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Exploring mechanisms of FGF signalling through the lens of structural biology

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Abstract

Fibroblast growth factors (FGFs) mediate a broad range of functions in both the developing and adult organism. The accumulated wealth of structural information on the FGF signalling pathway has begun to unveil the underlying molecular mechanisms that modulate this system to generate a myriad of distinct biological outputs in development, tissue homeostasis and metabolism. At the ligand and receptor level, these mechanisms include alternative splicing of the ligand (FGF8 subfamily) and the receptor (FGFR1–FGFR3), ligand homodimerization (FGF9 subfamily), site-specific proteolytic cleavage of the ligand (FGF23), and interaction of the ligand and the receptor with heparan sulphate cofactor and Klotho co-receptor.

Fibroblast growth factors (FGFs) are secreted protein ligands that act in a paracrine or endocrine fashion to carry out their pleiotropic functions in development, tissue homeostasis and metabolism. The mammalian FGF family comprises 18 ligands, which are grouped into five paracrine-acting subfamilies and one endocrine-acting subfamily on the basis of sequence homology and phylogenetic and structural analysis^{1,2} (FIG. 1a). FGF ligands signal through cell surface FGF receptor (FGFR) Tyr kinases, which are encoded by four distinct genes in mammals (*FGFR1–FGFR4*)³ (FIG. 1b). Ligand binding induces FGFR dimerization⁴ (FIG. 1c,d), which brings the intracellular receptor kinase domains in the correct proximity and orientation to each other such that transphosphorylation, and hence activation, of the kinases can occur^{5,6}. The activated receptor kinases, in turn, phosphorylate and activate their intracellular substrates, chief among which are FGFR substrate 2 α (FRS2 α) and phospholipase C γ 1 (PLC γ 1)^{7,8} (FIG. 2). Activated FRS2 α initiates downstream signalling through the RAS–MAPK pathway or the PI3K–AKT pathway, whereas the activation of PLC γ 1 leads to release of calcium ions from intracellular stores and activation of protein kinase C (PKC)^{7–11} (FIG. 2). Although the biological outcome of activation of these pathways by FGFs varies depending on the cellular context, the RAS–MAPK pathway mostly generates a mitogenic cell response, the PI3K–AKT pathway promotes cell survival and the PLC γ 1 pathway is thought to have a role in mediating cell motility^{7–11} (FIG. 2).

A common characteristic of FGFs is that they interact with heparan sulphate glycosaminoglycan chains of heparan sulphate proteoglycans¹², which are abundantly

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Competing interests statement

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present both at the cell surface and in the pericellular and extracellular matrix¹³ (FIG. 1b). Paracrine FGFs exhibit a much higher affinity for heparan sulphate than endocrine FGFs¹², and therefore, paracrine FGFs become immobilized in the pericellular and extracellular matrix near the site of their secretion and can only act on cells within the same organ. By contrast, owing to their poor affinity for heparan sulphate^{12,14}, endocrine FGFs can freely diffuse away from the cells secreting them and enter the blood circulation to reach their target cells in distant organs. Whereas paracrine FGFs require heparan sulphate as a cofactor to activate FGFR^{15,16} (FIG. 1c), endocrine FGFs rely on Klotho co-receptors to do so^{17–23} (FIG. 1b,d) (see below).

Paracrine FGFs have essential roles in virtually every step during embryonic development, including the induction and patterning of germ cell layers, the formation of body axes, the induction of organogenesis and morphogenesis and the patterning of tissues^{1,9,10,24}. These ligands also fulfil crucial functions in a number of homeostatic processes in the adult organism, most notably in the repair and remodelling of tissues^{25–28}. Endocrine FGFs regulate multiple metabolic processes, including phosphate, glucose and lipid metabolism^{29–32}. The broad range of FGF functions is reflected in the diversity of disorders caused by dysregulated FGF signalling, including developmental syndromes, metabolic disorders and cancer^{1,11,33,34}.

The structural information gained about the FGF signalling system in recent years has begun to illuminate the complexity of its molecular control, which is in place to fine-tune this system to enable it to produce specific signalling outputs in diverse biological contexts. This Review discusses, through the lens of structural biology, the molecular mechanisms by which the FGF signalling system is regulated extracellularly at the level of the ligand, the receptor, the heparan sulphate cofactor and the Klotho co-receptor. The specific mechanisms covered include autoinhibition of the ligand by homo-dimerization, ligand inactivation by site-specific proteolytic cleavage, modulation of the receptor-binding affinity of the ligand by alternative splicing and differences in ligand-induced receptor dimerization between different ligand–receptor pairs. We also discuss the mechanism by which Klotho co-receptors convert FGFRs into specific receptors for endocrine FGFs.

Regulation of FGF ligand activity

FGFs share a core homology domain of about 120 amino acids, which folds into a globular β -trefoil. Paracrine FGFs have a regular β -trefoil domain that is composed of 12 β -strands ($\beta 1$ – $\beta 12$)², whereas endocrine FGFs have an atypical β -trefoil due to the lack of the $\beta 11$ strand¹⁴. The conserved core domain is flanked by highly divergent amino-terminal and carboxy-terminal sequences, which have a key role in conferring distinct functional properties on FGFs^{35–39}. FGF activity is regulated by multiple mechanisms, including heparan sulphate binding, N-terminal alternative splicing, homodimerization and site-specific proteolytic cleavage of the FGF ligand. Among these, the interaction of FGFs with heparan sulphate is the most important in determining the biological activity of the ligand. It not only defines the mode of action of FGFs, that is, whether an FGF acts in a paracrine or endocrine fashion, but also contributes to the distinct biological activities of paracrine ligands within the same subfamily.

Interaction of FGF ligand with heparan sulphate

The interaction between heparan sulphate and paracrine FGFs serves, among other roles, to sequester ligand near the site of action, provide a reservoir for ligand storage, enhance ligand stability and limit the radius of ligand signalling. In this subsection, we discuss the emerging concept that differences in heparan sulphate-binding affinity among paracrine

FGFs, including ligands of the same subfamily, underlie the formation of different, ligand-specific gradients in the extracellular matrix, which produce distinct biological responses.

Heparan sulphate is a linear glycan composed of repeating disaccharide units of *N*-substituted glucosamine and glucuronic acid^{13,40}. *N*-sulphated domains of 5–10 disaccharide units in length that harbour various modifications alternate along the glycan chain with *N*-acetylated regions that are mostly unmodified^{13,40}. Modifications at *N*-sulphated domains include C5 epimerization of glucuronic acid to iduronic acid, 2-*O*-sulphation of glucuronic or iduronic acid and 3-*O*- and 6-*O*-sulphation of *N*-sulphated glucosamine (REFS 13,40). These modified sulphated domains are the principal binding sites for FGFs⁴⁰. Notably, the modification of heparan sulphate by sulphation and epimerization is regulated in a tissue-specific fashion, and hence different tissues can produce heparan sulphate chains with a distinct ‘fine structure’^{13,40}, which influences FGF activity.

