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Synthetic Proteins Potently and Selectively Bind the Oncoprotein Gankyrin, Modulate Its Interaction with S6 ATPase, and Suppress Gankyrin/MDM2-Dependent Ubiquitination of p53

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Abstract

Overexpression of the ankyrin repeat oncoprotein gankyrin is directly linked to the onset, proliferation and/or metastasis of many cancers. The role of gankyrin in multiple disease-relevant biochemical processes is profound. In addition to other cellular processes, gankyrin overexpression leads to decreased cellular levels of p53, through a complex that involves MDM2. Thus, inhibition of this interaction is an attractive strategy for modulating oncogenic phenotypes in gankyrin-overexpressing cells. However, the lack of well-defined hydrophobic small-molecule binding pockets on the putative ankyrin repeat binding face presents a challenge to traditional small-molecule drug discovery. In contrast, by virtue of their size and relatively high folding energies, synthetic gankyrin-binding proteins could, in principle, compete with physiologically relevant PPIs involving gankyrin. Previously, we showed that a shape-complementary protein scaffold can be resurfaced to bind gankyrin with moderate affinity ($K_D \sim 6 \mu\text{M}$). Here, we used yeast display high-throughput screening, error-prone PCR, DNA shuffling, and protein engineering to optimize this complex. The best of proteins bind gankyrin with excellent affinity ($K_D \sim 21 \text{ nM}$), selectively co-purify with gankyrin from a complex cellular milieu, modulate an interaction between gankyrin and a physiological binding partner (S6 ATPase), and suppress gankyrin/MDM2-dependent ubiquitination of p53.

Graphical abstract

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Associated Content

Supporting Information. Experimental details, isothermal titration calorimetry data for all experiments, PAGE gels of all purified proteins described in this work, sequences of all proteins described in this work. "This material is available free of charge via the Internet at <http://pubs.acs.org>."

Overexpression of the recently reported ankyrin repeat oncoprotein gankyrin (Figure 1A, orange) is directly linked to the onset, proliferation, and/or metastasis of many cancers, including breast(1, 2), liver(3), oral(4), pancreatic(5), and colorectal cancers(6). Additionally, gankyrin plays a prominent role in Ras-initiated tumorigenesis, which is operative in ~30% of all cancers.(7)

Consistent with most ankyrin repeat proteins(8), gankyrin does not have enzymatic activity. Disease-relevant processes originate from higher than normal cellular levels of gankyrin, resulting in abnormally high levels of protein-protein interactions (PPI's) involving this oncoprotein. For example, in cells that overexpress gankyrin, cyclin-dependent kinase 4 (CDK4) is bound by gankyrin at abnormally high levels. Through mechanisms that are not well understood, CDK4/gankyrin assembly increases the extent to which CDK4 phosphorylates retinoblastoma protein (Rb).(9, 10) Increased cellular levels of phosphorylated Rb (pRb) leads to over-activation of E2F transcription factors and aberrant E2F-dependent transcription (Figure 1B).(9) Additionally, gankyrin can bind to the E3 ubiquitin ligase MDM2, and in doing so, increases the extent to which p53 is ubiquitinated/polyubiquitinated (Figure 1C).(11, 12) Increased ubiquitination/polyubiquitination of p53 by the MDM2/gankyrin complex ultimately leads to p53 degradation in the proteasome and suppression of p53-dependent apoptosis. Collectively, these gankyrin-dependent processes result in genome instability and cancer. Finally, gankyrin is known to function as a chaperone for the formation of the 26S proteasome(13), where it also associates with the S6 ATPase sub-unit (referred to as S6 ATPase herein, Figure 1A, grey).(10, 14) It is believed that association between gankyrin and S6 ATPase facilitates delivery of polyubiquitinated p53 to the proteasome. Disease-relevant cellular processes resulting from increased cellular levels of PPIs that involve gankyrin make disruption of these assemblies an attractive therapeutic strategy.(15, 16)

