Uncoupling the Mitogenic and Metabolic Functions of FGF1 by Tuning FGF1-FGF Receptor Dimer Stability

Graphical Abstract

FGF1-FGFR dimer stability dictates mitogenic versus metabolic functions of FGF1

Highlights

- FGF1-FGFR dimer stability dictates mitogenic versus metabolic functions of FGF1
- Proliferative response requires robust and long-lived FGF1-FGFR dimerization
- Weak and transient FGF1-FGFR dimerization is sufficient for a metabolic response

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In Brief

Huang et al. report that quantitative differences in FGF-FGFR dimer stability give rise to different thresholds of intracellular signals to determine mitogenic versus metabolic activities of FGFs.
Uncoupling the Mitogenic and Metabolic Functions of FGF1 by Tuning FGF1-FGF Receptor Dimer Stability

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SUMMARY

The recent discovery of metabolic roles for fibroblast growth factor 1 (FGF1) in glucose homeostasis has expanded the functions of this classically known mitogen. To dissect the molecular basis for this functional pleiotropy, we engineered an FGF1 partial agonist carrying triple mutations (FGF1ΔHBS) that diminished its ability to induce heparan sulfate (HS)-assisted FGF receptor (FGFR) dimerization and activation. FGF1ΔHBS exhibited a severely reduced proliferative potential, while preserving the full metabolic activity of wild-type FGF1 in vitro and in vivo. Hence, suboptimal FGFR activation by a weak FGF1-FGFR dimer is sufficient to evoke a metabolic response, whereas full FGFR activation by stable and sustained dimerization is required to elicit a mitogenic response. In addition to providing a physical basis for the diverse activities of FGF1, our findings will impact ongoing drug discoveries targeting FGF1 and related FGFs for the treatment of a variety of human diseases.

INTRODUCTION

The mammalian fibroblast growth factor (FGF) family comprises 15 paracrine-acting and three endocrine-acting ligands that signal through four FGF receptor tyrosine kinases (FGFR1–FGFR4) and their alternatively spliced isoforms to govern a plethora of essential functions in mammalian development, metabolism, and tissue homeostasis (Beenken and Mohammadi, 2009; Goetz and Mohammadi, 2013; Kurosu et al., 2007; Ogawa et al., 2007; Urakawa et al., 2006). HS- or Klotho-dependent dimerization of extracellular domains of FGF by paracrine or endocrine FGFs juxtaposes the cytoplasmic tyrosine kinase domains in correct orientation/proximity to facilitate activation loop (A-loop) tyrosine transphosphorylation resulting in the activation of intracellular kinase domains (Mohammadi et al., 1996). This in turn leads to phosphorylation of intracellular substrates and activation of multiple downstream signaling pathways (Eswarakumar et al., 2005; Lemmon and Schlessinger, 2010; Schlessinger and Lemmon, 2003). Despite exerting pleiotropic functions, all FGFs rely on a common set of intracellular pathways including PLCγ/PKC, FRS2α/RAS-MAPK, Gab1/PI3 kinase/Akt, and CrkL/Cdc42-Rac pathways (Dailey et al., 2005; Eswarakumar et al., 2005; Kouhara et al., 1997; Larsson et al., 1999; Seo et al., 2009). Hence, the underlying molecular mechanisms of signaling specificity and/or diversity have remained elusive.

To gain insights into the molecular mechanisms regulating FGF signaling specificity/diversity, in this report, we analyzed the large body of accumulated structural data in our laboratory that includes eight binary paracrine FGF-FGFR complexes, an HS-assisted 2:2 FGF-FGFR dimer, and two endocrine FGFs (Beenken and Mohammadi, 2012; Goetz and Mohammadi, 2013). This analysis led us to posit a model whereby different biological responses of FGFs are manifestations of different thresholds in FGF-FGFR binding/dimerization, with corresponding differences in FGF transphosphorylation and activation. We tested our working model using FGF1, the founding member of the FGF family that is best known for its mitogenic activity on cells from a variety of tissue origins including liver, vasculature, and skin (Kan et al., 1989; Nabel et al., 1993; Wiedlocha et al., 1996). Recently, Jonker et al. (2012) discovered an unexpected metabolic role for FGF1 as a critical transducer of PPARγ signaling that mediates the proper coupling of nutrient storage to adaptive remodeling of adipose tissue. In a follow-up study, we showed that administration of exogenous FGF1 stimulates glucose uptake in an insulin-dependent fashion,
much like the endocrine-acting non-mitogenic FGF21 (Suh et al., 2014). The mixed mitogenic and metabolic activities of FGF1 make this ligand an ideal paradigm for dissecting the role of FGF-FGFR dimer stability in differentiating mitogenic versus metabolic functions of FGFs. Based on our detailed structural insights into HS-assisted paracrine FGF-FGFR dimerization (Mohammadi et al., 2005; Schlessinger et al., 2000), an FGF1 variant, termed FGF1\textsubscript{DHS}, with suppressed ability to induce HS-dependent FGFR dimerization was engineered. Detailed analysis of the mitogenic and metabolic properties of this FGF1 variant showed that it has greatly reduced proliferative potency and efficacy while retaining full metabolic efficacy of wild-type FGF1 (FGF1\textsubscript{WT}) both in vitro and in vivo. These data support our hypothesis that differences in FGF-FGFR binding affinity and dimer stability translate into differences in the magnitude of intracellular signals emanating from FGFRs to determine mitogenic versus metabolic cellular responses. Moreover, the results from this study pave the way for engineering safer FGF1 and other FGFs that solely possess metabolic activity.