Heparan sulphate mediates the formation of paracrine FGF gradients in the extracellular matrix^{36,41–43}, which determine cellular processes such as cell migration, cell proliferation and specification of cell fate during tissue morphogenesis and patterning in embryonic development. Recent studies on FGF7 subfamily ligands, which play essential parts in the branching morphogenesis of a number of glandular organs, have provided new insight into how these FGF gradients are formed and how the biological response to an FGF ligand is dictated by the shape of this gradient^{43–45}. FGF10 has greater binding affinity for heparan sulphate than its subfamily member FGF7 (REF. 43) (FIG. 3a). As a result, the diffusion of FGF10 in the extracellular matrix is more restricted than that of FGF7, and hence FGF10 forms a short, steep gradient close to its source, whereas FGF7 forms a long, shallow gradient spreading away from its source⁴³ (FIG. 3b). In explants of developing submandibular gland tissue, FGF10 causes elongation of epithelial gland buds, whereas FGF7 induces bud branching⁴³ (FIG. 3c,d). Reducing the heparan sulphate-binding affinity of FGF10 by replacing Arg187 in its heparan sulphate-binding site with Val, which is the corresponding residue in FGF7, converts FGF10 functionally into an FGF7-like morphogen⁴³ (FIG. 3a–c). By contrast, reducing the receptor-binding affinity of FGF10 affects the degree, but not the nature, of the morphogenetic activity of FGF10 (REF. 43). Thus, the differences in heparan sulphate-binding affinity between FGF7 and FGF10, which translate into differently shaped extracellular ligand gradients, alone account for the distinct morphogenetic activities of these ligands. Similarly, the aberrant activity of the FGF9 mutant carrying a N143T mutation⁴², a spontaneous dominant mutation causing Elbow knee synostosis (Eks) in mice⁴⁶, is due to altered heparan sulphate-binding affinity and hence altered gradient formation of the mutant FGF9 compared with wild-type ligand⁴².

The cells that secrete FGF ligand also produce heparan sulphate, the ‘fine structure’ of which is crucial for mediating the formation of an FGF gradient in the extracellular matrix. In addition, the ‘fine structure’ of the heparan sulphate produced by the cells that are responding to FGF is crucial for ligand signalling. This is suggested by the findings that abrogation of glycosaminoglycan biosynthesis selectively in mesenchymal cells of the developing eye, which secrete FGF10, leads to excessive diffusion of FGF10 through the extracellular matrix and, as a consequence, a lack of FGF10-induced budding of the prospective lacrimal gland epithelium⁴⁵. Moreover, the formation of the extracellular FGF10 gradient that results in induction of lacrimal gland budding requires heparan sulphate produced by the mesenchyme around the developing gland to be modified by *N*-sulphation but not 2-*O*- or 6-*O*-sulphation⁴⁵. By contrast, FGF10 signalling to the epithelium of the developing gland requires heparan sulphate produced by the epithelium to be modified by 2-*O*- and 6-*O*-sulphation⁴⁴. Thus, distinct modifications of heparan sulphate in the mesenchyme and epithelium may provide another layer of control for directional FGF signalling from the mesenchyme to the epithelium and vice versa (see below). In addition,

distinct hepa-ran sulphate modifications in different FGF-secreting cells, or in the same cell at different stages of embryonic development, may give rise to the formation of distinct gradients of specific FGF ligands, resulting in diverse biological responses.

Dimerization of FGF ligand

Unique among FGFs, ligands of the FGF9 subfamily undergo reversible homodimerization, which controls their biological activity^{36,38,42}. The crystal structures of the homodimers of FGF9 and FGF20 have illuminated the role of homodimerization as an autoinhibitory mechanism. In the structures, both the β -trefoil core and the N-terminal and C-terminal regions flanking the core participate in the dimer formation, with extensive interactions between the N-terminal and C-terminal regions of each monomer being the driving force^{36,38} (FIG. 4a). Notably, about half of the residues involved in ligand homodimerization are also predicted to mediate receptor binding^{36,38} (FIG. 4b). In other words, the ligand homodimer is incompatible with receptor binding, and hence the dimer must dissociate into monomers in order to enable ligand–receptor interactions. These structural findings suggest that homodimerization autoinhibits the activity of FGF9 subfamily ligands by downregulating the effective concentration of ligand monomer that can bind and activate the receptor. Consistent with this mechanism, mutations that disrupt contacts in the dimer interface of FGF9 or FGF20 cause a shift in the ligand monomer to dimer equilibrium in solution towards the monomeric ligand form³⁶. The mutated FGF9 and FGF20 ligands exhibit increased receptor binding and, as a consequence, increased biological activity compared with wild-type ligands³⁶. Notably, the extent of increased receptor binding caused by a dimer interface mutation correlates with the degree of ligand monomerization that is caused³⁶.

In addition to downregulating the concentration of monomeric ligand that is available to interact with receptors, homodimerization autoinhibits ligand activity by restricting the signalling radius of the ligand. The mutated FGF9 and FGF20 ligands, which have a reduced ability to homodimerize, also exhibit reduced binding affinity for heparan sulphate compared with wild-type ligands³⁶. As a result, the mutated ligands diffuse over a greater distance in the extracellular matrix than their wild-type counterparts and hence have a greater signalling radius than them³⁶. A recent study demonstrated the biological importance of homodimerization in the regulation of FGF9 activity⁴². This study identified an N143T mutation in FGF9 as the spontaneous mutation that causes a primarily skeletal phenotype in mice with Eks, a hallmark of which is the synostosis of elbow and knee joints^{42,46} (FIG. 4c). Replacement of Asn143, which lies in the FGF9 dimer interface, with Thr weakens FGF9 homodimerization⁴². As a consequence, the N143T mutant exhibits reduced binding affinity for heparan sulphate, and hence diffuses over a greater distance in developing tissue than wild-type FGF9 (REF. 42) (FIG. 4d). This hyperdiffusion leads to ectopic FGF9 signalling⁴², which underlies the Eks phenotype. These findings demonstrate that FGF ligand homodimerization enhances the binding affinity of ligand for heparan sulphate, thereby restricting ligand diffusion in the extracellular matrix and, as a consequence, limiting the signalling radius of the ligand. Taken together, ligand homodimerization autoinhibits the activity of FGF9 subfamily ligands at two levels: it inhibits ligand–receptor binding by occluding receptor-binding sites on the ligand, and it reduces the ability of the ligand to diffuse in the extracellular matrix by promoting ligand–heparan sulphate binding.