While structural details of the gankyrin/CDK4 and gankyrin/MDM2 complexes have not been reported, a structure involving gankyrin and the C-terminal portion of S6 ATPase is available (Figure 1A).(14) Consistent with most complexes involving an ankyrin repeat(8), the interaction occupies a large portion of the concave putative binding face. In particular, the binding interface between gankyrin and S6 ATPase occupies $\sim 2400 \text{ \AA}^2$, which is substantially larger than the observed average value of $\sim 1600 \text{ \AA}^2$ for a PPI.(17) Moreover, in contrast to the structural classes of proteins that are most commonly targeted by FDA-

approved small molecules (G-protein coupled receptors, enzymes, nuclear receptors, and ion channels), ankyrin repeat proteins lack well-defined and largely hydrophobic binding pockets on their putative binding face.(8) Collectively, the relatively featureless landscape of the concave putative binding face, which lacks well-defined hydrophobic binding pockets, and large area involved in ankyrin repeat PPI's, can present a challenge to traditional small-molecules (molecular weight <800 Da). For example, recent fragment-based efforts resulted in the discovery of a molecule that binds the Notch-1 ankyrin repeat with a dissociation constant (K_D) of ~10 mM.(18) While it is likely (if not probable) that small molecules could be developed to recognize relatively small portions of the concave gankyrin-binding face, it is unclear how potently (K_D <100 nM) and truly selective these molecules would be. Moreover, it is unclear if recognition of such a small surface on the gankyrin-binding face would result in physical disruption of physiologically-relevant PPI's.

An alternative strategy to potently recognizing, and disrupting large PPI's that lack well-defined hydrophobic small molecule binding pockets is to employ a synthetic protein (a protein that does not exist in Nature). In comparison to small molecules, the size, relatively complex three-dimensional surfaces, high folding energies (typically ~10–20 kcal/mol) and functional group diversity (by virtue of the proteinogenic amino acids) can allow proteins to potently and selectively recognize macromolecular surfaces that challenge—or entirely evade—small molecule discovery. Finally, while modern organic synthesis can enable relatively fast synthesis of simple small-molecules, the construction of small-molecules with structural complexities likely necessary for truly potent and highly-selective recognition of an ankyrin repeat binding face, and inhibition of a physiologically-relevant interaction, likely remains a complicated and laborious endeavor. In contrast, multiple high-throughput methods(19–22) often make the identification of new proteins that potently and selectively bind a disease-relevant macromolecule substantially simpler than the analogous small-molecule discovery process.

In previous work(16), we made use of an ankyrin repeat shape-complementary protein (Prb(23), Figure 2A, brown) as a scaffold for developing new gankyrin-binding proteins. Prb binds Pdar (Figure 2A, blue), an *in silico* designed thermostable ankyrin repeat with a largely hydrophobic binding face. We randomized eight residues on the ankyrin repeat shape-complementary surface of Prb (Figure 2B) to all possible proteinaceous amino acids using standard molecular biology methods. We then performed two-rounds of split-superpositive GFP reassembly(22), and *in cellulo* screen we recently reported, to identify new proteins that bind gankyrin in a complex cellular environment (*E. coli* cells). The best protein we identified (Gankyrin-Binding Protein 7, GBP7, Figure 2B) binds gankyrin with moderate affinity (K_D ~6 μ M).

From this starting point, we applied yeast display, error-prone PCR, DNA shuffling, and protein engineering to identify new gankyrin-binding proteins with dramatically improved affinity (K_D ~20 – 100 nM). Isothermal Titration Calorimetry (ITC) is used to provide the thermodynamic signature of these interactions, as well as measure the effect mutations in, near, or relatively far from the putative gankyrin-binding site have on binding affinity. The highest affinity protein binds gankyrin very tightly (K_D ~21 nM) and with exquisite selectivity in cell lysate. This protein is also able to modulate the PPI between gankyrin and

S6 ATPase, and dramatically suppress gankyrin/MDM2-dependent polyubiquitination of p53. The proteins described in this work represent the tightest gankyrin-binding reagents known to date; the highest affinity proteins bind gankyrin ~3-fold tighter than S6 ATPase, a physiological binding partner of gankyrin. Additionally, these proteins represent the only known protein modulators of gankyrin function (p53 ubiquitination).