**RESULTS AND DISCUSSION**

**Structural Analysis Identifies a Link between 1:1 FGF-FGFR Binding Affinity and 2:2 Dimer Stability and Differential Biological Activity of FGFs**

The existence of a relationship between the strength of FGF-FGFR binding/dimerization and the divergent biological activities of FGFs was first inferred from our structural exploration of the functional dichotomy of FGF8 isoforms. FGF8a and FGF8b are two alternatively spliced FGF8 isoforms that share an identical FGFR binding specificity profile yet elicit markedly different tissue patterning outcomes during brain development (Joyner et al., 2000). Through solving the crystal structure of FGF8b in complex with FGFR (Olsen et al., 2006) (PDB: 2FDB) (Figure 1A). We then showed that mutation of Phe-32 to alanine converts FGF8b to an FGF8a-like molecule with regard to FGFR binding affinity and morphogenetic potential (Olsen et al., 2006). Because 2:2 FGF-FGFR dimer stability is directly proportional to 1:1 FGF-FGFR binding affinity, these data suggest that FGF8b forms a more robust 2:2 FGF-FGFR dimer than FGF8a, which in turn implies that FGF8b is more potent in inducing FGFR transphosphorylation and activation than FGF8a. Moreover, because FGFR activation is a prerequisite for intracellular substrate phosphorylation and activation of downstream pathways, it follows that FGF8b should be transmitting robust/persistent signals, whereas intracellular signals of FGF8a should be weak and transient. These observations led us to postulate that differences in
the abilities of FGF8a and FGF8b to bind, dimerize, and activate FGFR1c translate into differences in the amplitudes of downstream signaling resulting in distinct biological responses. To test this hypothesis, we used size exclusion chromatography (SEC) coupled with multi-angle light scattering (MALS) (SEC-MALS) to compare the ability of FGF8a, FGF8b, and the F32A mutant derivative of FGF8b (FGF8bF32A) to promote HS-assisted FGFR1c dimerization in vitro. As shown in Figure 1B, FGF8b exhibited a greater capacity than FGF8a to dimerize the FGFR1c. Mutation of Phe-32 to alanine converted FGF8b to an FGF8a-like molecule with regard to FGFR binding affinity (Figure S1A) and FGFR dimerization capacity (Figure 1B). We further compared the stability of FGF8a-FGFR1c, FGF8b-FGFR1c, and FGF8bF32A-FGFR1c complexes in the absence and presence of HS by measuring their unfolding/melting temperatures (Tm) using a fluorescence dye-based thermal shift assay. Consistent with the higher affinity of FGF8b for FGFR1c measured in SEC-MALS experiments (Figure S1A), Tm of the FGF8b-FGFR1c complex was higher by 2.5°C than those of the FGF8a-FGFR1c and FGF8bF32A-FGFR1c complexes without HS (Figure S1B). The higher affinity of FGF8b for FGFR1c also manifested in ~11.5°C higher Tm of the FGF8b-FGFR1c-HS complex than those of FGF8a-FGFR1c-HS and the FGF8bF32A-FGFR1c-HS complexes (Figure 1C). Having confirmed the greater stability of the FGF8b-FGFR1c-HS complex relative to the FGF8bF32A-FGFR1c-HS complexes, we next compared side-by-side the abilities of FGF8a, FGF8b, and FGF8bF32A to bind, dimerize, and activate FGFR1c isoform ectopically expressed on the surface of BaF3 cells. Treatment of cells with increasing doses of FGF8a, FGF8b, and FGF8bF32A in the presence of HS showed that FGF8b is more potent than FGF8a in inducing phosphorylation of FGFR1c on the kinase A-loop tyrosines and downstream MAPK phosphorylation. Moreover, mutation of Phe-32 to alanine converted FGF8b to an FGF8a-like molecule with regard to activating the intracellular signaling pathway (Figure 1D).

