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Supplementary Discussion

β-Klotho binding energy is distributed along the length of $FGF21_{CT}$.

The affinity of FGF21 for sKLB was measured using MicroScale Thermophoresis (MST)[1], where the thermophoretic movement of fluorescently-labeled FGF21 (fl-FGF21) in solution is monitored upon sKLB binding. Fitting the normalized fluorescence intensities yields a dissociation constant (K_D) of 43.5 nM for FGF21 binding to sKLB (Extended Data Fig. 6a). This value agrees with measurements using SPR[2], and confirms earlier reports that FGF21 binds β-Klotho with high affinity in the absence of FGFR[3]. To measure binding of $FGF21_{CT}$ to sKLB, an MST-based competition assay was used, in which GST-fused $FGF21_{CT}$ (GST-$FGF21_{CT}$) was titrated into a constant sKLB/fl-FGF21 mixture (Extended Data Fig. 6c). Fitting of the thermophoresis changes arising from competition by GST-$FGF21_{CT}$ using the Hill equation yields an IC_{50} value of 704 nM, confirming that the C-terminal region of FGF21 is primarily responsible for the high-affinity binding of the ligand to sKLB. We also measured the binding affinities of other interactions essential for FGF21-induced stimulation of FGFR1c. MST data obtained using fluorescently labeled, extracellular ligand-binding region of FGFR1c ($FGFR1c_{D2D3}$) revealed that sKLB binds $FGFR1c_{D2D3}$ with a K_D value of approximately 1 μM (Extended Data Fig. 6b). By contrast, FGF21 binding to $FGFR1c_{D2D3}$ is too weak for precise K_D determination using MST (K_D > 10-100 μM) even though the canonical FGF1 binds to the same $FGFR1c_{D2D3}$ protein with K_D of around 1 μM[4,5]. These results are consistent with the failure of even very high FGF21 concentrations to activate cells expressing FGFR1c alone (Extended Data Fig. 7).

To determine whether binding of $FGF21_{CT}$ to sKLB is dominated by either the multi-turn element that docks on site 1 (D1) or the pseudosubstrate that binds to site 2 (D2), we generated mutations in both regions and assessed their effects on GST-$FGF21_{CT}$ binding to sKLB in the MST-based competition assay described above. First, we tested mutations expected to destabilize the internal structure of the multi-turn element in $FGF21_{CT}$ (Fig. 2e), specifically D192A and P193A mutations that will disrupt intramolecular hydrogen bonds that stabilize the D192-P193-L194-S195 β-turn. As anticipated, IC_{50} values measured for D192A or P193A-mutated GST-

$FGF21_{CT}$ variants were 10 to 20-fold higher than those for wild-type (Extended Data Fig. 6c). Second, we tested mutations that should disrupt central intermolecular interactions between the S-P-S pseudosubstrate region of $FGF21_{CT}$ and site 2 (D2) of β-Klotho. As shown in Extended Data Fig. 6d, replacing S204, S206 or Y207 in GST-$FGF21_{CT}$ with alanines causes an 8 to 10-fold increase in IC_{50}.

These data argue that binding of $FGF21_{CT}$ to β-Klotho involves both elements in $FGF21_{CT}$, and is mediated by their cooperative binding to both site 1 and site 2 in β-Klotho. Moreover, the results argue that both site 1 and site 2 must be occupied in order to maintain stable interactions between $FGF21_{CT}$ and β-Klotho. This conclusion, also clearly suggested by the crystal structure, is consistent with several previous results. For example, earlier studies using α-Klotho/β-Klotho chimeras showed that FGF21 binding requires that both D1 and D2 are from β-Klotho[6]. Similarly, proteolytic cleavage or truncation that removes the 10 C-terminal amino acids from FGF21 (which bind D2) leads to its inactivation *in vivo* and in cellular studies[2,7,8]. In parallel, we found using SPR and/or MST that loss of the 10 C-terminal amino acids from FGF21 abolishes its binding to β-Klotho, and that loss of D2 from β-Klotho abolishes its binding to wild-type FGF21 (data not shown). Thus, FGF21 binding to neither site 1 nor site 2 alone is sufficient for stable binding to β-Klotho, arguing that FGF21/β-Klotho complex formation is mediated by cooperation of multiple weak binding events, primarily to site 1 and site 2.

Mutating β-Klotho's FGF21-binding-interface impairs receptor activation but not Klotho/FGFR1 interactions.

We next investigated the effects of mutations in β-Klotho's two $FGF21_{CT}$-binding sites on the ability of FGF21 to stimulate FGFR1 activation in transfected L6 myoblasts (Extended Data Fig. 6e-g). L6 cells lack endogenous FGFRs and β-Klotho, but were engineered to express ectopically either human FGFR1c or β-Klotho alone, or to co-express FGFR1c and β-Klotho at matched levels. As expected, FGF21 only stimulates FGFR1c tyrosine phosphorylation in cells that co-express FGFR1c and β-Klotho (Extended Data Fig. 7), whereas FGF1 activates FGFR1c to similar levels regardless of β-Klotho's presence. Three independent mutations in site 1 on D1, replacing V392, T431, and M435 individually with their corresponding residues in α-Klotho (which does not bind FGF21), caused a substantial decrease in FGF21 stimulation of FGFR1c tyrosine phosphorylation (Extended Data Fig. 6f). Similarly, mutating key amino acids in the

pseudo-substrate binding site of D2 or site 2 (Y643, H646, E693, R696, R829 or R845) almost completely abolished FGF21-induced stimulation of FGFR1c, while leaving FGF1-induced stimulation of the receptor unaffected in the same cells (Extended Data Fig. 6g). Mutating F849, by contrast, which abuts the linker between the two parts of the $FGF21_{CT}$ ligand (Extended Data Fig. 6e), had relatively little effect (Extended Data Fig. 6g) on FGF21-induced receptor activation, consistent with the bipartite nature of the interface discussed above. Importantly in this analysis, we found that the β-Klotho mutations did not affect interactions between FGFR1c and β-Klotho as assessed by their levels in anti-FGFR1c immunoprecipitates (Extended Data Fig. 6f-g), arguing that FGF21 must activate a pre-existing FGFR1c/β-Klotho complex.

