Genetic insights into the mechanisms of Fgf signaling

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The fibroblast growth factor (Fgf) family of ligands and receptor tyrosine kinases is required throughout embryonic and postnatal development and also regulates multiple homeostatic functions in the adult. Aberrant Fgf signaling causes many congenital disorders and underlies multiple forms of cancer. Understanding the mechanisms that govern Fgf signaling is therefore important to appreciate many aspects of Fgf biology and disease. Here we review the mechanisms of Fgf signaling by focusing on genetic strategies that enable in vivo analysis. These studies support an important role for Erk1/2 as a mediator of Fgf signaling in many biological processes but have also provided strong evidence for additional signaling pathways in transmitting Fgf signaling in vivo.

The fibroblast growth factor (Fgf) family of signaling proteins includes 22 members that have been identified based on sequence homology. Eighteen of these Fgfs function as ligands, which bind four receptor tyrosine kinases (RTKs) in mice and humans. The four remaining Fgfs [Fgf11–14] are intracellular proteins that do not interact with Fgf receptors [Fgfrs] (Smallwood et al. 1996; Olsen et al. 2003). A fifth Fgfr-like protein [FgfrL1] has also been identified that lacks an intracellular tyrosine kinase domain and likely negatively regulates Fgfrs by sequestering ligand (for review, see Trueb et al. 2013). Fgf signaling is required throughout metazoans and is commonly studied in organisms ranging from cnidarians to humans (Tulin and Stathopoulos 2010). These studies indicate that Fgf signaling is required pleiotropically during development and also regulates multiple homeostatic and reparative functions in adults (Ornitz and Itoh 2015). Additionally, pathological activation of Fgfrs underlies many congenital disorders and cancer types. Several therapeutic strategies are currently being developed to modulate Fgfr signaling in various pathologies (Carter et al. 2015; Degirolamo et al. 2016). Understanding the mechanisms that govern Fgf signaling is therefore important to appreciate many aspects of Fgf biology and disease.

Many of the developmental functions of Fgf signaling seem to be conserved between mice and humans. This is evident by the striking phenotypic similarities between human congenital disorders caused by alterations in Fgf signaling and their corresponding mouse models. Conserved developmental requirements have been demonstrated in skeletal growth, palate closure, limb patterning, ear development, cranial suture ossification, neural development, and the hair cycle [Hebert et al. 1994; Rousseau et al. 1994; Shiang et al. 1994; Wilkie et al. 1995; Partanen et al. 1998; Chen et al. 1999; Li et al. 1999; Wang et al. 1999, 2005; Dode et al. 2003; Tsai et al. 2005; Gill and Tsai 2006; Mason 2007; Riley et al. 2007; Falardeau et al. 2008; Mansour et al. 2009; Stanier and Pauws 2012; Simonis et al. 2013; Higgins et al. 2014; Ornitz and Marie 2015]. These conserved developmental functions and accessible genetics make the mouse an excellent model for studying the mechanisms that Fgf signaling uses in vivo, which we discuss in this review. Valuable information pertaining to Fgf signaling has also been gained from studies of invertebrate organisms, Xenopus, and zebrafish, which have been reviewed elsewhere [Huang and Stern 2005; Itoh 2007; Dorey and Amaya 2010].

Ligand binding specificity

Ligand binding represents the first step in initiating the Fgfr signaling cascade. Fgfrs contain three extracellular immunoglobulin-like domains [Igl–IgIII] with an eight-residue acid box in the linker region between Igl and IgII [Lee et al. 1989]. Igl and the acid box play an inhibitory role in ligand–receptor complex formation [Kalinina et al. 2012], while IgII and IgIII cooperate in ligand binding. In Fgfr1–3, ligand binding specificity is largely determined by alternative splicing of the C terminus of the IgIII domain, which is encoded by either exon 8 or 9 to generate...
the Fgfrb or Fgfrc isoform (Fig. 1A, Johnson et al. 1991; Chellaiah et al. 1994; Ornitz et al. 1996; Zhang et al. 2006). These b and c isoforms are generally restricted to epithelial and mesenchymal tissues, respectively. In this way, alternative splicing of the receptors allows ligands to activate receptors in the adjacent mesenchymal or epithelial tissue without activating autocrine signaling (Fig. 1B,C; Miki et al. 1992; MacArthur et al. 1995; Min et al. 1998; Xu et al. 1998b). However, there are several exceptions to this general principle of paracrine signaling, as some biological processes depend on ligand–receptor interactions within the same tissue. For example, mesenchymal Fgfb influences development of both the epithelium and mesenchyme during lung development (del Moral et al. 2006; White et al. 2006). Additionally, Fgf20 is required in an autocrine fashion during development of the kidney and organ of Corti (Barak et al. 2012, Huh et al. 2012). Finally, a recent study has demonstrated that Fgfl0, expressed in the lung mesenchyme, engages Fgfr1b and Fgfr2b in the same tissue during the formation of lipofibroblasts (Al Alam et al. 2015). Proper splicing of the Fgfrb isoforms is achieved by a splicing complex that includes the epithelial-specific

Figure 1. Fgfr alternative splicing facilitates interactions between epithelial and mesenchymal tissues. (A) Alternative splicing of exons 8 and 9 generates b and c isoforms of Fgfr1–3, while exon 10 encodes an invariant transmembrane domain. (B-C) Fgf ligands expressed in epithelium engage Fgfrb isoforms in the adjacent mesenchyme (B), while ligands expressed in the mesenchyme activate Fgfrb isoforms in the epithelium (C). Paracrine signaling also depends on the heparan sulfate proteoglycan (HSPG) coreceptor. (D) Endocrine Fgf ligands use Klotho coreceptors rather than HSPGs. (Ex) exon, (TK) tyrosine kinase. The exon, Fgfr isoform, and cell type specificity are color coded, with blue and green representing epithelium and mesenchyme, respectively.

Esrp1 and Esrp2 RNA-binding proteins [Warzecha et al. 2009]. Accordingly, combined genetic ablation of Esrp1 and Esrp2 leads to aberrant splicing of Fgfr1–3b in vivo and causes defects in multiple epithelial contexts that require Fgf signaling (Bebee et al. 2015). Genetic disruption of specific b and c isoforms individually has demonstrated that Fgfr1 and Fgfr3 primarily function in the mesenchyme, while Fgfr2 is more important in epithelial contexts (Partanen et al. 1998; De Moerlooze et al. 2000; Hajihosseini et al. 2001; Eswarakumar et al. 2002; Zhang et al. 2004; Eswarakumar and Schlessinger 2007). However, each receptor also possesses functions in the reciprocal cell type.

Heparan sulfate proteoglycans (HSPGs) also regulate multiple properties of Fgf ligands and receptors [Ornitz 2000]. These cell surface and extracellular matrix macromolecules are composed of a protein core to which heparan sulfate (HS) glycosaminoglycan (GAG) disaccharide polymers are added [Nelson and Cox 2005]. HS molecules are differentially O- or N-sulfated in a tissue-specific manner, and these sulfation patterns facilitate distinct ligand–receptor associations [Guimond et al. 1993; Pye et al. 1998; Allen and Rapraeger 2003; Qu et al. 2011]. HSPG affinity influences ligand dispersal to shape morphogen gradients [Harada et al. 2009; Makarenkova et al. 2009; Qu et al. 2012]. These HS chains can also be cleaved to spread ligand between cells or release ligand sequestered by the extracellular matrix [Patel et al. 2007; Shimokawa et al. 2011].

The FGF19 subfamily lacks the ability to bind HSPGs, enabling them to escape the HS-rich cell surface and function as endocrine hormones (Fig. 1D; Itoh et al. 2015). The endocrine subfamily of Fgf ligands regulates multiple processes in the adult, including phosphate homeostasis, adipocyte metabolism, and bile acid synthesis [Shimada et al. 2004; Inagaki et al. 2005; Kharitonenkov et al. 2005; Schoenberg et al. 2011]. Two homologous proteins, Klotho and βklotho, serve as coreceptors in place of HSPGs to facilitate ligand–receptor interactions [Kurosu et al. 2006; Urakawa et al. 2006; Ogawa et al. 2007]. Recent studies suggest that modulating the homeostatic functions of Fgf signaling may be of therapeutic value in multiple pathologies [Degirolamo et al. 2016].