Comparative structural analysis of the paracrine and endocrine FGFs further emphasizes the notion that differences in the strength of FGF-FGFR binding and dimerization could underlie the divergent biological activities of FGFs. Modeling of endocrine FGF-FGFR complexes based on the crystal structures of paracrine FGF-FGFR complexes as templates reveals replacements of at least one residue in the respective receptor binding site of endocrine FGFs with residues that are less optimal for receptor binding (Figures 2A and 2B). Consistent with these structural observations, binding of endocrine FGFs to FGFR is barely detectable in vitro (Ibrahim et al., 2004; Khartononenkov et al., 2005; Mohammadi et al., 2005; Yie et al., 2009). This structural analysis implies that compared to paracrine FGFs such as FGF1, endocrine FGFs should have weaker ability in dimerizing and activating FGFRs and correspondingly should have weaker capacity to activate intracellular signaling pathways. To test our structural prediction, the lentiviral expression system was used to establish two BaF3 cell lines that ectopically co-express FGFR1c (a cognate FGR for all three endocrine FGFs) and the full-length transmembrane form of αKlotho (coreceptor for FGF23) or βKlotho (coreceptor for FGF19 and FGF21). The side-by-side comparison of the abilities of two different concentrations of FGF1WT, wild-type FGF21 (FGF21WT), wild-type FGF19 (FGF19WT), and wild-type FGF23 (FGF23WT) clearly showed that at both doses tested, all three endocrine FGFs were weaker than FGF1WT in inducing phosphorylation of FGFR1c on the kinase A-loop tyrosines and downstream MAPK phosphorylation (Figures 2C–2E). Importantly, compared to paracrine FGFs such as FGF1 that are potent mitogens for a variety of cell types, the endocrine FGFs completely lack FGF21 or have poor mitogenic activity (FGF19) (Figure S2A), which is consistent with previously published data (Kharitononenkov et al., 2005; Nakamura et al., 2011; Omitz et al., 1996; Suzuki et al., 2008; Zhang et al., 2006a). The higher mitogenic activity of FGF19 than FGF21 is congruent with the higher FGFR binding affinity of FGF19 relative to FGF21 (Wu et al., 2010).

Taken together, these data substantiate the existence of a direct link between the strength/stability of FGF-FGFR binding/dimerization and the selection of cellular outcome. Our results lead us to propose that while mitogenic activity would require strong FGF-FGFR binding and persistent dimerization, a metabolic response could be achieved with a weak FGF-FGFR binding and transient receptor dimerization.

The Metabolic Effect of FGF1 Can Be Uncoupled from Its Mitogenic Activity

FGF1, a classical FGF mitogen, was recently shown to also possess metabolic activity similar to the non-mitogenic, endocrine FGF21 (Suh et al., 2014). The mixed mitogenic and metabolic activities of FGF1 make this ligand an ideal paradigm for directly testing our hypothesis on the role of FGF-FGFR dimer stability in determination of cellular outcome. We and others have previously shown that in the absence of HS, the affinities of paracrine FGFs for their cognate FGFR are below the threshold necessary to induce sustained receptor dimerization (Delehedde et al., 2002; Makarenkova et al., 2009; Mohammadi et al., 2005; Schlessinger et al., 2000). HS simultaneously engages the HS binding sites of FGF and FGFR simultaneously with a resulting enhancement of 1:1 FGF-FGFR binding and stabilization of 2:2 FGF-FGFR dimers (Mohammadi et al., 2005; Schlessinger et al., 2000). Guided by our structural insights on HS-assisted FGF-FGFR dimerization (Figure 3A), we decided to engineer an FGF1 variant with a diminished ability to promote HS-assisted FGF1 dimerization. To this end, three key residues from the HS-binding site of FGF1 (namely Lys127, Lys128, and Lys133) were replaced with residues that are less optimal for HS binding (Lys127Asp, Lys128Gln, and Lys133Val; termed FGF1HBS) (Figure 3B). We postulated that as a result of compromised HS-binding affinity, FGF1HBS should possess a weaker ability to promote HS-assisted FGF1 dimerization and should therefore transmit weaker intracellular signals compared to the wild-type parent molecule.

To confirm our structural prediction, biotinylated heparin (a surrogate for HS) was captured onto a streptavidin containing surface plasmon resonance (SPR) biosensor chip, and increasing concentrations of FGF1WT and FGF1HBS were passed over the chip. SPR spectroscopy data confirmed that the FGF1HBS mutant sustained a substantial loss in HS binding affinity (Figure 3C). We next used SEC-MALS to compare the capacities of FGF1WT and FGF1HBS to induce FGFR1c

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dimerization in the absence and in the presence of a HS deca-
saccharide (Figures 3D and S3A). In the absence of HS, both 
FGF1WT and FGF1HBS formed stable 1:1 complexes with 
FGFR1c with identical calculated molecular weights (MWs) of 
37.7 kDa (FGF1WT) and 37.2 kDa (FGF1HBS), which were very 
close to the theoretical MW of 42.8 kDa (Figure S3A). As shown 
in Figure 3D, the HS decasaccharide caused stoichiometric 
dimerization of the FGF1WT-FGFR1c complex. The MW of the
dimeric species (89.19 kDa) measured by MALS matched closely with the theoretical MW for a 2:2:2 dimer (91.546 kDa). In contrast, in the presence of HS, the FGF1\textsuperscript{D\textsubscript{HBS}}-FGFR1c-HS complex eluted as a 64.75 kDa species, larger than a 1:1 complex but smaller than a 2:2 complex. These data indicate that compared to the 2:2:2 FGF1\textsuperscript{WT}-FGFR1c-HS dimer, the FGF1\textsuperscript{DHBS}-FGFR1c-HS dimer is less stable and interconverts between 2:2 and 1:1 complexes of FGF1\textsuperscript{DHBS}-FGFR1c in the timescale of size exclusion chromatographic analysis. To further drive this point home, we compared the stability of FGF1\textsuperscript{WT}-FGFR1c and FGF1\textsuperscript{D\textsubscript{HBS}} complexes in the presence and absence of HS by measuring their unfolding temperatures (Tm) using a fluorescence dye-based thermal shift assay (Figures 3E and S3B). In agreement with the SEC-MALS data (Figure S3A), in the absence of HS, both FGF1\textsuperscript{WT}-FGFR1c and FGF1\textsuperscript{D\textsubscript{HBS}}-FGFR1c exhibited similar Tms. In the presence of...
HS, however, Tm of the FGF1<sup>ΔHBS</sup>-FGFR1c-HS was ~13°C lower than that of FGF1<sup>WT</sup>-FGFR1c-HS. These data that are harmonious with the SEC-MALS data (Figure 3D) demonstrate the reduced stability of FGF1<sup>ΔHBS</sup>-FGFR1c-HS complex relative to the FGF1<sup>WT</sup>-FGFR1c-HS. Hence, this engineered FGF1<sup>ΔHBS</sup> variant constituted a tool for the exploration of the role of FGF1-FGFR dimer stability in the regulation of mitogenic and metabolic responses elicited by FGF1.