Results

Yeast Display Screening Reveals Potent Synthetic Gankyrin-Binding Proteins

We began with GBP7, a synthetic protein we recently reported that binds gankyrin with moderate affinity ($K_D \sim 6 \mu\text{M}$).⁽¹⁶⁾ We performed rounds of yeast display screening⁽²⁰⁾, error-prone PCR⁽²⁴⁾, DNA shuffling⁽²⁵⁾, and protein engineering to optimize this interaction. Other than the putative ankyrin repeat binding face, which we previously matured for gankyrin affinity by split-superpositive GFP reassembly, it was unclear which residues in GBP7 should be mutated to improve affinity. Thus, we started by incorporating mutations in an unbiased manner using error-prone PCR (ep-PCR), and identified the tightest binders by yeast display. Using a commercially available ep-PCR kit (GeneMorph II Random Mutagenesis Kit, Agilent) we prepared a library of GBP proteins. Sequencing 30 library members after a single round of ep-PCR indicated that approximately 4 nucleic acid mutations occurred per gene. Following standard methods, we performed yeast display to identify proteins with improved affinity. Briefly, a library of gankyrin-binding proteins (generated by ep-PCR) was displayed on EBY100 *saccharomyces cerevisiae*. Displayed GBPs contain a C-terminal *myc* tag. Thus, cells can be incubated with a commercially available FITC-labelled anti-*myc* antibody and display efficiency of folded library members can be measured by flow cytometry. The yeast display library was incubated with varied concentrations of gankyrin that was first biotinylated with BirA using standard methods⁽²⁶⁾, then complexed with a streptavidin-phycoerythrin conjugate. Since the emission profiles of FITC and phycoerythrin are orthogonal, relative phycoerythrin:FITC levels can be measured by flow cytometry, and those yeast with the highest levels of bound gankyrin were sorted by Fluorescence Activated Cell Sorting (FACS). Following the first round of yeast display and FACS, enriched yeast was grown to confluence over 3 days and plasmid DNA was extracted (Yeast Plasmid Miniprep II, Zymoprep). Library DNA was either used in further diversification or sequenced.

The library generation and screening protocol to optimize gankyrin-binding affinity and selectivity is shown in Figure 3A. In yeast display rounds 1–4, library generation was achieved by ep-PCR. In each round, the level of gankyrin was decreased (round 1: 1000 nM; round 2: 500 nM; round 3: 100 nM; round 4: 50 nM) to ensure that only the tightest gankyrin-binding proteins were enriched to the next round of screening. In the fifth round, no diversification reaction was done; however, we challenged gankyrin-binding proteins by adding unlabeled off-target ankyrin repeat proteins. The enriched library of yeast displayed gankyrin-binding proteins was incubated with 50 nM gankyrin-phycoerythrin and 1 μM each of Pdar⁽²³⁾, a synthetic ankyrin repeat that is bound by the scaffold protein Prb, and p16INK4a, an ankyrin repeat protein that competes with gankyrin for binding to CDK4⁽⁹⁾, were added to the screening solution. Thus, any yeast displayed protein that bound gankyrin

in round 5 selectively did so in the presence of 20 equivalents each of unlabeled off-target protein. Flow cytometry data from each round is shown in Figure 3B; enriched cells are highlighted by encapsulation in the dashed box.

