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Modulation of the FGF14:FGF14 homodimer interaction through short peptide fragments

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Abstract

Fibroblast growth factor 14 (FGF14) is a member of the intracellular FGF (iFGFs) family and a functionally relevant component of the neuronal voltage-gated Na⁺ (Nav) channel complex. Through a monomeric interaction with the intracellular C-terminus of neuronal Nav channels, FGF14 modulates Na⁺ currents in an Nav isoform-specific manner serving as a fine-tuning regulator of excitability. Previous studies based on the highly homologous FGF13 homodimer crystal structure have proposed a conserved protein:protein interaction (PPI) interface common to both Nav channel binding and iFGF homodimer formation. This interface could provide a novel target for drug design against neuronal Nav channels. Here, we provide the first in-cell reconstitution of the FGF14:FGF14 protein complex and measure the dimer interaction using the split-luciferase complementation assay (LCA). Based on the FGF14 dimer structure generated *in silico*, we designed short peptide fragments against the FGF14 dimer interface. One of these fragments, FLPK aligns with the pocket defined by the β 12-strand and β 8- β 9 loop, reducing the FGF14:FGF14 dimer interaction by 25% as measured by LCA. We further compared the relative interaction strength of FGF14 wild type homodimers with FGF14 hetero- and homodimers carrying double N mutations at the Y153 and V155 residues, located at the β 8- β 9 loop. The Y153N/V155N double mutation counteracts the FLPK effect by increasing the strength of the

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CONFLICT OF INTEREST

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dimer interaction. These data suggest that the β 12 strand of FGF14 might serve as scaffold for drug design against neuronal FGF14 dimers and Nav channels.

Keywords

Fibroblast growth factors; hot-spots; protein:protein interaction; split-luciferase assay; voltage-gated sodium channels; peptides

INTRODUCTION

The pore-forming α subunit of the voltage-gated Na^+ (Nav) channel (Nav1.1-Nav1.9) provides the basis for excitability in neurons and cardiac cells. Upon membrane depolarization, these channels open, inactivate and subsequently close allowing a rapid influx of Na^+ ions that mediate the rising and the initial decay phase of the action potential [1–5]. In the brain, up- or down-regulation of specific Nav channel α isoforms are associated to a plethora of channelopathies and neurological disorders, including epilepsy, neurodegeneration, demyelinating disorders, migraine, post-traumatic brain injury, and mental illnesses [6–10], diseases that are all in need of targeted therapeutics. Compounds targeting Nav channels are widely used in the clinical setting, but the lack of Nav isoform specificity of these drugs is a source of side effects and remains a significant barrier in neuropharmacology [11–13]. More specific compounds targeting non-conserved channel domains are therefore highly desirable.

Native Nav channels are found in complex with multiple accessory proteins bound to the intracellular domains of the pore-forming α -subunit [5, 14, 15]. However, only few of these protein-protein interactions (PPI) produce functional outcomes on Na^+ currents and cell firing. Among those are the PPI complexes formed by the iFGFs with the C-terminal tail of Nav channels [16–21]. The iFGFs, including FGF11-FGF14, are highly homologous in sequence (~45%) and fold, consisting of a well-conserved 12-stranded β -trefoil structure [21, 22]. Despite this degree of homology, though, the iFGF regulatory effect on Na^+ currents is factor-dependent and specific for each Nav channel isoform [22, 23]. This functional specificity suggests non-conserved structural properties at each iFGF:Nav pair interface placing the iFGFs in a category of promising molecular targets for the development of new Nav isoform specific drugs [24].

In addition to forming high-affinity monomeric complexes with the Nav channel C-tail, iFGFs can exist as homodimers. *In vitro* structure-function studies based on purified proteins have proposed a conserved interface mediating both iFGF:iFGF and iFGF:Nav channel complexes [21]. Because of this structural overlap, the iFGF monomer interface reconstituted from full length iFGF proteins could serve as an accurate template for designing peptides and/or small molecules targeting the iFGF:Nav channel complex.

In the central nervous system (CNS), FGF14 is highly abundant and is required for action potential firing and synaptic plasticity of neurons [25]. In heterologous expression systems, FGF14 has been shown to control Na^+ current amplitude and voltage-dependence of activation and/or steady-state inactivation of the neuronal Nav1.1, Nav1.2, and Nav1.6

channels [19, 26]. In animal models, deletion, mutations or overexpression of FGF14 disrupt Nav channel sub-cellular targeting, modify Na⁺ currents and alter neuronal excitability in the hippocampus and cerebellum [17–19, 27]. In humans, inherited mutations of FGF14 have been linked to spinocerebellar ataxia 27 (SCA27), a complex motor-cognitive disorder [28–30], and SNPs in the FGF14 gene linked to schizophrenia [31] and depression [32], indicating a critical role of FGF14 in the brain. FGF14-based interventions modulating the FGF14:Nav channel complex could therefore be of great therapeutic value for diseases of the CNS.