Ligand–receptor binding affinities are therefore determined by multiple properties, including alternative splicing of the receptor, the presence of specific HSPG modifications, and the expression of Klotho coreceptors. Several fundamental studies have determined each ligand’s receptor specificity in vitro using mitogenic assays or by directly measuring complex affinities [MacArthur et al. 1995; Ornitz et al. 1996; Kurosu et al. 2006; Olsen et al. 2006; Zhang et al. 2006; Ogawa et al. 2007]. A comprehensive review of ligand–receptor binding specificities has been discussed recently elsewhere [Ornitz and Itoh 2015].

Fgfrs function individually and in combination

All of the Fgf ligands and receptors have been genetically knocked out in mice, producing phenotypes at virtually every stage of life, from the preimplantation blastocyst...
to the adult organism. The phenotypes caused by genetic disruption of the Fgf ligands have been extensively reviewed elsewhere [Ornitz and Itoh 2015]. Fgfr knockout phenotypes have demonstrated that these receptors have both essential and redundant roles throughout development. Fgfr1−/− mutant mice fail to undergo the epithelial-to-mesenchymal transition required for mesoderm formation [Deng et al. 1994; Yamaguchi et al. 1994; Ciruna et al. 1997; Ciruna and Rossant 2001; Hoch and Soriano 2006]. However, this phenotype is dependent on genetic background, since Fgfr1 was shown to regulate primitive endoderm formation on a 129S4 genetic background, while the same null allele caused mesoderm defects on a mixed genetic background [Hoch and Soriano 1997; Ciruna and Rossant 2001; Hoch and Soriano 2006]. Other studies have also demonstrated that ear defects caused by an ENU-induced mutation in Fgfr1 are also modified by genetic background [Pau et al. 2005; Calvert et al. 2011]. For Fgfr2, different targeting strategies have produced distinct phenotypes. Deletion of exons 9–12 (Fgfr2Δ9−12 allele) or exon 5 (Fgfr2Δ5 allele) caused perimplantation lethality, likely due to defects in extraembryonic lineages [Arman et al. 1998; Blak et al. 2007]. Fgfr2 mutants that lack exons 7–9 (Fgfr2Δ7−9 allele) or exons 8–10 (Fgfr2Δ8−10 allele) die around embryonic day 10 (E10) and exhibit defects in limb induction, chorioallantoic fusion, and the labyrinth component of the placenta [Xu et al. 1998b; Yu et al. 2003]. The Fgfr2Δ7−9/Δ7−9 phenotype was consistent across different genetic backgrounds [Hoch and Soriano 2006; Brewer et al. 2015]. Other studies have also demonstrated that second site modifiers do not underlie this phenotypic discrepancy (Xu et al. 1998b).

Intracellular signaling

Fgfrs engage multiple signaling pathways, including Erk1/2, PI3K/Akt, Plcγ, Pkc, and Stats. This is achieved mostly through an adaptor-mediated mechanism in which the receptor recruits nonenzymatic proteins that function as a scaffold to engage additional signaling proteins (Fig. 2). Fgfr-rerecruited proteins, their known signaling capabilities, and their in vivo significance are discussed below.

Engagement of Fgfrs leads to the phosphorylation of several Frs. Frs2 and Frs3 are myristyl-anchored membrane adaptor proteins that bind the juxtamembrane domain of Fgfrs ([Table 1; Kouhara et al. 1997; Xu et al. 1998a; Dhalluin et al. 2000; Ong et al. 2000]). This interaction is mediated by the phosphotyrosine-binding domain of the Frs (Fgf-regulated substrate) family of adaptor proteins engages Erk1/2 downstream from Fgfrs.

Several additional contexts have been shown to require signaling through multiple Fgfrs. Here, Fgfrs are largely thought to function as homodimers in vivo. However, two studies have provided biochemical evidence that suggests that Fgfrs are capable of forming heterodimers. First, Fgfr2 is capable of phosphorylating Fgfr1 intracellular tyrosines [Bellot et al. 1991]. Second, an Fgfr1 dominant-negative (Fgfr1DN) allele that lacks the cytoplasmic tail is capable of suppressing activation of Fgfr1−3 [Ueno et al. 1992]. The absence of the cytoplasmic tail prevents receptor transphosphorylation following ligand binding and therefore results in a nonproductive dimerization event. The ability of the Fgfr1DN protein to suppress activation of Fgfr2 and Fgfr3 therefore suggests that Fgfr1 is capable of forming a heterodimer with other Fgfrs [Ueno et al. 1992]. However, the Fgfr1DN construct could inhibit activation of wild-type Fgfrs by sequestering ligand without forming a heterodimer. These studies have been conducted using overexpression assays in cultured cells or Xenopus oocytes. The existence of Fgfr heterodimers in vivo at endogenous expression levels therefore remains to be demonstrated.

Figure 2. Schematic representation of Fgfr signaling functions. (A) Fgfrs are capable of engaging Erk1/2 through multiple mechanisms, including the Frs2, Shb, and Crk adaptor proteins as well as Plcγ. For simplicity, Crkl, Crkll, and CrkL adaptor proteins are referred to as Crk. Please see the text for further discussion of the role of these signaling proteins. (B) Fgfrs are also capable of engaging several additional signaling pathways, including PI3K/Akt, Pkc, Src, Stat1, p38, and Jnk.
Erk1/2 activation in vivo. Also indicated that Frs2 is required for Fgf-mediated (Gotoh et al. 2004b).

During embryonic development, Fgf-mediated Erk1/2 activation is decreased in the extraembryonic ectoderm at E6.5 and E7.5, indicating that the two adaptor proteins share similar functions that have not been documented. Fgf-mediated Erk1/2 activation in many tissues, while Frs3 overexpression is capable of rescuing defects in anterior/posterior patterning (Gotoh et al. 2005). Erk1/2 activation is decreased in the extraembryonic ectoderm (E6.5 extraembryonic ectoderm). Chimeric analysis demonstrated that Frs2 cells accumulate at the primitive streak (Gotoh et al. 2005). This phenotype was also observed in Fgfr1 mice, since this receptor is required to initiate the epithelial-to-mesenchymal transition required to form mesoderm (Yamaguchi et al. 1994, Ciruna et al. 1997, Ciruna and Rossant 2001).

While Frs2 is an important mediator of Fgfr signaling, several studies have indicated that Frs2 regulates only a subset of Fgfr functions. Mice containing amino acid substitutions in Fgfr1 that prevent Fgfr1–Frs2 binding die at birth with multiple developmental defects, including cleft palate, post-axial polydactyly, hypoplasia of multiple midfacial and posterior pharyngeal arch derivatives (Hoch and Soriano 2006). However, some studies have indicated that Frs2 regulates only a subset of Fgfr functions. Mice containing amino acid substitutions in Fgfr1 that prevent Fgfr1–Frs2 binding die at birth with multiple developmental defects, including cleft palate, post-axial polydactyly, hypoplasia of multiple midfacial and posterior pharyngeal arch derivatives (Hoch and Soriano 2006). Another strategy to recapitulate the phenotype of Fgfr1−/− mice failed to recapitulate the phenotype of Fgfr1−/− mice, indicating that Frs2 and/or Frs3 are required for only a subset of Fgfr1 signaling functions in vivo (Hoch and Soriano 2006).

Several phenotypes caused by loss of Frs2 function have been also indicated that Fgfr1 is required for Fgf-mediated Erk1/2 activation in vivo. Fgfr1 mice are recovered at the expected Mendelian frequency at E6.5 and have defects in anterior/posterior patterning (Gotoh et al. 2005). Erk1/2 activation is decreased in the extraembryonic ectoderm of Fgfr1−/− mutants at E6.5 (Gotoh et al. 2005). Fgfr2 is expressed in the extraembryonic ectoderm of (Ciruna and Rossant 1999) and is believed to engage Erk1/2 through Frs2 in this tissue. However, similar defects have not been documented in Fgfr2 mice, suggesting that Fgfr2 may function downstream from multiple Fgfrs or other RTKs in the E6.5 extraembryonic ectoderm. Chimeric analysis demonstrated that Fgfr1−/− cells accumulate at the primitive streak (Gotoh et al. 2005). This phenotype was also observed in Fgfr1−/− mutants, since this receptor is required to initiate the epithelial-to-mesenchymal transition required to form mesoderm (Yamaguchi et al. 1994, Ciruna et al. 1997, Ciruna and Rossant 2001).

