FGF signaling regulates development by processes beyond canonical pathways

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FGFs are key developmental regulators that engage a signal transduction cascade through receptor tyrosine kinases, prominently engaging ERK1/2 but also other pathways. However, it remains unknown whether all FGF activities depend on this canonical signal transduction cascade. To address this question, we generated allelic series of knock-in \( \text{Fgfr1} \) and \( \text{Fgfr2} \) mouse strains, carrying point mutations that disrupt binding of signaling effectors, and a kinase dead allele of \( \text{Fgfr2} \) that broadly phenocopies the null mutant. When interrogated in cranial neural crest cells, we identified discrete functions for signaling pathways in specific craniofacial contexts, but point mutations, even when combined, failed to recapitulate the single or double null mutant phenotypes. Furthermore, the signaling mutations abrogated established FGF-induced signal transduction pathways, yet FGF functions such as cell–matrix and cell–cell adhesion remained unaffected, though these activities did require FGFR kinase activity. Our studies establish combinatorial roles of \( \text{Fgfr1} \) and \( \text{Fgfr2} \) in development and uncouple novel FGFR kinase-dependent cell adhesion properties from canonical intracellular signaling.

**Keywords:** FGF; craniofacial development; neural crest; ERK1/2; cell adhesion

Supplemental material is available for this article.

Received July 26, 2020; revised version accepted October 13, 2020.

Classic models of receptor tyrosine kinase activation involve ligand binding, receptor dimerization, transactivation of the kinase domain, phosphorylation of intracellular tyrosines, and binding of effectors that orchestrate activation of downstream signaling pathways [Simon et al. 1991; Lemmon and Schlessinger 2010]. Different thresholds in dimer strength and stability may also come into play, as well as signaling dynamics engaged by each downstream pathway [Vasudevan et al. 2015; Zinkle and Mogami 2018; Li and Elowitz 2019]. For FGFRs, where downstream pathways have been particularly well studied, numerous lines of evidence point to ERK1/2 as the main effector of FGF signaling [Lanner and Rossant 2010; Brewer et al. 2016]. Although characterization of effector binding to RTKs provides critical insights on signaling specificity, assessing relative pathway significance requires in vivo validation. A previous analysis showed that knock-in \( \text{Fgfr1} \) point mutations disrupting binding of multiple signaling effectors, alone or in combination, did not recapitulate the \( \text{Fgfr1}^{-/-} \) phenotype despite eliminating ERK1/2 outputs, suggesting involvement of additional FGF effectors [Brewer et al. 2015].

In mammals, 22 FGFs have been identified by sequence homology, with 18 acting as secreted ligands for four FGF receptors [FGFR1–4] [Ornitz and Itoh 2015; Brewer et al. 2016]. Both \( \text{Fgfr1} \) and \( \text{Fgfr2} \) play critical roles in early development. \( \text{Fgfr1} \)-null mutants fail to gastrulate and exhibit a defect in epithelial to mesenchymal transition required for mesoderm formation [Deng et al. 1994; Yamauchi et al. 1994; Ciruna and Rossant 2001]. Recent studies of \( \text{Fgfr1} \) and \( \text{Fgfr2} \) mutants, however, have documented an earlier genetic background-dependent role for \( \text{Fgfr1} \) in primitive endoderm and trophectoderm development [Hoch and Soriano 2006; Brewer et al. 2015; Kurowski et al. 2019] and a combined role for \( \text{Fgfr1} \) and \( \text{Fgfr2} \) in both of these lineages [Kang et al. 2017; Molotkov et al. 2017; Kurowski et al. 2019]. Null mutants for \( \text{Fgfr2} \) exhibit embryonic lethality at E10.5 associated with placenta deficiency and exhibit multiple additional defects including the absence of limb bud development [Xu et al. 1998; Yu et al. 2003; Molotkov et al. 2017].
Additional evidence further supports a role for Fgfr1 and Fgfr2 in craniofacial development, as conditional mutagenesis of Fgfr1 in cranial Neural Crest Cells (cNCCs) or of Fgfr2 in the epithelium leads to facial or palatal clefting (Rice et al. 2004; Hosokawa et al. 2009; Wang et al. 2013; Brewer et al. 2015), while deletion of both receptors in cNCCs prevents midface closure (Park et al. 2008). The development of the face involves the coordination of multiple morphogenetic processes including the formation of the frontonasal, maxillary, and mandibular processes, and their convergence at the midline. The pharyngeal arches (PA) that appear on each side of the future head are largely composed of cNCC-derived mesenchyme covered by surface ectoderm. The primitive mouth is flanked rostrally by the frontonasal prominence [FN], laterally by the maxillary [Mx] processes, and caudally by the mandibular [Man] processes. By embryonic day [E] 10.5, the FNP wraps around the nasal pits, which separate the medial [MNP] from the lateral [LNP] nasal processes. Rapid growth of the Mx and LNP then pushes the MNP to converge and the midface to close. Paracrine signaling between the facial ectoderm and the underlying cNCC-derived mesenchyme is particularly important for craniofacial morphogenesis, implicating FGF8 in mandibular development (Trumpp et al. 1999; Shigetani et al. 2000) and midface integration (Griffin et al. 2013). However, the signaling mechanisms by which FGFs regulate craniofacial development have not been elucidated, making this an excellent model system for interrogation.

FGFs are known to regulate cell proliferation and survival through canonical RTK signaling and regulation of gene expression, but are also known to regulate cell–matrix [Meyer et al. 2012] or cell–cell adhesion [Rasouli et al. 2018; Sun and Statopoulos 2018; Kurowski et al. 2019] through other well-established mechanisms. It remains unclear whether all activities engaged by the FGF receptors are dependent on activation by FGFs, on signaling through the kinase domain, or whether the receptors can engage cell adhesion receptors through interactions of their extracellular domains or by acting as scaffolds in specific cell surface compartments. Our previous results on signaling pathways operating downstream of FGFR1 [Brewer et al. 2015], although suggestive of additional signaling pathways, were difficult to interpret due to expression of FGFR2. The in vivo functions of FGFR2 signaling effectors are still largely unknown, however evidence to date indicates that signaling through FRS2 is not required during development [Eswarakumar et al. 2006; Sims-Lucas et al. 2009]. In this work, we use constitutive signaling and kinase dead mutations as well as conditional mutations in the craniofacial mesenchyme, to understand which pathways coordinate FGF signaling at a developmental and biochemical level. We found that signaling mutations for each receptor disrupt classical signal transduction pathways in the absence of the other receptor, but do not recapitulate the null phenotypes. We furthermore show that FGF activity is kinase-dependent and identify FGF outputs independent of canonical signaling that help reconcile the gap in our phenotypic analyses.

Results

Fgfr1 and Fgfr2 genetically interact in craniofacial development

To interrogate FGFR1/2 functions in development and their role in supporting each other’s activity, and to establish a baseline to study cell signaling mutations, we initially investigated how loss of both receptors in cNCCs influences craniofacial development. We found both receptors extensively coexpressed in the cNCC-derived mesenchyme and overlying epithelia, using fluorescent Fgfr1 and Fgfr2 reporter alleles [Molotkov et al. 2017]. Fgfr1 expression was observed primarily in the mesenchyme [Fig. 1A, green arrow]. In contrast, strong Fgfr2 expression was seen in the epithelia except for a small domain surrounding the nasal pit [Fig. 1A, yellow asterisk]. Weaker widespread Fgfr2 expression was also observed within the mesenchyme [Fig. 1A, red arrow], indicating that both Fgfr1 and Fgfr2 are coexpressed in multiple regions.

Conditional null alleles (henceforth denoted cKO) of Fgfr1 and Fgfr2 were combined with Wnt1Cre drivers active in NCCs [Danielian et al. 1998; Lewis et al. 2013]. Both Fgfr1−/− and Fgfr2−/− conditional null alleles create framework mutations early in the coding region likely leading to nonsense mediated decay, and if not, only a short peptide fragment unable to interact with ligands or any known effectors [Hoch and Soriano 2006; Molotkov et al. 2017]. Throughout this work, all Fgfr1−/−, Fgfr2−/−, and Cre driver alleles were analyzed on a 129S4 cosogenic background, to avoid phenotypic variations that might be attributable to second-site modifiers. At E18.5, Fgfr1cKO/cKO embryos displayed a fully penetrant facial cleft while Fgfr2cKO/cKO mutants had no overt phenotype. Loss of Fgfr2 significantly enhanced the phenotype of Fgfr1cKO/cKO conditional mutants, and Fgfr1cKO/cKO;Fgfr2cKO/cKO double mutants exhibited severe agenesis of most NCC-derived craniofacial structures including the frontal and nasal bones, nasal cartilage, maxilla, and mandible [Fig. 1B, Supplemental Fig. S1A]. Both Fgfr1cKO/cKO;Fgfr2cKO/cKO and Fgfr1cKO/cKO;Fgfr2cKO/cKO embryos exhibited defects in mandible development, affecting proximal structures including angular and coronoid processes [Supplemental Fig. S1B]. FGFR1 and FGFR2 are thought to function predominantly in mesenchymal or epithelial contexts, respectively, since Fgfr1c mutants recapitulate many aspects of the Fgfr1−/− phenotype [Partanen et al. 1998], and Fgfr2b mutants are reminiscent of Fgfr2−/− embryos [De Moerlooze et al. 2000]. However, our results suggest that both receptors function coordinately within the neural crest and combined loss of Fgfr1 and Fgfr2 together in cNCCs leads to a significantly more severe defect than loss of either receptor alone.