To gain structure-function insights on the FGF14:FGF14 dimer that could guide future interventions against neuronal Nav channels, we have combined the split-luciferase complementation assay (LCA) with molecular modeling and *in silico* studies. We designed FGF14 model-based peptide fragments inhibiting the FGF14:FGF14 dimer interaction and tested the effect of these peptide fragments on the FGF14:FGF14 homodimer interaction when reconstituted in live cells. *In silico* studies predict that one short peptide fragment, FLPK, aligns to the β 12 strand and β 8- β 9 loop region at the FGF14 monomer:monomer interface, reducing significantly the dimer interaction. The FLPK effect is abolished upon N double mutations of the Y153 and V155 from the β 8- β 9 loop in both hetero- and homo FGF14 mutant dimers. Previous studies have shown that these same Y153 and V155 residues modulate the FGF14:Nav1.6 complex formation [33], confirming structural overlap between iFGF homodimers and iFGF:Nav channel interfaces and suggesting that the β 12 strand and the β 8- β 9 loop region of FGF14 might be part of a PPI pocket that could serve as target for drug development against Nav channels.

MATERIALS AND METHODS

Materials

D-luciferin was purchased from Gold Biotechnology (St. Louis, MO) and prepared as a 30 mg/ml stock solution in phosphate buffer saline (PBS). This solution was stored in a –20° freezer.

DNA Constructs Preparation

Mammalian expression vectors coding for N-terminal [pcDNA3.1-V5_HIS TOPO; FRB-N-terminal luciferase fragment (FRB-NLuc)] and C-terminal [pEF6-V5_HIS TOPO] were used to generate the CLuc-FGF14 construct was by replacing FKBP with neuronal FGF14 (1b isoform) in the CLuc-FKBP fusion vector, as described before [33, 34]. Briefly, CLuc-FGF14 was engineered by PCR amplification of the FGF14 open reading frame (nt 1–855) using a 5' primer containing a BsiWI site up to a linker region and a 3' primer containing a NotI site and ligated into the CLuc vector. To generate the FGF14-NLuc construct, FGF14 (1b isoform) was similarly replaced with FRB in the FRB-NLuc construct using PCR amplification and ligation into BamHI at the 5' end and BsiWI at the 3' end. The FGF14^{Y153N/V155N} mutants were engineered similarly to CLuc-FGF14 and FGF14-NLuc using pQBI-FGF14^{Y153N/V155N}-GFP as a template in the PCR reaction [17, 21]. All constructs were verified by DNA sequencing. The full length firefly luciferase was cloned into the pGL3-CMV plasmid.

Cell Culture and Transient Transfections

HEK293 cells were incubated at 37°C with 5% CO₂ in medium composed of equal volumes of Dulbecco modified essential medium (DMEM) and F12 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin. Transfections were performed in 24-well CELLSTAR^R tissue culture plates (Greiner Bio-One, Monroe, NC) at 4.5x10⁵ cells per well and incubated overnight to produce monolayers at 90%–100% confluence. The cells were then transiently co-transfected with pairs of plasmids (usually 1 µg per plasmid/well) to achieve an equal ratio of protein production or a single plasmid along with a carrier DNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to manufacturer's instructions.

Peptide Synthesis and Delivery

Custom made peptides (acetylated group on the N-terminal and amide group on the C-terminal) were synthesized by the American Peptide Company (Sunnyvale, CA). Peptides were dissolved in phosphate buffer saline (PBS) at a concentration of 10 mM and delivered to HEK293 cells in conjunction with cDNA during transient transfection to a final concentration of 10 µM. Mixtures of peptides (40 µM) along with cDNA plasmids (1–2 µg) or Lipofectamine 2000 alone were first incubated each in 50 µl DMEM+ F12 media for 5–6 min, and then combined and allowed to stand for 20 minutes. The combined 100 µl mixtures were then gently vortexed for 30 seconds and dispensed into individual wells of a 24-well plate containing 4.5x10⁵ cells/well seeded with 100 µl DMEM+F12 media for 6 hours. The final concentration of the peptides for the time of transfection was 10 µM into a 200 µl final volume of DMEM+F12 media. Six hours later, 800 µl media (DMEM+F12+FBS +antibiotics) was added to maintain cells in culture. PBS alone mixed with cDNA and Lipofectamine 2000 was used as a negative control.

Bioluminescence Assays

24–48 hours post-transfection, cells were detached from 24-well plate by 0.04% trypsin:EDTA mixture dissolved in PBS, centrifuged and seeded in white, clear-bottom CELLSTAR[®] µClear[®] 96-well tissue culture plates (Greiner Bio-One, Monroe, NC) at ~10⁵ cells per well in 200 µl of medium. The cells were incubated for 24 h and then the growth medium was replaced with 100 µl of serum-free, phenol red-free DMEM/F12 medium (Invitrogen). The reporter reaction was initiated by automatic injection of 100 µl of substrate solution containing 1.5 mg/mL of D-luciferin dissolved in PBS using a SynergyTM H4 Multi-Mode Micro plate Reader (Biotech, Winooski, VT). Luminescence readings were initiated after 3 s of mild plate shaking and performed at 2 min intervals for 20 min with integration times of 0.5 s. Cells were maintained at 37°C throughout the measurements.