### Table 1. Fgfr intracellular binding sites

| Protein | Fgfr1 | Fgfr2 | Fgfr3 | Fgfr4 | Reference |
|---------|-------|-------|-------|-------|-----------|
| CrkL    | Y463  | N.D.  | N.D.  | N.D.  | Xu et al. 1998a, Raffioni et al. 1999, Dhalluin et al. 2000, Ong et al. 2000, Eswarakumar et al. 2006 |
| CrkI    | Y463  | No    | N.D.  | N.D.  | Hart et al. 2001, Moon et al. 2006, Seo et al. 2009 |
| Shb     | Y766  | N.D.  | N.D.  | N.D.  | Cross et al. 2002 |
| Plcy (SH2) | Y766 | Y769  | Y760  | Yes   | Mohammadi et al. 1991, Peters et al. 1992, Raffioni et al. 1999, Kong et al. 2002, Ceridono et al. 2005 |
| Grb14   | Y766/Y776 | N.D. | N.D. | N.D. | Reilly et al. 2000, Browaey-Poly et al. 2010, Ezzat et al. 2013 |
| Stat1   | Yes | N.D.  | Y724c | Y724c | Hart et al. 2001, Krejci et al. 2008 |
| Stat3   | Y677d | Yesd | Y724c | Y390e | Hart et al. 2001, Krejci et al. 2008, Dudka et al. 2010, Uliganathan et al. 2015 |
| Src     | Y730  | Yes   | N.D.  | N.D.  | Schuller et al. 2008, Dudka et al. 2010 |
| Grb2 (SH3) | No | 807–821 | N.D.  | N.D.  | Ahmed et al. 2010, 2013, Lin et al. 2012, Timsah et al. 2014 |
| P85     | Y734  | Yes   | Y760  | Y754  | Vainikka et al. 1996, Salazar et al. 2009, Francavilla et al. 2013 |

Summary of known intracellular protein interactions of Fgfr1–4. Residue numbers indicate validated binding sites in each Fgfr. Protein–protein interactions mediated by unknown residues are indicated as “yes,” while negative results are shown as “no.” SH2 and SH3 refer to protein interactions mediated by Src homology 2 or 3 domains of the respective proteins. (N.D.) Potential interactions that have not been documented.

aY463 is not conserved in Fgfr3.
bFgfr–Stat3 interaction depends on Fgfr1K656E and Fgfr4K656E mutations.
cFgfr3–Stat1 interaction is increased in Fgfr3K650E mutations.
dFgfr–Stat3 interaction depends on overexpression of Fgfr1 or Fgfr2.
eStat3–Stat interaction depends on the Fgfr4C88E mutation.
containing amino acid substitutions that prevented only Frs2 from binding Fgfr1 [Hoch and Soriano 2006; Brewer et al. 2015]. This phenotypic disparity is likely due in part to allele design, since the Fgfr1<sup>Δ<sup>474</sup></sup> allele relied on a partial cDNA knock-in strategy that failed to completely recapitulate normal expression or function of Fgfr1 [Hoch and Soriano 2006].

Surprisingly, mice containing L424A and R426A mutations in Fgfr2<sup>Fgfr2<sup>R</sup></sup> allele that disrupt Frs2 binding are viable [Eswarakumar et al. 2006; Sims-Lucas et al. 2009]. Fgfr2–Frs2 signaling is therefore dispensable for embryonic development. However, Fgfr2 signaling through Frs2 is required for multiple phenotypes in a mouse model of Crouzon syndrome [Eswarakumar et al. 2006]. Mice heterozygous for a constitutively active allele of Fgfr2<sup>Fgfr2<sup>C342Y</sup></sup> allele containing the C342Y mutation approximate Crouzon syndrome, which is characterized by premature fusion of cranial sutures [craniosynostosis]. Mice containing three amino acid substitutions, C342Y, L424A, and R426A [Fgfr2<sup>CLR</sup> allele], designed to disrupt Fgfr2–Frs2 binding in the constitutively active receptor were phenotypically indistinguishable from wild-type littermates. This result indicates that Fgfr2 signaling requires Frs2 during pathologic cranial suture ossification. Mice homozygous for the activated allele of Fgfr2 exhibited several additional phenotypes, including cleft palate, trachea, and elbow/knee joints. Fgfr2<sup>CLR</sup> mice displayed no defects at the knee or elbow joints but retained the cleft palate and tracheal phenotypes of the Fgfr2<sup>C342Y/CLR</sup> mutant [Eswarakumar et al. 2006]. This context-specific attenuation of Fgfr2<sup>C342Y/CLR</sup> phenotypes may be due to differential requirements for Frs2 signaling in each developmental process or distinct threshold effects between the palate, trachea, and elbow/knee joints.

Engineering Fgfrs to disrupt their ability to activate Frs2 provides a major advantage in understanding the relative importance of this signaling protein downstream from specific Fgfrs. This is because Frs2 binds a number of RTKs in addition to Fgfrs that include Trks, Ret, Alk, and Vegfrs [Rabin et al. 1993; Ong et al. 1996, 2000; Dhaluin et al. 2000; Kurokawa et al. 2001; Melillo et al. 2001; Degoutin et al. 2007; Chen et al. 2014b]. Loss of Frs2 therefore alters signaling downstream from multiple RTKs, making the phenotypes of Frs2<sup>−/−</sup> mutants difficult to attribute to an individual RTK. This point is emphasized by a series of studies focused on kidney development. Fgfr2, Frs2, and Ret are each required for kidney development [Schuchardt et al. 1994; Zhao et al. 2004; Sims-Lucas et al. 2009]. However, Fgfr2 mutants that lack the ability to bind Frs2 develop normal kidneys, while Ret mutants that are unable to signal through Frs2 recapitulate the kidney defects observed when Frs2 is conditionally disrupted in the ureteric bud [Jijiwa et al. 2004; Zhao et al. 2004; Sims-Lucas et al. 2009]. Therefore, specifically uncoupling Frs2 signaling from Fgfr2 or Ret helped to clarify the contribution of each receptor’s signaling function in kidney development. More information on how Fgf signaling regulates kidney development can be found in several recent review articles [Bates 2011; Trueb et al. 2013].

**Shp2 is a critical mediator of Frs2-dependent Erk1/2 activation**

Shp2 is a tyrosine phosphatase that also functions as an adaptor protein downstream from multiple RTKs. Frs2-mediated Fgf signal transduction is reinforced by the constitutive Shb–Shp2 complex [Fig. 2A; Cross et al. 2002]. Following receptor activation, Shb binds the Fgfr tyrosine kinase domain, enabling Shp2 to bind Fgfr2 [Cross et al. 2002]. Shp2 is also tyrosine-phosphorylated following Fgf treatment, which allows Shp2 to bind Grb2–Sos and engage the Ras–Erk1/2 signaling pathway in PC12 cells [Hadari et al. 1998]. Of note, prolonged Erk1/2 activation depends on Grb2 that is recruited by Shp2 rather than Grb2 that binds Frs2 directly [Hadari et al. 1998]. It is not known whether the phosphatase function of Shp2 is involved in this or another process in Fgfr-mediated signal transduction, but this phosphatase activity is required for sustained Fgf-mediated Erk1/2 activation [Hadari et al. 1998].

Genetic studies also indicate that Shp2 is an important mediator of Frs2-dependent functions downstream from Fgfrs. To better understand the requirement of specific signaling functions of Frs2, mutations were engineered into Fgfr2 that disrupt the ability of this adaptor protein to interact with Shp2 [Fgfr2<sup>Δ<sup>204</sup></sup> allele] or Grb2 [Fgfr2<sup>Δ<sup>570</sup></sup> allele] [Gotoh et al. 2004a]. Analysis of mice engineered with these mutations demonstrated that the Frs2–Shp2 protein complex is required for Fgf-dependent lens placode induction but that Frs2–Grb2 binding was dispensable for eye development [Faber et al. 2001; Gotoh et al. 2004a]. Fgfr2<sup>Δ<sup>204/Δ570</sup></sup> mutant mice also exhibited decreased Erk1/2 activation during lens induction, indicating that Frs2–mediated Erk1/2 activation depends on Shp2 binding [Gotoh et al. 2004a]. Similar experiments have demonstrated the importance of Shp2 in Fgf-mediated closure of the optic fissure [Cai et al. 2013]. Conditional loss of both Fgfr1 and Fgfr2 in the optic vesicle caused ocular coloboma. This phenotype was also observed when both Fgfr2 and Ptpn11 [the gene encoding murine Shp2] were conditionally disrupted in the optic vesicle or when the Fgfr2–Shp2 protein complex was disrupted in a Ptpn11-deficient background in Frs2<sup>Δ<sup>204/Δ570</sup></sup> mice. This result indicates that Shp2-independent functions of Frs2 are not sufficient for closure of the optic fissure [Cai et al. 2013]. Additionally, loss of Fgfr1 and Fgfr2 or Fgfr1<sup>−/−</sup> and Ptpn11 was rescued by introducing a constitutively active Kras<sup>G12D</sup> allele, indicating that Fgfr signaling primarily depends on Erk1/2 in this context. Collectively, these studies support the model that Frs2-mediated Erk1/2 activation depends on Shp2 in Fgf-mediated developmental processes.