The anterior part of the craniofacial skeleton, including the maxilla and mandible, nasal cartilage, Meckel’s cartilage, frontal bone, and anterior cranial base [ethmoid and sphenoid bones], are derived from NCCs. Skeletal preparations revealed an anteriorly truncated skull due to loss of the nasal cartilage in Fgfr1cKO/cKO;Fgfr2cKO/cKO mutants at E14.5 [Supplemental Fig. S1C]. Meckel’s
Figure 1. Defects in craniofacial morphogenesis in Fgfr1/2 double mutants. (A) Spatial domain of Fgfr1 and Fgfr2 expression in facial prominences at E10.5. GFP and mCherry immunohistochemistry were used to detect expression from Fgfr1-GFP and Fgfr2-mCherry reporter alleles. GFP expression was primarily restricted to the mesenchyme (green arrow). Although mCherry expression was restricted to the epithelium, many cells in the mesenchyme also express mCherry (red arrow). mCherry expression was down-regulated in the epithelium lining the nasal pit (yellow asterisk). (B) Inferior view (mandibles removed) of alcian blue/alizarin red staining of mouse skulls at E18.5 showed a wider midline separation and hypoplastic MNP, LNP, and maxillary and mandibular prominences (Fig. 1C, yellow asterisk). Fgfr1cKO/cKO;Fgfr2cKO/cKO double mutants exhibited the most severe defect, with agenesis of medial and proximal structures as well as severe reduction of the mandible. A) Alisphenoid, (Bs) basioccipital, (Bo) basi-sphenoid, (M) maxillary, (Nc) nasal cartilage, (P) palatal, (PM) premaxillary, (ppMx) palatal process maxillary, (Pt) pterygoid, (Pa) palate, (T) tympanic bulla. (C) Conditional Fgfr1-Fgfr2 null mutant embryos carrying the ROSA26mT/mG reporter analyzed in whole mount. Schematic of reporter mouse design showing GFP [green] expression upon Cre expression in cNCCs [green]. (Top) At E9.5, Fgfr1cKO/cKO;Fgfr2cKO/cKO;ROSA26mT/mG embryos showed reduced GFP fluorescence and had hypoplastic pharyngeal arches PA1 and PA2 (yellow arrow). Fgfr1cKO/cKO;Fgfr2cKO/cKO and Fgfr1cKO/cKO;Fgfr2cKO/cKO mutants appeared normal at this stage. Frontal view at E10.5 (bottom) shows a wide midline separation in both Fgfr1cKO/cKO;Fgfr2cKO/cKO;ROSA26mT/mG and Fgfr1cKO/cKO;Fgfr2cKO/cKO;ROSA26mT/mG mutants (yellow asterisk).

Craniofacial defects in Fgfr1 and Fgfr2 conditional mutants are associated with cell death

At E9.5 and E10.5, Fgfr1cKO/cKO;Fgfr2cKO/cKO;ROSA26mT/mG double mutants developed hypoplastic pharyngeal arches with reduced NCC lineage GFP+ cells [Fig. 1C]. Although GFP+ NCCs were distributed throughout their migration streams at E9.5, the PA1 and PA2 arches appeared hypoplastic in double mutants [Fig. 1C, yellow arrow]. By E10.5, Fgfr1cKO/cKO;Fgfr2cKO/cKO;ROSA26mT/mG double mutants were morphologically indistinguishable from wild-type embryos [Fig. 1C, yellow asterisk]. At E10.5, Fgfr1cKO/cKO;Fgfr2cKO/cKO;ROSA26mT/mG mutants showed a 50% reduction in the number of GFP+ cells relative to controls. Micro-CT analysis at E18.5 further demonstrated reduced ossification of NCC-derived structures in the anterior skull and mandible [Supplemental Fig. S1A]. Skeletal differentiation is a multistep process starting with formation of cartilage progenitors prior to terminal differentiation into bone. To investigate the role of FGF signaling during skeletal differentiation, we analyzed the expression of chondrogenic (Col2a1) and osteogenic (Col10a1) markers, first at E14.5 when cartilage progenitors are formed, and then at E17.5 when terminal differentiation is largely complete [Supplemental Fig. S1D]. At E14.5, Col2a1 was expressed in a broader domain in Fgfr1cKO/cKO;Fgfr2cKO/cKO;ROSA26mT/mG double mutants [Supplemental Fig. S1D], suggesting that skeletal differentiation is initiated but delayed in double null mutants. By E17.5, while Col2a1 was expressed at similar levels in both controls and Fgfr1cKO/cKO;Fgfr2cKO/cKO;ROSA26mT/mG double mutants, Col10a1 was undetectable in double null mutants [Supplemental Fig. S1D], indicating a block in terminal differentiation. The observation that FGF signaling is important for skeletal differentiation is consistent with previous studies of differentiation in long bones [Karuppaiah et al. 2016].
controls [Fig. 2B]. We did not observe a significant reduction in either Fgfr1^+/KO,Fgfr2^+KO,Rosa26^+mT/mG or Fgfr1^KO/+;Fgfr2^+KO,Rosa26^+mT/mG or Fgfr1^KO/^KO,Fgfr2^+KO,Rosa26^+mT/mG mutants, suggesting that the skeletal defects observed at E14.5 or E17.5 result from both reduction in NCC numbers as well as an FGF-dependent skeletal differentiation defect.

A reduction in NCC numbers in the midface might occur due to reduced proliferation, increased cell death, or both. Cell proliferation remained unaffected (Supplemental Fig. S2B); however, we observed increased cell death both. Cell proliferation remained unaffected (Supplemental Fig. S2B); however, we observed increased cell death both. To interrogate signaling mechanisms in vivo, we generated an allelic series of Fgfr2 signaling mutants. We con- trolled Fig. S2B); however, we observed increased cell death both. Cell proliferation remained unaffected (Supplemental Fig. S2B); however, we observed increased cell death both. To interrogate signaling mechanisms in vivo, we generated an allelic series of Fgfr2 signaling mutants. We con- trolled Fig. S2B); however, we observed increased cell death both. Cell proliferation remained unaffected (Supplemental Fig. S2B); however, we observed increased cell death both.

An allelic series of Fgfr2 signaling mutations

To interrogate signaling mechanisms in vivo, we generated an allelic series of knock-in point mutations at the Fgfr2 locus preventing binding of effectors to the receptor [Fig. 3A,B, Supplemental Fig. S3A–C], similar to previous Fgfr1 mutations [Brewer et al. 2015]. The Fgfr2^+Fgfr2^+C, and Fgfr2^C,C mutations were designed to disrupt binding of FGR2c, CRK-L, and PLCγ/GB14, respectively. We also generated compound Fgfr2^CPCG and Fgfr2^CPCG signaling mutants by combining multiple signaling mutations. To validate the disruption of effector binding, 3T3 cells were transfected with triple FLAG-tagged cDNAs of FGFR2c isoforms for each signaling mutant. We con- firmed disruption of FRS2, CRK-L, and PLCγ binding in Fgfr2^CPCG, Fgfr2^CPCG, Fgfr2^CPCG, and Fgfr2^CPCG mutations, respectively, via communoprecipitation and Western blot analysis [Fig. 3B].

