20A–Fam20C heterodimer suggests that Fam20C uses an almost identical set of residues to interact with itself and Fam20A (Figs. 1b, 3c, Supplementary Fig. 4B). However, Fam20A contains several unique features that make it a more efficient Fam20C-interactor. For example, Ile214A, Ile255A, and Leu365A are unique to Fam20A (Supplementary Fig. 2), and contribute to the formation of an optimized hydrophobic surface for interacting with Fam20C (Fig. 1b, Supplementary Fig. 4B). Lys324A and Tyr327A are also unique to Fam20A and mediate additional interactions with Fam20C in the middle of the Fam20A–Fam20C interface (Supplementary Fig. 2, Fig. 1b, Supplementary Fig. 4B). Fam20C is expressed ubiquitously, whereas Fam20A expression is restricted to specific tissues, particularly ameloblasts and the lactating mammary gland20,30,35, where high Fam20C activity is likely required for phosphorylating enamel matrix and milk proteins. Thus, we propose that the Fam20C homodimer plays a “house-keeping” function, whereas Fam20A is employed when Fam20C activity is in high demand.

The Fam20A–Fam20C tetramer. Our previous biochemical data suggested that Fam20A and Fam20C form a heterotetramer consisting of two molecules each of Fam20A and Fam20C20,21. However, this state is not present in the human Fam20A–Fam20C crystal. In an attempt to address this discrepancy, we solved the structure of the Fam20A–drFam20C complex that is crystallized in a different space group (Table 1). A four-leaf clover-shaped tetramer is clearly present in this structure (Fig. 4a), formed by two Fam20A–drFam20C heterodimers similar to the human Fam20A–Fam20C heterodimer shown in Fig. 1a. A helix in the N-terminal region of drFam20C (Na2drFam20C), which is conserved in human Fam20C (Supplementary Fig. 2), plays a pivotal role in mediating the tetramer formation.

In light of this Fam20A–drFam20C tetramer structure, we realized that the absence of a human Fam20A–Fam20C tetramer in the crystal is also due to an unexpected obstructing ATP molecule, besides crystal packing effects. Both Fam20A–Fam20C and Fam20A–drFam20C only crystallized in the presence of ATP, likely because ATP significantly stabilizes Fam20A21. In both structures, an ATP molecule (ATP-1) is bound to Fam20A in an inverted orientation, as we previously reported21. In the human Fam20A–Fam20C crystal, an additional ATP (ATP-2) is bound to Fam20A perpendicularly to ATP-1 (Supplementary Fig. 5A), and is incompatible with tetramer formation because it would sterically occlude the Na2 helix in Fam20C (Supplementary Fig. 5B). In agreement with this observation, excess ATP converts the human Fam20A–Fam20C tetramer to a dimer in solution (Supplementary Fig. 5C).

To assess the functional importance of the tetramer, we designed two Fam20A mutants: E299G/I300S and K129A/P357G, Supplementary Fig. 2) is monomeric (Supplementary Fig. 5C). In agreement with this observation, excess ATP converts the human Fam20A–Fam20C tetramer to a dimer in solution (Supplementary Fig. 5C).

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N-linked glycosylation (Asn298-Gly299-Ser300). Given that Asn298 is at the center of the Fam20A−drFam20C tetramer interface (Fig. 4a), the bulky glycan addition is predicted to hinder tetramer formation. Lys129, Arg132, and Arg136 probably interact with the Nα2 helix in the tetramer (Fig. 4a), and are also involved in binding to ATP-2 in the Fam20A−Fam20C structure (Supplementary Fig. 5A). These two mutants reduced and abolished human Fam20A−Fam20C tetramer formation, respectively, as shown by size exclusion chromatography (Supplementary Fig. 5D). Nevertheless, both mutants activated Fam20C at levels comparable to WT Fam20A in vitro (Fig. 4b) and in cells (Fig. 2c). These data suggest that while a Fam20A−Fam20C heterodimer is required for Fam20A to enhance Fam20C activity, further assembly of a tetramer is dispensable for activation. The functional significance of ATP-2 remains unclear, and we cannot rule out the possibility that it is a crystallization artifact.