Alternative splicing of FGF ligand

Unlike other FGFs, the biological activity of ligands of the FGF8 subfamily is regulated by alternative splicing. The genes encoding FGF8 and FGF17, but not the *FGF18* gene, are alternatively spliced to generate ligand isoforms with N termini of varying length and sequence^{47–50}. In humans, there are four isoforms of FGF8 (FGF8a, FGF8b, FGF8e and

FGF8f)⁴⁸ and two isoforms of FGF17 (FGF17a and FGF17b) (FIG. 5a). The importance of N-terminal splicing in regulating the biological activity of these ligands has been demonstrated by studies on the roles of FGF8a and FGF8b in midbrain and hindbrain patterning. Both splice isoforms are expressed by the isthmus organizer, which is a signalling centre within the anterior neural plate that directs the patterning of midbrain and anterior hindbrain (the cerebellum)^{47,51}. When ectopically expressed in the neural plate of chick embryos, FGF8a induces an expansion of midbrain tissue into the fore-brain region, whereas FGF8b induces cerebellum formation in regions of prospective midbrain and caudal forebrain⁵¹ (FIG. 5b). Likewise, in mouse embryos, ectopic expression of FGF8a in the midbrain causes an overgrowth of midbrain tissue, whereas FGF8b transforms the midbrain into cerebellum^{52,53}. FGF8a and FGF8b also differ in their ability to induce mesoderm formation^{54,55}, and mutations at the spliced N-terminal region of FGF8 found in patients with idiopathic hypogonadotropic hypogonadism impair the biological activity of the affected isoforms⁵⁶. These findings illustrate the biological significance of N-terminal alternative splicing.

The crystal structure of FGF8b in complex with FGFR2c has unveiled the molecular mechanism by which N-terminal splicing regulates the biological activity of FGF8 (REF. 37) (FIG. 5c). In the structure, hydrophobic residues from the N-terminal G helix and the $\beta 4$ – $\beta 5$ loop of FGF8b bind to a hydrophobic groove in the D3 domain of FGFR2c³⁷. Notably, among the FGF8b residues that interact with the receptor groove, a single residue, Phe32, is from the isoform-specific N-terminal sequence³⁷. As FGF8a lacks this sequence, the number of hydrophobic contacts is reduced in an FGF8a–receptor complex as compared to an FGF8b–receptor complex, and hence FGF8a binds more weakly to receptors than FGF8b. Indeed, replacement of Phe32 with Ala in FGF8b reduces its receptor-binding affinity to a similar level to that of FGF8a³⁷, and converts FGF8b functionally into an FGF8a-like ligand³⁷ (FIG. 5b). Thus, N-terminal alternative splicing regulates ligand activity by modulating the binding affinity of ligand for receptor, and differences in receptor-binding affinity underlie the distinct biological activities of the FGF8 splice isoforms. It is likely that differences in receptor-binding affinity among the members of other ligand subfamilies also contribute to the distinct biological activities of those ligands.

Proteolytic cleavage of FGF ligand

Specific proteolytic processing of FGF ligands also regulates their activity. FGF23, which functions as a hormone that controls phosphate and vitamin D metabolism^{30,31}, is inactivated by proteolytic cleavage at the ¹⁷⁶Arg-Xaa-Xaa-Arg¹⁷⁹ motif (where Xaa represents any amino acid) that is located at the boundary between its β -trefoil core domain and its 72 amino acid long C-terminal tail^{57,58}. The Arg-Xaa-Xaa-Arg motif is recognized by proprotein convertases that cleave specifically at basic amino acid residues and belong to the family of subtilisin-like Ser endoproteases⁵⁹. These convertases cleave secretory proteins in subcellular organelles such as the Golgi complex, at the cell surface or in the extracellular matrix, and in most cases they convert a protein precursor into an active protein⁵⁹. The cleavage occurs C-terminally to the second Arg residue of the Arg-Xaa-Xaa-Arg motif⁵⁹, and can be inhibited by *O*-glycosylation of Ser or Thr residues within or adjacent to the cleavage site⁶⁰. Although the endoprotease that cleaves FGF23 is unidentified, recent studies have provided insight into the mechanism by which proteolytic cleavage inactivates FGF23 (REF. 35). It was shown that FGF23 binds to preformed binary complexes of FGFR and α -Klotho co-receptor³⁵. The ligand interacts with a *de novo* binding site that is generated at the composite receptor–co-receptor interface in these binary complexes³⁵. The region on FGF23 that binds to this *de novo* site was mapped to the 72 amino acid long C-terminal tail, which follows the β -trefoil core domain³⁵. Thus, the N-terminal fragment of proteolytic cleavage (Tyr25 to Arg179, which includes the β -trefoil

core domain) is metabolically inactive⁵⁷ because it lacks the binding site for the FGFR- α -Klotho complex. The C-terminal proteolytic fragment (Ser180 to Ile251), however, can compete with full-length FGF23 for binding to the FGFR- α -Klotho complex to antagonize the metabolic activity of FGF23, because this fragment contains the binding site for the FGFR- α -Klotho complex³⁵.

These findings suggest a dual mechanism by which proteolytic cleavage at the Arg-Xaa-Xaa-Arg motif inactivates FGF23; the cleavage removes the binding site for the FGFR- α -Klotho complex from FGF23 and concomitantly generates an endogenous inhibitor of FGF23. Inhibition of this proteolytic cleavage by missense mutations within the Arg-Xaa-Xaa-Arg motif in FGF23 leads to accumulation of full-length, bioactive FGF23, causing renal phosphate wasting disease in humans^{57,58,61}. Conversely, enhanced FGF23 cleavage due to impaired *O*-glycosylation of FGF23 leads to a deficit in full-length FGF23, which manifests as hyperphosphatemia and soft tissue calcification in humans^{62,63}. Therefore, specific proteolytic processing of FGF23 is essential for regulating the activity of this hormone. It is currently not known whether FGF19 and FGF21, the other two members of the endocrine FGF subfamily, undergo proteolytic processing in order to be inactivated.

Interestingly, ligands of the FGF7 subfamily contain an Arg-Xaa-Xaa-Arg cleavage motif in the N-terminal region preceding the β -trefoil core domain, and cultured mammalian cells secrete both full-length FGF7 ligand and the C-terminal fragment that results from proteolytic cleavage at the Arg-Xaa-Xaa-Arg motif⁶⁴. However, whether specific proteolytic cleavage of FGF7 subfamily ligands occurs *in vivo* and is crucial for regulating the activity of these ligands is currently unknown. Proteolytic processing may also have a role in limiting the activity of FGF8 subfamily ligands. This is suggested by the findings that high temperature requirement A1 (HTRA1), a *Staphylococcus aureus* V8-like Ser-endoprotease, cleaves FGF8, and that loss of *htra1* gene function in zebrafish embryos leads to an increase in *fgf8* gene expression and defects in dorsoventral patterning reminiscent of those caused by ectopic FGF8 signalling⁶⁵.

Regulation of FGFR function

A prototypical FGFR consists of three extracellular immunoglobulin-like domains (D1–D3), a single-pass transmembrane domain and an intracellular Tyr kinase domain² (FIG. 1b). The ectodomain region encompassing D2, D3 and the D2–D3 linker is necessary and sufficient for ligand binding² (FIG. 1b,c). Specificity of ligand binding by FGFR1, FGFR2 and FGFR3 is primarily determined by alternative splicing in the D3 domain of these receptors, which generates FGFRb and FGFRc isoforms^{37,39,66–72}. This splicing event is fundamental to the establishment of directional FGF signalling between epithelial and mesenchymal tissues. FGF–FGFR binding induces FGFR dimerization⁴, which juxtaposes the intracellular kinase domains of the receptors so that one kinase can phosphorylate and hence activate the other^{5,6}. Subtle differences in receptor dimerization are induced by distinct ligands, and these may translate into differences in receptor activation that yield distinct biological responses.

