Resurfaced Shape Complementary Proteins That Selectively Bind the Oncoprotein Gankyrin

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Materials and Methods

Materials

All chemicals obtained from Sigma-Aldrich unless specified
LB Miller Broth- Fisher
5-alpha chemically competent *E. coli*- NEB
BL21 (DE3) chemically competent *E. coli*- NEB
BL21-Gold *E. coli* (DE3)- Stratagene
Agar- Fisher
Carbenicillin- GoldBio Technology
Restriction Enzymes- NEB
Kanamycin- GoldBio Technology
L-arabinose- Goldbio Technology
Isopropyl-β-D-1-thiogalactopyranoside (IPTG)- GoldBio Technology
Quick Ligation Kit- NEB
Q5 High Fidelity DNA Polymerase- NEB
Oligonucleotides- IDT
10β Electrocompetent *E. coli*- NEB
Gene Pulser/Micro Pulser Cuvettes 1mm- Biorad
Maxiprep and Miniprep Kits- OMEGA
Nickel Coated Plates- Pierce
HRP-conjugated anti-DDDDK antibody- Abcam
Odessey Blocking Buffer-LI-COR
TMB One Substrate- Promega
PageRuler Prestained Protein Ladder- Thermo Scientific
15% Ready Gel precast gels- Biorad

All water was obtained from a Milli-Q water purification system.

Instrumentation

MoFlo Flow Cytometer and High Speed Cell Sorter with a solid state iCyt 488nm laser.
Gene Pulser Xcell- Biorad
Sonifer W-350 cell disruptor-Branson
J2-21 centrifuge-Beckman Coulter
MJ mini gradient thermal cycler- Biorad
iTC200- GE Healthcare/MicroCal
VP-Capillary DSC- GE Healthcare/MicroCal
Molecular imager gel doc XR+ system- Biorad
Innova 42/42R incubator shakers- New Brunswick Scientific
SynergyMx Microplate Reader-BioTek

Experimental Data

Library Preparation

Plasmids containing Pdar and Prb were generously provided by Professor David Baker (University of Washington). The construct NspGFP-Prb was amplified using oligonucleotides, digested with restriction enzymes NcoI and PacI, and ligated into pre-cut pETDuet-1 vector to serve as the backbone for the library. NspGFP-Prb pETDuet-1 was cut with restriction enzymes AatII and BglII for library insertion, resulting in 20 µg digested plasmid. The library insert was amplified using the saturation mutagenesis primers:

5-CGGATAAAATTGAGGTCGT*AGATC T*GTTTGTG**NNK**CCG**NNKNNK**GCG **NNKNNK**TTCGTTGTCTATGCCATCAAGAAAGGG, and

5-TTCCTTGGCTT*GACGTC*CAGC**MNNMNNMNN**ATATGTGTTGTACTGAAA CCATACCACTTTTGC.

where N represents a 25% mix each of adenine, thymine, guanine, and cytosine nucleotides; and K represents a 50% mix each of thymine and guanine nucleotides, and M represents M represents a 50% mix each of adenine and cytosine.

NNK codons were used to remove 2 of 3 possible stop codons, while maintaining all 20 amino acids. The library insert was cut with AatII and BglII and ligated into pre-cut NspGFP-Prb pETDuet-1 plasmid in its entirety, resulting in ~12 µg library plasmid DNA. Entire library was transformed in 48 batches by electroporation into 2.4 ml of electrocompetent 10βs (NEB) using 1mm cuvettes at 1.7 keV. Cells were allowed to recover in 1L pre-warmed SOC for 1hr. Library size was calculated by serial dilution, resulting in a library of ~5.0×10^9. Carbenicillin was added to a working concentration of 100 µg/mL and culture was grown overnight at 37 ºC. Library plasmid DNA was recovered the following day using a maxiprep kit (OMEGA). Gankyrin-CspGFP pBAD was prepared by amplifying Gankyrin with oligonucleotides, digested with NcoI and AatII, and ligated into pre-cut pBAD containing the link-CspGFP construct.

Preparation of Electrocompetent *E. coli*

The Gankyrin-CspGFP pBAD construct was transformed into chemically competent BL21-Gold (DE3). Cells were made electrocompetent following standard procedures. Efficiencies of > 4.0×10^8 cfu/µg DNA were achieved regularly using ~250 ng library DNA.