Substrate recognition mechanism of Fam20B. Human Fam20C and Fam20B are 42% identical within the kinase domain yet Fam20B is a glycan kinase that regulates proteoglycans.
biosynthesis. To gain insights into the evolution of the Fam20 kinases, we searched annotated animal genomes by Position-Specific Iterative Basic Local Alignment Search Tool (PSI-BLAST) for Fam20 homologs. Several lower animals encode a single Fam20 protein (Fig. 5a). We previously demonstrated that the single Fam20 homolog in *C. elegans* is a Fam20C-like kinase despite its previous annotation as Fam20B. In contrast, the single Fam20 proteins in basal animals such as sponge (*Amphimedon queenslandica*, aqFam20) and hydra (*Hydra magnipapillata*, hmFam20) are apparently Fam20B-like because they exhibit robust activity for the Gal-β1-4Xyl-β1 disaccharide (Gal-Xyl hereafter, Fig. 5b), and do not phosphorylate protein substrates such as OPN, casein, and enamelin (Fig. 5c). In fact, xylose phosphorylation within the tetrasaccharide linker region of CS proteoglycans was detected in hydra prior to the discovery of the Fam20B xylosylkinase activity. Similarly to human Fam20B, aqFam20 and hmFam20 are monomers (Supplementary Fig. 3D).

To further understand the structural difference between Fam20B and Fam20C, we sought to determine the crystal structure of Fam20B. Although human Fam20B can be purified to homogeneity, it failed to crystallize despite extensive trials. hmFam20 crystallized readily, but the initial crystals diffracted poorly. Treatment of the purified hmFam20 with endoglycosidase F3 to remove the majority of the N-linked glycans significantly improved crystal quality and diffraction. The hmFam20 structure was subsequently determined at 2.2 Å resolution (Table 1). The overall structure of hmFam20 resembles that of Fam20C, and can be superimposed onto ceFam20 with a root-mean-square deviation of 1.5 Å over 342 aligned Cα atoms. There are four disulfide bonds in hmFam20 (Cys207-Cys222, Cys212-Cys215, Cys267-Cys340, Cys341-Cys400), which all align with the four disulfide bonds in Fam20C. To explore the substrate recognition mechanism of Fam20B, we also determined the structure of hmFam20 in complex with Gal-Xyl (Table 1, Fig. 6a). Although AMP-PNP, a nonhydrolyzable ATP analog is also present in the crystal solution, electron densities can only be clearly discerned for the adenosine moiety. The phosphate groups display weak electron densities and are not modeled. A comparison of the hmFam20 structure with the Mn/ADP-bound ceFam20 reveals the ATP-binding site is highly conserved between Fam20B and Fam20C (Fig. 6a). Asp152 occupies the position of Glu213ceFam20 and forms an ion pair with Lys133. Although Glu218ceFam20, instead of Glu213ceFam20, aligns with the ion pair Glu in canonical kinases (such as Glu91 in PKA), Glu213ceFam20 is nevertheless important for the kinase activity of Fam20C, and Fam20A is a pseudokinase partly...
because it contains a Gln instead of an acidic Asp/Glu at this position. The Gal-Xyl is accommodated next to the ATP-binding site, in a pocket formed by Thr114, Gln115, Tyr148, Glu149, Gly150, Tyr151, Tyr214, Tyr253, His301, and Lys321 (Fig. 6b).