Western Blot

Transfected HEK293 cells were washed with cold PBS. Subsequently, 50 µl of lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1% NP-40) and 1 µl Protease inhibitor cocktail (set #3, Calbiochem, Billerica, MA) were added. Cell extracts were collected, sonicated for 20 sec and centrifuged at 4°C, 13,000 g for 15 min adding 2x sample buffer containing 50 mM tris (2-carboxyethyl) phosphine (TCEP). Mixtures were heated for 10 min at 55°C and resolved

on 4–15% polyacrylamide gels (BioRad, Hercules, CA). Resolved proteins were transferred to PVDF membranes (Millipore, Bedford, MA) for 2 hours at 4°C and blocked in tris-buffered saline (TBS) with 3% nonfat dry milk and 0.1% Tween-20. Membranes were then incubated overnight in blocking buffer containing the anti-luciferase goat polyclonal antibody (Promega, Madison, WI) or anti-calnexin rabbit polyclonal antibody (Cell Signaling Technology, Danvers, MA). Washed membranes were incubated with donkey anti-goat or goat anti-rabbit-HRP (1:5000) and visualized with ECL Advance Western Blotting Detection kit (GE Healthcare, Piscataway, NJ); protein bands were visualized using FluorChem® HD2 System and analyzed with AlphaView 3.1 software (ProteinSimple, Santa Clara, CA).

Immunoprecipitations

HEK293 cells were transiently transfected with pQBI-FGF14-6xmyc and pQBI-FGF14-GFP plasmids (5ug per plasmid). The following day, cells were washed twice with PBS and lysed in lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1% NP-40), and protease inhibitor mixture (set #3, Calbiochem, Billerica, MA) was added immediately before cell lysis. Cell extracts were collected and sonicated for 20 s and centrifuged at 4°C, at 13,000 × g for 15 min. Supernatants were collected and mixed with rabbit anti-myc agarose beads (Sigma), treated with 10 uM FLPK peptide and incubated overnight at 4°C with agitation. After washing five times with lysis buffer, 2× sample buffer (Bio-Rad, Hercules, CA) containing 50 mM TCEP “tris(2-carboxyethyl) phosphine” was added. Lysates were then heated for 10–15 min at 70 °C and resolved on 7.5% or 4–15% polyacrylamide gradient gels (Bio-Rad, Hercules, CA). Resolved proteins were transferred to PVDF membranes (Millipore, Bedford, MA) for 2 h at 4°C and blocked in tris-buffered saline (TBS) with 3% nonfat dry milk and 0.1% Tween-20. Membranes were then incubated in blocking buffer containing an anti-GFP (1:1000) or monoclonal mouse anti-myc (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA) antibody overnight at 4°C. Washed membranes were incubated with secondary goat anti-mouse or goat anti-rabbit-HRP (1:5000) and visualized with ECL Advance Western Blotting Detection kit (GE Healthcare, Piscataway, NJ); protein bands were imaged using FluorChem® HD2 System and analyzed with AlphaView 3.1 software (ProteinSimple, Santa Clara, CA).

Data Analysis

Relative luminescence values (RLU) measured by Synergy H4™ Multi-Mode Microplate Reader were tabulated by well position and time point into Microsoft Excel. Signal intensity for each well was calculated as a mean value of peak luminescence measured at three adjacent time points; the calculated values were expressed as percent of mean signal intensity in the control samples from the same experimental plate. Statistical values are calculated as mean and standard error of the mean (mean ± SEM), unless otherwise specified. The statistical significance (*p<0.05) of different groups was determined by Student's t-test, one-way ANOVA with *post-hoc* Bonferroni's method or Kruskal–Wallis one-way ANOVA on ranks with *post-hoc* Dunn's method using Sigma Stat software (Jandel Inc., San Jose, CA). Graphs were plotted in Origin 8.6 Software (Origin Lab Corporation, Northampton, MA).

Molecular Modeling

The FGF14:FGF14 homodimer model was built with the FGF13 dimer crystal structure (3hbw) as a template [21] by aligning the sequence and structure of the FGF14 monomer model [29] to the FGF13 monomers in the FGF13 dimer crystal structure. The Y153N/V155N mutations were carried *in silico* within the UCSF-Chimera molecular modeling suite [35]. The sequence alignment of the FGF14 model [29] to the FGF13 monomers in the FGF13 dimer crystal structure (3hbw) was performed with the MatchMaker algorithm [36] by superimposing both structures created with a pairwise sequence alignment using the Needleman-Wunsch and BLOSUM-62 algorithms. The structure of the FGF14 homodimer, as well as of the FGF14^{Y153N/V155N} hetero- and homodimer mutant structures were energy minimized with the Amber's Antechamber module implemented in UCSF-Chimera [37, 38]. Before minimizing the FGF14 homodimer structure, the FGF14 monomer:monomer interface was optimized with the DockPrep algorithm [39, 40], also implemented in the UCSF Chimera suite.