Fluorescence Activated Cell Sorting (FACS)

2 µg library DNA was transformed by electroporation into 400 µl BL21-Gold *E. coli* containting the Gankyrin-CspGFP pBAD construct in 8 batches. Cells were rescued in

250 ml pre-warmed 2XYT and allowed to recover for 1hr at 37ºC. Kanamycin (50 µg/mL) and carbenicillin were added to the culture and grown until an OD_{600} of 0.5 was reached. Cells were brought to room temperature and induced with 0.2% arabinose and 1 mM IPTG. Growth was continued for 6 hrs at 30 °C, then cells were pelted and washed with ice-cold PBS 3x and resuspended in PBS. Cells were sorted by FACS at a rate of 9-11,000 events/second using single sort mode for 5 hrs, setting the GFP^+ gate above the negative control (uninduced). ~800 GFP^+ cells were sorted into fresh 2XYT and allowed to recover for 12 hrs at 37 °C before the addition of antibiotics, and growth was continued overnight. Cells were inoculated into fresh 2XYT containing antibiotics and induced (as described above) when OD_{600} reached 0.5. Growth was continued for 6 hrs at 30 °C, then prepared for FACS as described above. Cells were sorted a 2^{nd} time at a rate of ~5,000 events/second for 30 minutes, taking the top 10% of the GFP^+ population. Cells were rescued and grown as described above. Plasmid DNA was harvested the following day using a miniprep kit (OMEGA).

Identification of Library Members

Plasmid DNA from the 2^{nd} round sort was used as a template for PCR. Prb mutants were amplified, digested with XhoI and PacI, and ligated back into a pre-cut pETDuet vector containing NspGFP. After transformation into chemically competent 5αs, individual colonies were picked, grown up, plasmid purified, and sequenced (GENEWIZ). Unique sequences were named Gankyrin Binding Protein (GBP) 1-30 and separately transformed into BL21-Gold containing Gankyrin-CspGFP pBAD via electroporation. Each unique GBP was verified for binding with split-GFP reassembly.

Protein Purification

Constructs were cloned into pETDuet using restriction enzymes BamHI and PacI, resulting in N-terminally His_6 tagged proteins and transformed into BL21s (DE3). Cells were grown in 2 L LB cultures containing carbenicillin at 37 °C to OD600= ~0.6 and induced with 1 mM IPTG at 25 °C overnight. Cells were then collected by centrifugation, resuspended in phosphate buffer (20 mM Sodium Phosphate pH 7.4, 150 mM NaCl, 2.5 mM BME) and stored at -20 °C. Frozen pellets were thawed and sonicated for 2 minutes. The lysate was cleared by centrifugation (15000 rpm, 30 min.) and the supernatant was mixed with 1 mL of Ni-NTA agarose resin for 1 hour. The resin was collected by centrifugation (4950 rpm, 5min.). The resin was washed with 50 mL of buffer and 20 mM imidazole. The protein was then eluted with 5 mL buffer containing 400 mM imidazole. The proteins were dialyzed against buffer and analyzed for purity by SDS-PAGE. Purified proteins were quantified using absorbance at 280nm.

ELISA

Separately, Gankyrin, Pdar, and the Notch-1 ankyrin repeat domain were cloned into MCS2 of pETDuet-1 with FLAG tags using restriction enzymes NdeI and PacI. Prb and GBPs were cloned into MCS1 of pETDuet-1 using restriction enzymes BamHI and

HindIII, resulting in N-terminal His_6-tagged proteins. Completed constructs were transformed into BL21s (DE3). Cells containing the co-expressed pair were inoculated and induced as described previously. Cells were spun down and resuspended in lysis buffer (100 mM potassium glutamate, 20 mM Hepes pH 7.5), lysed by sonication, and spun down to remove cell debris. Cleared lysates were incubated on clear Ni-NTA coated plates for 1 hr at room temperature and washed 3x with 200 µL wash buffer (100 mM potassium glutamate, 20 mM Hepes pH 7.5, 0.05% Tween-20, 0.01 mg/mL BSA). HRP-conjugated mouse anti-DDDDK antibody in LiCor Blocking Buffer was incubated for 1 hr at room temperature, followed by 4x 200 µL washes. Colorimetry was developed using TMB-One substrate and absorbance was measured at 655nm on a plate reader.