Fig. 4 The Fam20A–Fam20C tetramer. a The crystal structure of Fam20A–drFam20C reveals a four-leaf clover-shaped tetramer formed by two Fam20A–drFam20C heterodimers. The side chain of Asn298A is shown. The positions of Lys129A, Arg132A, and Arg136A in the Nα2 helix are highlighted in red. The side chains of these three residues are not clearly visualized in the structure. b The E299G/I300S and K129A/R132A/R136A mutants of Fam20A activate Fam20C at levels comparable to WT Fam20A in vitro.

Fig. 5 Phylogenetic analyses of the Fam20 proteins. a A diagram depicting the presence of different Fam20 proteins in representative species. b The Fam20 proteins from sponge (aqFam20) and hydra (hmFam20) phosphorylate the Gal-Xyl disaccharide with efficiencies similar to human Fam20B (hsFam20B). Error bars represent the standard deviation of three independent experiments. c Only human Fam20C phosphorylates enamelin, casein, and OPN, as revealed by 32P autoradiography.
Fam20C dimer emerged with a change in substrate preference. The hmFam20 structure enabled us to consider how Fam20B may have emerged in higher animals. Inspection of the hmFam20 structure (Fam20B-like) and the ceFam20 structure (Fam20C-like) reveals three regions in Fam20C that lead to a change in substrate preference. First, the K33-K36 loop of Fam20C contains a D/N-F/H-F/S/T-D motif (Clash1; Figs. 7a, b). Within this motif, the F-Y-F (Phe207ceFam20, Tyr208ceFam20, Phe209ceFam20, Fig. 7c) cluster together by strong hydrophobic/π interactions, and the invariant Asp (Asp211ceFam20) is anchored to an Arg (Arg214ceFam20). In the context of these structural restraints, the F/S/T would clash with Gal-Xyl (Phe209ceFam20, Ser210ceFam20, Fig. 7c). Second, the K86-K87 loop of Fam20C contains a C-D/S-Y-Y-C motif (Clash2). The two Cys that form a disulfide bond (Cys273ceFam20-Cys277ceFam20) are spaced by three residues, as opposed to two residues found in Fam20B (Fig. 7a). Consequently, a Tyr is pushed down and would hinder the binding of Gal-Xyl (Tyr275ceFam20, Fig. 7c). Finally, an Arg in the K81-K9 loop of Fam20C (Clash3) replaces a critical Lys in Fam20B (Fig. 7a), and the more extended side chain of the Arg would prevent disaccharide binding (Fig. 7c). Indeed, changing the corresponding Lys to Arg in human Fam20B (K312R, Fig. 6c) completely abolishes kinase activity.