RESULTS

Reconstitution of the CLuc-FGF14:FGF14-NLuc Homodimer Complex in Live Cells

We applied the split-luciferase complementation assay (LCA) in live cells to reconstitute the FGF14:FGF14 homodimer protein complex in a physiological microenvironment. Fig. 1A illustrates the molecular engineering strategy used to generate the two constructs bearing the complementary N-terminus (NLuc) and C-terminus (CLuc) fragments of the *Photinus* Firefly luciferase[33, 41] fused to the FGF14 wild type molecule in either 5' or 3' position (CLuc-FGF14 and FGF14-NLuc respectively). In this reporter system, the two luciferase fragments are brought in close proximity by the corresponding FGF14 protein driving full reconstitution of the luciferase enzyme and consequent light production upon addition of the D-luciferin substrate (Fig. 1B). The resulting luminescence signal is as a measure of relative binding strength and/or stability of the protein complex. The CLuc-FGF14 and FGF14-NLuc constructs were transiently expressed in HEK293 cells using lipid-based transfection methods. Upon initiation of the assay by addition of the substrate, a strong luminescence signal (RLU), reaching a steady-state maximal value after ~12–15 minutes and persisting to the end of each experiment, was detected indicating a robust assembly of the FGF14:FGF14 dimer complex in live cells (Fig. 1C, n=10 independent experiments, black circle). In contrast, the luminescence response produced by cells transfected with CLuc-FGF14 alone was negligible and used as a background reference (Fig. 1C, D, open circles). These results indicate that LCA is a suitable method for the reconstitution and characterization of the FGF14:FGF14 homodimer in the physiological microenvironment of living cells.

Molecular Model of the FGF14:FGF14 Dimer

To gain structural insights into the FGF14 dimer interface, we generated a homology model of the FGF14 dimer from the FGF14 monomer model proposed by Van Swieten [29] and the FGF13 homodimer crystal structure as a template [21]. Two FGF14 monomers were each aligned by sequence and structure to the FGF13 monomers in the FGF13 dimer crystal structure. The FGF14 dimer structure was further energy minimized to optimize the monomer:monomer interface (Fig. 2B, C). From the 146 common amino acid residues

resolved in the FGF13 homodimer crystal structure and common for both FGF13 and FGF14 structures only 28 are not conserved, giving an 81% sequence identity between the two molecules (Fig. 2A). All the amino acid residues that are part of the β 8- β 9 loop holding the Y153 and the V155 residues are conserved, pointing to a stabilizing function of the loop in both the FGF13 and FGF14 homodimer (Fig. 2B, C). Comparison of the FGF13 and FGF14 dimer models shows that Y153 in FGF14 and Y151 in FGF13 are extending at the monomer:monomer interface holding the dimers macromolecular organization and structure. The orientation of the amino acid side chain of the FGF14:Y153 and FGF13:Y151 differs, indicating a flexibility element which can cause structural divergence between the two iFGFs (Fig. 2B).

Model-Based Peptide Design

To further characterize the FGF14:FGF14 interaction interface we used the molecular model of the FGF14 dimer as a template to design three short peptides (4 amino acids long) against the FGF14 β 12 C-terminal strand and the β 8- β 9 loop at the monomer:monomer interface. The FLPK and PLEV peptides correspond to two specific consecutive areas of the β 12 sheet (FLPKPLEV) at the monomer:monomer interface, while the EYYV mimics the exposed YYV sequence of the β 8- β 9 loop (Fig. 3A). As the FGF14 monomers are organized head-to-toe in the dimer structure (Fig. 2B), the designed short peptides are expected to compete with the monomer:monomer interaction by blocking the respective pockets on the FGF14 dimer interface.

In-cell activity of small peptides against the FGF14:FGF14 dimer

FLPK, PLEV and EYYV were synthesized in their acetylated form and tested for their activity against the FGF14 dimer assembly in cells. To ensure efficient intracellular delivery, peptides were introduced into cells at the time of transfection using lipid-based methods. HEK293 cells expressing CLuc-FGF14 and FGF14-NLuc along with individual peptide fragments were assayed with LCA (Fig. 4A). The most significant reduction of the luminescence response occurred in the presence of 10 μ M FLPK (75 ± 3 %, $n=20$, $***p < 0.001$, one way ANOVA, *post-hoc* Bonferroni), while mild, but non-statistically significant effects were detected with 10 μ M PLEV (87 ± 4 %, $n=28$ $*p=0.080$), and with 10 μ M EYYV (89 ± 4 %, $n=24$, $p=0.28$) compared to control cells (Fig. 4B). To rule out non-specific interactions of these peptides with the luciferase enzyme, the peptides were tested against full length firefly luciferase. HEK293 cells were transiently transfected with pLG3-CMV-full length firefly luciferase and peptides delivered while performing transfection. None of these peptides had any statistically significant effect on the firefly luciferase activity compared to control (FLPK, 95 ± 18 %, $p=0.804$, Fig. 4C; PLEV, 75 ± 8 %, $p=0.0478$; EYYV, 99 ± 14 %, $p=0.96$; for all groups $n=6$, data for PLEV and EYYV are not shown). To further support the notion that FLPK peptide can reduce the FGF14:FGF14 dimer formation, we have performed co-immunoprecipitation from lysates of HEK293 cells transiently transfected with FGF14-GFP and FGF14-6xmyc and treated with either FLPK peptide (10 μ M, dissolved in H₂O) or vehicle (H₂O). The FGF14-6xmyc:FGF14-GFP complex was co-immunoprecipitated using anti-myc agarose beads. As illustrated in Fig. 4D, the fraction of FGF14-GFP co-immunoprecipitating with FGF14-6xmyc was significantly lower in cells treated with FLPK peptide compared to vehicle (~44% compared to 100% control).

Altogether, these results corroborate the hypothesis that FLPK can interfere with the FGF14 dimer formation.