We next compared the mitogenic potencies of FGF1<sup>WT</sup> and FGF1<sup>ΔHBS</sup> using NIH 3T3 fibroblasts, which are known to endogenously express several FGFR isoforms including FGFR1c, FGFR4, HSPGs, and Klotho (Li et al., 1994). As shown in Figure 3F, relative to FGF1<sup>WT</sup>, FGF1<sup>ΔHBS</sup> sustained a major loss in mitogenic activity. Regardless of how high concentrations were used, FGF1<sup>ΔHBS</sup> was incapable of achieving the maximal response elicited by FGF1<sup>WT</sup>. A dose-response comparison of FGF1<sup>WT</sup> and FGF1<sup>ΔHBS</sup> showed that FGF1<sup>ΔHBS</sup> is at least an order of magnitude weaker than FGF1<sup>WT</sup> in inducing A-loop tyrosine phosphorylation of FGFRs (Figure 3G). In agreement with the receptor phosphorylation data, FGF1<sup>ΔHBS</sup> had a diminished ability to induce FRS2α and ERK phosphorylation, a major substrate of FGFRs on tyrosine 196, and downstream ERK activation (Figure 3G).

We further compared the stability of cell surface FGF-FGFR dimers induced by FGF1<sup>WT</sup> and FGF1<sup>ΔHBS</sup> by analyzing the intensity and duration of FGFR phosphorylation on A-loop tyrosines and activation of downstream FRS2α/ERK pathway kinetically. As shown in Figure 3H, treatment of cells with FGF1<sup>WT</sup> led to the appearance of a signal for A-loop tyrosine phosphorylated FGFR as early as 2 min and the signal continued to intensify until 15 min. By 30 min, the signal intensity diminished but still remained above that observed in the vehicle-treated cells. In contrast, in FGF1<sup>ΔHBS</sup>-treated cells, a weak phosphorylation of FGFR1 was observed after only 5 min, and the signal completely dissipated by 30 min. The signals for phosphorylated FRS2α and ERK followed the same time-dependency trend as that of the FGFR A-loop phosphorylation. Combined with the HPLC-MALS and fluorescence dye-based thermal shift data, these cell-based experiments show that FGF1<sup>WT</sup> is capable of promoting stable and long-lived FGFR dimerization and correspondingly generating robust and persistent intracellular signaling. By contrast, FGF1<sup>ΔHBS</sup> can only induce weak FGFR activation and trigger short-lived downstream intracellular signaling.

Having determined that FGF1<sup>ΔHBS</sup> is less mitogenic than FGF1<sup>WT</sup>, we next compared side-by-side the metabolic activities of FGF1<sup>WT</sup> and FGF1<sup>ΔHBS</sup> in rat hepatoma cell line (H4IIE) and the differentiated 3T3L1 adipocytes. H4IIE endogenously express several FGFR isoforms including FGFR1c, FGFR4, HSPGs, and Klotho (Li et al., 1994), the obligatory coreceptor of endocrine FGFs but lack Klotho (Li et al., 1994). The obligatory coreceptor of endocrine FGFs but lack Klotho. However, upon differentiation to adipocytes, they also express Klotho and thus become responsive to FGF19 and FGF21. Therefore, we also included FGF21<sup>WT</sup> and FGF19<sup>WT</sup> as controls in this assay. As shown in Figures 4A and 4B, despite its major loss in mitogenic activity, FGF1<sup>ΔHBS</sup> retained the full capacity of FGF1<sup>WT</sup> to stimulate glucose uptake in both H4IIE and 3T3L1 adipocytes. Remarkably, the metabolic activity of FGF1<sup>ΔHBS</sup> was similar to those of endocrine FGFs (Figures 4A and 4B). We also compared the abilities of FGF1<sup>WT</sup>, FGF1<sup>ΔHBS</sup>, FGF21<sup>WT</sup>, and FGF19<sup>WT</sup> to induce phosphorylation of FRS2α/ERK pathway. Consistent with the results obtained using transfected BaF3 cells (Figure 2) in both H4IIE and 3T3L1 adipocyte cell lines, FGF1<sup>ΔHBS</sup> exhibited a significant loss in the ability to induce phosphorylation of FGFR on A-loop tyrosines, with activation of the FRS2α-RAS-MAPK pathway essentially behaving more like an FGF19 and FGF21 molecule (Figures 4C and 4D). Apparently, the amplitude/strength of FGFR activation and ensuing downstream intracellular signaling generated by FGF1<sup>ΔHBS</sup> is comparable to...
that induced by the endocrine FGFs, is below the threshold necessary for a mitogenic signal but still sufficient for a metabolic response to occur. These in vitro data corroborate our model that differences in FGF-FGFR binding affinity and dimer stability translate into differences in the magnitude of intracellular signals emanating from FGFRs to determine cellular responses. Specifically, a metabolic response can be achieved with transient receptor dimerization and partial receptor activation, whereas mitogenic activity requires persistent FGFR dimerization and full activation.