Interestingly, some of these changes correlate with the capacity of Fam20C to dimerize. The D/N-F/H-F and the S/T residues in the D/N-F/H-F/Y-Y-C motif are involved in the homodimer interface of ceFam20 and drFam20C (Asn205ceFam20, Phe207ceFam20, Ser210ceFam20, Asp274drFam20C, Phe276drFam20C, Ser279drFam20C, Supplementary Fig. 2; Fig. 3c). The corresponding residues in human Fam20C are also involved in forming the Fam20A–Fam20C heterodimer (Asp250A-Phe252A, Ile255A; Asp298C, Phe300C; Fig. 1b). Similarly, one of the Tyr in the C-D/S-Y-Y-C motif is located in the ceFam20 dimer interface (Tyr276ceFam20, Supplementary Fig. 2), and is exploited by human Fam20A and Fam20C for the formation of the Fam20A–Fam20C heterodimer (Tyr321A, Tyr369C, Fig. 1b). Therefore, it appears that emergence of the capacity for Fam20C to form dimers was concomitant with its divergence from the Fam20B glycan kinase during evolution. Changes in other places, especially the appearance of the F-x-S-P motif in the N-lobe insertion, further stabilize the Fam20C homodimers (Phe260ceFam20-Pro263ceFam20, Phe330drFam20C, Pro333drFam20C, Supplementary Fig. 2, Fig. 3c) and the Fam20A–Fam20C heterodimer (Phe306A-Pro309A, Phe354C, Pro357C, Fig. 1b).
protein involved in a range of cellular functions, often by mediating becoming increasingly clear that these proteins are actively From a broader perspective, pseudoenzymes are prevalent not compatible with disaccharide binding in Fam20C are highlighted using red regions that are critical for substrate selectivity. Residues that are for catalysis, while several positively charged amino acids that are potentially involved in substrate binding reside in the Kβα3 loop and the Kβα6 loop (Supplementary Fig. 7). In the Fam20A alone structure, these two loops interact with another Fam20A molecule to form a Fam20A homodimer (Supplementary Fig. 7A) that is distinct from the Fam20A–Fam20C heterodimer and Fam20C homodimer. When Fam20A is in complex with Fam20C, these two loops move significantly to mediate the heterodimer interaction (Supplementary Fig. 7B). Interestingly, in this state, the two loops in Fam20A become very Fam20C-like (Supplementary Fig. 7C). Whether Fam20C can undergo similar conformational changes is unknown, but the sequence similarity between Fam20A and Fam20C (Supplementary Fig. 2) suggest that the corresponding regions in Fam20C could be inherently dynamic. The Kβα3 loop of Fam20C contains Glu307C (equivalent to Glu213 in Fam20A mentioned above) that is essential for catalysis, while several positively charged amino acids that are known to stabilize the conformation of the two loops discussed above. Fingolimod, a structural analog of sphingosine and a clinically approved drug for treating multiple sclerosis, also stimulates Fam20C kinase activity. Further understanding the mechanisms by which Fam20C is regulated may facilitate the design of better therapeutics for patients carrying non-lethal Fam20C mutations. Our work also shed light on the evolution of the Fam20 kinases. We show that the Fam20B xylosylkinase activity and specificity are evolutionarily conserved, and can be traced back further than the Fam20C protein kinase in the animal kingdom (Fig. 5). Sponges are considered the oldest animal phylum, and we find that the single Fam20 protein in sponge is Fam20B-like. Interestingly, although the sponge glycans are structurally distinct from typical CS and HS glycosaminoglycans, homologs of XylT, GalT-I, GalT-II, and GlcAT appear to exist in A. queenslandica (NCBI protein IDs: XP_011404143, XP_003384378, XP_003384004, XP_003386699). This suggests that A. queenslandica is capable of producing the tetrasaccharide linker with a phosphorylated xylose residue. Hydra is the most basal animal that contains classic CS and HS glycosaminoglycans. The single Fam20 protein in hydra is also Fam20B-like, which likely plays a similar role in regulating proteoglycan synthesis as its ortholog in higher animals. In contrast, the single Fam20 in C. elegans is a protein kinase like Fam20C. Consistently, only unphosphorylated xylose was detected in the tetrasaccharide linker of C. elegans CS proteoglycan. A close examination based on the information gained in this study suggests that the Fam20 proteins in other nematodes are also Fam20C-like, although some are still annotated as the Fam20B glycosaminoglycan xylosylkinase (such as the Fam20 in the parasitic worm Strongyloides ratti,
NCBI ID: CEF70981.1). Most higher animals encode both Fam20B and Fam20C in their genomes. For example, fruit fly (Drosophila melanogaster) has one copy each of Fam20B and Fam20C. Although their functions are not yet characterized, xylose phosphorylation within the tetrasaccharide linker region of CS and HS proteoglycans has also been demonstrated in Drosophila, and this activity can probably be ascribed to Fam20B. Sea urchin (Strongylocentrotus purpuratus) contains one Fam20B (NCBI protein ID: XP_011661062) and two Fam20C (XP_791445, XP_011661640). However, Fam20C appears to have been lost in tunicates such as Ciona intestinalis (Fig. 5a). Although the incomplete nature of the Ciona genome makes this observation less certain, another tunicate, Oikopleura dioica, also seems to have only Fam20B, and no Fam20C. In contrast, both Fam20B and Fam20C are present in other invertebrate chordates such as Branchiostoma (XP_002593700, XP_002660109) and Saccoglossus (XP_006822578, XP_002731199). Among vertebrates, the expansion of Fam20C is found in fish. For example, zebrafish has three copies of Fam20C (XP_509298166, XP_688892, XP_001345757) (Fig. 5a). Fam20A is also first found in fish13. In conjunction with our biochemical and structural observations, these phylogenetic analyses suggest that the monomeric Fam20B glycan kinase might have preceded the appearance of Fam20C in evolution. The appearance of Fam20C protein kinase is associated with dimer formation, in which the two protomers stabilize each other’s conformations. Fam20A is probably derived from Fam20C given their close sequence similarity, and has lost bona fide kinase activity but acquired a specific Fam20C-activator function.