***In Silico* Modeling of FLPK**

Of the three designed peptides against the 12 amino acid residues of the β 8–9 loop and the β 12 strand of the FGF14 dimer interface, FLPK had the most pronounced effect on the monomer:monomer interaction. Therefore, we proceeded to define the pockets at the FGF14 dimer interface adjacent to the β 12 strand FLPK sequence (Fig. 3A). To achieve this we aligned the FLPK sequence to the FGF14 dimer interface by creating a script in the Match-align algorithm plug-in of UCSF Chimera [35, 36] (Fig. 3B). In our model, the FLPK fragment aligned well in the region defined by the β 8– β 9 loop and the FLPK sequence of the β 12 strand, which implies that the pocket defined by Y153, Y154, V155 and by F196, L197, P198, K199 might be at the FGF14 dimer interface. Thus, FLPK might act as a competitive inhibitor targeting the β 8– β 9 loop and the β 12 strand preventing the FGF14 dimer formation. This prediction is confirmed by our in cell studies showing a significant reduction of ~25% in the monomer:monomer interaction upon FLPK treatment (Fig. 4A–B).

Impact of Mutations at Y153 and V155 on FGF14:FGF14 Homodimer Stability

Previous structure-function studies derived from the crystal structure of the FGF13 dimer propose that Y151 and V153 located at FGF13 are part of the surface conserved in the core domain of all iFGFs that mediates PPI at both the iFGF:iFGF and the iFGF:Nav channel interfaces [21]. To examine the impact of Y153 and Y155 on the FGF14 dimer assembly, CLuc-FGF14 and FGF14-NLuc constructs bearing Y153N/V155N double mutations (Fig. 5A) were transiently transfected in HEK293 cells to form either FGF14^{Y153N/V155N}:FGF14^{Y153N/V155N} homodimers or FGF14^{Y153N/V155N}:FGF14 heterodimers (Fig. 5B). Both hetero- and homodimer mutants could be reconstituted in live cells giving rise to a strong luminescence signal (Fig. 5B–C). One way ANOVA with *post-hoc* Bonferroni comparisons of RLU across the three different pairs of constructs, revealed that the strength of interaction for the FGF14^{Y153N/V155N}:FGF14 heterodimer was not different from the FGF14:FGF14 dimer complex ($109 \pm 8\%$, $n=16$ independent experiments, $p=0.08$), while the one of the FGF14^{Y153N/V155N}:FGF14^{Y153N/V155N} homodimer was significantly increased ($136 \pm 8\%$, $n=11$, $***p<0.001$, Fig. 5C). To rule out that the observed changes in luminescence across the experimental groups were driven by changes in the protein expression level, Western blot analysis was performed on total cell lysates from cells transfected with either hetero- or homodimer FGF14 mutants. As shown in Fig. 5D, an anti-goat luciferase antibody that recognizes both CLuc- and NLuc fragments revealed two bands corresponding to a predicted molecular weight of either ~75 kD for the FGF14 constructs carrying the NLuc- fragment or ~50 kD for the FGF14 constructs carrying the CLuc- fragment. The band intensity corresponding to the two fragments was visually indistinguishable across conditions and quantification of CLuc/NLuc band intensity showed no statistical difference across the three conditions ($n=4$, $p=0.88$, one way ANOVA, Fig. 5D). These results indicate that Y153 and V155 are “hot-spots” and play a key role in the stability and structural organization of the FGF14:FGF14 dimer interface as shown by increased interaction strength upon mutations of these residues. Furthermore, these data combined with previous results showing that the FGF14^{Y153N/V155N} double mutation affects

the FGF14:Nav1.6 complex assembly [33], confirm the hypothesis that the FGF14:FGF14 and the FGF14:Nav1.6 interfaces overlap.

Role of the Y153 and V155 residues at the FGF14:FGF14 Dimer Interface

The mechanism of action of the Y153N and V155N mutations at the FGF14 dimer interface was also predicted *in silico* by creating models of the FGF14 hetero- and homodimer mutants. In Fig. 6A, the ribbon and surface representation of FGF14 wild type are shown. Y153 and V155 residues are shown as magenta and purple. When the Y153N and V155N mutations are introduced only on one FGF14 monomer, the FGF14^{Y153N/V155N}:FGF14 monomer:monomer interface is not significantly disturbed due to the head-to-toe orientation of the monomers and the presence of the Y153 in the wild type monomer which preserves the heterodimer structure (Fig. 6B). On the other hand, mutating Y153 and V155 in both FGF14 monomers heavily affects the dimer stability, resulting in a collapse of the structure seen as a tighter packing of the two monomers in the FGF14^{Y153N/V155N} homodimer (Fig. 6C). This ‘tighter packing’ is due to the lack of the bulk tyrosine phenol ring in the Y153N mutation, which stabilizes the homodimer interface in the wild type FGF14. The V155N mutation further ‘smooths’ the surface corresponding to the β 8- β 9 loop reinforcing the interaction between the two FGF14^{Y153N/V155N} monomers. The *in silico* model for the FGF14^{Y153N/V155N} homodimer correlates with the ~36% significantly increased interaction between the two monomers compared to the wild type dimer assessed by LCA (Fig. 5B–C).