Lysate Ni-NTA Pull-down Assay

Ankyrin repeats (Gankyrin and Pdar) were cloned into MCS of pETDuet-1 using restriction enzymes BamHI and HindII, resulting in N-terminal His_{6x}-tagged proteins. Prb and GBPs were cloned into MCS2 of pETDuet-1 using the restriction enzymes NdeI and PacI. Completed constructs were transformed into BL21s (DE3). Cells containing the co-expressed pair were inoculated and induced as described previously. Cells were spun down and resuspended in lysis buffer (100 mM potassium glutamate, 20 mM Hepes pH 7.5), lysed by sonication, and spun down to remove cell debris. Cleared lysate was incubated with 100 µL Ni-NTA agarose resin for 1 hour. Ni-NTA agarose was washed with 5mL lysis buffer and 5mL lysis buffer with 20 mM imidazole. Proteins were eluted with lysis buffer containing 400 mM imidazole. The pull-down was analyzed by SDS-PAGE.

Isothermal Titration Calorimetry

Isothermal titration calorimetry was performed in collaboration with GE Healthcare using a MicroCal iTC200 calorimeter maintained at 25 °C. All proteins were purified as described previously and dialyzed extensively in phosphate buffer (20 mM sodium phosphate pH 7.4, 150 mM NaCl, 2.5 mM BME). Gankyrin was placed in the sample cell at a concentration of 20 µM, and 200 µM GBP5 or GBP7 were titrated in 2 µL increments (16 injections total) at 160 sec intervals using a stirring speed of 750 rpm. Data were analyzed using Origin7.0 (MicroCal, iTC200) using a one set of sites binding model for fitting.

Differential Scanning Calorimetry

DSC was performed in collaboration with GE Healthcare using a MicroCal VP-Capillary DSC system. All proteins were purified as described previously and dialyzed extensively in phosphate buffer (20 mM sodium phosphate pH 7.4, 150 mM NaCl, 2.5 mM BME). DSC experiments were performed using 1 mg/mL of Prb, GBP5, and GBP 7 in phosphate buffer containing BME. Temperature scanning was performed from 20 to 95 °C at a scan rate of 60 °C/hr using passive feedback mode.

Figure S1. Overlay of Pdar and Gankyrin. Superposition of Pdar (blue) and Gankyrin (orange) result in a Root Mean Squared Deviation (RMSD) value of 0.69 Å. PDB files were loaded into pymol and aligned.

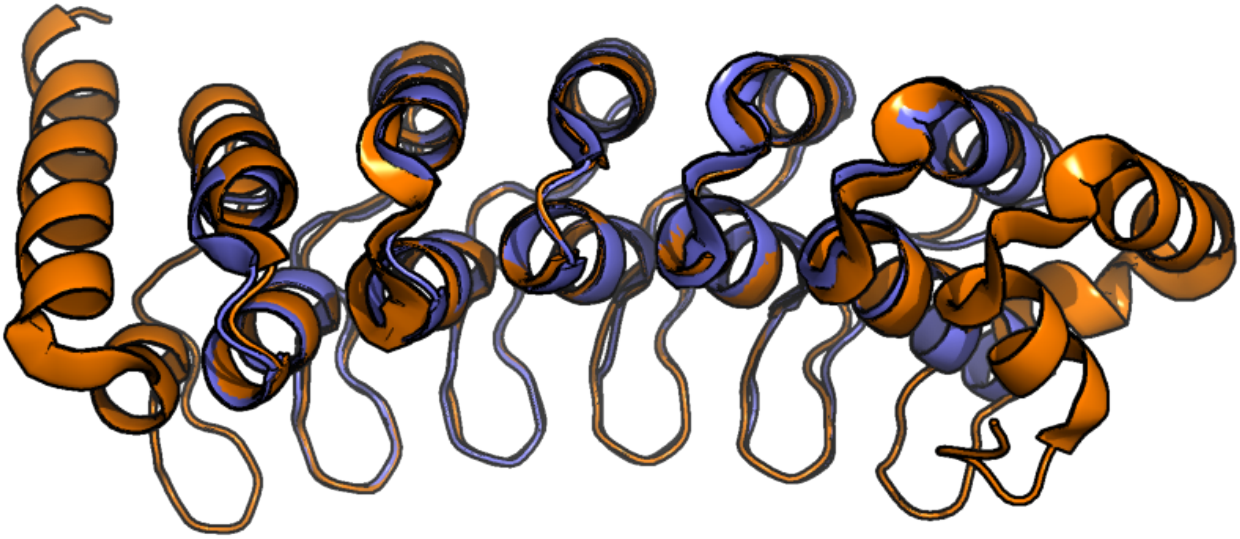

Figure S2. Sequence homology of the concave binding faces of Pdar and Gankyrin. Only ~12% sequence homology exists for the residues on the concave binding face and loop regions of Pdar (top, blue) and Gankyrin (bottom, orange), over the region covered by Prb.