Comparison of the Mitogenic and Hyperplastic Activity of FGF1WT with FGF1ΔHBS in Normal Mice
To corroborate our in vitro findings, we assessed the mitogenic activity of FGF1WT and FGF1ΔHBS in vivo. To this end, normal C57BL/6J mice were injected either intraperitoneally (0.5 mg/kg body weight for 3 months) or intravenously (2.0 mg/kg body weight for 1 month) with FGF1WT and FGF1ΔHBS every other day, and the mice livers were analyzed for signs of hyperplasia by immunohistochemical staining using PCNA and Ki67 and western blotting. Irrespective of the routes of administration, FGF1WT caused a clear increase in the number of hyperproliferating cells in the liver of mice. In contrast, there was no increase in the number of hyperproliferating cells in the livers of FGF1ΔHBS-treated mice over that of the PBS-treated control (Figures 5A–5F). Alongside the in vivo experiment, we also compared the abilities of FGF1WT and FGF1ΔHBS to induce activation of FGFRs in primary liver cells by measuring the levels of induction of the FRS2α/ERK pathway. The data showed that FGF1ΔHBS exhibited a significant loss in the ability to induce FRS2α and ERK phosphorylation (Figures 5G and 5H).

Effects of FGF1WT and FGF1ΔHBS on Blood Glucose Levels and Insulin Sensitivity in db/db Mice
We next compared the metabolic activities of FGF1WT and FGF1ΔHBS in a diabetic mouse model (db/db). An acute injection of FGF1WT and FGF1ΔHBS (0.5 mg/kg body weight) significantly lowered blood glucose levels with effects lasting up to 24 hr post-injection (Figure 6A). Moreover, this effect was dose-dependent in both FGF1WT and FGF1ΔHBS treatment groups (Figure 6B), consistent with previously published data (Suh et al., 2014). The blood glucose levels of db/db mice acutely treated with FGF1WT or FGF1ΔHBS remained lower throughout the glucose tolerance test (GTT) (Figures 6C and 6D). Furthermore, mice treated with either FGF1WT or FGF1ΔHBS showed a marked improvement in insulin sensitivity as measured by an insulin tolerance test (ITT) (Figures 6E and 6F).

We next studied the effects of chronic administration of FGF1WT and FGF1ΔHBS by injecting db/db mice with 0.5 mg/kg every other day for 4 weeks. As recently shown for FGF1WT (Suh et al., 2014), FGF1ΔHBS caused sustained glucose lowering (Figure 6G) with minimal changes in body weight (Figure 6H). Notably, both FGF1WT and FGF1ΔHBS were able to normalize blood glucose levels in db/db mice after 4 weeks of treatment (Figure 6G). Importantly, as previously shown for FGF1WT (Suh et al., 2014), FGF1ΔHBS did not induce hypoglycemia in both normoglycemic (healthy) chow-fed mice (Figure S4A) and diabetic mice even at a high dose (Figures S4B and S4C). FGF1ΔHBS had no effect on blood glucose levels in streptozotocin (STZ)-induced type 1 diabetic mice demonstrating that FGF1ΔHBS mediates its glucose-lowering effects in an insulin-dependent fashion (Figure S4D).

Chronic Effects of FGF1WT and FGF1ΔHBS on Hepatic Lipid and Glucose Metabolism in db/db Mice
We also analyzed the effects of FGF1WT and FGF1ΔHBS on hepatic lipid and glucose metabolism. As expected, db/db mice had markedly enhanced hepatic steatosis as evident by hematoxylin and Oil Red O staining (Figure S5A). Chronic treatment of db/db mice with FGF1WT and FGF1ΔHBS showed that both ligands are capable of attenuating this hepatic steatosis (Figure S5A). Consistent with our histological findings, chronic FGF1WT and FGF1ΔHBS treatment also significantly reduced hepatic levels of triglycerides in the livers of db/db mice (Figure S5B). Notably, these values approached those of lean, PBS-treated littermates (db/m) (Figure S5B). The decreased lipid accumulation in hepatic tissues implies a decrease in lipogenesis (lipid synthesis) and lipid storage in both FGF1WT and FGF1ΔHBS variant treatment groups. This was confirmed by the finding of lateral reduction in multiple lipogenic gene expression at both protein (Figures S5D–S5G) and mRNA (Figures S5H–S5J) levels in hepatic tissues.