We first evaluated whether Fgfr2 signaling mutant alleles could partially or completely recapitulate the E10.5 Fgfr2^−− placenta and limb phenotype [Xu et al. 1998; Yu et al. 2003; Molotkov et al. 2017]. Surprisingly, all signaling allele mutants were at least partially viable and fertile as homozygotes [Supplemental Table S1]. Fgfr2^+F/F, Fgfr2^CPCG, and Fgfr2^CPCG mutants were rescued by the concomitant disruption of CRK-L binding site in Fgfr2^CPCG/CPCG mutants, suggesting opposite roles for these effectors in mediating FGF2 signaling. Skeletal prepara- tions at birth revealed a kinked tail phenotype for Fgfr2^CPCG/CPCG [7/9] and Fgfr2^CPCG/CPCG [6/12] neonates [Supplemental Fig. S4B]. To further assess the effect of our signaling mutations in vivo, we crossed Fgfr2 signaling mutant mice with a null allele generated from the Fgfr2 cKO strain. Fgfr2^−−, Fgfr2^−−, and Fgfr2^CPCG/CPCG mice were viable. In contrast, hemizygous Fgfr2^−− and Fgfr2^CPCG/CPCG mutant mice were recovered in expected Mendelian ratios at E18.5, but died at birth [Supplemental Table S1]. None of the Fgfr2^−− and Fgfr2^CPCG/CPCG neonates were able to suckle, as evidenced by the absence of an abdominal milk spot, possibly as a result of cranial nerve defects since Fgfr2^−− and Fgfr2^CPCG/CPCG E10.5 mutant embryos exhibited decreased trigeminal nerve projections into facial prominences [Supplemental Fig. S4C].

Both Fgfr2^F/F and Fgfr2^CPCG/CPCG mice developed peri- ocular lesions in the eye starting at postnatal day P15–P21, associated with a defect in lacrimal gland development [Supplemental Fig. S4D,E]. In mice, lacrimal gland development starts at E13.5 by an epithelial invagination into the surrounding mesenchyme, and progresses by branching morphogenesis to become a fully functional organ by P7. FGF10-FGFR2 signaling plays a critical role during this process where it regulates proliferation in epithelial cells [Steinberg et al. 2005; Garg et al. 2017]. Both Fgfr2^F/F and Fgfr2^CPCG/CPCG mutants showed loose clusters of acinar cells, which populate the distal end of the ducts, and occupied a much smaller area at P7. A significant reduction in the size of the lacrimal gland was also brought about by reduced number of branches and smaller lengths of the tubes in the Fgfr2^F/F and Fgfr2^CPCG/FPCG signaling mutants [Supplemental Fig. S4F,G].

The observation that all Fgfr2 signaling mutants were viable and the fact that the Fgfr2^CPCG/CPCG mutants do not recapitulate the Fgfr2^−− phenotype raised the possibility that a critical downstream adaptor might still interact with the FGFR2CPCG receptor. We data-mined a recent proteomic screen identifying FGFR2C dependent phosphorylation events [Francavilla et al. 2013] and identified IRS2 [insulin receptor substrate] as a possible new putative FGFR2 binding partner. IRS2 belongs to the same superfamily of adaptor proteins as FRS2 and shares a similar protein architecture, with membrane targeting and PTB [phosphotyrosine binding] domains, as well as a C-terminal tail containing multiple tyrosine phosphorylation sites [Supplemental Fig. S4H]. IRS2 bound weakly to both WT and FGFR2CPCG receptors in primary MEFs, independent of FGF stimulation [Supplemental Fig. S4I]. However, we failed to observe a genetic interaction between IRS2^−− and Fgfr2^CPCG/CPCG mutant mice [data not shown], indicating that IRS2 is a not a critical missing effector of FGFR2 signaling in vivo.
Combined Fgfr1/2 signaling mutations do not recapitulate the null phenotypes

Because Fgfr1 and Fgfr2 are coexpressed in cNCCs, we reasoned that discrete functions of signaling pathways downstream from one receptor could be masked by the presence of the other, wild-type receptor. To test this hypothesis, we first analyzed compound conditional hemizygous Fgfr1F and Fgfr1FCPC mutations over the Fgfr1cKO conditional null allele and in the absence of Fgfr2. At E16.5, Fgfr1F/cKO;Fgfr2−/cKO and Fgfr1FCPC/cKO;Fgfr2−/cKO conditional mutants developed severe agenesis of the midface structures, but the phenotype was not as severe as in Fgfr1cKO/cKO;Fgfr2−/cKO mutants (Fig. 3C). In addition, the nasal cartilage and the mandible were more severely affected in Fgfr1FCPC/cKO;Fgfr2−/cKO conditional mutants compared with Fgfr1F/cKO;Fgfr2−/cKO conditional mutants at E16.5 and E18.5 (Fig. 3C; Supplemental Fig. S5A; Supplemental Table S2), highlighting a specific role for FGF-driven CRK and PLCγ signaling in craniofacial development.

Figure 2. Craniofacial defects in double mutants are associated with cell death. (A) Fgfr1cKO/cKO;Fgfr2−/cKO;ROSA26mT/mG mutants showed reduced GFP fluorescence in the PA1 (yellow arrow), PA2 and migratory stream at E10.5 suggesting reduced number of neural crest lineage cells. (B) Flow sorting was used to quantify the percentage of GFP+ cells in the facial prominences at E10.5 embryo across various genotypes. The percentage of GFP+ cells in Fgfr1cKO/cKO;Fgfr2−/cKO;ROSA26mT/mG double mutants was reduced by half as depicted in the bar graph. The proportion of GFP+ cells remain unchanged in Fgfr1cKO/cKO;Fgfr2−/cKO;ROSA26mT/mG mutants although they exhibited observable phenotypic defects. (C) Apoptosis was examined at E10.5 by TUNEL. Increased TUNEL positive cells were observed in Fgfr1cKO/cKO;Fgfr2−/cKO mutants in the lateral nasal process (LNP) compared with controls. Fewer TUNEL positive cells were observed in the medial nasal process (MNP). (D) Quantitation of TUNEL positive foci across different mutant genotypes show a 40-fold increase in cell death in Fgfr1/2 double mutants in the LNP (the asterisk inside the bar graph represents significance level compared with control) at E10.5. (E) Inferior view (mandibles removed) of alcian blue/alizarin red-stained mouse skulls in Fgfr1cKO/cKO;Fgfr2−/cKO;Bim−/+ and Fgfr1cKO/cKO;Fgfr2−/cKO;Bim−/− embryos showed partial rescue of medial structures, the nasal cartilage (NC) and palate (PL) process, premaxilla (PMX), maxilla (MX), and basiphendoid (BS) and palatal shelves (PS) in Bim−/− mutants. The black bar measures midline separation. Average midline separation (mm) is reduced by twofold (N = 4, p = 0.0109) in Fgfr1cKO/cKO;Fgfr2−/cKO;Bim−/+ as represented in the graph. (F) Frontal views of control Fgfr1+/cKO;Fgfr2+/cKO;Bim−/+ and Fgfr1cKO/cKO;Fgfr2−/cKO;Bim−/+ embryos at E17.5 showing partial phenotypic rescue. The black bar indicates intercanthal distance. (G) Cell death levels observed in conditional double mutants were partially rescued in Bim−/+ mutants at E10.5. More TUNEL+ cells were observed in the LNP in Fgfr1cKO/cKO;Fgfr2−/cKO;Bim−/+ and Fgfr1cKO/cKO;Fgfr2−/cKO;Bim−/− compared with corresponding Fgfr1cKO/cKO;Fgfr2−/cKO;Bim−/+ and Fgfr1cKO/cKO;Fgfr2−/cKO;Bim−/− counterparts.
We next turned to Fgfr2 signaling mutants, and analyzed their phenotype over the Fgfr2cKO conditional null allele and in the absence of Fgfr1. Fgfr1cKO;Fgfr2cKO embryos also developed more severe midline fusion and mandible defects than Fgfr1cKO;Fgfr2cKO controls. Anterior skeletal structures such as the nasal cartilage, premaxilla and maxilla, tympanic bulla, and sphenoid bones were either severely reduced or absent. A striking reduction of the mandible was observed along with a complete loss of Meckel’s cartilage (Fig. 3D). We were unable to perform conditional mutagenesis with the Fgfr2FCPG allele as multiple lox sites were retained during the generation of this allele. Similar to Fgfr1cKO;Fgfr2cKO mutants, the frontal bone (F), nasal cartilage (NC), squamosal bone (SQ), tympanic bulla (T), maxilla (MX), pterygoid (Pt), and mandible (MD) were affected. Defects in mandible, squamosal, and pterygoid bones were exacerbated in Fgfr1FCPG/cKO;Fgfr2cKO mutants. Most severe defects were observed in Fgfr1cKO/cKO;Fgfr2cKO/cKO null mutants, where we observed a reduction in alizarin red staining, reduction of mandible, loss of nasal cartilage, and a wider mid-facial clefting (depicted by red bars). Midfacial clefting was quantified (in millimeters) across various genotypes for signaling mutants and is represented in the graph. Scale bar, 1 mm.