Alternative splicing in the D3 domain of FGFR

Ligand-binding specificity of FGFR1, FGFR2 and FGFR3 is regulated by alternative splicing in the D3 domain of these receptors^{37,39,66–72}. The N-terminal portion of the D3 domain is encoded by exon 7 (also known as exon IIIa), whereas the C-terminal portion is encoded by one of two mutually exclusive exons, exon 8 (also known as exon IIIb) or exon 9 (also known as exon IIIc)^{66,68,69}. This alternative splicing is largely tissue-specific, with the expression of the FGFRb splice isoform generally being restricted to epithelial cell lineages and the expression of the FGFRc splice isoform by and large limited to

mesenchymal cell lineages^{69,70,73}. These receptor splice isoforms exhibit a distinct ligand-binding specificity profile^{67,69–72}. The epithelial FGFRb isoforms specifically bind FGF ligands that are secreted from mesenchymal tissue and, conversely, the mesenchymal FGFRc isoforms preferentially interact with FGF ligands that are secreted from epithelial tissue⁷⁴. This reciprocal expression of receptor splice isoforms and cognate ligands creates specific paracrine FGF signalling loops between the epithelium and the mesenchyme, which are crucial for orchestrating developmental processes and also for regulating tissue homeostasis in the adult^{75–81} (FIG. 6a). In the developing lung, for example, bidirectional FGF signalling between the epithelial layer and the surrounding mesenchyme underlies the coordination of mesenchymal proliferation and epithelial branching that governs normal lung organogenesis. Specifically, FGF9 secreted by the epithelium stimulates proliferation of the mesenchyme⁷⁶, whereas FGF10 secreted by the mesenchyme acts on the epithelium to induce budding and branching^{78,79}. Notably, the reciprocal expression of receptor splice isoforms and cognate ligands not only establishes directional paracrine FGF signalling but safeguards against illegitimate autocrine FGF signalling.

The crystal structures of ligand-bound FGFR2b and FGFR2c splice isoforms have provided molecular insight into how alternative splicing in the D3 domain controls ligand-binding specificity^{37,39,82} (FIG. 6b–d). In the structure of FGF10 in complex with its cognate receptor, FGFR2b, FGF10 residues bind to a cleft in the D3 domain of FGFR2b³⁹. One side of this cleft is formed by the FGFR2b splice isoform-specific $\beta C'$ – βE loop. At the ligand–D3 domain cleft interface, unique hydrogen bonding contacts between Asp76 of FGF10 (a residue specific to FGF10 and other FGF7 subfamily ligands) and Ser315 of FGFR2b (a residue specific for FGFR2b) are crucial in determining the binding specificity of FGFR2b towards FGF10 (REF. 39) (FIG. 6b,c). The crucial role of the Asp76–Ser315 interaction for the ligand-binding specificity of FGFR2b is underscored by the finding that replacement of Ser315 in FGFR2b with Ala, the corresponding residue of FGFR2c, greatly reduces the ability of FGFR2b to bind FGF7 (REF. 83). FGF2, a member of the FGF1 subfamily, specifically binds to FGFR2c. The crystal structure of the FGF2–FGFR2c complex shows that, similar to the mode of FGF10 binding to the D3 domain of FGFR2b, FGF2 residues interact with the D3 domain cleft of FGFR2c, which is formed in part by the receptor isoform-specific $\beta C'$ – βE loop⁸² (FIG. 6d). As is the case for FGF10–FGFR2b binding, unique hydrogen bonding contacts between FGF2 and the $\beta C'$ – βE loop of FGFR2c determine binding specificity. These are the hydrogen bonds formed between Gln65 of FGF2, a residue unique to FGF2, and Asp321 of FGFR2c, an isoform-specific residue of the $\beta C'$ – βE loop of the receptor (FIG. 6d). Together, the structural data show that the alternative splicing in the D3 domain of FGFR2 confers ligand-binding specificity by changing the sequence of key ligand-binding residues or ligand-binding pockets.

Receptor mutations that disrupt ligand-binding specificity set by alternative splicing cause developmental skeletal disorders. The D321A mutation in FGFR2c, for example, causes Pfeiffer syndrome^{84,85}, an autosomal dominant disorder characterized by premature synostosis of cranial sutures. The mutation greatly reduces the binding affinity of FGFR2c for FGF2 and, at the same time, enables FGFR2c to interact with the non-cognate ligand FGF10⁸⁶. The loss in binding affinity of the mutant receptor for FGF2 can be explained by the loss of highly specific hydrogen bonding contacts between Asp321 in FGFR2c and Gln65 in FGF2 (REF. 82) (FIG. 6d). The concomitant gain in the ability of the mutant receptor to bind FGF10 is likely due to the loss of steric clashes and electrostatic repulsion between Asp321 of FGFR2c and Asp76 of FGF10, which preclude binding of FGF10 to the wild-type receptor. By disrupting ligand-binding specificity, the D321A mutation enables autocrine FGF signalling in the mesenchyme, and such illegitimate signalling in the developing cranial suture leads to craniosynostosis as seen in Pfeiffer syndrome. The effects of the mutation of key ligand-binding residues in the receptor, similarly to the D321A

mutation in FGFR2c, underscore the central role of alternative splicing in regulating the specificity of FGF signalling.

Ligand-induced dimerization of FGFR

Paracrine FGFs require heparan sulphate as a cofactor to robustly bind FGFR and promote the formation of a stable FGF–FGFR signal transducing complex at a ratio of 2:2 (REF. 4) (FIGS 1c,7a). In the complex, each ligand interacts with both receptors, and the two receptors also interact directly with each other. These multivalent ligand–receptor and receptor–receptor contacts are fortified by heparan sulphate, which simultaneously engages ligand and receptor in the complex. At the primary ligand–receptor interaction site, an extensive network of hydrogen bonds is formed between the ligand, the receptor D2–D3 linker and the receptor D3 domain^{4,37,39,82,87–89} (FIG. 7b,c). This hydrogen-bonding network confers structural rigidity on the interface between the ligand and the D3 domain and D2–D3 linker of the receptor, and its composition and geometry thus determine the orientation of the D3 domain and the D2–D3 linker in the receptor dimer. Comparative analysis of the crystal structures of FGF–FGFR complexes has identified three receptor residues and two ligand residues as key constituents of the hydrogen-bonding network (FIG. 7b). The receptor residues, an Arg residue in the D2–D3 linker and an Asp and a Gln residue in the $\beta\text{B}'$ – βC loop of the D3 domain, are conserved among mammalian FGFRs. The ligand residues, a Glu residue in the β8 strand and an Asp in the β9 strand, are nearly completely conserved among the mammalian FGFs. Importantly, these receptor and ligand residues also form entropically favourable intramolecular hydrogen bonds with similarly conserved residues. In ligand–receptor complexes containing this conserved core of hydrogen-bonding interactions at the interface between the ligand and the D3 domain and D2–D3 linker of the receptor, the orientation of the D3 domain and D2–D3 linker is similar. Accordingly, the distance between the C-terminal membrane insertion points of the D3 domains in a dimer of ligand-bound receptors is similar among these complexes (FIG. 7a). . This is the case for complexes of FGFR with FGF1 subfamily ligands or FGF10 (REFS 4,39,82,87–89).