Following the first 5 rounds of yeast display screening we sequenced 10 clones and performed an initial assessment of gankyrin-binding by an Enzyme-Linked Immunosorbent Assay (ELISA). Briefly, biotinylated gankyrin was immobilized onto streptavidin-coated plates (Pierce). Solutions containing 25 nM C-terminally FLAG-tagged GBPs (GBP7.1-GBP7.10) were incubated with immobilized gankyrin, then washed 3 times. Following incubation with an anti-FLAG-HRP antibody (Abcam), and after subsequent washing steps, TMB-One HRP substrate (Promega) was added and relative amounts of complex in each well was measured by colorimetric analysis on a plate reader. As shown in Figure 4A, all of the proteins we tested exhibit appreciable affinity for gankyrin. However, 2 of the clones (GBP7.5 and GBP7.7) were the most potent gankyrin-binding proteins and thus were investigated further. To provide more quantitative feedback on the effectiveness of our yeast display screening, the binding affinity, thermodynamic signature, and stoichiometry of these new protein-protein interactions were characterized by ITC. As shown in Figure 4B, GBP7.5 and GBP7.7 bind gankyrin with significantly improved affinities ($K_D \sim 139$ and ~ 125 nM, respectively), compared to our starting point GBP7 ($K_D \sim 6 \mu\text{M}$), representing a ~ 46 -fold improvement in affinity.

To further optimize the complex, we performed DNA shuffling on the entire sub-library of clones that were enriched in round 5, and screened the shuffled protein library by yeast display. We sequenced 25 clones and performed an initial assessment of gankyrin-binding by ELISA. The best performing clones are shown in Figure 4C. Similar to the previous ELISA experiment, all of the proteins exhibited appreciable affinity for gankyrin; however, 2 of the clones (GBP7.15 and GBP7.17) were the most potent gankyrin-binding proteins, and thus the complex between these proteins and gankyrin was further characterized by ITC. As shown in Figure 4D, DNA shuffling generated proteins with significantly improved affinity. GBP7.15 and GBP7.17 bind gankyrin with low nanomolar dissociation constants ($K_D \sim 62$ and 42 nM, respectively): a ~ 120 -fold improvement from the initial complex involving GBP7. The observed changes in enthalpy (ΔH) and entropy ($-\Delta S$) for the PPI involving gankyrin and GBP7.15 were $-8.9(\pm 0.2)$ and $-0.9(\pm 0.2)$ kcal/mol, respectively. The observed changes in enthalpy (ΔH) and entropy ($-\Delta S$) for the PPI involving gankyrin and GBP7.17 were $-13.5(\pm 0.5)$ and $3.5(\pm 0.5)$ kcal/mol, respectively.

Mutational Analysis of New Gankyrin-Binding Proteins

Of the 25 clones we sequenced from the enriched gankyrin-binding library, three mutations, N55Y (which is near the putative ankyrin repeat binding face), D79G and R132C (both relatively distant from the putative ankyrin repeat binding face) were found in all 25 sequenced clones (Figure 5A and Figure 5B, boxed). In order to measure the importance of each mutation in gankyrin recognition, we prepared a library of reversion mutants and measured their affinity for gankyrin by ITC. Reverting the cysteine at position 132 back to arginine decreased affinity ~ 2.8 -fold, compared to GBP7.17 (Figure 5C). The G79D reversion mutant bound gankyrin with no appreciable loss in affinity (Figure 5D).

Interestingly, mutating tyrosine 55 back to asparagine had a much more dramatic effect, and resulted in ~22.5-fold lower affinity for gankyrin (Figure 5E). This makes sense, since residue 55 is relatively close to the putative gankyrin-binding face and might directly engage gankyrin.

Among the residues that were initially optimized in our previous work to generate the starting protein GBP7, one (H110R) was mutated in GBP7.17 as a result of ep-PCR and DNA shuffling. Additionally, a Y93C mutation was found near the initially optimized binding face. To measure the importance of these residues, we made reversion mutants and measured their affinity for gankyrin by ITC. Interestingly, converting cysteine 93 back to tyrosine only lowered affinity for gankyrin by ~2.2-fold, compared to GBP7.17 (Figure 5F). Additionally, reverting arginine 110 back to histidine only lowered affinity by ~1.5-fold (Figure 5G).