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Similar to Fgfr1cKO;Fgfr2cKO mutants, morphological defects in Fgfr1cKO;Fgfr2cKO and Fgfr1FCPG/cKO;Fgfr2cKO embryos arose as early as E10.5 and were accompanied by cell death in the LNP [Supplemental Fig. S5B–E]. Fgfr1FCPG/cKO;Fgfr2cKO embryos and, to a lesser extent, Fgfr1cKO;Fgfr2cKO embryos developed hypoplastic nasal prominences.
In iFNPs was diminished in both for PI3K/AKT and PLCγ (P38 and JNK) (Brewer et al. 2016). For ERK1/2, but also and STAT3) and two less well-characterized pathways total FGF-engaged pathways (ERK1/2, PI3K/AKT, PLCγ). We then eliminated These cells express predominantly iFNPs cells (Supplemental Fig. S6A). enchyme markers was similar between primary and immortalized E10.5 frontonasal prominence cell lines (iFNPs) by crossing to tent mortalized FNP cells (iFNPs) (Supplemental Fig. S6A). We next revisited cell signaling pathways in Fgfr1FCPG/FCPG mutants (Brewer et al. 2015). We used CRISPR/Cas9 to create Fgfr1FCPG/FCPG;Fgfr2CRISPR-KO iNPs cells, and found that ERK1/2, pAKT, and PLCγ activation were eliminated in these cells (Fig. 4B). Fgfr1FCPG/FCPG;Fgfr2RIPR-KO iNPs cells also showed abrogation of pSTAT3 activation. Interestingly, P38 activation and JNK activation were only modestly affected (Supplemental Fig. S7B). Nonetheless, taken together, these results indicate that the most severe signaling mutation combinations in Fgfr1 and Fgfr2 broadly abrogate classic signal transduction pathways for each receptor.

Fgfr1F/F;Fgfr2F/F mutant embryos were not recovered at E10.5. Similar hypoplastic nasal and mandibular prominence defects were observed in Fgfr1FCPG/FCPG;Fgfr2FCPG/FCPG compound mutants (Supplemental Fig. S5C). Interestingly, Fgfr1FCPG/FCPG;Fgfr2FCPG/FCPG mutants survived to E8.0 and still formed mesoderm, as evidenced by T and Fgfr8 staining [Fig. 3F], in contrast to Fgfr1−/−;Fgfr2−/− double-null mutants, which fail at implantation (E5.5) on the same genetic background (Kurowski et al. 2019). These results indicate that signaling mutations in both Fgfr1 and Fgfr2 interact genetically during development, but that the combination of the most severe signaling mutations fails to recapitulate the double null mutant phenotype.

Fgfr1/2 signaling mutations abrogate signal transduction cascades

FGFs activate numerous signaling pathways upon ligand stimulation [Brewer et al. 2016]. To evaluate intracellular pathway activation downstream of wild-type FGR2, FGR2F, FGR2FCPG, and FGR2FCPG, we generated immortalized E10.5 frontonasal prominence cell lines (iFNPs) by crossing to Ink44a/Arf mutants. Similar to a previous study with palatal mesenchymal cells [Fantazzu and Soriano 2017], we found that expression of facial mesenchyme markers was similar between primary and immortalized FNP cells (iFNPs) (Supplemental Fig. S6A). These cells express predominantly Fgfr1 and to a lesser extent Fgfr2, but no Fgfr3 or Fgfr4 (Supplemental Fig. S6B). We then eliminated Fgfr1 expression by CRISPR/Cas9 mutagenesis leaving Fgfr2 as the sole receptor, and interrogated activation by Fgfr1, which gave more robust responses than Fgfr8 (Supplemental Fig. S6C), of four pivotal FGF-engaged pathways [ERK1/2, PI3K/AKT, PLCγ, and STAT3] and two less well-characterized pathways (P38 and JNK) [Brewer et al. 2016]. For ERK1/2, but also for PI3K/AKT and PLCγ, robust activation seen in WT iFNPs was diminished in both Fgfr2F or Fgfr2FCPG/CPCG iFNPs, but only eliminated in Fgfr2FCPG/FCPG mutant cells [Fig. 4A]. Our results thus indicate that both FRS2 and CRKL/PLCγ binding is necessary for activation of these pathways, as for Fgfr1 [Brewer et al. 2015]. STAT3, P38, and JNK activation were also abrogated in Fgfr2FCPG/FCPG iFNPs, with variable effects in Fgfr2FCPG iFNPs and Fgfr2FCPG/CPCG iFNPs [Supplemental Fig. S7A]. For P38 we found that ERK1/2, pAKT, and PLCγ activation were eliminated in these cells (Fig. 4B). Fgfr1FCPG/FCPG;Fgfr2CRISPR-KO iNPs cells also showed abrogation of pSTAT3 activation. Interestingly, P38 activation and JNK activation were only modestly affected (Supplemental Fig. S7B). Nonetheless, taken together, these results indicate that the most severe signaling mutation combinations in Fgfr1 and Fgfr2 broadly abrogate classic signal transduction pathways for each receptor.

Fgfr2 function requires its kinase activity

The lack of more severe phenotypes in Fgfr2 signaling mutants raised the possibility that FGFR2 acts independent of kinase activity. To address this question, we generated a K517A kinase dead (KD) mutation in the ATP binding site at the Fgfr2 locus (Fig. 3A; Supplemental Fig. S3A, B, D; Hanks et al. 1988; Bellot et al. 1991). Fgfr2−/− heterozygous embryos [27/60] showed no obvious defects at E10.5. Fgfr2−/KD heterozygotes appeared normal during later developmental stages up to P0, but fewer than expected [24/78] were recovered after weaning. They exhibited no limb or craniofacial abnormalities, but significantly more severe semidominant defects in lacrimal gland development than Fgfr2F/F and Fgfr2FCPG/CPCG mutants at P15 [Supplemental Fig. S4D–F].

No Fgfr2KD/KD homozygotes were recovered at birth [Supplemental Table S1]. Morphological examination of Fgfr2KD/KD embryos at E10.5 showed characteristic Fgfr2−/− phenotypes, with absence of limb buds, incomplete connection of the allantois to the ectoplacental cone, and dilated pericardium [Fig. 5A], suggesting that FGFR2 broadly operates in a kinase-dependent fashion. Moreover, since the Fgfr2b constitutive mutation leads to similar limb phenotypes but no placental insufficiency [De Moerlooze et al. 2000], and phenotypes in both tissues are observed in Fgfr2−/− or Fgfr2−/KD mutants, FGR2 activity must be kinase-dependent in both mesenchymal and epithelial contexts. However, Fgfr2−/KD embryoids exhibited additional phenotypes including severe posterior truncations and craniofacial defects [Fig. 5A]. We tested for complementation between Fgfr2−/KD and Fgfr2−/− alleles to evaluate phenotypic differences. Fgfr2−/− embryoids showed absence of limb buds and defects in the chorio-allantoic junction, along with a dilated pericardium, similar to Fgfr2−/− embryoids, and posterior truncations defects similar to Fgfr2−/KD embryoids [Fig. 5A]. In contrast, defects in the forebrain, medial and lateral nasal prominences, and maxillary and mandibular prominences appeared less severe than in Fgfr2−/− mutants [Fig. 5B–D].
The semidominant effects in Fgfr2\(^{KD}\) heterozygous mutants during postnatal development in the lacrimal gland, and more severe phenotypes in Fgfr2\(^{KD}\)/Fgfr2\(^{KD}\) mutants relative to the null affecting craniofacial and mesoderm development might be due to the ability for FGFRs to form heterodimers (Bellot et al. 1991; Ueno et al. 1992), although these have never been demonstrated in vivo in the absence of over-expression. The semidominant effects that we observed suggest that the Fgfr2\(^{KD}\) allele not only inactivates Fgfr2 but also suppresses Fgfr1 activity through FGFR2\(^{KD}\):FGFR1 heterodimers, wherever they are coexpressed. This would explain why Fgfr2\(^{KD}\) mutants still generally resemble Fgfr2\(^{−/−}\) mutants, which are lethal at a similar stage, rather than Fgfr1\(^{−/−}\) mutants [Brewer et al. 2015; Molotkov et al. 2017; Kurowski et al. 2019]. While signaling might occur through heterodimers, this mechanism cannot fully account for the discrepancy between FCPG and null phenotypes for either receptor, as Fgfr1\(^{FCPG}/FCPG\);Fgfr2\(^{−/−}\) double mutants develop until E8.0 with a significant degree of mesoderm formation, whereas Fgfr1;Fgfr2 double null mutants have a more severe defect at implantation [Molotkov et al. 2017; Kurowski et al. 2019]. Alternatively, the FGFR2\(^{KD}\) receptor could act as a dominant negative by titrating/sequestering ligand away from functional receptors, if it is distributed differently in the cell, but this is unlikely as Fgfr2\(^{1/FCPG}\) mutants do not show dominant-
Cell matrix and cell adhesion properties are retained beyond canonical signaling