Hepatic glycogen synthesis and breakdown play an important role in modulating blood glucose levels (Satelli and Kahn, 2001). Accordingly, the effect of chronic treatment of FGF1WT and FGF1ΔHBS on hepatic glycogen levels was determined using periodic acid-Schiff staining (PAS) and a colorimetric assay. As shown in Figures S5A and SSC, compared to their lean littermates (db/m), db/db mice had elevated hepatic glycogen levels, which was consistent with a previous report (Zhang et al., 2006b). In addition, hepatic glycogen levels were further increased following treatment of db/db mice with FGF1WT and FGF1ΔHBS (Figures S5A and SSC). These observations demonstrate that chronic treatment of db/db mice with FGF1WT and FGF1ΔHBS lowers glucose levels by increasing insulin sensitivity to thereby manifest in elevated hepatic glycogen synthesis and storage.

Concluding Remarks
In conclusion, we show that the mitogenic and the metabolic activity of FGF1 can be uncoupled by surgically dampering FGF1-FGFR dimer stability through triple point mutations that diminish FGF1–HS affinity. The FGF1ΔHBS sustained a loss in the ability to promote HS-dependent FGFR dimerization to a level that practically eliminated its mitogenic activity without compromising its metabolic function. To begin to gain insights into the impacts of FGF1-FGFR dimer stability on the choice of downstream intracellular pathways, we performed SILAC-based phosphoproteomics on 3T3L1 fibroblasts (a pre-adipocyte cell line) that were stimulated with FGF1WT or FGF1ΔHBS. Analysis of the phosphorylation of FGFR, its direct intracellular substrates, and further downstream molecules reveals a quantitative change in overall net phosphorylation and activation of FGFR and correspondingly weaker phosphorylation of downstream effector proteins between FGF1WT- and FGF1ΔHBS-treated cells (Figure S6). These findings support the concept that different thresholds of receptor
dimerization translate into different degrees of FGFR activation and downstream “signal flow” that in turn manifest in different cellular outcomes (i.e., mitogenesis versus metabolism) (Figure 7). The threshold model is further corroborated by a side-by-side comparison of the dimerization ability and mitogenic versus metabolic potentials of FGF1WT, FGF1ΔNT1, FGF1ΔHBS, and FGF1ΔNT2. As shown in Figure S7, these four FGF1 molecule show a gradient of dimerization capacity in the following order: FGF1WT > FGF1ΔNT1 > FGF1ΔHBS > FGF1ΔNT2. Consistent with our threshold model, as the dimerization strength decreases, the mitogenic potential dissipates first before a decrease in metabolic response can be observed. This trend between

Figure 5. Mitogenic Activity of FGF1WT and FGF1ΔHBS on Liver Tissue In Vivo

(A and D) Microscopic images of mouse liver tissue sections stained for proliferating cell nuclear antigen (PCNA) and Ki67. Liver tissue was isolated from normal C57BL/6J mice intraperitoneally treated with FGF1WT or FGF1ΔHBS (0.5 mg/kg body weight) every other day for 3 months (A) and from normal C57BL/6J mice intravenously treated with FGF1WT or FGF1ΔHBS (2.0 mg/kg body weight) every other day for 1 month (D), respectively. Note the increased number of PCNA- and Ki67-positive cell nuclei in liver tissue from FGF1WT-treated mice compared with liver tissue from FGF1ΔHBS-treated mice. Data are representative of 11 mice from each group. Scale bar, 50 μm.

(B and C) Immunoblot analysis (B) and its corresponding densitometric analysis (C) for PCNA and Ki67 protein expression in liver tissues from mice intraperitoneally treated with 0.5 mg/kg body weight FGF1WT or FGF1ΔHBS every other day for 3 months.

(E and F) Immunoblot analysis (E) and its corresponding densitometric analysis (F) for PCNA and Ki67 protein expression in liver tissues from mice intravenously treated with 2.0 mg/kg body weight FGF1WT or FGF1ΔHBS every other day for 1 month. Data in (B), (C), (E), and (F) are presented as mean ± SEM (n = 11). *p < 0.05, ***p < 0.001 versus vehicle control; ##p < 0.01, ###p < 0.001 versus FGF1WT.

(G and H) Immunoblots showing the activation of FRS2α/ERK pathway induced by FGF1WT and FGF1ΔHBS in primary liver cell line. (H) Quantification of western blot (G) by densitometric analysis. Data from three independent measurements are presented as mean ± SEM. ***p < 0.001 versus PBS buffer control, ##p < 0.01, ###p < 0.001 versus FGF1WT.

See also Figure S2.
dimerization strength and the ensuing signaling outcome of FGF1 molecules further validates our “threshold” model (Figure 7).