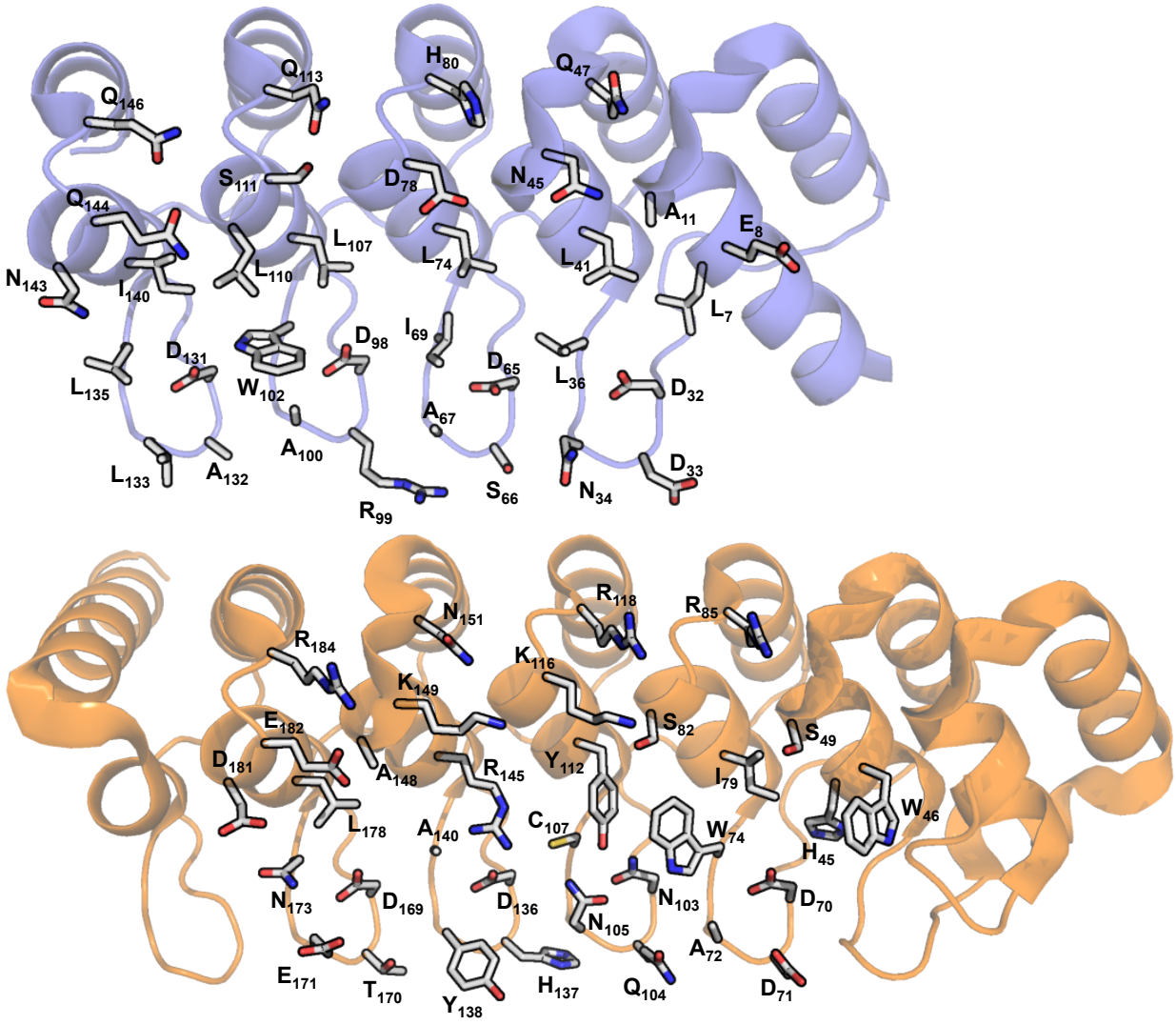

Figure S3. Structural homology with the oncoprotein gankyrin. Ankyrin repeat proteins Pdar (blue, 0.69 Å RMSD) and Notch-1(green, 1.27 Å RMSD) exhibit high structural homology with the oncoprotein gankyrin (orange).