Since signaling mutant cells showed near complete inactivation of classic RTK signaling activities, but the corresponding mutant mice failed to recapitulate the null mutant phenotype, we reasoned that some function engaged by FGF signaling must be retained in the most severe FCPG mutants. Previous lines of evidence have implicated FGF signaling in the control of cell–matrix [Meyer et al. 2012] or cell–cell adhesion [Rasouli et al. 2018; Sun and Stathopoulos 2018; Kurowski et al. 2019]. We first examined Fgfr1;Fgfr2-dependent cell spreading/migration on extracellular matrix across a wound, using primary Fgfr1<sup>−/−</sup>;Fgfr2<sup>−/−</sup>;ROSA26<sup>mT/mG</sup>, Fgfr1<sup>FCPG</sup>;Fgfr2<sup>−/−</sup>;ROSA26<sup>mT/mG</sup>, or Fgfr1<sup>−/−</sup>;Fgfr2<sup>−/−</sup>;ROSA26<sup>mT/mG</sup> iFNP cells upon growth factor stimulation. Spreading of control cells over the wound over 12 h was comparable upon FGF, PDGF, and serum-stimulated conditions. Interestingly, Fgfr1<sup>FCPG</sup>;Fgfr2<sup>−/−</sup>;ROSA26<sup>mT/mG</sup> double-mutant cells failed to spread into the wound area in response to FGF, while responses to PDGF and serum were normal [Fig. 6A].

Defects in migration arise from impaired focal adhesion formation during cell spreading and cell–matrix interaction. However, it is unclear what role FGF signaling plays during this process. We first investigated the role of FGFR1 in focal adhesion formation by looking at Paxillin localization by immunofluorescence as cells spread in Fgfr1<sup>−/−</sup>;Fgfr2<sup>−/−</sup>;ROSA26<sup>mT/mG</sup>, Fgfr1<sup>FCPG</sup>;Fgfr2<sup>−/−</sup>;ROSA26<sup>mT/mG</sup>, Fgfr1<sup>−/−</sup>;Fgfr2<sup>−/−</sup>;ROSA26<sup>mT/mG</sup> double null mutant FNP cells upon treatment with FGF, PDGF, or serum over a 3-h period. Both GFP<sup>+</sup> Fgfr1<sup>−/−</sup>;Fgfr2<sup>−/−</sup>;ROSA26<sup>mT/mG</sup> control cells and Fgfr1<sup>FCPG</sup>;Fgfr2<sup>−/−</sup>;ROSA26<sup>mT/mG</sup> mutant cells formed numerous Paxillin-enriched focal adhesions during PDGF- and FGF-stimulated cell spreading that resembled serum-enriched conditions. Double null mutant cells, however, failed to form any Paxillin<sup>+</sup> foci upon FGF treatment and subsequent cell spreading, but still responded normally to PDGF and serum [Fig. 6B; Supplemental Fig. S8A]. Western blot analysis showed reduced pFAK and total FAK levels in Paxillin<sup>−/−</sup>–<sup>−</sup> cell lines compared with Paxillin<sup>+</sup>−<sup>−</sup> cells (Fig. 6C; Supplemental Fig. S8C,D). We further examined the role of FGFR2 in cell–matrix adhesion by analyzing Fgfr1<sup>−/−</sup>;Fgfr2<sup>−/−</sup>;ROSA26<sup>mT/mG</sup>, Fgfr1<sup>−/−</sup>;Fgfr2<sup>−/−</sup>;ROSA26<sup>mT/mG</sup>, and Fgfr1<sup>−/−</sup>;Fgfr2<sup>−/−</sup>;ROSA26<sup>mT/mG</sup> iFNP cells and found that they spread and formed Paxillin<sup>+</sup> focal adhesions in response to PDGF, or FGF, in contrast to Fgfr1<sup>−/−</sup>;Fgfr2<sup>−/−</sup> iFNP cells [Fig. 6C; Supplemental Fig. S8D]. Because FGFR2 broadly acts in a kinase-dependent fashion, we next tested the behavior of Fgfr1<sup>−/−</sup>;Fgfr2<sup>−/−</sup> iFNP cells and found that they failed to spread and form focal adhesions in response to FGF1, but responded normally to PDGF or serum [Fig. 6C; Supplemental Fig. S8D,E]. We furthermore tested the effect of a pan-FGFR kinase inhibitor, Infigratinib, at various doses found to inhibit FGF induced ERK1/2 activation. We likewise observed a defect in cell spreading and focal adhesion formation in response to FGF1, but responded normally to PDGF or serum [Supplemental Fig. S8F]. Taken together, these observations indicate that although FGFR1<sup>FCPG</sup> and FGFR2<sup>FCPC</sup> lose most FGF-dependent intracellular kinase signaling outputs, they still retain functions pertaining to cell–matrix interactions and that cell spreading and
stabilization of cell–matrix interactions are actively governed by FGF signaling to its receptors. We also explored whether Fgfr1FCPG cells freshly dissociated from the embryo established stable cell–cell contacts comparable with control cells. We found extensive adherens junctions among Fgfr1+/cKO;Fgfr2+/cKO;ROSA26mT/mG control cells and Fgfr1FCPG/cKO;Fgfr2cKO/cKO;ROSA26mT/mG cells, marked by localized β-catenin along cell boundaries (Fig. 7A,B; Supplemental Fig. S8G). In contrast, double null mutant cells showed far fewer cell–cell contacts with no localized β-catenin accumulation, suggesting that contacts are either unstable or do not mature (Fig. 7A,B). Last, we examined cell–cell contacts in vivo, in the E11.5 LNP (Fig. 7C). GFP+ cNCCs in the mesenchyme exhibited extensive cell–cell contacts within the LNP in both control and Fgfr1FCPG/cKO;Fgfr2cKO/cKO;ROSA26mT/mG embryos. Strikingly, GFP+ cNCCs in the double null mutant LNP were mostly isolated and interspersed, although cell contacts in the MNP remained unaffected (Fig. 7C). Taken together, these results indicate that the most severe signaling mutations in Fgfr1 and Fgfr2 still retain cell–matrix and cell–cell interactions otherwise lost in the nulls, while abrogating classic signal transduction pathways.

Discussion

In this work, we sought to address the signaling mechanisms through which FGFs function in development. Because FGFs are known to regulate craniofacial development (Trumpp et al. 1999; Rice et al. 2004; Hosokawa et al. 2009; Griffin et al. 2013; Wang et al. 2013; Brewer et al. 2015), we used the craniofacial mesenchyme as a
model to assess the phenotypic consequences of various disruptions in FGF signaling. First, we showed that Fgfr1;Fgfr2 conditional NCC mutants exhibit a near total lack of mandible development in addition to a midface integration defect, a phenotype considerably more severe than previously noticed [Park et al. 2008], possibly due to the 129S4 congenic background used throughout this study. Second, although we identified tissue-specific requirements for individual signaling pathways in craniofacial development, signaling mutants that disrupt the established signal transduction cascade do not phenocopy null mutants. Last, we demonstrated additional, noncanonical FGF signaling outputs that seemingly function independent of classic FGF signal transduction pathways.

Despite the established activity of FGFs as mitogens in many cell types, we did not detect a significant change in cell proliferation in Fgfr1\(^{1\text{COI} \times 1\text{COI}}\);Fgfr2\(^{1\text{COI} \times 1\text{COI}}\) double null mutant embryos. However, we observed high levels of apoptosis in conditional double null mutants, suggesting that this process might be involved in establishing the overall mutant phenotype. Increased cell death has previously been observed in the branchial arches of hypomorphic or conditional Fgfr8 mutants [Trumpp et al. 1999; Griffin et al. 2013]. Cell death was highest in the LNP, which normally together with the maxillary prominence expand considerably and push cells toward the midline. To functionally test the role of cell death, we crossed a null mutant allele for Bim, which antagonizes anti-apoptotic members of the BCL2 family, into the double conditional null background. This resulted in decreased cell death accompanied by partial rescue of frontal structures, highlighting a critical role for FGF-mediated cell survival during craniofacial development. How FGF signaling might regulate cell survival remains to be determined, but BIM is a known target of phosphorylation by several MAP kinases, particularly ERK1/2, which phosphorylates BIM and targets it for ubiquitination and proteasomal degradation [Clybouw et al. 2012]. JNK and PI3K/akt activation are also known to affect BIM levels [Lei and Davis 2003]. Cell survival through BIM may therefore be regulated by FGF since several of these signaling pathways are engaged by FGR1 and FGR2 [Breuer et al. 2016].