Loss of Ptpn11 causes phenotypes reminiscent of decreased Fgf signaling in other developmental processes as well. Genetic disruption of Ptpn11 causes gastrulation defects that are reminiscent of Fgfr<sup>Δ<sup>204</sup></sup> mutant phenotypes [Deng et al. 1994; Yamaguchi et al. 1994; Saxton et al. 1997]. Conditional deletion of Ptpn11 in neural crest cells or Fgf8 in the facial epithelium also leads to agenesis of multiple craniofacial structures [Trumpp et al. 1999; Nakamura et al. 2009]. These phenotypes are consistent
with the concept that Shp2 is required in multiple developmental contexts regulated by Fgf signaling.

**The Crk family of adaptor proteins engages Erk1/2 and Jnk downstream from Fgfrs**

The Crk gene encodes two distinct proteins (named CrkI and CrkII) by alternative splicing [Feller 2001]. These proteins function as adaptors and form multiprotein complexes via their SH2 and SH3 domains [Feller 2001]. CrkII binds the juxtamembrane domain of activated Fgfr1 at phosphotyrosine 463 to mediate Erk1/2 and Jnk activation [Table 1; Larsson et al. 1999]. Disruption of the Fgfr1–CrkII complex also decreases Fgfr1-mediated Frs2 tyrosine phosphorylation in vitro, suggesting that CrkII enhances Frs2 activation by Fgfrs [Larsson et al. 1999]. Therefore, Crk-mediated activation of Erk1/2 downstream from Fgfrs may be Frs2-dependent to some extent. CrkII activates Jnk by recruiting Cas and activating the C3G, Rap1 axis [Fig. 2B; Larsson et al. 1999]. A related protein, Crk-like (CrkL), contains amino acid composition, domain structure, and functional similarities to CrkI and CrkII [Feller 2001]. CrkL also interacts with phosphotyrosine 463 of Fgfr1 and phosphotyrosine 466 of Fgfr2 with a greater affinity than Crk proteins [Seo et al. 2009]. CrkL has been shown to engage the Erk1/2 pathway independently of Ras through Rac1, Cdc42, and Pak [Fig. 2A; Seo et al. 2009].

Genetic analysis suggests that CrkL signaling is required to mediate Fgfr8-dependent development of the pharyngeal arches [Moon et al. 2006]. While Fgfr8+/− or CrkL−/− heterozygous mutants exhibit normal development of the pharyngeal arches, Fgfr8−/−; CrkL−/− compound heterozygous mice have defects in multiple pharyngeal arch derivatives, including the vasculature, cardiac outflow tracts, thymus, and parathyroid glands. The penetrance and severity of these defects are enhanced in Fgfr8−/−; CrkL−/− mutants. Decreased Erk1/2 activation was observed in the pharyngeal arches and correlated with the severity of gene dosage and phenotypic outcome, suggesting that CrkL is required for Fgfr8-mediated Erk1/2 activation. Alterations in Fgfr8 and CrkL gene dosage also affected development of the femur, palate, and mandible, suggesting that CrkL is required for Fgfr8-mediated signaling in multiple developmental contexts. These phenotypes are likely dependent on multiple Fgfrs, since CrkL interacts with both Fgfr1 and Fgfr2 in mouse embryonic fibroblasts [Moon et al. 2006]. Mice engineered to disrupt the ability of Fgfr1 to signal through Crk adaptor proteins were viable and fertile without developmental or homeostatic defects [Brewer et al. 2015]. This result may be consistent with the hypothesis that multiple Fgfrs signal through Crk adaptor proteins in the pharyngeal arches and other Fgf-dependent developmental contexts.

Erk1/2 is required in many Fgf-mediated developmental processes

Surprisingly, despite being activated by many cell surface receptors, Erk1/2 is phosphorylated at high levels in discrete tissues during development rather than being uniformly activated [Corson et al. 2003]. Erk1/2 activation is decreased in many of these contexts when embryos are cultured in Fgfr inhibitors, suggesting that Fgf signaling is a major driver of Erk1/2 activation in multiple developmental processes [Corson et al. 2003]. Additionally, Fgf, Fgfr, and Erk1/2 loss-of-function phenotypes are often similar, suggesting that Fgfrs primarily signal through Erk1/2 in vivo. Fgfrs have been shown to function through Erk1/2 in many biological processes, and several of these are discussed below.

**Fgfr4–Erk1/2 signaling regulates primitive endoderm specification**

The preimplantation blastocyst is composed of an inner cell mass surrounded by trophectoderm [Fig. 3]. The inner cell mass is made up of primitive endoderm and epiblast cells, which give rise to the yolk sac and embryo, respectively. Initially, cells of the inner cell mass express both epiblast and primitive endoderm markers [Plusa et al. 2008]. These lineages subsequently become restricted to an epiblast or primitive endoderm cell fate as the expression of lineage-specific genes becomes mutually exclusive [Plusa et al. 2008]. This cell fate decision can be modulated to generate an inner cell mass completely composed of primitive endoderm or epiblast cells by culturing embryos in exogenous FGF4 or a Fgfr inhibitor, respectively [Fig. 3; Yamanaka et al. 2010]. Mek inhibition also results in an inner cell mass devoid of primitive endoderm [Nichols et al. 2009], indicating that Erk1/2 signaling is also required for primitive endoderm formation and thus is likely responsible for mediating Fgfr function.

Genetic studies have also supported the model that Fgf uses Erk1/2 in the formation of primitive endoderm. Fgfr4−/− mutants fail to express primitive endoderm markers at implantation and die around this time [Feldman et al. 1995; Goldin and Papaioannou 2003; Kang et al. 2013]. Genetic disruption of Fgfr1 shifts the composition of the inner cell mass in favor of the epiblast lineage and

![Figure 3](image.png)
produces fewer primitive endoderm cells [Brewer et al. 2015]. Fgfr2 is also thought to mediate this process, since this receptor is expressed in the primitive endoderm, and some Fgfr2 \(^{-/-}\) mutants die at implantation [Arman et al. 1998; Blak et al. 2007]. This raises the possibility that Fgfr1 and Fgfr2 function together during primitive endoderm formation, although this possibility needs to be tested. Genetic loss of Mapk1 or Mapk3 [the genes encoding Erk2 and Erk1, respectively] has not been associated with primitive endoderm defects to date [Pages et al. 1999; Saba-El-Leil et al. 2003]. However, it is possible that Erk1 and Erk2 function redundantly in this context. The inner cell mass of Grb2 \(^{-/-}\) mutants is also composed entirely of epiblast cells and is devoid of primitive endoderm [Chazaud et al. 2006]. Grb2 associates with Frs2 and Shp2, allowing Fgfrs to engage the Ras–Erk1/2 pathway through Sos [Fig. 2A; Ong et al. 1996; Kouhara et al. 1997; Hadari et al. 1998]. Grb2 is also capable of engaging PI3K downstream from Fgfrs [Fig. 2B; Ong et al. 2001], suggesting that loss of PI3K activation may also contribute to the failure of Grb2 \(^{-/-}\) mutants to form primitive endoderm. However, no defects in primitive endoderm have been associated with decreased PI3K activity in the blastocyst [Brachmann et al. 2005; Riley et al. 2005, 2006].