By contrast, two of the key residues in the hydrogen- bonding network seen in these complexes are not conserved in FGF8 subfamily ligands, namely the Asp and a Tyr residue in the β9 strand. These substitutions, together with other divergent residues, account for an altered composition and geometry of the hydrogen-bonding network in the FGF8b–FGFR2c complex, and hence for a different orientation of the D3 domain and D2–D3 linker in comparison to the other FGF–FGFR complexes³⁷ (FIG. 7c). As a result of the different domain orientation, the C-terminal membrane insertion points of the D3 domains are closer to each other in a dimer of FGF8b-bound receptors than in the dimers of receptors bound to other FGF ligands³⁷ (FIG. 7a). Such differences in the spatial positioning of the receptor ectodomains among different FGF–FGFR complexes may translate into differences in the juxtapositioning of the intra-cellular receptor kinase domains and distinct signalling outputs. In the case of an FGF8 signalling complex, the kinase domains would be closer to each other than in other FGF signalling complexes. This might generate the more intense or persistent signal that might be required for FGF8 functions such as the organizer activity at signalling centres that drive brain and limb development.

Klotho co-receptors in FGF signalling

Endocrine FGFs depend on Klotho co-receptors for signalling because compared to paracrine FGFs, these ligands have intrinsically low binding affinity for both heparan sulphate and FGFR^{14,90}. Their low affinity for heparan sulphate enables these FGFs to signal in an endocrine fashion, whereas their low affinity for FGFR safeguards against nonspecific off-target signalling. Klotho co-receptors, which include α -Klotho, β -Klotho and γ -Klotho, are members of the Klotho subfamily^{91–94} of family 1 β -glycosidases^{95–97}.

Klotho co-receptors contain an extracellular domain composed of 1 (γ -Klotho) or 2 (α -Klotho and β -Klotho) β -glycosidase-like domains, a single-pass trans-membrane domain and a short cytoplasmic tail^{91–93} (FIG. 1b). α -Klotho is required for FGF23 signalling^{19,20,23} and β -Klotho is required for signalling by FGF19 and FGF21 (REFS 17,18,21,22). γ -Klotho may function as an additional co-receptor in FGF19 signalling⁹⁸, but *in vivo* evidence for such a role is lacking. Expression of α -Klotho and β -Klotho co-receptors is restricted to the target tissues of endocrine FGFs⁹⁸, where these co-receptors constitutively associate with cognate FGFRs of endocrine FGFs^{18,19,21,23}. When bound to α -Klotho or β -Klotho co-receptors, FGFRs exhibit high affinity for endocrine ligands^{18,19,21,23,35,90}. Although this role of α -Klotho and β -Klotho is fairly well established, recent findings have provided new insight into the mechanism by which these co-receptors convert FGFRs into specific receptors for endocrine FGFs⁹⁹.

Similarly to ligands of the FGF4, FGF8 and FGF9 subfamilies^{2,74}, α -Klotho and β -Klotho preferentially bind to the FGFRc splice isoforms of FGFR1, FGFR2 and FGFR3 (REFS 19,21,23,99,100). This raised the possibility that the binding site for α -Klotho and β -Klotho co-receptors on FGFR may overlap with the binding site for these paracrine FGFs. Indeed, it was shown that α -Klotho and β -Klotho engage the conserved hydrophobic groove in the D3 domain of FGFRc isoforms⁹⁹, which is also used by FGF8 subfamily ligands for receptor binding³⁷ (FIG. 5c). Thus, the complex formation of α -Klotho and β -Klotho co-receptors with FGFRc iso-forms and FGFR4, which also contains the hydrophobic groove in its D3 domain, obscures the binding site for FGF8 subfamily ligands and, as a result, inhibits signalling by these ligands⁹⁹. This mechanism is likely to be biologically relevant, as α -Klotho and β -Klotho co-receptors and FGF8 subfamily ligands are co-expressed in several adult tissues, such as the liver, kidney and mature ovarian follicles^{18,23,101–103}. Notably, α -Klotho and β -Klotho co-receptors are also co-expressed with other paracrine FGFs in adult tissues^{25,104} and might also inhibit signalling by these FGFs by binding to FGFRs at a site that overlaps with that of these ligands. Together, the findings support the concept of a dual mechanism by which α -Klotho and β -Klotho convert FGFRs into specific receptors for endocrine FGFs; these co-receptors not only enhance the binding affinity of FGFRs for endocrine ligands but concomitantly suppress the binding of paracrine FGFs, such as FGF8 subfamily ligands, to FGFRs. In essence, Klotho co-receptors modify the ligand-binding specificity of FGFRs in favour of endocrine FGFs. This co-receptor function probably evolved to dedicate FGFRs in target cells of endocrine FGFs exclusively to endocrine FGFs, avoiding any interference from paracrine FGFs.

Yet another role for Klotho co-receptors in endocrine FGF signalling is suggested by a recent study demonstrating that heparan sulphate is dispensable for the metabolic activity of endocrine FGFs⁹⁰. This study showed that endocrine FGF mutants devoid of residual heparan sulphate binding have the same capacity as the native ligands to activate FGFR in the presence of α -Klotho or β -Klotho co-receptor and to elicit metabolic effects *in vivo*⁹⁰. Thus, heparan sulphate is not a component of the endocrine FGF signal transduction unit (FIG. 1d), which in turn implies that Klotho co-receptors fulfil the same role in the formation of the endocrine FGF signalling complex as heparan sulphate does in the formation of the paracrine FGF signalling complex. This suggests that Klotho co-receptors promote FGFR dimerization upon endocrine FGF binding, which is required for FGFR kinase activation.

Conclusion and perspective

Recent advances have improved our understanding of the structural basis of FGF signalling and provided a glimpse at the complexity of molecular control that is in place for this essential signalling system. Importantly, the structural findings suggest that there may be no

functional redundancy among FGF ligands. This is supported by genetic studies that demonstrate that the gene knockout of one FGF ligand cannot be compensated for by any other member of the same FGF subfamily. For example, FGF10-deficient mouse embryos show agenesis of the lungs^{78,79}, aplasia of the salivary glands¹⁰⁵ and hypoplasia of the mammary glands¹⁰⁶, whereas mouse embryos lacking FGF7 exhibit defects in ureteric bud growth and branching in the developing kidney¹⁰⁷. The distinct phenotypes caused by these gene knockouts reflect that, although FGF7 and FGF10 are closely related members of the same subfamily and both branching morphogens, these ligands cannot substitute for one another. Perhaps even more strikingly, FGF7 and its subfamily member FGF22 both promote the differentiation of presynaptic terminals on dendrites of CA3 pyramidal neurons in the hippocampus¹⁰⁸. However, the two ligands promote the formation of functionally distinct synapses¹⁰⁸, and neither ligand can replace the other. Hence, future studies should be directed at identifying novel ligand-specific functions of FGF signalling.