FGFs are best known for activating signal transduction cascades, most prominently ERK1/2 [Lanner and Rossant 2010; Breuer et al. 2016]. Ever since the classic studies on the Sevenless receptor [Simon et al. 1991], we have expected RTK function to depend mostly on canonical signal transduction, but, surprisingly, we found that mutations in Fgfr1 and Fgfr2, which broadly eliminate such outputs, fail to recapitulate the Fgfr1\(^{−}\)/ Fgfr2\(^{−}\) phenotypes. Interestingly, some residual P38 activation was still observed in Fgfr1\(^{FCPG}\)/Fgfr2\(^{FCPG}\); Fgfr2\(^{CRISPR-KO}\) cells. P38 may thus represent a critical FGF1 effector in primitive endoderm [Thamodaran and Bruce 2016], which does not develop well in Fgfr1\(^{−}\)/ Fgfr2\(^{−}\) mutants, in contrast to FGR1\(^{FCPG}\)/Fgfr2\(^{CRISPR-KO}\) embryos that survive to E10.5 [Breuer et al. 2015]. The near complete abrogation of multiple signal transduction outputs in the most severe Fgfr1 and Fgfr2 signaling alleles indicates that we have interrogated relevant cell signaling pathways and implies the existence of noncanonical functions not impacted by our signaling mutations. For FGR2 in particular, the phenotypic gap between Fgfr2\(^{FCPG}\)/Fgfr2\(^{FCPG}\) and Fgfr2\(^{−}\)/ Fgfr2\(^{−}\) mutant embryos could either be due to heretofore unrecognized kinase-dependent signaling activity or to a kinase-independent function. To distinguish between these possibilities, we generated an inactivating Fgfr2\(^{KD}\) allele by introducing a mutation in the ATP binding site of the kinase domain [Hanks et al. 1988; Bellot et al. 1991]. Although such kinase dead mutations have been introduced in cells or organisms ectopically, to our knowledge this is the first such knock-in allele generated in an RTK gene. This Fgfr2 mutation resulted in lethality at E10.5, with defects in limb outgrowth and chorio-alantoic junction, reminiscent of the Fgfr2\(^{−}\)/ Fgfr2\(^{−}\) mutant phenotype. The fact that this mutation recapitulates hallmark Fgfr2\(^{−}\)/ Fgfr2\(^{−}\) mutant phenotypes supports the model that FGR2 broadly operates in a kinase-dependent fashion.

A wide body of literature has implicated FGRs in cell adhesion through interactions of the extracellular domain, which remains untouched in any of our signaling mutations, with cell adhesion molecules [Williams et al. 1994; Francavilla et al. 2009]. FGF ligand binding to FGRs is known to involve a third party, heparan sulfate proteoglycans [Rapraeger et al. 1991; Yayon et al. 1991; Endo et al. 2012], which in turn can interact with integrins, regulating cell–matrix adhesion [McQuade et al. 2006; Moser et al. 2009; Geiger and Yamada 2011]. Consistent with a critical role for FGRs, we found that cell–matrix adhesion was unperturbed in cells carrying the most severe signaling mutations, but lost in the absence of FGRs following FGF stimulation. Because FGF ligand still promotes cell–matrix adhesion in cells derived from Fgfr1\(^{−}\) Fgfr2\(^{−}\) mutant embryos, the activity of cell adhesion receptors must be enhanced independent of traditional signalizing outputs. The cell adhesion defect was also observed in Fgfr1\(^{CRISPR-KO}\). Fgfr2\(^{KD-KD}\) cells and in cells treated with an FGR-specific kinase inhibitor, suggesting a possible model in which the adhesion complex is recruited by FGR1/2 and further modified directly or indirectly by an unknown molecule/kinase. Defects in FGR-dependent adhesion could also result in the induction of anoikis [Frisch and Francis 1994], a process previously shown to be regulated by Bim [Maitheux et al. 2007], potentially linking our observed adhesion defects and increase in cell death in the LNP. Last, focal adhesion assembly and phosphorylation defects have also been observed in Fgfr1\(^{−}\)/ Fgfr2\(^{−}\) keratinocytes [Meyer et al. 2012], suggesting that engagement of FGF signaling has a broad function in regulating cell adhesion in both mesenchymal and epithelial contexts. The involvement of RTK signaling with cell adhesion processes might be more general, as other receptor tyrosine kinases like PDGFRs have been found to bind integrins [Borges et al. 2000], and PDGFR signaling mutations have also failed to recapitulate null mutant phenotypes [Klinghoffer et al. 2002; Tallquist et al. 2003].

Both FGR1 and FGR2 are also known to interact through their extracellular domain acid box with various cell adhesion molecules such as cadherins [Williams
et al. 1994; Kon et al. 2019), critical players in cNCC migration (Scarpa et al. 2015). Consistent with a role for FGFRs in mediating cell–cell adhesion through cadherins, we observed that cells derived from the most severe cell signaling mutants made strong β-catenin-positive cell contacts, in contrast to double null mutant cells. We also observed fewer contacts in vivo, where double null mutant cells showed very limited interactions in the
LNP. FGF signaling regulates E-cadherin localization, and in the absence of Fgfr1, E-cadherin polarization is affected in mural trophoderm [Kuwoski et al. 2019]. Drosophila mesoderm [Sun and Stathopoulos 2018], and zebrafish cardiomyocytes [Rasouli et al. 2018]. FGF signaling also regulates cadherin switching during epithelial to mesenchymal transition, including neural crest cell delamination [Sun et al. 1999; Ciruna and Rossant 2001; Nieto et al. 2016]. Taken together, our results indicate that FGFRs regulate cell adhesion, and possibly other processes, through mechanisms beyond their classic signaling cascades [Fig. 7D]. Additional genetic, biochemical, and cell biological studies may identify further noncanonical roles for these receptors beyond their traditional activities in signal transduction.

Materials and methods

Generation of knock-in mice

Four distinct targeting vectors carrying the Fgfr2 F, Fgfr2 C, Fgfr2 PG, and Fgfr2 KD mutations were generated. The Fgfr2 KD targeting vector was generated by cloning a short homology arm (1.7-kb region between exons 9 and 10) and a long homology arm (5.1 kb, spanning exon 10) into PGKneolox2DTA.2 (Hoch and Soriano 2006). To allow recombineering into SW105 bacteria, the neo cassette was subsequently replaced by PGKEm7neo flanked by FRT sites, which contain both a eukaryotic and a prokaryotic promoter. Similarly, for the Fgfr2 C targeting vector, we cloned a long homology arm (5.3-kb region spanning exon 11) and a short homology arm (1.7-kb region between exons 11 and 12) into PGKneolox2DTA.2 and used a PGKEm7neo flanked by both FRT and LoxP sites for recombineering. For the Fgfr2 PG targeting vector, we cloned a short homology arm (1.9-kb region 5′ of exon 19) and a long homology arm (5.4 kb spanning exon 19 and 3′-UTR) into PGKneolox2DTA.2 and used a PGKEm7neo flanked by FRT sites for recombineering. For Fgfr2 KD targeting vector, we cloned short homology arm (1.9-kb Smal to MfeI, spanning exon 12) and a 3.7-kb-long homology arm (MfeI to BclI, spanning exon 13) into PGKneolox2DTA.2 (Hoch and Soriano 2006). Details of regions corresponding to homology arms are in Supplemental Table S3.

For all four alleles, allele-introduced mutagenesis (SDM) was performed using Phusion polymerase [NEB #M0530]. Nucleotide substitutions introduced by SDM in exon10 for Fgfr2 F allele [introduces an XmaI site], exon11 for Fgfr2 C allele [introduces an ScaI site], exon19 for Fgfr2 PG allele [introduces an EcoRI site] and in exon12 for Fgfr2 KD allele [introduces an AluI site] are provided in Supplemental Figure S3B. All introduced mutations were verified by sequencing.