**Fgfr1 functions through Erk1/2 in the segmentation clock**

Fgf signaling also functions through Erk1/2 during axial elongation and periodic somite formation. Axial elongation depends on cell movements in the presomitic mesoderm that facilitate posterior outgrowth [Hubaud and Pourquié 2014]. Modulating the activity of Fgf8, Fgfr1, or Erk1/2 influences cell movements and therefore axis elongation in zebrafish and chicks [Dubruille et al. 2001; Sawada et al. 2001; Delfini et al. 2005]. In mice, an Fgf8 gradient observed in the presomitic mesoderm was shown to correlate with a gradient of Akt activity, raising the possibility that Fgfr1 also functions through PI3K in this context [Dubruille and Pourquié 2004]. However, pharmacological inhibition of PI3K did not affect cell movements or axial elongation in chicks, suggesting that PI3K activity is dispensable for Fgf-mediated axial elongation in this species [Delfini et al. 2005]. No functional interrogation of PI3K activity in murine axial elongation has been described to date.

Periodic somite formation involves oscillating activity of multiple signaling pathways [Dubruille and Pourquié 2004]. During this process, the most anterior presomitic mesoderm condenses and forms somites. Erk1/2 activity oscillates, but a similar oscillating expression has not been described for Fgf ligands or receptors [Niwa et al. 2011]. Instead, Fgf8 is present in a gradient throughout the presomitic mesoderm, while Sprouty2/4 and Dusp4/6 feedback inhibitors oscillate and may regulate Erk1/2 activity [Dequeant et al. 2006; Niwa et al. 2007; Hayashi et al. 2009]. In addition, Shp2 oscillations have been described, suggesting that constant receptor activation and differential expression of this signaling protein may also contribute to oscillating Erk1/2 activity [Dequeant et al. 2006]. Conditional disruption of Fgf4 and Fgf8 or Fgfr1 produces characteristic segmentation defects in which expression of cyclic genes is lost and presomitic mesoderm prematurely differentiates into disorganized somite structures, resulting in truncation of the embryo’s posterior end [Niwa et al. 2007; Wahl et al. 2007; Naiche et al. 2011]. Similarly, inhibition of Fgfrs or Erk1/2 results in reduced random cell motility and abolished expression of cyclic genes belonging to multiple pathways [Delﬁni et al. 2005; Niwa et al. 2007; Benazeraf et al. 2010]. Collectively, these results indicate that Fgf functions through Erk1/2 in axial elongation and periodic somite formation.

**Fgf8–Erk1/2 is required for development of the facial prominences**

A functional requirement for Erk1/2 activity downstream from Fgf signaling has also been demonstrated in the developing pharyngeal arches. Erk1/2 is highly activated in an Fgfr-dependent fashion in the pharyngeal arches [Corson et al. 2003]. Additionally, conditional inactivation of Fgf8 in the ectoderm of the first pharyngeal arch or Mapk1 and Mapk3 in the neural crest-derived mesenchyme produces similar phenotypes, characterized by agenesis of the maxillary and mandibular prominences and clefting of the nasal prominences [Trumpp et al. 1999; Newbern et al. 2008; Griffin et al. 2013]. Conditional inactivation of Fgfr1 in the neural crest-derived mesenchyme produced a milder phenotype of midline facial clefting and normal development of the mandible [Tromovic et al. 2003; Wang et al. 2013; Brewer et al. 2015]. Combined deletion of Fgfr1 and Fgfr2 in neural crest cells did produce a more severe facial cleft, although these mutants still fail to recapitulate the facial agenesis caused by conditional loss of Fgf8 [Park et al. 2008]. Therefore, Fgf8 likely functions through multiple Fgfrs to engage Erk1/2 during development of the pharyngeal mesenchyme.

**Fgf–Erk1/2 signaling regulates epithelial–mesenchymal interactions in the limb**

Reciprocal Fgf signaling between the epithelium and mesenchyme during limb development is also mediated by Erk1/2. Fgf10 expressed in the limb bud mesenchyme activates Fgfr2b in the presumptive apical ectodermal ridge (AER), which in turn induces Fgf8 expression in the AER [Fig. 4; Min et al. 1998; Xu et al. 1998b; De Moerlooze et al. 2000]. Fgf4, Fgf9, and Fgf17 are subsequently expressed in the AER together with Fgf8, and these ligands engage Fgfr1c and Fgfr2c in the mesenchyme to reinforce Fgf10 expression [Fig. 4; Mariani et al. 2008; Yu and Ornitz 2008]. In this way, reciprocal Fgf signaling regulates induction and proximal/distal patterning of the limb. Consequently, genetic disruption of Fgf10 or Fgfr2b results in complete agenesis of the limbs [Min et al. 1998; Xu et al. 1998b; De Moerlooze et al. 2000]. Conditional ablation of Fgf8 and Fgf4 or of Fgfr2 in the epithelium also causes a near complete agenesis of the hindlimb [Sun et al. 2002; Yu and Ornitz 2008]. A less dramatic phenotype was observed in the forelimbs, characterized by missing distal elements [Sun et al. 2002; Yu and Ornitz 2008]. The difference in severity between the forelimb and
hindlimb defects may be attributed to the activity of the Msx2-Cre driver, which is observable in the hindlimb at an earlier stage than the forelimb (Sun et al. 2000). Activated Erk1/2 is observable in the limb bud mesenchyme, and AER and has been shown to depend on Fgfr signaling (Corson et al. 2003). Hindlimb agenesis accompanied by distal forelimb defects is also observed in conditional mutants that lack Mapk1 in the embryo proper (Fremin et al. 2015). It is not known why loss of Mapk1 produces more severe defects in hindlimb development, although this may suggest that forelimbs and hindlimbs require different levels of Erk1/2 signaling. It is also unknown whether disruption of both Mapk1 and Mapk3 would recapitulate the limb agenesis phenotypes reported in Fgfr10−/− and Fgfr2b−/− mutants (Min et al. 1998; Xu et al. 1998b; De Moerlooze et al. 2000).

**Fgfr3 functions through Erk1/2 to inhibit chondrocyte hypertrophic differentiation**

In growth plate chondrocytes, Fgfr3 functions through Erk1/2 to regulate postnatal hypertrophic differentiation. Fgfr3 limits long bone growth by inhibiting chondrocyte proliferation and hypertrophic differentiation (Colvin et al. 1996; Deng et al. 1996). Loss of Fgfr3 function therefore causes long bone overgrowth, while activating mutations in Fgfr3 cause skeletal dwarfism (Colvin et al. 1996; Deng et al. 1996; Naski et al. 1998; Chen et al. 1999; Li et al. 1999; Wang et al. 1999). Similarly, genetic inactivation of Mapk1 and Mapk3 in chondrocytes causes long bone overgrowth (Sebastian et al. 2011). Transgenic expression of a constitutively active Map2k1 allele (the gene encoding Mek1) in chondrocytes also causes skeletal dwarfism associated with fewer hypertrophic chondrocytes but normal chondrocyte proliferation (Murakami et al. 2004). Activation of Mek1 is also capable of rescuing the long bone overgrowth caused by loss of Fgfr3, indicating that Erk1/2 functions downstream from Fgfr3 to regulate long bone growth through hypertrophic differentiation [Murakami et al. 2004]. Some studies have also proposed that Erk1/2 regulates Fgfr3-mediated inhibition of chondrocyte proliferation [Raucci et al. 2004; Krejci et al. 2008]. However, other studies have suggested that this process is mediated by Stat1 (Sahni et al. 1999, 2001; Murakami et al. 2004).

Fgfr3 functions through Erk1/2 to inhibit chondrocyte hypertrophic differentiation [Murakami et al. 2004]. Some studies have also proposed that Erk1/2 regulates Fgfr3-mediated inhibition of chondrocyte proliferation [Raucci et al. 2004; Krejci et al. 2008]. However, other studies have suggested that this process is mediated by Stat1 (Sahni et al. 1999, 2001; Murakami et al. 2004).