In summary, we have solved a series of crystal structures, including that of the human Fam20A-Fam20C complex and a Fam20B ortholog in complex with its disaccharide substrate. Our results have provided a complete set of structural templates for the future study of this family of kinases, offered a deeper understanding of their regulation and substrate specificity, and revealed a unique example of protein evolution.

Methods

Cell culture. S21 and High Five cells, originally purchased from Invitrogen, were maintained in non-modified shaker at 27°C in the SIM SF medium and the SIM HF medium (Sino Biological Inc.), respectively. U2OS cells, originally purchased into plasmids by a PCR-based method, and the mutant proteins were purified by anion exchange chromatography (Resource 5), eluted using a 50–500 mM NaCl salt gradient in 20 mM Tris- HCl pH 8.0, followed by size exclusion chromatography (Superdex increase 200), eluted in 10 mM Hepes, pH 7.5, 100 mM NaCl. His6-MBP-hsFam20B was digested with TEV protease, and endoglycosidase H and then puriﬁed on N-linked glycans. Untagged and deglycosylated hmFam20 was then puriﬁed by cation exchange chromatography (Resource 5), eluted using a 50–500 mM NaCl salt gradient in 20 mM MES, pH 6.5; followed by size exclusion chromatography (Superdex 200 16/600), eluted in 10 mM Hepes, pH 7.0, 150 mM NaCl.

Crystallography. All crystals were grown at 20°C using the hanging drop or sitting drop vapour diffusion method. To crystallize the Fam20A–Fam20C complex, Fam20A was incubated with human Fam20C or drFam20C in the presence of 0.25 mM ATP on ice for 1 h, and then passed through a size exclusion column (Superdex increase 200). The purified complexes in 20 mM Hepes pH 7.5, 100 mM NaCl, and 0.25 mM ATP were then concentrated to about 6 mg per ml. The human Fam20A–Fam20C complex was crystallized in 2% 1,4-Dioxane, 0.1 M (NH4)2SO4, 0.1 M Bis-TRIS propane, pH 7.0, and 8% Polyethylene glycol monomethyl ether 2000. Apo hmFam20 was crystallized in 1.5 M NaH2PO4/K2HPO4, pH 6.9, 10 mM MgCl2, 5 mM AMP-PNP, and 2 mM Gal1-4Xylβ1-O-benzyl. All crystals were transferred into the crystallization solution plus 20% glycerol and flash-frozen in liquid nitrogen for data collection.

Data collection and structure determination. The crystal diffraction data were collected at the Shanghai Synchrotron Radiation Facility (beamline BL17U) and the National Facility for Protein Science Shanghai (beamline BL19U). The data were processed using HKL2000 (HKL Research). All structures were solved by the molecular replacement method using Phaser62, with the ceFam20 and hsFam20A structures (PDB IDs: 4KQA and T9WR34) as search models. The structural model was then manually built using Coot63 and refined using Phenix4. Five percent randomly selected reflections were used for cross-validation64. Final structures were validated with the MolProbity program in the Phenix and the wwpdb server46.