Effect of FLPK on FGF14 Homo- and Heterodimer Mutants

All three peptides were tested in cells for their effect on the FGF14 wild type homodimers, but only FLPK showed a significant activity inhibiting the FGF14 monomer:monomer interaction. Given that FLPK aligns in a pocket adjacent to the β 8- β 9 loop holding the Y153 and V155, one expectation is that the activity of FLPK would be influenced by the same N mutations that disrupt the FGF14 dimer organization (Fig. 5B–C). To test this hypothesis, cells expressing the FGF14^{Y153N/V155N}:FGF14 heterodimer or the FGF14^{Y153N/V155N}:FGF14^{Y153N/V155N} homodimer with FLPK were processed for LCA. As illustrated in Fig. 7, FLPK was inactive against both the hetero- and the homodimer. The luminescence response detected from the FGF14^{Y153N/V155N}:FGF14 heterodimer and the FGF14^{Y153N/V155N}:FGF14^{Y153N/V155N} homodimer in the presence of FLPK was 90.8 ± 5.1 % and 98.3 ± 3.3 %, respectively, and both values were statistically different from the corresponding FGF14:FGF14 homodimer condition in the presence of FLPK ($n=20$, $p<0.05$ for FGF14^{Y153N/V155N}:FGF14 vs. $n=12$, $p<0.01$ for FGF14^{Y153N/V155N}:FGF14^{Y153N/V155N}). These results were complemented with the *in silico* modeling of the FLPK on the FGF14 interface shown in Fig. 3. The modeling predicts that FLPK obstructs the interaction of the Y153 from the β 8- β 9 loop of one FGF14 monomer with the opposing FGF14 in the homodimer (Fig. 8). Mutating the Y153 residue to N might reduce the accessibility of FLPK to this pocket creating a tighter interaction between the two monomers, thus preventing the effect of FLPK on the dimer assembly.

DISCUSSION

In this study we characterize for the first time the FGF14:FGF14 homodimer interface by combining an in-cell approach designed to reconstitute the dimer complex in live cells with an *in silico* approach based on the FGF14 homodimer structure calculated with the FGF13 dimer crystal structure as a template. To identify potential critical regions at the FGF14:FGF14 interface we designed three short peptides, matching two consecutive areas on the β 12 strand (FLPKPLEV) and the adjacent β 8- β 9 loop (EYYV), which are located at the homodimer interface. These peptides were tested for in-cell activity with LCA and data showed that the FLPK peptide significantly reduces the formation of the FGF14 homodimer. In our homology model the FLPK β 12-strand mimic fragment aligned well in the region defined by the β 8- β 9 loop, which includes Y153 and V155.

To determine whether Y153 and V155 acted as “hot-spots” at the FGF14:FGF14 dimer and whether were required for FLPK activity, we examined the effect of the FGF14^{Y153N/V155N} double mutation on the FGF14:FGF14 homodimer stability and the impact of these mutations on the activity of FLPK. Using LCA we found that the conversion of Y153 and V155 into N increases the stability/relative binding strength of the two monomers presumably by inducing a tighter packed dimer. The role of the two Y153/V155 “hot spots” was further confirmed by comparing the strength of interaction of hetero- and homodimers carrying double Y153N and V155N mutations using *in silico* models and predicting that the β 8- β 9 loop encompassing Y153 and V155 and the FLPK sequence of the β 12 strand would be adjacent and critical for the FGF14 monomer:monomer interaction. Mutating both Y153 and V155 to N abolished the FLPK effect in cells indicating that its activity depends on these residues and suggesting a synergy of action between the β 12 area and the β 8- β 9 loop.

Introducing short peptide fragments that mimic the modeled FGF14 dimer interface and modulate the monomer:monomer interaction in cells further corroborates the idea that Y153/V155 of the β 8- β 9 loop and the β 12 strand of the FGF14 C-terminal tail are critical for the FGF14:FGF14 homodimer assembly and that are integral parts of the dimer interface. Furthermore, these results combined with previous studies showing that Y153 and V155 affect the FGF14:Nav1.6 channel interaction [33] and are “hot-spots” at this interface provide evidence of overlaps between the FGF14:FGF14 and the FGF14:Nav1.6 interfaces, confirming prior structural studies [21, 33]. Overall, these results identify the β 12-strand FLPK sequence and the β 8- β 9 loop containing Y153/V155 as modulatory PPI sites and potential druggable targets against the FGF14:FGF14 and the FGF14:Nav1.6 channel interface [33].

We also observed a small, but not statistically significant effect of PLEV and EYYV on the FGF14 dimer. The EYYV and the PLEV sequences are structurally adjacent to the FLPK sequence on the FGF14:FGF14 dimer interface. Thus, longer peptides such as FLPKPLEV or YYVFLPKP or cyclic peptide derivatives could act as more potent modulators of the FGF14:FGF14 dimer and/or FGF14:Nav complex. Future medicinal chemistry and pharmacological efforts along with structural resolution of the peptide/s and their conformers in solution and/or docked to various FGF14 protein complexes will be needed to

test this hypothesis and will be required to identify peptide variants suited for selective *in vivo* interventions against the dimer versus the FGF14:Nav channel complex.

Despite their high sequence homology and similar 3D fold, iFGFs differ in their binding affinity for Nav channels and the way they modulate Nav channel currents [19, 21]. Divergence in function typically translates in structural variations at the protein interface responsible for specific macromolecular interactions. From the *in silico* comparison between the two FGF13 and FGF14 dimer structures structural differences in the side chains of residues at the β 12 strand and the β 8- β 9 loop region holding the Y153 in FGF14 and the Y151 in FGF13 were evident. These differences might be the basis of specialized functions towards Nav channels and could translate into highly targeted drug-based interventions against each iFGF:Nav channel pair.