Although it is established that Klotho co-receptors convert FGFRs into specific receptors for endocrine FGFs, a full understanding of the molecular basis for this functional conversion awaits the determination of the crystal structure of a ternary complex of endocrine FGF with FGFR and Klotho co-receptor. A ternary complex structure may also unveil how Klotho co-receptors promote FGFR dimerization upon endocrine FGF binding and provide clues as to why an endocrine FGF signalling complex sends a weaker signal than a paracrine FGF signalling complex^{18,23}. Importantly, this crystal structure would also offer a template for the structure-based design of endocrine FGF agonists or antagonists for the treatment of metabolic disorders.

Moreover, although it is known that paracrine FGFs cooperate with one another to direct developmental processes, it has yet to be explored how paracrine FGF signalling is integrated with endocrine FGF signalling to regulate metabolism. The latest discoveries on the roles of FGF1 and FGF21 in the dynamic remodelling and metabolic activity of adipose tissue may set the stage for future investigations in this direction. Both FGF1 and FGF21 are induced postprandially in gonadal white adipose tissue by the nuclear hormone receptor PPAR γ (peroxisome proliferator activated receptor- γ)^{26,109}, which is a major regulator of adipocyte differentiation and metabolic function¹¹⁰. FGF1 is required for the remodelling of adipose tissue to adjust to fluctuations in nutrient availability²⁶, and this process is influenced by FGF21 (REFS 109,111). Notably, as part of a positive feedback loop, FGF21 stimulates PPAR γ activity in adipocytes¹⁰⁹, raising the intriguing possibility that FGF21 regulates FGF1 signalling in adipose tissue through PPAR γ . Nuclear hormone receptor signalling might therefore serve as a mechanism that integrates the functions of endocrine FGFs and paracrine FGFs in the control of metabolic homeostasis.

Lastly, key questions remain in regard to the molecular basis of FGF signal transduction specificity. For example, what determines the speed, extent and duration of transphosphorylation of FGFR kinases in a ligand-bound FGFR dimer? Moreover, how do the speed, extent and duration of kinase transphosphorylation contribute to the generation of distinct signalling outputs? Future studies addressing these questions and more are poised to unravel new layers of molecular control, which operate to fine-tune FGF signalling. Answering these questions may not only advance our understanding of how FGFs generate specific signalling outputs in enormously diverse biological contexts but also pave the way for new treatments for disorders caused by dysregulated FGF signalling.

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Glossary

Paracrine	Refers to a mode of signalling in which the cell responding to a signalling molecule is near the cell secreting the molecule
Endocrine	Refers to a mode of signalling in which the cell responding to a signalling molecule is far away from the cell secreting the molecule
Transphosphorylation	The process by which one kinase molecule in a kinase dimer phosphorylates the other (also referred to as autophosphorylation)
Proteoglycans	Protein–glycan conjugates that consist of a core protein to which one or more glycosaminoglycan chains are attached
Morphogenesis	Process of cell movement during embryonic development that controls the size, shape and patterning of tissues and organs
β-trefoil	Portion of a protein that consists of 12 β -strands arranged into 3 similar sets of 4-stranded β -sheets
Epimerization	Process by which an epimer of a molecule is converted into its stereoisomeric counterpart
Submandibular gland	Gland located under the mandible bone that secretes saliva into the mouth
Elbow knee synostosis	(Eks). Mouse skeletal phenotype characterized by bone fusion at elbow and knee joints
Lacrimal gland	Gland of the eye that produces tear fluid to keep the eye lubricated
Idiopathic hypogonadotropic hypogonadism	Hereditary disorder characterized by the failure of sexual maturation and infertility due to deficiency of gonadotropin-releasing hormone
Renal phosphate wasting disease	Inherited or acquired condition characterized by excessive renal excretion of phosphate due to impaired tubular reabsorption
Hyperphosphatemia	Increase in blood phosphate levels above normal

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Figure 1. The FGF signalling system

a | The 18 mammalian fibroblast growth factor (FGF) ligands are listed, grouped by subfamily and mode of action. The ligand which each subfamily is named after is boxed in orange. The crystal structures of FGF2, a prototypical paracrine FGF (Protein Databank identifier (PDB ID): 1FQ9)⁴, and FGF19, an endocrine FGF (PDB ID: 2P23)¹⁴ are shown. The conserved globular core domain consists of 12 β-strands in FGF2 and 11 β-strands in FGF19. Residues from the loop connecting β-strand 1 and β-strand 2 and from the region between β-strand 10 and β-strand 12 comprise the heparan sulphate-binding site (shown in blue). The conformation of the FGF19 heparan sulphate-binding site differs from the conserved conformation of the heparan sulphate-binding site in paracrine FGFs, which are represented here by FGF2. **b** | The domain architecture of a prototypical FGF receptor and Klotho co-receptor are shown. Alternative splicing in the D3 domain of FGFR1, FGFR2 and FGFR3 generates FGFRb and FGFRc isoforms with distinct ligand-binding specificity^{37,39,6672}. FGFRs interact with cell surface heparan sulphate via their D2 domain. Heparan sulphate is the obligatory cofactor for paracrine FGF signalling. A schematic of an

heparan sulphate proteoglycan and the crystal structure of an heparan sulphate octasaccharide (PDB ID: 1FQ9)⁴ are shown. **c** | The crystal structure of the 2:2:2 ternary complex of FGF2, FGFR1c and heparan sulphate (PDB ID: 1FQ9)⁴ as well as a schematic of the paracrine FGF signal transduction unit based on this structure are shown. In the ternary complex, each FGF ligand interacts extensively with one receptor through the primary ligand-binding site comprising D2, D3 and the D2 D3 linker of the receptor. Each ligand also binds to the adjoining receptor in the complex through a secondary ligand-binding site on the D2 domain, which is adjacent to the site mediating the receptor receptor interaction. Heparan sulphate promotes the formation of the 2:2 paracrine FGF FGFR complex through concomitant interaction with both ligand and receptor. **d** | A schematic of two working models for the endocrine FGF FGFR Klotho co-receptor signal transduction unit is shown. A recent study on the ternary complex formation between FGF21, FGFR1c and β -Klotho supports the 1:2:1 model¹¹² rather than the 2:2:2 model. AB, acid box; TMD, transmembrane domain. The FGF19 structure in part **a** is reproduced, with permission, from REF. 14 © (2007) American Society for Microbiology.