In vitro kinase assay. The kinase assays were performed as previously described65,66. Fam20C kinase assays were performed in 50 mM Hepes, pH 7.0, 60 mM NaCl, 10 mM MgCl2, 0.5 mg per ml BSA, and 100 μM [γ-32P]ATP (specific activity 5000 cpm per pmol) using OPN, casein, or human ENAM (173 cpm per pmol) as substrate. The kinase reactions were initiated by the addition of recombinant hsFam20C (40 nM), ceFam20 (40 nM), or a combination of hsFam20C (40 nM) and hsFam20A (40 nM); incubated for 10 min at 30°C, terminated by the addition of SDS-PAGE buffer plus 20 mM EDTA; and then boiled. The reaction mixtures were then separated by SDS-PAGE and visualized by Coomassie staining. Incorporation was detected by autoradiography. Uncropped images of gels and blots are shown in Supplementary Fig. 8.

Fam20B xylosylkinase assays were carried out in 50 mM Hepes, pH 7.5, 10 mM MnCl2, 100 μM Galβ1-4Xylβ1-O-benzyl, 100 μM [γ-32P]ATP (specific activity 5000 cpm per pmol) and 40% ammonium sulfate at 20°C for 20 min. Reactions were terminated with 40 mM EDTA and 10 mM ATP, and loaded onto Sep-Pac C18 cartridges ( Waters) pre-equilibrated with 0.2 M (NH4)2SO4. The columns were washed with 2 ml of 0.2 M (NH4)2SO4 three times, and the disaccharides were eluted with 1 ml methanol. Incorporated radioactivity was measured by liquid scintillation counting (Tri-Carb 2810TR, PerkinElmer).

Analytical ultracentrifugation. Sedimentation velocity experiments were carried out on a Beckman XL-1 Analytical Ultracentrifuge. Purified hsFam20C-hsFam20C (−0.8 mg per ml, 400 μl) in 20 mM Hepes, pH 7.5, 100 mM NaCl, with or without 1 mM ATP was spun at 50,310 × g for 20 h, and the 280 nm absorbance data were recorded. Data analysis was performed using SEDEX67.

Cell-based Fam20C activation assay. For co-expression experiments, U2OS cells were grown in a six-well plate format to ~50–50% confluency. Cells were co-transfected with 0.75 μg pCCF-Fam20C (WT or mutants), 0.75 μg pcDNA-Fam20A-HA (WT or mutants), and 1.5 μg pcDNA-OPN-V5 using 6 μl X-tremeGENE-9 (Roche). Conditioned media were harvested 40–48 h later, centrifuged at 3500 x g for 5 min to remove the cell debris, further cleared at 8000 x g for 30 min, and then exposed to immunoprecipitation. V5-tagged proteins were immunoprecipitated using anti-V5 rabbit (Millipore, AB3792) and protein-G agarose (Pierce, 20399), washed three times with PBS, and eluted with SDS loading buffer. To analyze the total intracellular proteins (total extract), cells were washed with PBS, lysed with 200 μl of SDS loading buffer, boiled, and then used for immunoblotting. Proteins were separated by SDS-PAGE, transferred to PVDF membranes, blocked in 5% milk, and probed with anti-FLAG M2 (mouse, Sigma, F3165), anti-V5 (mouse, Invitrogen, R690-25), and anti-HA (mouse, sigma, F1199).
Quantification and statistical analysis. The quantitative values obtained in the ligand screens were analyzed in Origin spreadsheets with the embedded basic statistical functions (mean, standard deviation).

Data availability. Atomic coordinates and structural factors have been deposited in the Protein Data Bank with accession codes 5YH3, 5YH2, 5XOM, and 5XO0 for Fam20A-Fam20C, Fam20A-drFam20C, drFam20C, hmFam20, and disaccharide-bound hmFam20, respectively. Other data are available from the corresponding author upon reasonable request.

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