CONCLUSION

PPI interfaces are emerging targets for pharmacological interventions especially in the CNS, where selectivity and specificity are vital for developing drugs with limited side effects. PPI are flexible and structural adaptive [42–45]. However, a general strategy for a rapid characterization of PPI interfaces is still being sought. The combined methodology presented here including a combination of in-cell split-luciferase assays, molecular modeling, and model-based peptide design responds to an urgent need in the field of molecular pharmacology of providing an integrated method for characterizing PPI interfaces. When applied to the study of iFGF:iFGF and iFGF:Nav channel complexes, this methodology will provide a rapid method to predict and screen for “hot-spots”, map interacting surfaces, and identify druggable pockets that can guide drug design against Nav channels with applicability to channelopathies and other Nav channel-related disorders.

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ABBREVIATIONS

FGF14	Fibroblast growth factor 14
Nav channel	Voltage-gated sodium channel
PPI, Protein	rotein interactions
LCA	Luciferase complementation assay
PBS	Phosphate buffer saline
N	Asparagine
Y	Tyrosine
Ac-FLPK-NH₂	Acetyl-Phenyl alanine-Leucine-Proline-Lysine-Amide
Ac-PLEV-NH₂	Acetyl-Proline-Leucine-Glutamic acid-Valine-Amide

Ac-EYYV-NH₂

Acetyl-Glutamic acid-Tyrosine- Tyrosine-Valine-Amide

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Fig. 1. In-cell reconstitution of the FGF14:FGF14 homodimer complex using the split-luciferase complementation assay (LCA)

A. Schematic representation of FGF14 constructs expressing either the CLuc (398–550) or the NLuc (2–416) fragment of firefly *Photinus pyralis* luciferase. A flexible linker (grey) spaces the FGF14 protein from the luciferase fragments. **B.** Spontaneous association of CLuc-FGF14 and FGF14-NLuc brings in close proximity the two halves of the luciferase fragments, allowing the enzyme to reconstitute and to emit light in the presence of the D-luciferin substrate. **C.** Luminescence (RLU) corresponding to the assembly of CLuc-FGF14 and FGF14-NLuc (black circle) is detected in HEK293 cells upon addition of D-luciferin at time zero (n=10 independent experiments). Cells expressing CLuc-FGF14 alone (open circle) produced negligible luminescence signal. **D.** Summary bar graph represents % maximal luminescence response of the FGF14:FGF14 homodimer versus background. Data are mean $\pm$ SEM. The statistical significance between the two groups was assessed using by Student's *t*-test, n=10, ****p*<0.001.

Fig. 2. Model of the FGF14 homodimer

A. Sequence and structure alignment of the FGF14 homodimer with the FGF13 homodimer crystal structure as a template. The FGF13 N terminal – R64 and C terminal – L212 are indicated with blue circles. The FGF14 N terminal – P63 and C terminal – Y206 are indicated with green circles. The FGF14 and FGF13 sequences FLPK, PLEV and NYVY are indicated with dashed boxes in orange, yellow and purple, respectively. The position of FGF14-Y153 and corresponding FGF13-Y151 from the β 8- β 9 loop are indicated with a dark grey dashed oval. The conservation and charge variation (red is negative, blue is positive) of the FGF14 and FGF13 amino acid residues, and the root mean square deviation (RMSD) between the C_{α} atoms of the FGF14 and FGF13 polypeptide chains are shown. **B.** Ribbon representation of the FGF14 dimer structure (green) superimposed with the FGF13 dimer crystal structure (3hbw, blue). The FGF14 β 12 strand is indicated in orange and the FGF13 β 12 strand in yellow. The FGF14 Y153 and V155 residues side chains at the monomer:monomer interface are shown in magenta and purple, respectively. The corresponding FGF13 Y151 and V153 residues side chains are shown in blue and magenta, respectively. **C.** Surface representation of the FGF13 homodimer (light blue) superimposed with the surface representation of the FGF14 homodimer in green. The FGF14 - Y153 residue surface, defining the monomer:monomer interface is colored in magenta. Part of the V155 residue side chain exposed at the FGF14 monomer:monomer interface is visible and colored in purple.

Fig. 3. Peptide mapping on the FGF14 surface

A. Mapping of the FLPK (orange), PLEV (yellow) and EYYV (purple) sequences on the FGF14 structure. The FGF14 secondary structure is presented as β strands in green, random coils in light blue and α -helices in orange. The N- and C-termini are indicated. The FGF14 surface is shown in light grey. **B.** Alignment of the FLPK peptide (dark grey) to the FGF14 β -12 strand. The position of the FLPK residues is indicated with circled letters. The FLPK (orange), PLEV (yellow) and EYYV (purple) sequence of FGF14 are indicated.