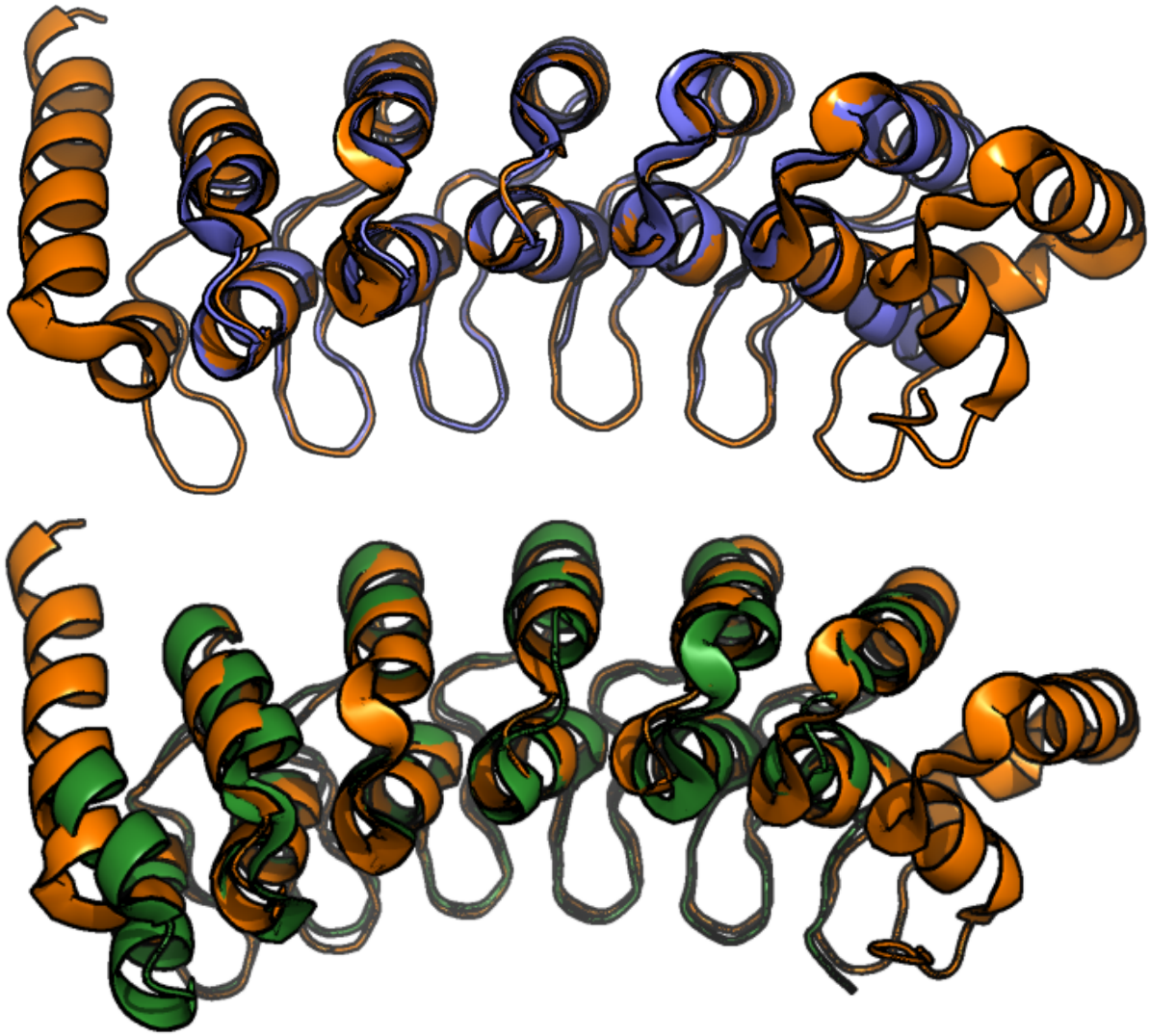

Figure S4. Concave binding face of Notch-1. The concave binding face residues of Notch-1, which share only 9% (3/33) sequence homology with Gankyrin over a regions that is covered by the scaffold protein, Prb.