The targeting vectors for Fgfr2 F [linearized with NotI], Fgfr2 C [linearized with Xhol], Fgfr2 PG [linearized with NotI] and Fgfr2 KD [linearized with NotI] were electroporated into 129S4 AK7 ES cells. For generating the allelic series of signaling mutations, ES cells were targeted first with the C targeting vector generating Fgfr2 KD mutant cells. After verifying for correct targeting events, the neo cassette was removed by transient transfection with PGKCrebpA, leaving a single LoxP site behind [Supplemental Fig. S3A]. Fgfr2 KD ES cells were then targeted using the PG targeting vector generating either Fgfr2 PG or Fgfr2 KD mutant cells, as determined by breeding of the chimeras to ROSA26 Fpino mice [Raymond and Soriano 2010]. After verifying for correct targeting events, the neo cassette was removed by transient transfection with PGKFlpobpA [Raymond and Soriano 2007], leaving both an FRT site and a LoxP site behind [Supplemental Fig. S3A]. Additional clonal screening was performed to identify correctly targeted ES cell clones for each allele, and phenotypes were confirmed in independent mouse lines were generated from independent ES cell clones for each allele, and phenotypes were confirmed in independent mouse lines were generated from independent ES cell clones for each allele, and phenotypes were confirmed in

Mouse strains

All animal experimentation was conducted according to protocols approved by the Institutional Animal Care and Use Committee of the Icahn School of Medicine at Mount Sinai. Fgfr1 KO/KO, Fgfr2 KO/KO, Fgfr1-GFP, and Fgfr2-mCherry were previously described [Hoch and Soriano 2006; Molotkov et al. 2017]. Fgfr1 signaling mutations [Brewer et al. 2015] are referred to as Fgfr1 KD, Fgfr1 KD, and Fgfr1 KD. Genotyping primers listed in Supplemental Table S4 were used to identify Fgfr2 WT band 553 bp, mutant band 655 bp, Fgfr2 PG (WT band 377 bp; mutant band 614 bp), and Fgfr2 KD (note: PCR is followed by Alu1 restriction digestion; WT band 350 bp; mutant band 300 bp) alleles. The F, C, or PG primers were all able to genotype Fgfr2 PG/KO mice.

Generation of Fgfr2-FLAG3x expression vector and stable 3T3 expression lines

An Fgfr2 isoform “c” cDNA isoform was PCR amplified from primary MEFs derived from Fgfr2 KO/KO, Fgfr2 KG/KO, and Fgfr2 KD/KD, and Fgfr2 KG/KO and subsequently digested with HindIII and Xhol. The fragments were cloned in the pcDNA expression vector and sequence verified. Linearized pcDNA-FGFR2 plasmids were transfected in 3T3 cells cultured in DMEM.
supplemented with 10% calf serum with 50 U/mL each penicillin and streptomycin. Stable clones were selected in 500 μg/mL G418. Ten clones from each construct (FGFR2 WT-FLAG3x, FGFR2 PG-FLAG3x, FGFR2 CPG-FLAG3x, FGFR2 F-FLAG3x, or FGFR2 CPG-FLAG3x) were expanded and assessed for FLAG expression by Western blot. Clones expressing high FGFR2-FLAG levels were selected for further analysis.

Coimmunoprecipitation and Western blotting

Stable 3T3 cells expressing FGFR2 WT, FGFR2 PG, FGFR2 CPG, FGFR2 F, or FGFR2 CPG-FLAG3x were serum-starved (0.1% calf serum supplemented DMEM) overnight, stimulated for 15 min with 50 ng/mL FGF1 [PeproTech 450-33A] or FGF8b [PeproTech 100-25B] and 5 μg/mL heparin (Sigma H3149), and lysed in ice-cold NP-40 lysis buffer (20 mM Tris HCl at pH 8, 137 mM NaCl, 10% glycerol, 1% Nonidet [NP-40], 2 mM EDTA, 25 mM β-glycerol phosphate, 1 mM Na3VO4, 10 mM NaF, 1× x Complete, EDTA-free protease inhibitor cocktail [Sigma 11836153001]). Eight-hundred micrograms of cell lysates was subsequently used for immunoprecipitation with anti-FLAG M2 magnetic beads (Sigma M8823) using the manufacturer’s protocol. We incubated lysates with anti-FLAG M2 magnetic beads overnight at 4°C followed by five washes with lysis buffer, and precipitated proteins were eluted in Laemml buffer (10% glycerol, 2% SDS, 0.002% bromophenol blue, 0.062M Tris- HCl at pH 6.8) containing 10% β-mercaptoethanol, heated for 5 min at 95°C, separated by SDS-PAGE, and analyzed by Western blots.

Western blot analysis was performed according to standard protocols using horseradish peroxidase-conjugated secondary antibodies (1:10,000 dilution) developed by chemiluminescent HRP substrate. Primary antibodies were used at the following dilutions for Western blotting: FGFR2 (1:500 dilution, Abcam ab109372), CRKL (1:500 dilution, Santa Cruz Biotechnology sc319), FRS2 (1:500 dilution, Santa Cruz Biotechnology sc8318), FLAG M2 (1:500 dilution, Sigma F1804), phospho-PLCγ1 (1:1000 dilution; CST 9212), phospho-PLCγ1 (1:1000 dilution; CST 4511), p85 (1:500 dilution, CST 9212), phospho-PLCγ1 (Y783, 1:200 dilution; CST 2821), PLCγ1 (1:1000 dilution, CST 2822), pNCK (1:1000 dilution, CST 4671), phospho-STAT3 (Y705, 1:500 dilution; CST 9145) STAT3a (1:1000 dilution, CST 8768), phospho-FAK (1:1000 dilution; CST 3283), FAK (1:1000 dilution, CST 3285), IRS2 (1:500 dilution; CST 3089), β-catenin (1:1000 dilution, CST 8480), and Paxillin (1:1000 dilution, Abcam ab32084). For signaling pathways analyzed, the blots were quantified for three independent biological replicates using ImageLab 6.0 analysis tool. Ratio of average of mean intensity value of pERK1/2, pAKT, pPLCγ1, pSTAT3, pβ-catenin, and Paxillin to GADPH s standard deviation was plotted for each pathway.

Cell derivation and culture conditions

Primary iFNPs were generated by dissecting the maxillary and nasal prominences of E11.5 Fgfr1 Crispr-KO;Fgfr2 −/−, Fgfr1 Crispr-KO;Fgfr2 CPG/CPG, Ink4a−/−, and E9.5 or E11.5 Fgfr2 CPG/CPG;Ink4a−/− embryos in PBS. The tissue was dissociated with 0.125% Trypsin-EDTA [Thermo Fisher 25200] and cultured in DMEM [Sigma D5796] supplemented with 20% BSA [HyClone SH30396.03], 50 U/mL each penicillin and streptomycin ( Gibco P0781) on fibronectin-coated (Sigma FC010) plates (0.5 μg/cm²). Cells were subsequently split 1:5 through for at least five passages before immortalized cell lines were obtained. Cells were allowed to grow until subconfluent. All experiments were performed between passage 15 and 25. We used PX459 V2.0 vector [Addgene plasmid 62988] to CRISPR out either Fgfr1 or Fgfr2 and create Fgfr1-null, Fgfr2-null, or Fgfr1/Fgfr2-double-null cells. gRNA sequences for Fgfr1 and Fgfr2 were selected using the CHOPCHOP gRNA design web tool and were cloned using the oligonucleotides (Supplemental Table S4), as previously described [Ran et al. 2013]. Plasmids were transfected in respective iPNS cells cultured in DMEM supplemented with 10% calf serum [HyClone SH30072.03] with 50 U/mL each penicillin and streptomycin. Stable clones were selected in 5 μg/mL Puromycin (Sigma P8833). Clones were verified [homozygous deletion of exon 6 for Fgfr1 and deletion exon 5 for Fgfr2, which also introduces a frameshift mutation] using PCR [Supplemental Table S4]. Prima- ry MEFs were derived from E12.5 wild-type mouse embryos. Embryos were eviscerated and after removing the head, remaining tissue was chopped into 1-mm pieces and incubated in 1 mL of Trypsin-EDTA (0.25%) for 30 min with intermittent shaking. Ten milliliters of DMEM/10% calf serum was added and the mixture was allowed to pass through a cell strainer. Cells collected from each embryo were plated in 0.2% gelatin-coated 15-cm plates.

Cell-matrix interactions

To study cell–matrix interactions, wild-type serum starved cells in suspension were treated with FGF1 [50 ng/mL FGF1, 5 μg/mL heparin], with or without Infigratinib [BGJ398, Selleckchem S2183], an Fgfr1/2/3 kinase inhibitor. Equal numbers of cells were plated on fibronectin-coated coverslips and cell spreading was quantified over a 90-min period during which cells attached to the matrix. The average number of cells per field was quantified for each treatment. Similar experiments were carried out with Fgfr1 Crispr-KO;Fgfr2 −/−, Fgfr1 Crispr-KO;Fgfr2 CPG/CPG, and Fgfr1 Crispr-KO;Fgfr2 CPG/CPG iFNPs and cell spreading was assessed and quantified upon treatment with either FGF1, PDGF-AA, or serum treatment. Paxillin focal adhesion formation (upon 3-h treatment) in various conditions was further used to assess cell–matrix interactions.