**PI3Ks contain p85 regulatory and p110 catalytic subunits that function as heterodimers (Thorpe et al. 2015). Fgfrs activate the PI3K/Akt pathway through Frs2. This occurs through Grb2-mediated recruitment of Gab1 independently of Ras [Fig. 2B; Ong et al. 2001]. However, disruption of the Fgfr1–Frs2 protein complex fails to reduce Fgfr1-mediated phosphorylation of Akt, suggesting that Fgfrs may also possess an Frs2-independent mechanism to engage this pathway [Hoch and Soriano 2006; Brewer et al. 2015]. Fgfr1–4 have been shown to recruit p85 directly [Table 1], although here p85 is thought to function independently of the PI3K/Akt pathway [Fig. 6A, below; Salazar et al. 2009; Francavilla et al. 2013].**

**PI3K is required for Fgfr-mediated development of GnRH-secreting neurons, which regulate the production of gonadotropin to control puberty and gametogenesis. In humans, loss-of-function mutations in FGF8 and FGFR1 cause hypogonadism that is characterized by stunted puberty and infertility [Dode et al. 2003; Pitteloud et al. 2006; Falardcu et al. 2008]. Transgenic mice that express a dominant-negative allele of Fgfr1 exhibit delayed puberty and compromised fertility and have fewer GnRH-expressing neurons with less projections (Tsai et al. 2005; Gill and Tsai 2006). Decreases in fertility have also been documented in mice that conditionally lack Ptk3r1 [the murine gene encoding p85α] in GnRH-expressing neurons [Acosta-Martinez et al. 2009]. Conditional loss of Mapk3 and Mapk1 in these neurons had no affect on fertility [Wierman et al. 2012]. Studies in chicks have also demonstrated that pharmacological inhibition of Fgfrs or PI3K signaling affected GnRH migration in ovo, but this process was not altered by Mek inhibition [Hu et al. 2013]. Collectively, these studies suggest that Fgfr1 mediates GnRH neural migration through PI3K signaling.**

In the eye, Fgfr2 is required for cell survival and differentiation of the lens. Conditional disruption of Fgfr2 in the lens results in increased cell death, which can be rescued by concurrent loss of Pten, a negative regulator of the PI3K/Akt pathway. However, disruption of Pten failed to rescue subsequent differentiation defects observed in Fgfr2 conditional mutants. These results suggest that Fgfr2 functions through the PI3K/Akt pathway to regulate cell survival and that additional pathways are involved in differentiation [Chaffee et al. 2016].

**Plcγ functions in Fgfr1-mediated vertebral patterning and Fgfr4-induced cardiac hypertrophy**

Plcγ binds the Fgfr1 C-terminal tail at phosphotyrosine 766 via the Plcγ SH2 domain (Table 1; Mohammadi
et al. 1991; Peters et al. 1992). Subsequent tyrosine phosphorylation of Pocy results in activation of the enzyme and hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP2) into diacylglycerol (DAG) and inositol [1,4,5] triphosphate (IP3) (Mohammadi et al. 1992; Peters et al. 1992). IP3 is soluble and diffuses to the endoplasmic reticulum, where it binds IP3 receptors to release Ca2+ from the endoplasmic reticulum. The resulting elevated cytosolic Ca2+ concentration, in cooperation with the membrane-bound DAG, activates Pkc (Fig. 2B; Huang et al. 1995).

It has been proposed that Plcy also contributes to Erk1/2 activation by acting at the level of Raf1, based on the analysis of Y766F mutations engineered in Fgfr1 to abrogate Fgfr1–Plcy binding (Huang et al. 1995). Another study has demonstrated that Shb also interacts with Fgfr1 at phosphorylated Y766 to recruit Shp2 (Cross et al. 2002). The Shb–Shp2 protein complex is required for maximal activation of Frs2 and recruitment of additional Grb2 molecules (Hadari et al. 1998; Cross et al. 2002). This may provide an alternative, Plcy-independent mechanism by which Fgfr1 phosphotyrosine 766 is required for maximal Erk1/2 activation.

Fgfr1–Plcy signaling negatively regulates the duration of Fgfr signaling by initiating internalization of the receptor in vitro [Sorokin et al. 1994]. This model was also supported in vivo by generating mice harboring a Y766F amino acid substitution (Fgfr1Y766F) that prevents Plcy from binding the receptor [Partanen et al. 1998]. Fgfr1Y766F/Y766F mice exhibit posteriorization of the vertebral column, while Fgfr1Y766F/+ mice present a similar phenotype with lower penetrance. The opposite homeotic transformation (vertebral anteriorization) is present in mice homozygous for an Fgfr1 hypomorphic allele (Fgfr1hypo/hypo) and transheterozygous mice containing hypomorphic and null alleles (Fgfr1hypo/−). This indicates that Fgfr1Y766F is a semidominant, gain-of-function mutation and that Plcy or downstream pathway members such as Pkc may act as negative regulators of Fgfr1 [Partanen et al. 1998].

Fgfr4 functions through Plcy in cardiomyocytes during disease progression of left ventricular hypertrophy [LVH] [Faul et al. 2011; Grabner et al. 2015]. Fgfr23 functions as an endocrine hormone to regulate phosphate homeostasis and is found at high levels in individuals with chronic kidney disease [Faul et al. 2011]. Elevated levels of Fgfr23 cause LVH by activating Fgfr4–Plcy signaling independently of Frs2–Erk1/2 [Faul et al. 2011; Grabner et al. 2015]. Additionally, pharmacological inhibition of Plcy prevented Fgfr23-induced hypertrophy of neonatal rat ventricular cardiomyocytes to a greater extent than Mek inhibition in vitro [Faul et al. 2011]. Genetic disruption of Fgfr4 also prevented LVH and Plcy activation in an Fgfr23-dependent model of chronic kidney disease [Grabner et al. 2015]. LVH and Plcy activation were observed in mice homozygous for a G385R-activating mutation in Fgfr4, collectively indicating that Fgfr4 signaling is necessary and sufficient for LVH pathogenesis [Grabner et al. 2015]. Plcy functions in LVH pathogenesis by regulating Ca2+ and the calcineurin/NFAT pathway, a potent inducer of cardiac hypertrophy [Molkentin et al. 1998; Faul et al. 2011; Grabner et al. 2015].

Pkcδ is required during ossification

Pkcς is a family of serine threonine kinases organized into three categories based on mechanisms of activation [Hage-Sleiman et al. 2015]. Conventional [c] Pkcs ([α, β, and γ]) are activated by DAG, Ca2+, and phorbol esters, while novel (n) Pkcs ([δ, ε, η, and θ]) are activated by DAG and phorbol esters but not Ca2+. Atypical [α] Pkcs ([ι, ι, and μ]) are activated by protein–protein interactions rather than secondary messengers (Hage-Sleiman et al. 2015). Fgfrs engage Pkc through Plcy (Fig. 2), although little is known about this signaling function in vivo. In osteoblast cell lines, FGFR2 enhances Runx2 expression and DNA-binding activity in a Pkc-dependent fashion [Kim et al. 2003]. Inhibition of individual Pkc isoforms indicates that this process primarily relies on Pkcδ [Niger et al. 2013]. Accordingly, Pkcδ−/− mutant mice exhibit delayed ossification of many skeletal structures, although Pkcδ activity is thought to be downstream from the noncanonical Wnt pathway in this context [Tu et al. 2007]. Similar delays in ossification have also been reported in Fgfr18−/− mutant mice, suggesting that Fgfr18 could initiate a Pkcδ-dependent pathway in osteogenesis [Liu et al. 2002; Ohbayashi et al. 2002]. This hypothesis is speculative, however, as Pkcδ activity has not been evaluated in Fgfr18−/− mutants to determine whether this signaling pathway is regulated by Fgfr signaling.

Adaptor protein Grb14

Grb14 was identified as an Fgfr1-binding protein in a yeast two-hybrid screen [Reilly et al. 2000]. This interaction is mediated by the Grb14 SH2 domain and C-terminal Fgfr1 phosphotyrosines 766 and 776 (Table 1; Fig. 2B). Overexpression of Grb14 inhibited FGF2-mediated proliferation, suggesting that Grb14 functions as a negative regulator of Fgfr signaling [Reilly et al. 2000]. It has been proposed that Grb14 inhibits Fgfr signaling by preventing recruitment and activation of Plcy to phosphotyrosine 766 [Browaeys-Poly et al. 2010]. Grb14−/− mice are viable and fertile with metabolic phenotypes that are generally attributed to alterations in signaling through the insulin receptor [Cooney et al. 2004]. It is therefore not known whether Grb14 contributes to Fgfr-mediated biological processes in vivo.