Supplementary References

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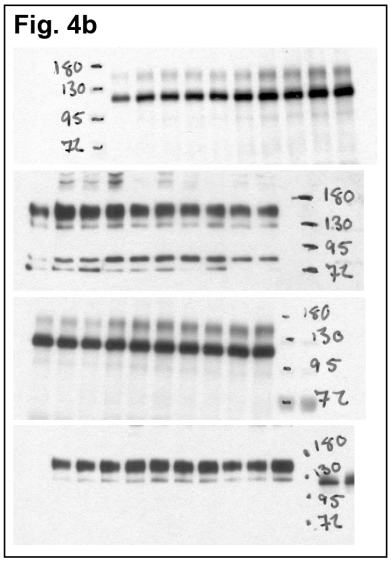

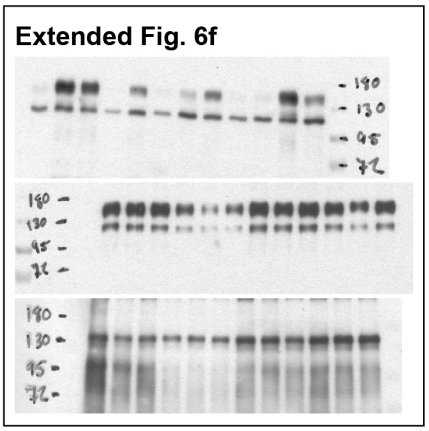

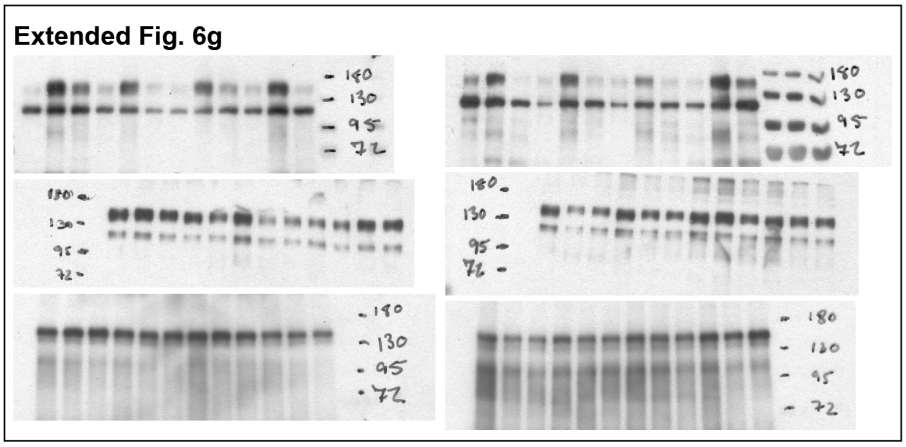

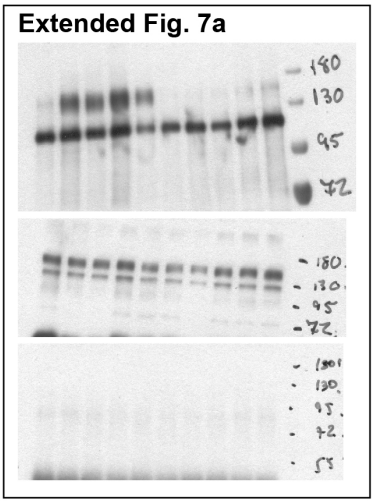

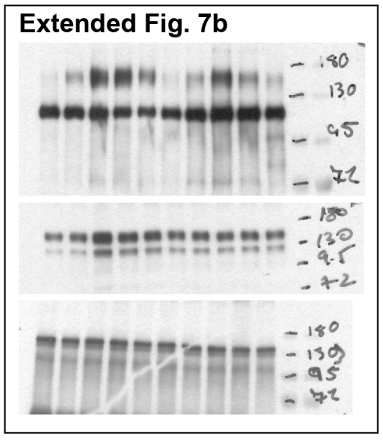

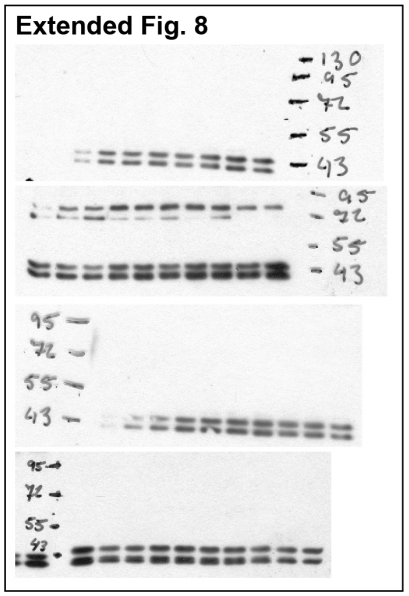

Supplementary Figure 1. | Raw images of immunoblots