Skeletal preparations

Embryos at E14.5, E16.5, or E18.5 embryos were skinned, eviscerated, fixed in 95% ethanol overnight, and stained (0.015% Alcian blue, 0.005% Alizarin red, 5% glacial acetic acid, in 70% ethanol) overnight at 37°C. Skeletons were then cleared in 1% KOH and transferred to decreasing concentrations of KOH in increasing concentrations of glycerol until clear.

Acetocarmine and hematoxylin and eosin staining

Freshly harvested tissue was fixed in 4% PFA at 4°C overnight, followed by dehydration in 70% ethanol. For acetocarmine staining, tissues were incubated in 0.5% acetocarmine [Sigma C1022, 0.5 g of carmine stain dissolved in 100 mL of boiling 45% acetic acid for 15 min], followed by destaining in 70% ethanol for 1 min and 1% acetic acid [1% HCl in 70% ethanol] for 2 min and 5% acetic acid [5% HCl in 70% ethanol] for 1 min. For hematoxylin and eosin staining, freshly harvested tissues were dissected in PBS, and fixed in 4% PFA followed by dehydration through a graded ethanol series, and embedded in paraffin. 5-μm sections were cut. After deparaffinization and rehydration, sections were stained with Harris-modified hematoxylin [Sigma HHS16], followed by a 10-sec wash in acid-alcohol (1% [v/v] HCl in 70% EtOH), followed by counterstaining with 1% eosinY.
Scratch assays

Cells were seeded onto glass cover slips coated with 5 μg/mL human plasma fibronectin purified protein. At ~90%–100% confluency, cells were scratched with a P1000 pipet tip, washed with PBS and incubated in fresh medium containing either 0.1% FBS, 10% FBS, 50 ng/mL FGF1, and 5 μg/mL heparin or 10 ng/mL PDGF-AA supplemented DMEM for 12 h.

Immunofluorescence and antibodies

For immunostaining whole-mount embryos were fixed in 4% paraformaldehyde solution [PFA] in PBS overnight and washed with PBS five times, permeabilized with 0.5% Triton X-100 in PBS for 30 min, and blocked in 5% BSA for 2 h at room temperature. Primary anti-neurofilament antibody [DSHB clone 2H3] was used at a 1:20 dilution in 5% BSA in PBST; embryos were incubated overnight at 4°C. The next day, embryos were washed four times in PBST and incubated with anti-mouse HRP-conjugated secondary antibodies at a 1:1000 dilution for 4 h at room temperature followed by washing in PBST four times and signal was developed using ImmPACTDAB kit [Vector Laboratories SK4105]. For whole-mount immunofluorescence at E7.5, embryos were fixed overnight in 4:1 methanol:DMSO. Primary antibodies for Eomes [Abcam #ab23355; 1:100 dilution] and Cdx2 [1:100 dilution, Biogenex MU392A] were used. For immunostaining cells, cells were fixed for 10 min in 4% PFA in PBS at room temperature. Cells/tissues were subsequently processed for immunofluorescence analysis as detailed above using anti-paxillin primary antibody [1:250 dilution, Abcam 23345] with Alexa647 conjugated phalloidin [1:40 dilution]. For immunofluorescence on sections, antibodies for GFP [1:100 dilution], mCherry [1:100 dilution] and SMA [1:100 dilution] was used. Embryos were stained with DAPI following fixation as previously described [Sandell et al. 2012]. Cells and tissues were photographed using a Leica SP5 confocal microscope, or a Hamamatsu C11440 camera fitted to a Zeiss Observer Z1 microscope. Epifluorescence was imaged using a Zeiss Axioplan fitted to a ProgRes CT3 camera.

In situ hybridization

Labeled antisense RNA probes were synthesized for Alx3, Msx1, Six3, Nkx2.1, Fgf8, Shh, Col2a1, Col10a1, and Meox1. Digoxigenin-labeled antisense probes were generated as described, and mRNA in situ hybridization on paraffin sections for chromogenic detection was performed using standard protocols.

Micro-CT imaging

Micro-CT imaging of the skulls were performed using a SkyScan 1172 scanner [Bruker]. The mouse heads were dissected and fixed in 10% neutral buffered formalin and washed and stored in PBS at 4°C. The skull bones were scanned with settings of 50 kV, 500 μA, 10-μm pixel resolution, 0.3° rotation steps, and four frames average imaging with a 0.5-mm Al filter at the Micro-CT Core, School of Dentistry, New York University, New York. The acquired X-ray projections were reconstructed using the Imaris software [Oxford Instruments].

Cell proliferation assay

For EdU labeling in mice, pregnant females were injected intraperitoneally with 100 mg/kg body weight of EdU. EdU detection was carried out as per the manufacturer’s instruction for Click-iT EdU cell proliferation kit [Thermo Fisher Scientific #C10340].

TUNEL assay

Sections were deparaffinized and were rehydrated in PBS, followed by post fixation in 4% PFA. In situ cell death detection kit [Roche 12156792910], TMR red user protocol was used to detect cell death. The number of TUNEL-positive foci were normalized to the number of DAPI-stained nuclei to quantify the extent of cell death for MNP and LNP across various genotypes. TUNEL and DAPI channels from LNP and MNP were manually cropped individually from acquired images and background fluorescence was reduced using brightness, contrast, and γ settings equally for all images. Fgfr1+/−cKO;Fgfr2+/−cKO and Fgfr1+/-cKO; Fgfr2-KO/cKO mutants did not show significant numbers of TUNEL-positive foci/pixel, in contrast to Fgfr1+/-cKO; Fgfr2-KO/cKO mutants.

RT-qPCR

Cells were lysed, and mRNA was extracted according to Qiagen RNacasy kit [Qiagen 74106] standard protocol. cDNA was synthesized using a 2:1 ratio of random primers to Oligo(dT) with SuperScript IV RT [Thermo Fisher 18090050]. qPCR was performed with PerfeCTa SYBR Green FastMix for iQ [WWR 101414-264] with Bio-Rad iQ5 multicolor real-time PCR detection system and analyzed with Bio-Rad iQ5 optical system software [version 2.0]. Cycling conditions were as follows: step 1, 3 min at 95°C; step 2, 10 sec at 95°C; step 3, 30 sec at 60°C; repeats steps 2 and 3 for 40 cycles. Proper amplification was confirmed using a melting curve and by running samples on a gel to ensure that the correct size band was obtained. Graphs were made using Microsoft Excel and Prism. Primer sequence for respective genes used for RT-qPCR analysis is listed below.

β-Catenin quantification

Cells were stained and imaged using a Leica SP5 confocal microscope under identical conditions. Stacks were then background subtracted using a 100px rolling ball function in ImageJ. The average pixel intensity along cell / cell junctions was measured in a single z-plane per junction using a 15-pixel line width in ImageJ for the EGFβ and β-catenin channels.

Quantitative and statistical analysis

Statistical analysis was performed using GraphPad Prism6.0 and Microsoft Excel. Values are presented as mean ± standard deviation. The statistical significance was determined using a Student’s t-test with Holm-Sidak method.

Acknowledgments

We thank Jia Li and Chantel Dixon for technical assistance; Kevin Kelley for stable tissue culture facilities; Elaine Fuchs for helpful insights into cell adhesion mechanisms; Jerry Chipuk for conversations about cell death; and our laboratory colleagues, Stu Aaronson, Rob Krauss, and Sergei Sokol for critical comments on the manuscript. We thank the New York University School of Dentistry Micro-CT Core and the Mt. Sinai Flow Cytometry and...
Microscopy facilities for assistance and advice. C.J.D. was supported by F32 DE026678 from National Institutes of Health (NIH)/National Institute of Dental and Craniofacial Research (NIDCR). This work was supported in part by the Tisch Cancer Institute at Mount Sinai [P30 CA196521 Cancer Center Support Grant, for access to Mt. Sinai cores] and by grant ROI DE022778 from NIH/NIDCR to P.S.

Author contributions: P.S., A.T.R., and P.M. conceived the project and planned the experimental approach. A.T.R. generated the bulk of the craniofacial morphogenesis, biochemical, and cell adhesion studies. P.M. performed the initial characterization of signaling mutants. J.R.B. obtained the initial craniofacial phenotypes. C.C. generated all of the signaling mutant targeting constructs. C.J.D. performed embryo and cell imaging. P.S. generated the KD targeting constructs and all of the mouse strains, and directed the project. Data were analyzed by A.T.R., P.M., C.J.D., and P.S. The manuscript was written by A.T.R. and P.S. with edits from all authors.

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