Fig. 4. Effect of FGF14 model-based peptides on the FGF14:FGF14 homodimer assembly
A. Percent of maximal luminescence response corresponding to the assembly of CLuc-FGF14 and FGF14-NLuc in the presence of FLPK (circle), PLEV (inverted-triangle), EYYV (upward-triangle), and control (square) detected in HEK293 cells upon addition of D-luciferin at time zero. **B.** Summary bar graph represents % maximal luminescence response corresponding to the assembly of CLuc-FGF14 and FGF14-NLuc in the presence of either PBS control ($100 \pm 2\%$), 10 μM FLPK ($75 \pm 3\%$), 10 μM PLEV ($87 \pm 4\%$), 10 μM EYYV ($89 \pm 4\%$). Data are mean $\pm$ SEM. The statistical significance between the three groups was assessed using one-way ANOVA *post-hoc* Bonferroni ($n=20-28$ experiments, $***p<0.001$). **C.** HEK293 cells were transiently transfected with full-length firefly luciferase with FLPK (10 μM) or vehicle (PBS) and expressed as % luciferase activity compared to control (PBS); data are mean $\pm$ SEM representing four replicates from six independent experiments ($n=6$). Statistical significance between the four groups was assessed using student *t*-test; ns=non-significant. **D.** Western blot analysis of cell lysate and co-immunoprecipitated fraction (IP:myc) of FGF14-6xmyc and FGF14-GFP. Treatment with 10 μM FLPK reduced the FGF14-6xmyc : FGF14-GFP complex formation.

Fig. 5. Y153N/V155N mutations modify the FGF14:FGF14 homodimer formation

A. A schematic representation of the CLuc- and NLuc- constructs carrying the Y153N/V155N mutations is illustrated. **B.** Luminescence (RLU) corresponding to the assembly of CLuc-FGF14 and FGF14-NLuc (black circles), CLuc-FGF14^{Y153N/V155N} and FGF14-NLuc (orange circles), and CLuc-FGF14^{Y153N/V155N} and FGF14^{Y153N/V155N}-NLuc (yellow circles) is detected in HEK293 cells upon addition of D-luciferin at time zero (n=11–16 independent experiments). **C.** Summary bar graph represents % maximal luminescence response of each pair normalized to the CLuc-FGF14:FGF14-NLuc homodimer response. Data are mean $\pm$ SEM. The statistical significance between the three groups was assessed using Kruskal–Wallis one-way ANOVA on ranks with *post-hoc* Dunn's method (n=11–17 independent experiments, ** p <0.01; *** p <0.001). **D.** Western blots of whole-cell extracts (equal amount of protein per lane) from cells transfected with CLuc-FGF14 wild type + FGF14-NLuc (lane 1, from left), CLuc-FGF14^{Y153N/V155N} + FGF14-NLuc (lane 2), CLuc-FGF14^{Y153N/V155N} + FGF14^{Y153N/V155N}-NLuc (lane 3). Western blots were probed with a polyclonal anti-luciferase antibody which recognizes different epitopes on the NLuc and CLuc fragments (~75 kD and 50 kD, respectively); immunodetection of calnexin is used as loading control. **E.** Densitometry analysis was determined by taking the ratio of CLuc and NLuc band intensity. Calnexin was used as loading control; the expression level of CLuc-FGF14^{Y153N/V155N} was comparable across conditions one-way ANOVA (n=4, p values = 0.88); data are mean $\pm$ SEM.

Fig. 6. Model of FGF14^{Y153N/V155N} hetero- and homodimer

A. Ribbon and surface representation of the FGF14:FGF14 homodimer structure after energy minimization. The two FGF14 wild type monomers are colored light (chain A) and dark green (chain B), respectively. The side chains/surface of the Y153 is colored in magenta and the one of V155 in purple, on both chains. **B.** Ribbon and surface representation of the FGF14^{Y153N/V155N}:FGF14 heterodimer structure. The FGF14^{Y153N/V155N} monomer (chain B) is colored in orange, and the FGF14 wild type monomer (chain A) is colored in light green. The Y153 and V155 on the FGF14 wild type monomer are colored in magenta, respectively. The side chains/surface of Y153N and V155N on the FGF14^{Y153N/V155N} monomer are colored in cyan. **C.** Ribbon and surface representation of the FGF14^{Y153N/V155N} homodimer structure in which the two monomers are colored in orange (chain A) and orange-red (chain B), respectively. The side chains/surface of the mutated Y153N and V155N is colored in cyan on both chains. All FGF14 homo and heterodimers are oriented the same way as in Fig. 2.

Fig. 7. The Y153N and V155N mutations prevent activity of FLPK

A. Luminescence (RLU) corresponding to the assembly of CLuc-FGF14 and FGF14-NLuc (black circles), CLuc-FGF14^{Y153N/V155N} and FGF14-NLuc (orange circles), and CLuc-FGF14^{Y153N/V155N} and FGF14Y153N^{V155N}-NLuc (yellow circles) in the presence of FLPK is detected in HEK293 cells upon addition of D-luciferin at time zero. **B.** Summary bar graph represents % maximal luminescence response of each pair normalized to the corresponding dimer control (PBS) response. Data are mean $\pm$ SEM. The statistical significance between the three groups was assessed using one-way ANOVA with *post-hoc* Bonferroni method (n= 16–20 experiments, * p <0.05; ** p <0.01).

Fig. 8. FLPK align to the FGF14 monomer interface

The FLPK peptide is presented as an electrostatic surface (positive red, negative–blue, neutral–dark grey). The FGF14 surface is in light grey.