Like FGF1, FGF19 also possesses “mixed” metabolic and “mitogenic” activities although mitogenic activity of FGF19 is inferior to that of FGF1 (Figure S2). In an effort to eliminate the undesired mitogenic effect of FGF19, Wu et al. (2010) at Amgen previously engineered a nonmitogenic FGF19 chimera wherein four residues from N terminus of FGF19 were replaced with the corresponding three residues from FGF21. This chimera was, however, shown to retain wild-type FGF19-like capacity to modulate glucose and bile acid metabolisms. The selective loss in mitogenic activity was attributed to a selective loss in binding of the chimera to FGFR4 that was proposed to mediate the mitogenic activity of FGF19. By contrast, the chimera retained the ability to bind FGFR1c, which was proposed to mediate metabolic actions of FGF19. However, based on our structural analysis, these N-terminal alterations would reduce interactions of FGF19 with both FGFR1c and FGFR4 such that reduced affinity and correspondingly reduced dimerization ability of this chimera underlies the non-mitogenic character of the chimera. Reminiscent of our results, Swanson et al. (2015) recently reported a therapeutic lectin that retains its full antiviral activity but is devoid of the undesired mitogenic activity. An engineered point mutation diminishes the glycan binding affinity of this lectin variant below the threshold necessary for formation of multivalent lectin-glycoprotein complexes that give rise to mitogenesis. However, the remaining glycan binding affinity of the variant was still sufficient to allow the mutated lectin to bind to viral glycan chains and hence exert antiviral effects. Hence, it appears that pleiotropic functions of other classes of proteins may also be manifestations of quantitative differences in the binding interactions with cognate binding partners. Full proof of this concept should open up distinctive horizons in engineering a repertoire of biologics with unique properties for use in both basic and translational research.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification
All the wild-type and mutated FGF1 (FGF1WT, FGF1NT1, FGF1NT2, and FGF1NT2), wild-type FGF8b and its mutation (FGF8bDHB), and wild-type FGF8a were expressed in E. coli (BL21) and purified as described in the Supplemental Experimental Procedures.
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**Figure 7. Schematic Representation of the “Threshold” Model**

The quantitative differences in the strength of FGF-FGFR binding and stability of dimerization underlie the proliferative and metabolic activities of the FGF ligands. Top: shows the correlation between the FGF-FGFR dimerization strength and the corresponding strengths of the intracellular signal. FGF1 and FGF2 have strong capacity to bind and dimerize FGFR1c and result in a strong intracellular signaling. FGF19, FGF1-NT2, FGF1-mHBB, FGF21, and FGF1-NT2 have a gradually weaker intracellular signal due to weaker dimerization strengths. FGF1-NT2 has almost no ability to bind/dimerize FGFR1c and hence produces no significant intracellular signaling. Bottom: shows the threshold of FGFR dimerization strength that determines the nature of signaling response. Sustained FGF-FGFR binding and dimerization is necessary to elicit strong intracellular signaling required to produce a mitogenic response (red trace) and weak FGF-FGFR binding and dimerization by a weak agonist leads to dampened intracellular signaling that is sufficient for a metabolic response (blue trace).

See also Figures S6 and S7.

**SPR Spectroscopy**

Real-time biomolecular FGF1-heparin interactions were analyzed with a BIAcore 2000 system (GE Healthcare) in HBS-EP buffer (10 mM HEPES-NaOH, 50 mM NaCl, 3 mM EDTA, and 0.005% [v/v] polysorbate 20) at 25°C. A heparin chip was prepared by immobilizing biotinylated heparin (Sigma-Aldrich) on flow channels of a research grade streptavidin chip (GE Healthcare). The control flow channel was left blank. Increasing concentrations of FGF1WT or FGF1mHBB were injected over the chip. The heparin chip was washed three times with 100 mM NaCl and 10 mM HEPES in PBS. Each experiment contained 60 μL of 200-fold diluted 5,000-fold stock solution, Bio- Rad, 50 μM FGFR1c in 25 mM HEPES pH 7.5, 150 mM NaCl with and without different ligands were made in duplicate in PCR strips at a final volume of 20 μL. The temperature gradient was carried out in the range of 4°C–95°C at 1°C/min with a real-time PCR system (Bio-Rad). Fluorescence was recorded as a function of temperature in real time. The melting temperature (Tm) was calculated with StepOne software v2.2 as the maximum of the derivative of the resulting SYPRO Orange fluorescence curves.

**BaF3 Cell Line Establishment and Stimulation**

The murine pro-B BaF3 cell lines overexpressing FGFR1c wild-type was generated as described in the Supplemental Experimental Procedures. The cells were starved for 5 hr in FBS/IL-3 free RPMI 1640 medium followed by 10 min stimulation with FGFB8a, FGFB8b, or FGFB8bF32A, respectively. Heparin was added to the cell culture medium to a final concentration of 5 μg/mL before stimulating the cells with the FGF ligands. BaF3 cell lines coexpressing FGFR1c and αKlotho or αKlotho and βKlotho were established by infecting BaF3-FGFR1c cell line with lentivirus containing αKlotho or βKlotho gene. After infection, the cell lines were selected in medium containing both neomycin and hygromycin for 10 days to get stably infected cells. The stimulation was done as described above with FGF21, FGF23, or FGF19 as ligands.

**Cell Culture, Adipocyte Differentiation, Glucose Uptake**

3T3-L1 preadipocytes, NIH 3T3 cells, and rat hepatoma cell H4IIE (American Type Culture Collection) were cultured as described previously (Kharitonenkov et al., 2005; Kurosawa et al., 2007). Full details are described in the Supplemental Experimental Procedures.

**Mitogenicity Assay**

For mitogenicity assay in vitro, NIH 3T3 cells and rat hepatoma cell H4IIE were grown to reach the mid-logarithm time and transferred to a 96-well plate (5 × 10^3/well), starved for 24 hr in DMEM without FBS, and stimulated with indicated concentration of FGF ligands for 48 hr. Next, the number of viable cells was determined by a Cell Growth Determination Kit (MTT-based) from Sigma-Aldrich.