Stat1 functions downstream from Fgfr3 to inhibit chondrocyte proliferation

Stats are a family of proteins that bind transmembrane receptors and function in the nucleus as transcription factors. Stats are tyrosine-phosphorylated, often by Jak nonreceptor tyrosine kinases, which allows them to dimerize and translocate to the nucleus. In vitro studies have demonstrated that Stat1, Stat3, and Stat5 can be activated by Fgfrs [Hart et al. 2000; Deo et al. 2002; Yang et al. 2009; Dudka et al. 2010].
Fgfr3 functions through Stat1 to regulate chondrocyte proliferation during postnatal endochondral ossification. During this process, chondrocytes proliferate, exit the cell cycle, and undergo hypertrophic differentiation (Ornitz and Marie 2015). Fgfr3 signaling regulates bone growth by inhibiting both chondrocyte proliferation and hypertrophic differentiation (Colvin et al. 1996; Deng et al. 1996). Therefore, loss of Fgfr3 causes skeletal overgrowth, while activating mutations in Fgfr3 cause skeletal dwarfism in mice and humans (Colvin et al. 1996; Deng et al. 1996; Naski et al. 1998; Chen et al. 1999; Li et al. 1999; Wang et al. 1999). It has been proposed that Fgfr3 uses Stat1 to inhibit chondrocyte proliferation and Erk1/2 to restrict hypertrophic differentiation (Murakami et al. 2004). Stat1 is activated by FGF1 treatment in primary chondrocytes and is required for FGF1-mediated growth arrest in these cells (Sahni et al. 1999). Genetic loss of Stat1 also rescues the shortening of long bones induced through transgenic overexpression of FGF2 in mice by restoring normal proliferation rates (Sahni et al. 2001). Intriguingly, genetic loss of Stat1 restores normal chondrocyte proliferation in mice expressing an activating Fgfr3G374R mutant allele but does not restore normal long bone length (Murakami et al. 2004).

STAT3 binds phosphorytrosine 677 of FGFR1 in cell lines containing genomic amplification of the receptor but not in cells that express FGFR1 at endogenous levels (Table 1; Dudka et al. 2010). Thus, FGFR1-mediated activation of Stat3 may represent a cancer-specific signaling function of FGFR1. Consistent with this hypothesis, Stat3 is dispensable for Fgfr1-mediated murine facial morphogenesis (Brewer et al. 2015). Similarly, Stat3 preferentially binds a germline G388R variant of FGFR4 that has been associated with multiple cancer types (Ulaganathan et al. 2015). Here, the FGFR4G388R allele alters the transmembrane domain of FGFR4, creates a membrane-proximal STAT3-binding site, and facilitates increased STAT3 activation (Ulaganathan et al. 2015). Therefore, both FGFR1 and FGFR4 possess cancer-specific signaling functions through Stat3.

p38 functions in Fgfr2-mediated pathological skin and bone development

The p38 serine threonine kinases represent a family of MAPKs activated by cellular stress and several growth factors. FGFI or FGF18 treatment is capable of activating p38 in chondrocyte cell lines (Shimoaka et al. 2002; Raucci et al. 2004). Little is known about the mechanism by which Fgfrs engage the p38 pathway, although it has been shown to depend on Ras (Tan et al. 1996). p38 contributes to some aspects of congenital disorders caused by activating alleles of Fgfr2. Beare-Stevenson cutis gyrata syndrome is caused by constitutively active mutations in FGFR2 and is associated with craniosynostosis, epidermal hyperplasia, and other skin and skeletal abnormalities in humans (Beare et al. 1969; Stevenson et al. 1978; Hall et al. 1992). Many of these phenotypes were also observed in mice engineered with the analogous mutation (Fgfr2Y394C allele) (Wang et al. 2012). p38 activity was higher in both the skin and skull of Fgfr2Y394C/+ mutants, while Erk1/2 activity was elevated only in the skull. In utero inhibition of p38 allowed for normal skin development by restoring epidermal proliferation to wild-type levels but did not attenuate the skull defects caused by constitutive Fgfr2 signaling. Treatment with a Mek inhibitor did not modify the skin or skull phenotypes in mutant mice (Wang et al. 2012), suggesting that signaling through p38 but not Erk1/2 is required downstream from Fgfr2Y394C during epidermal hyperplasia.

Other studies have evaluated the requirement for specific signaling pathways during pathologic endochondral ossification in a mouse model of Apert syndrome (Yin et al. 2008; Chen et al. 2014a). Apert syndrome is caused by S252W- or P253R-activating mutations in FGFR2 and is characterized by craniosynostosis and syndactyly (Wilkie et al. 1995). Fgfr2S252W/+ and Fgfr2P253R/+ mice are smaller than their wild-type counterparts, with decreased bone length and mass (Yin et al. 2008; Chen et al. 2014a). Both p38 and Erk1/2 activity was higher in bone mesenchymal stem cells derived from mutant mice, and inhibition of either pathway was capable of restoring normal length in cultured long bones (Yin et al. 2008; Chen et al. 2014a). The craniosynostosis associated with Apert syndrome is Erk1/2-dependent and can be prevented or treated using a Mek inhibitor (Shukla et al. 2007, Yin et al. 2008).

Fgfr4 functions through Jnk to regulate bile acid synthesis

The Jnk serine threonine kinases represent a family of MAPKs activated by cellular stress and growth factors. Treatment of FGFI9 on primary human hepatocytes represses the rate-determining enzyme of bile acid synthesis, CYPZA1, in a JNK-dependent fashion (Holt et al. 2003). Additionally, Fgfr4+/− mice exhibited higher levels of bile acid production and Cypza1 expression, while transgenic mice expressing a constitutively active allele of Fgfr4 in the liver had decreased bile acid synthesis and lower Cypza1 expression and exhibited greater Jnk activity (Yu et al. 2000, 2005). Fgfr1 has been shown to engage Jnk through the Crk adaptor proteins C3G and Rap1 (Fig. 2B, Larsson et al. 1999). It is not known whether Fgfr4 uses a similar mechanism to activate Jnk.

Utilization of signaling functions

Fgfrs possess many signaling functions, raising the question of whether these effectors work individually or in combination. For example, Frs2 appears to be important in Fgfr1-mediated mesoderm formation (Gotoh et al. 2005), while CrkL has been implicated in pharyngeal arch development downstream from Fgfr1 and Fgfr2 (Moon et al. 2006). Does this suggest that Fgfrs use distinct effectors to activate Erk1/2 during different biological processes? Several studies addressing these questions have demonstrated that Fgfrs use diverse signaling mechanisms throughout development.
Fgfr1 requires the cumulative effect of multiple signaling effectors that converge on downstream pathways [Fig. 5, left panel]. This model was developed by analyzing the phenotype of mice with knock-in mutations designed to disrupt the ability of Fgfr1 to bind and therefore activate a subset of signaling functions. Loss of the ability to engage Frs2, Crk proteins, or Plcγ individually produced only subtle defects relative to the Fgfr1-null phenotype [Partanen et al. 1998; Hoch and Soriano 2006; Brewer et al. 2015]. Importantly, loss of individual signaling functions influenced similar Fgfr1-mediated developmental processes, most notably anterior/posterior patterning of the thoracic vertebrae [Partanen et al. 1998; Brewer et al. 2015]. Disruption of multiple signaling functions simultaneously produced more severe developmental defects, including developmental retardation, posterior truncations, and agenesis of the second pharyngeal arch, indicating that Fgfr1 uses these signaling functions additively [Brewer et al. 2015]. Similarly, Erk1/2 activation was only modestly decreased in primary cells when Fgfr1 was unable to engage CrkL, Plcγ, and Grb14 collectively or Frs2 individually [Hoch and Soriano 2006; Brewer et al. 2015]. Combined disruption of these signaling functions led to decreases in Erk1/2 activation that were similar to complete loss of Fgfr1 function [Brewer et al. 2015]. This supports the idea that Erk1/2 is engaged downstream from Fgfr1 through the combination of multiple effectors [Fig. 5, left panel]. Plcγ is also likely to be engaged through multiple mechanisms by Fgfr1, since this signaling molecule is activated both directly by the receptor and in an Frs2-dependent fashion [Brewer et al. 2015]. Additionally, Akt phosphorylation was not decreased by mutations engineered to disrupt the Fgfr1–Frs2 protein complex, suggesting that Fgfr1 also possesses additional mechanisms to activate the PI3K/Akt pathway [Hoch and Soriano 2006; Brewer et al. 2015].