Figure 2. FGF signalling pathways

Binding of fibroblast growth factor (FGF) to the FGF receptor (FGFR) induces FGFR dimerization, which juxtaposes the intracellular Tyr kinase domains of the receptors so that kinase activation by transphosphorylation can occur^{5,6}. Activated FGFR kinase in turn activates its intracellular substrates by phosphorylation, setting in motion distinct but potentially interactive signalling pathways that generate diverse cellular responses⁹¹¹. Major substrates of FGFR kinase are FGFR substrate 2α (FRS2α), which is constitutively associated with the receptor kinase, and phospholipase Cγ1 (PLCγ1)^{7,8}. Activated FRS2α binds the adaptor protein growth factor receptor-bound 2 (GRB2)¹¹³. GRB2 then recruits either the guanine nucleotide exchange factor son of sevenless (SOS) (a) or the adaptor protein GRB2-associated binding protein 1 (GAB1) (b) to the signalling complex. Recruited SOS activates RAS GTPase, which initiates activation of the MAPK cascade¹¹³ (a), whereas recruited GAB1 leads to PI3K-mediated activation of AKT kinase (also known as protein kinase B)¹¹⁴ (b). Activated MAPK translocates from the cytoplasm to the nucleus, where it phosphorylates and hence activates immediate early gene transcription factors, such as FOS to induce transcription of specific genes (a). The outcome of this pathway is primarily cell proliferation but can also lead to cell differentiation, cell migration or another cellular response⁹¹¹. Activated AKT kinase, on the other hand, inactivates pro-apoptotic effectors such as the BCL-2 antagonist of cell death (BAD) and forkhead box class O (FOXO) transcription factors, thereby promoting cell survival^{115,116} (b). Recruitment and phosphorylation of PLCγ1 by FGFR kinase initiates a distinct signalling pathway that is thought to have roles in cell migration and cell differentiation and that can influence the RAS–MAPK and PI3K–AKT pathways (c). Activated PLCγ1 catalyses the hydrolysis of the membrane phospholipid phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)(P)₂) into diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP₃)⁸. DAG activates protein kinase C (PKC), which in turn activates its substrates by phosphorylation, including the myristoylated Ala-rich C kinase substrate (MARCKS), a regulator of cell motility¹¹⁷. IP₃ stimulates the release of calcium ions from intracellular stores, and this triggers the activation of calcium-dependent proteins such as the phosphatase calcineurin. Activated calcineurin induces nuclear translocation of the transcription factor nuclear factor of

activated T cells (NFAT), which stimulates the expression of proteins essential for cell motility¹¹⁸. BAX, BCL-2-associated X protein; MAPKK, MAPK kinase; PDK, phosphoinositide-dependent protein kinase.

Figure 3. Heparan sulphate-binding affinity determines the morphogenetic activity of FGF7 subfamily ligands

a | Structural model of heparan sulphate binding to fibroblast growth factor 7 (FGF7) and FGF10. FGF7 (Protein Databank identifier (PDB ID): 1QQK)¹¹⁹ and FGF10 (PDB ID: 1NUN)³⁹ were superimposed onto FGF2 in the FGF2–FGFR1c–heparan sulphate complex (PDB ID: 1FQ9)⁴. The heparan sulphate octasaccharide from this complex and the α -carbon traces of the heparan sulphate-binding regions of FGF7 (orange) and FGF10 (teal) are shown. Note that Arg187 of FGF10, a crucial residue for heparan sulphate-binding affinity, makes hydrogen bonds (dashed black lines) with the heparan sulphate octasaccharide, whereas Val174, the corresponding residue of FGF7, cannot interact with the cofactor. **b** | Diffusion profiles of FGF10, FGF7 and an FGF10 mutant with reduced heparan sulphate-binding affinity (FGF10^{R187V}) in the extracellular matrix. Heparan sulphate beads loaded with fluorescently labelled FGF ligand were embedded in a laminin-rich hydrogel (also known as Matrigel), and ligand diffusion was monitored by measuring fluorescence intensity along a line passing through the centre of the bead. The dashed circles indicate the radii of ligand diffusion; FGF10 forms a short, steep gradient, FGF7 forms a long, shallow gradient and the FGF10 mutant (FGF10^{R187V}) diffuses similarly to FGF7. **c** | Epithelial growth response of developing submandibular gland tissue to FGF10, FGF7 and FGF10^{R187V} mutant. Explants of prebranched submandibular gland epithelial buds were embedded in a Matrigel matrix and exposed to FGF ligand. FGF10 causes elongation of the epithelial gland buds, whereas FGF7 and FGF10^{R187V} induce bud branching. **d** | Model for the regulation of branching morphogenesis by paracrine FGFs with different heparan sulphate-binding affinity. An FGF ligand with high binding affinity for heparan sulphate has a restricted diffusion range in the extracellular matrix (red sector), and hence can only signal to cells (red circles) closest to the FGF source (black asterisk). This leads to elongation of an epithelial gland bud towards the FGF source. Conversely, an FGF with low heparan sulphate-binding affinity can diffuse over a greater distance in the extracellular matrix, reaching cells further from the FGF source. The activation of cells both close to and distant from the FGF source results in branching of an epithelial gland bud. Image in part **a** is

modified and the images in parts **b**, **c**, and **d** are reproduced, with permission, from REF. 43
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Figure 4. Autoinhibition of FGF9 by homodimerization

a | The structure of the crystallographic fibroblast growth factor 9 (FGF9) homodimer (Protein Databank identifier (PDB ID): 1IHK)³⁸. The dimer interface can be divided into two regions (black circles), one containing residues of the β -trefoil core domain and the other containing the amino-terminal and carboxy-terminal regions flanking the core domain. Extensive hydrophobic and hydrogen-bonding contacts between the N-terminal and C-terminal regions of the two monomers in the dimer drive homodimerization. Note that Asn143, which is spontaneously mutated in mice with Elbow knee synostosis (Eks), is located in the core region of the dimer interface. **b** | The molecular surface of an FGF9 monomer (dark yellow; PDB ID: 1IHK)³⁸ is shown with residues that exclusively mediate ligand dimerization depicted in light blue, and residues that mediate dimerization and are also predicted to bind FGF receptors shown in dark blue. Note that the bifunctional residues account for about half of the residues that form the ligand dimer interface (red boundary). **c** | Joint synostosis in developing limb tissue caused by the N143T mutation leading to Eks. Forelimbs of a newborn mouse with Eks and a newborn wild-type mouse are shown. The elbow joints (marked by arrows) are also shown at greater magnification. In the forelimb of the mouse with Eks, humerus (h) and radius (r) bones are fused together at the prospective elbow joint, and humerus and ulna (u) bones are barely separated by cartilaginous joint tissue. **d** | Hyperdiffusion of the FGF9 mutant that causes Eks in developing limb tissue in mice. Beads loaded with FGF9 or the FGF9^{N143T} mutant were implanted into forelimb buds of an *Fgf9*^{-/-} embryo around the initiation stage of joint synostosis, and ligand diffusion was detected immunochemically. As FGF9^{N143T} exhibits reduced binding affinity for heparan

sulphate, it diffuses further than wild-type FGF9. s, scapula. The image in partc is reproduced, with permission, from REF. 46 © (2002) Springer, and the image in partd is reproduced, with permission, from REF. 42 © (2009) Macmillan Publishers Limited. All Rights Reserved.