For mitogenicity assay in vivo, male C57BL/6J mice (2-month-old) were treated with vehicle (PBS), FGF1WT, or FGF1mHBB (0.5 mg/kg or 2.0 mg/kg body weight) every other day. After the indicated time (0.5 mg/kg, 3 months; 2.0 mg/kg, 1 month) of treatment, the mice were sacrificed and the liver tissues were collected for immunohistochemical staining and western blot assay of hepatic proliferation.

**In Vivo Protocol**

All experimental animals were from the Model Animal Research Center of Nanjing University, China, or Jackson Laboratory, and the protocols used in these studies were approved by the Animal Care and Use Committee of Wenzhou Medical University, China or the University of Louisville, USA. The full details of animal feeding, grouping, and administration were described in the Supplemental Experimental Procedures.

**Western Blot Analysis**

The above-cultured 3T3-L1 adipocytes, NIH 3T3 cells, or H4IIE were starved for 12 hr, stimulated with a different dose of FGF1WT and FGF1mHBB for 20 min, and then lysed for future use. The liver and adipose tissues of the male db/db and db/db mice were collected and lysed after 28-day treatment with or without FGF1WT and FGF1mHBB. Forty micrograms of lyase proteins from 3T3-L1 adipocytes, NIH 3T3, H4IIE cells, or liver tissues were separated using 8%–12% SDS-PAGE and electrotransferred onto a nitrocellulose membrane. The details of protein blots are described in the Supplemental Experimental Procedures.

**RNA Extraction, cDNA Synthesis, and qRT-PCR**

Total RNA was extracted from liver and adipose tissues with TRizol reagent (Invitrogen). The details of cDNA synthesis and qRT-PCR are described in the Supplemental Experimental Procedures.

**Fluorescence Dye-Based Thermal Shift Assay**

Thermal stability assays were performed with the SYPRO Orange dye as a fluorescent probe (diluted 200-fold from a 5,000-fold stock solution, Bio-

**HPLC-MALS Analysis**

An inline HPLC (Waters 1500 pump with 2498 UV detector and 2707 autosampler)-MALS (Wyatt miniDawn-Treos and Optilab rEX) system was used to study complex assembly of FGF1 and FGF8 variants with the ligand-binding domain of FGFR1c. A Superdex 200 10/300 GL SEC column (GE Healthcare) was equilibrated and run at a flow rate of 0.25 mL/min for 5 column volumes of 2x PBS. Each experiment contained 60 μL of each individual component (ligand, receptor with or without decasaccharide [Dp10, Iduron]) before being brought up to the final injection volume of 50 μL with 2x PBS. Light scattering and refractive index data were manually aligned to the UV spectra in the ASTRA software.

**Figure 7. Schematic Representation of the “Threshold” Model**
Tissue Preparation, Histopathological Evaluation, Immunohistochemistry

Tissues were fixed overnight in 4% paraformaldehyde and embedded in paraffin. After deparaffinization and rehydration, the paraffin sections (5 μm) were subjected to H&E or immunohistochemical staining. For immunocytochemical staining, paraffin sections were stained with primary antibodies (rabbit polyclonal to PCNA [1:1,000], rabbit polyclonal to Ki67 [1:500]) from Abcam overnight at 4°C. After washing, sections were incubated with horseradish peroxidase conjugated secondary antibody against rabbit and developed with DAB (3,3-diaminobenzidine) developing system (Vector Laboratories), counterstained with hematoxylin and observed under light microscopy.

Metabolite Analysis

Hepatic lipid accumulation was evaluated by Oil Red O staining in accordance with the standard procedure. Triglyceride content in liver was measured using a standard procedure. Glycogen in liver was evaluated by periodic acid–Schiff staining and a Glycogen Assay kit (Abcam) in accordance with the standard procedure or the manufacturer’s instructions, respectively.

Phosphopeptide Quantitation Using SILAC

The details of HILIC fractionation preparation, liquid chromatography-mass spectrometry (LC-MS), and protein identification and quantitation are described in the Supplemental Experimental Procedures.

Statistical Analysis

The in vitro experiments were performed three times with triplicate samples for each individual experiment. Data obtained from the animal study were obtained from five mice or six rats. All data were expressed as the mean ± SEM and subjected to statistical analysis by one-way or two-way ANOVA and Student’s t test using statistical software NASAQ: SPSS (SPSS Inc.). Furthermore, p < 0.05 was considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2017.06.063.

AUTHOR CONTRIBUTIONS

The overall hypothesis of this work was conceived and formulated by M.M. Z.H., Z.H., Y.T., L. Song, D.J.C., T.A.N., L.C., X.L., and M.M. participated in the experimental design of the project and interpretation of the data. Z.H., Y.T., J.G., Y. Liu, J.N., L.Z., Q.L., J.D., and Y.L generated the data. Z.H., L. Srinivasan, and M.M. wrote the manuscript. As the joint corresponding authors, X.L. and M.M. had full access to all the data in the study and take full responsibility for the integrity of the data and the accuracy of the data analysis.

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