Optimizing the Protein-Protein Interaction

Three mutations on or near the designated gankyrin-binding face differentiate the two best performing GBPs (GBP7.15 and GBP7.17). Both an E112V and Y93H mutation are found in GBP7.15, and these residues reside within the originally matured gankyrin-binding face. Slightly outside of this, we observe a E57K mutation in GBP7.15. As stated previously, a Y93C mutation was found in our best performing GBP (GBP7.19). However, reversion of this residue did not dramatically lower binding, suggesting residue 93 plays a less prominent role in stabilizing a complex with between gankyrin and GBP7.17. Given this, we focused on integrating the E112V and/or E57K mutations found in GBP7.15 into our best performing protein (GBP7.17), and measuring how each change alters affinity for gankyrin.

Replacing the glutamic acid at position 112 in GBP7.17 with the valine that is found in GBP7.15 does not appreciably improve affinity for gankyrin (Figure 5H). In contrast, mutating the negatively charged glutamic acid at position 57 in GBP7.17 with the positively charged lysine that is found in GBP7.15 results in a mutant with significantly improved affinity (K_D ~21 nM, Figure 5I). This protein is referred to as GBP7.19 herein. This is consistent with our earlier finding that the Y55N reversion mutation resulted in dramatically lowered affinity for gankyrin. Collectively, these two results suggest that the surface of GBP7.19 displaying residues 55 and 57 might directly engage gankyrin, and as a result, the chemical makeup of these residues can dramatically effect complex stability with gankyrin. The observed changes in enthalpy (ΔH) and entropy ($-\Delta S$) for the PPI involving gankyrin and GBP7.19 were $-13.8(\pm 0.3)$ and $3.3(\pm 0.2)$ kcal/mol, respectively. Addition of the E57K and E112V mutation to the GBP7.17 scaffold does not appreciably improve affinity, compared to GBP7.19 (K_D ~22 nM, Figure 5J). Owing to its affinity for gankyrin, we focused on measuring the selectivity of the GBP7.19/gankyrin interaction, and the ability of GBP7.19 to modulate a physiologically-relevant gankyrin-dependent PPI and a gankyrin-dependent and disease-relevant biochemical process.

GBP7.19 binds gankyrin with exquisite selectivity, when expressed in *E. coli* with His₆-tagged gankyrin, the two proteins co-elute following nickel-NTA purification (Figure 5K, lane 2). Appreciable levels of other co-purified proteins is not observed, demonstrating the selectivity of the gankyrin/GBP7.19 binding interaction – even in a complex cellular milieu.

A Synthetic Gankyrin-Binding Protein Inhibits the Gankyrin/S6 ATPase Protein-Protein Interaction

Any therapeutic utility of gankyrin-targeted reagents requires tight and selective recognition of this oncoprotein. In this regard, the proteins described above are excellent candidates. However, modulation of disease-relevant gankyrin activity likely requires physical disruption or inhibition of physiological PPI's. As stated above, gankyrin is known to bind CDK4 and MDM2, leading to increased Rb phosphorylation and p53 polyubiquitination, respectively.(9, 11) Unfortunately, challenges exist when trying to express recombinant CDK4 or MDM2 in *E. coli*. Gankyrin is also known to bind the C-terminal portion of S6 ATPase, a proteasomal subunit (Figure 1A).(14) However, both the C-terminal portion of S6 ATPase, and the full-length protein do not express in a soluble form in *E. coli* –soluble S6 ATPase can only be expressed concomitantly with gankyrin.(14)

We recently grafted the C-terminal ATPase subdomain of S6 onto the C-terminal ATPase subdomain of FtsH, an *E. coli* derived protein, and showed that this new protein (FtsH-S6 ATPase, Figure 6A) expresses well in *E. coli*, is folded in solution, and binds gankyrin with excellent affinity ($K_D \sim 67$ nM, Figure 6B).(27) Since FtsH-S6 ATPase expresses independent of gankyrin, this surrogate for the C-terminal subunit of S6 ATPase can be used to determine if GBP7.19 modulates the gankyrin/S6 ATPase interaction, and physically disrupts this complex.