Similar allelic series of signaling mutations have not yet been described for other Fgfrs. However, Fgfr3 may use a distinct mechanism in growth plate chondrocytes that relies on differential signaling to inhibit proliferation and differentiation [Fig. 5, right panel]. Stat1 is used by Fgfr3 in order to increase expression of p21 and inhibit chondrocyte proliferation [Sahni et al. 1999, 2001; Murakami et al. 2004]. Fgfr3 then functions through Erk1/2 to restrict hypertrophic differentiation [Murakami et al. 2004]. In contrast to Fgfr1, Fgfr3 may therefore use differential signaling functions during distinct developmental processes. Similar studies of Pdgfrs have also demonstrated that Pdgfrα has distinct requirements for individual signaling functions, while Pdgfrβ requires the additive effect of multiple pathways [Tallquist et al. 2000, 2003; Klinghoffer et al. 2002]. This supports the notion that evolutionarily related RTKs can function through distinct mechanisms.

**Figure 5.** Fgfrs use diverse mechanisms of signaling. Fgfr1 functions through multiple effectors that converge on downstream Erk1/2 signaling in multiple contexts, including axial elongation and development of the pharyngeal arches. In contrast, Fgfr3 uses differential signaling during distinct cellular responses in growth plate chondrocytes. Here, Stat1 is engaged to limit chondrocyte proliferation, and Erk1/2 is subsequently used to inhibit hypertrophic differentiation.

**Ligand-specific cellular responses**

An emerging theme in Fgf signaling is that cellular responses are often encoded in the identity of the ligand. Different Fgf ligands can therefore initiate distinct developmental responses in the same tissue. This is achieved through multiple mechanisms, some of which function by initiating distinct properties of intracellular signaling.

Culture of lung explants with FGF7 or FGF10 generates cyst-like or branched structures by inducing proliferation or migration, respectively [Fig. 6A, Belluscio et al. 1997; Francavilla et al. 2013]. These ligands induce distinct tissue morphologies by initiating different kinetics of FGFR2b signaling [Francavilla et al. 2013]. FGF10 but not FGF7 stimulation induces phosphorylation of intracellular Tyr734 on FGFR2b. Phosphorytrosine 734 functions as a docking site for p85 bound to SH3BP4, which enables receptor recycling back to the cell surface and sustained receptor activation. Mutation of Y734F switches the kinetics of FGF10-activated FGFR2b to a transient signal and the structure of lung explants to resemble an FGF7-induced morphology [Francavilla et al. 2013].

An alternative mechanism has been proposed in the submandibular and lacrimal glands based on each ligand’s HSPG affinity [Fig. 6B]. Here, FGF7 or FGF10 produces branched or elongated structures in explants [Steinberg et al. 2005; Makarenkova et al. 2009]. FGF7 binds HSPGs with a lower affinity than FGF10, allowing FGF7 to diffuse more extensively through the tissue, while FGF10 forms sharp gradients restricted to the tips [Igarashi et al. 1998; Makarenkova et al. 2009]. These gradients influence the spatial pattern of proliferation within the tissue to regulate morphogenesis. Mutation of the FGF10 HSPG-binding domain functionally mimics FGF7 HSPG-binding
properties to generate diffuse gradients and branched tissue structures (Makarenkova et al. 2009).

Distinct Fgf8 isoforms have also been shown to initiate different developmental programs that correlate with each isoform’s receptor affinity. Eight Fg8 isoforms (Fgf8a through Fgf8h) are generated by alternative splicing in mice (Crossley and Martin 1995; MacArthur et al. 1995). Fgf8a and Fgf8b induce distinct cellular responses when ectopically expressed in the chick neural plate. Fgf8a expression causes an expansion of the midbrain into the presumptive forebrain, while Fgf8b switches the fate of the midbrain to the cerebellum (Sato et al. 2001). Similar phenotypes have also been observed in transgenic mice that ectopically express Fgf8a or Fgf8b in the midbrain [Lee et al. 1997; Liu et al. 1999a]. It is not known whether the Fg8 isoforms induce distinct cellular programs by initiating different intracellular signaling profiles. However, Fgfr2c forms a larger hydrophobic interface with Fgf8b than Fgf8a, providing a greater affinity of the ligand to the receptor (Olsen et al. 2006). It therefore seems possible that Fgf8b may initiate a stronger signal than Fgf8a.

In the hippocampus, Fgf7 and Fgf22 have been shown to differentially promote the formation of inhibitory or excitatory presynaptic terminals, respectively [Fig. 6C, Terauchi et al. 2010]. Mice genetically lacking Fgf7 or Fgf22 therefore have fewer inhibitory or excitatory synapses on hippocampal CA3 pyramidal neurons. Altering the balance of inhibitory/excitatory synapses influences the predisposition to experimentally induced epileptic seizures. Loss of Fgf7 therefore causes increased seizure susceptibility, while loss of Fgf22 protects against seizures. The ability to induce different synapse identities is dependent on differences in each ligand’s receptor affinity (Terauchi et al. 2010; Dabrowski et al. 2015). Fgf7 primarily engages Fgfr2b, while Fgf22 can activate both Fgfr2b and Fgfr1b [Zhang et al. 2006]. Genetic disruption of Fgfr2b caused decreases in inhibitory and excitatory synapses, while loss of Fgfr1b prevented only excitatory synapse development (Dabrowski et al. 2015). This supports the model that the differential presynaptic responses to each ligand are dependent on activation of distinct receptor profiles.

Of course, some Fgf ligands are likely to initiate similar cellular programs. For example, multiple ligands are expressed in the AER to regulate induction and proximal/distal patterning of the limb (Lewandoski et al. 2000; Sun et al. 2000, 2002; Mariani et al. 2008). Nevertheless, these studies collectively demonstrate that Fgf ligands can also induce distinct biological responses. This can be achieved by influencing intracellular signaling kinematics, altering the pattern of cellular responses within a tissue, or engaging distinct Fgfrs.

Considerations and future directions

Many genetic and pharmacological studies have demonstrated that Erk1/2 mediates many Fgfr functions in diverse biological contexts. However, these in vivo studies do have limitations to consider. One difficulty of these studies is the inability to definitively connect an individual RTK to specific signaling pathways in vivo. A commonly used approach is to determine whether reducing pathway activity phenocopies loss of a given RTK. However, results from this approach may be difficult to interpret, as many biological contexts require signaling through multiple RTKs. For example, the labyrinth compartment of the placenta requires Fgfr2, Met, Egfr, Igf1r, and many signaling pathways, including p38a, Erk1/2, and Akt [Liu et al. 1993; Bladt et al. 1995; Sibilia and Wagner 1995; Threadgill et al. 1995; Xu et al. 1998b, Adams et al. 2000, Mudgett et al. 2000; Hatano et al. 2003; Yang et al. 2003, 2005; Fremin et al. 2015]. Since Fgfr2 is capable of engaging all of these pathways, it is difficult to know whether Fgfr2-associated placental defects are simply due to decreases in Erk1/2 signaling or whether these phenotypes are the result of lowering the activity of multiple signaling pathways. Similarly, it seems likely that phenotypes caused by decreased Erk1/2 activity alter signaling downstream from multiple RTKs. This problem is challenging given
Fgfr4 functions through Plc- 
pendent signaling pathways used by Fgfrs. For example, 
cussed several studies that have identified Erk1/2-inde- 
regulated biological processes, this review has also dis- 
may therefore be helpful in overcoming this issue.

biochemical inhibition or dominant-negative constructs 
broadly expressed. Additional strategies that rely on phar-

eight Src family kinases in mammals, four of which are 
alyzed in vivo, since Fgfr1 seems to primarily function 
ient signaling functions in vivo. However, there is also strong evidence implicating Erk1/2-

dependent signaling functions in vivo. Additionally, 
quentative differences in the magnitude or duration 
of Erk1/2 activation may be used to instruct diverse cellular responses. Collectively, these studies have provided new insights into signal transduction, informed the developmental etiologies of many congenital disorders, and may form the basis to develop novel therapeutic strategies.

Conclusion

In the many years that have followed the identification of Fgfrs, multiple studies have shed light on the diversity of Fgfr signaling mechanisms in numerous developmental and homeostatic processes. In many biological contexts, Fgfr signaling functions through the Erk1/2 pathway, although there is also strong evidence implicating Erk1/2-

dependent signaling functions in vivo. Additionally, quantitative differences in the magnitude or duration of Erk1/2 activation may be used to instruct diverse cellular responses. Collectively, these studies have provided new insights into signal transduction, informed the developmental etiologies of many congenital disorders, and may form the basis to develop novel therapeutic strategies.

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