Figure 5. N-terminal alternative splicing regulates the biological activity of FGF8

a | Sequence alignment of the amino-terminal regions of the mature human fibroblast growth factor 8 (FGF8) splice isoforms. Residues that make up the secondary structure elements known for FGF8b (the N-terminal G helix and the $\beta 1$ strand) are indicated. Phe32 and Val36 of the FGF8b G helix (shaded orange) are key residues that interact with the D3 domain of FGF receptor (FGFR) c isoforms. Mutations at Pro26 and Phe40 of FGF8f (shaded grey) have been associated with idiopathic hypogonadotropic hypogonadism in humans⁵⁶. **b** | FGF8 splice isoforms possess distinct abilities to transform midbrain (Mid) into cerebellum (Cb) in chick embryos. Shown are dorsal views of developing chick brains transfected on the right side (represented by green fluorescence in the inset in the first image) with plasmids encoding FGF8a, FGF8b or FGF8b^{F32A} or empty vector (control). The asterisk to the right of the midline of the brain (dashed red line) marks transformation of midbrain into cerebellum, and the arrows point to the expansion of midbrain tissue. FGF8b, but not FGF8a, is able to transform midbrain into cerebellum, and mutation of Phe32 to Ala in FGF8b abrogates this isoform-specific patterning ability. **c** | Crystal structure of the FGF8b–FGFR2c complex (Protein Databank identifier: 2FDB)³⁷. A view of the whole structure and a close-up view of the ligand receptor D3 domain interface are shown. Phe32 and Val36 from the G helix and Phe93 from the $\beta 4$ – $\beta 5$ loop of FGF8b engage a hydrophobic groove in the D3 domain of FGFR2c (coloured yellow). Note that Phe32 is the sole isoform-specific residue of FGF8b that interacts with the receptor groove. Residues that are specific to the FGFRc splice isoforms of FGFR1, FGFR2 and FGFR3 mainly form this hydrophobic

groove, thus the groove is unique to the FGFRc isoforms. Note that Klotho co-receptors also engage this receptor groove. Di, diencephalon; Tel, telencephalon. The image in part **b** is reproduced, with permission, from REF. 37 © (2006) Cold Spring Harbor Laboratory Press.

Figure 6. Alternative splicing confers ligand-binding specificity on FGFR

a | Fibroblast growth factor (FGF) ligands secreted from mesenchymal tissue exhibit binding specificity for epithelial FGF receptor (FGFR) b isoforms, whereas ligands secreted from epithelial tissue specifically bind to mesenchymal FGFRc isoforms. These paracrine FGF signalling loops are essential for controlling developmental processes⁷⁵⁸¹. **b** | D3 domain sequence alignment of FGFR2b and FGFR2c. The location of the β -strands in the D3 domain of FGFR2c are indicated by brackets, and the dashed line across the alignment marks the junction between the common amino-terminal portion and the spliced carboxy-terminal portion of the D3 domain. Isoform-specific residues of the receptor $\beta C'$ - βE loop that are crucial in determining ligand-binding specificity are shaded orange. Boxes indicate the positions of mutations in FGFR2c that cause skeletal disorders in humans. **c** | The ligand-receptor D3 domain interface in the FGF10-FGFR2b structure (Protein Databank identifier (PDB ID): 1NUN)³⁹ is shown, along with a view of the whole structure. The ligand is coloured orange, the receptor D3 domain is coloured green (common N-terminal

portion) and teal (alternatively spliced C-terminal portion). A network of hydrogen bonds (dashed lines) is formed between FGF10 and the isoform-specific $\beta C'$ – βE loop of FGFR2b. Within this network, the hydrogen bonds between Asp76 of FGF10 (a residue unique to FGF10 and other FGF7 subfamily ligands) and Ser315 of FGFR2b (an isoform-specific residue of the $\beta C'$ – βE loop) determine binding specificity. The hydrogen-bonding network is buttressed by hydrophobic contacts between Phe146 of FGF10 and Ile317 of FGFR2b.^d | The ligand–receptor D3 domain interface in the FGF2–FGFR2c structure (PDB ID: 1EV2)⁸² is shown. Ligand and receptor are coloured as in part c. Among the hydrogen bonds (dashed lines) that are formed at the ligand–receptor $\beta C'$ – βE loop interface, unique bonds between Gln65 of FGF2 and Asp321 of FGFR2c dictate binding specificity. Mutation of Asp321 to Ala, which causes Pfeiffer syndrome in humans, overrides the binding specificity of FGFR2c by eliminating steric clashes and electrostatic repulsion between Asp76 of FGF10 and Asp321 of FGFR2c. This enables FGF10 to bind to the mutant receptor and signal illegitimately through FGFR2c. Hydrophobic contacts between Tyr82, Val97 and Phe102 of FGF2 and Val317 of FGFR2c strengthen the hydrogen bonding interactions at the ligand receptor $\beta C'$ – βE loop interface.

Figure 7. Ligand-dependent differences at the FGF–FGFR interface differentially regulate FGFR dimerization

a | Superimposition of a 2:2 fibroblast growth factor 8b–FGF receptor 2c (FGF8b–FGFR2c) complex onto a 2:2 FGF2 – FGFR2c complex. The α -carbon traces of the complexes are shown. For the FGF8b–FGFR2c complex model, FGF8b from the FGF8b–FGFR2c structure (Protein Databank identifier (PDB ID): 2FDB)³⁷ was superimposed onto each of the two FGF2 ligands in the 2:2:2 FGF2–FGFR1c–heparan sulphate complex (PDB ID: 1FQ9)⁴. The FGF2–FGFR2c model was created in a similar manner using FGF2 from the FGF2–FGFR2c structure (PDB ID: 1EV2)⁸². Note the differences in the orientation of the receptor D3 domain and D2–D3 linker between the two dimers of ligand-bound FGFR2c, which translate into differences in the spatial distance between the carboxy-terminal membrane insertion points of the D3 domains. **b** | The interface between the ligand and the D3 domain and D2–D3 linker of the receptor in the FGF2–FGFR2c structure (PDB ID: 1EV2)⁸² is shown. The ligand is coloured orange, the receptor is coloured green (D2–D3 linker and common amino-terminal portion of the D3 domain) and teal (alternatively spliced C-terminal portion of the D3 domain). The network of hydrogen bonds (dashed lines) formed at this interface is highly conserved among complexes of FGFR with FGF1 subfamily ligands or FGF10. Its key constituents comprise Glu105 and Asn113 of FGF2 and Arg251, Asp283 and Gln285 of FGFR2c. Most of these residues also form entropically favourable intramolecular hydrogen bonds with similarly conserved residues, such as Glu105 and Asn113 of FGF2 with Tyr115 of FGF2. **c** | The interface between the ligand and the D3 domain and D2–D3 linker of the receptor in the FGF8b–FGFR2c structure (PDB ID: 2FDB)³⁷ is shown. Ligand and receptor are coloured as in part **b**. The composition and geometry of the network of hydrogen bonds (dashed lines) formed at this interface differ from that observed in the FGF2–FGFR2c complex (see, part **b**). For example, Asn113 and Tyr115 of FGF2 are replaced with Thr and Leu, respectively, in FGF8b. The side chain of

Glu131 in FGF8b, the corresponding residue to Glu105 in FGF2, adopts a different rotamer conformation compared to that in the FGF2–FGFR2c complex as it makes an intramolecular hydrogen bond with Lys176, a residue specific to FGF8 subfamily ligands. A total of four hydrogen bonds unique to the FGF8b–FGFR2c complex (dashed red lines) are formed at this interface between FGF8b and FGFR2c. Images in parts **b** and **c** are modified, with permission, from REF. 37 © (2006) Cold Spring Harbor Laboratory Press.