The ability of our tightest gankyrin-binding protein (GBP7.19) to modulate the FtsH-S6 ATPase/gankyrin interaction was initially tested by ITC. As stated above, FtsH-S6 ATPase binds gankyrin with excellent affinity(27) ($K_D \sim 67$ nM, Figure 6B). However, when FtsH-S6 ATPase is titrated into a gankyrin/GBP7.19 complex, we observe dramatically decreased affinity (Figure 6C). This finding suggests that GBP7.19 likely binds the concave face of gankyrin and therefore is in a position to block the gankyrin/S6 ATPase interaction. The dramatic change in enthalpy ($\Delta H = -29$ kcal/mol for the titration of S6 ATPase into gankyrin; $\Delta H = -14$ kcal/mol for the titration of S6 ATPase into pre-complexed gankyrin / GBP7.19) also suggests an altered mode of binding between S6 ATPase and gankyrin, possibly due to GBP7.19 blocking a portion of the protein-protein interface found in the native S6 ATPase / gankyrin complex.

A Synthetic Gankyrin-Binding Protein Suppresses Gankyrin/MDM2-Dependent Ubiquitination of p53

We next set out to determine if GBP7.19 modulates gankyrin- and MDM2-dependent p53 ubiquitination – a principal disease-relevant role for this oncoprotein. As stated above, gankyrin binds the E3 ubiquitin ligase MDM2, and in doing so increases the extent to which p53 is polyubiquitinated.(12, 28) Ultimately, this leads to decreased cellular levels of p53 and suppression of p53-dependent apoptosis. We used a commercially available *in vitro* assay (MDM2/p53 Ubiquitination Kit, BostonBiochem) to measure p53 ubiquitination without gankyrin, with gankyrin, or with gankyrin and varied concentrations of GBP7.19. Briefly, p53 was mixed with the ubiquitination cocktail (reaction buffer, E1, E2, MDM2, Mg^{2+} -ATP, ubiquitin) and native p53 was measured by Western blot. As expected, only ~68% of native p53 is present following the ubiquitination reaction (Figure 6D and Figure

6E, lane 2), compared to a control experiment where no ubiquitin is added (Figure 6D and Figure 6E, lane 1). Adding 1 equivalent of gankyrin to the ubiquitination cocktail (relative to MDM2) resulted in a significant reduction in the level of native (unubiquitinated) p53 (Figure 6D and Figure 6E, lane 3), compared to the ubiquitination reaction that lacks gankyrin (Figure 6D and Figure 6E, lane 2). Addition of GBP7.19 results in decreased ubiquitination of p53. When 0.25, 0.5, or 1 equivalents of GBP7.19 is added to the ubiquitination cocktail (relative to gankyrin and MDM2), we observe a concentration-dependent and dramatic increase in the levels of native p53 (Figure 6D and Figure 6E, lanes 4–6). One equivalent of GBP7.19 completely inhibits p53 ubiquitination. One possible explanation for this observation is that GBP7.19 binds the gankyrin/MDM2 complex (as opposed to displacing MDM2), and the ternary complex is unable to perform the ubiquitination reaction.

Conclusion

Gankyrin is a recently reported ankyrin repeat oncoprotein whose overexpression is directly linked to the onset, proliferation, and/or metastasis of a number of cancers. In forming a complex with MDM2 gankyrin increases the extent to which p53 is polyubiquitinated, leading to suppression of p53-dependent apoptosis and cell cycle deregulation in gankyrin overexpressing cells.

Collectively, the findings in this paper can be summarized as follows: using error-prone PCR, DNA shuffling, yeast display screening, and protein engineering, we were able to dramatically optimize a PPI involving the oncoprotein gankyrin and synthetic gankyrin-binding proteins (GBPs). The most active protein we identified (GBP7.19) bind gankyrin very tightly ($K_D \sim 21$ nM), modulate a physiologically-relevant interaction involving gankyrin and S6 ATPase, and dramatically decrease gankyrin/MDM2-dependent ubiquitination of p53, a principal disease-relevant function of gankyrin.

The proteins reported in this paper represent the most potent gankyrin-binding reagents known to date, establish GBP7.19 as a viable tool to study gankyrin-dependent cellular processes, and may represent an intermediate point in the development of a gankyrin-targeted protein therapeutic. Efforts toward the later goal are currently underway and will be reported in due course.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(A) Crystal structure of the gankyrin/S6 ATPase complex (PDB: 2DVW). Gankyrin is colored orange; the C-terminal subunit of S6 ATPase is colored in gray. (B) Under normal conditions, p16INK4a binds cyclin-dependent kinase 4 (CDK4) and modulates phosphorylation of Rb. In gankyrin overexpressing cells, gankyrin binds CDK4, which leads to increased cellular levels of phosphorylated Rb (pRb), which in turn leads to activation of E2F-dependent transcription. (C) Gankyrin forms a complex with the E3 ubiquitin ligase MDM2, and this complex increases the extent to which p53 is polyubiquitinated, ultimately leading to p53 degradation and suppression of p53-dependent apoptosis.

Figure 2.

(A) Crystal structure of the Pdar (light blue)/Prb (light brown) complex (PDB: 3Q9N). (B) Residues on the putative ankyrin repeat binding face of Prb (highlighted in green) that were mutated to generate gankyrin-binding protein 7 (GBP7). The residues on the putative ankyrin repeat binding face of Prb and GBP7 are shown in (B).

Figure 3.

(A) Strategy for the development of potent (low nanomolar dissociation constant) Gankyrin-Binding Proteins (GBP) from GBP7. GBP7, which binds gankyrin with moderate affinity ($\sim 6 \mu\text{M}$ dissociation constant), was diversified by error-prone PCR and highest affinity library members were enriched by Fluorescence Activated Cell Sorting (FACS), using yeast display. The enriched library was further diversified by a second round of error-prone PCR and highest affinity binders were enriched by FACS. Error-prone PCR and FACS screening was done for a total of 4 rounds. Enriched library members from the fourth round were then screened by yeast display / FACS for selective recognition of gankyrin in a solution containing 20 equivalents of off-target ankyrin repeats (Pdar and p16INK4a). Following this round of yeast display / FACS, the enriched library was scrambled by DNA shuffling and the resulting protein library was screened for gankyrin affinity by yeast display / FACS. Following sequencing of enriched gankyrin-binding proteins, we combine common beneficial mutations in an effort to further optimize affinity for gankyrin. (B) Flow cytometry data from screening of error-prone PCR generated libraries (rounds 1–4), the screen for selective recognition of gankyrin (round 5) and screening of the DNA shuffling generated library (round 6).

Figure 4.

(A) Enzyme-Linked Immunosorbent Assay (ELISA) data for gankyrin-binding proteins 7.1–7.10, which were generated by error-prone PCR and identified in screening rounds 1–5. (B) Isothermal titration calorimetry (ITC) analysis of the protein-protein interactions involving gankyrin and GBP7.5 or GBP7.7. (C) ELISA data for gankyrin-binding proteins 7.11–7.18, which were generated from DNA shuffling and identified in screening round 6. (D) ITC analysis of the protein-protein interactions involving gankyrin and GBP7.15 or GBP7.17. Stated K_D 's are the mean of three independent experiments, with standard deviation error.

Figure 5.

(A and B) GBP7.15 and GBP7.17, which were identified as the tightest gankyrin-binding proteins following screening of the DNA shuffled library. Green colored residues were generated in our earlier work as a result of split-superpositive GFP screening, and were thus found in the starting protein (GBP7). Red colored mutations were generated in this work, as a result of error-prone PCR and DNA shuffling. (C–G) Isothermal titration calorimetry data for five reversion mutants of GBP7.17, which provided information on which residues are most critical to gankyrin recognition. (H–J) Isothermal titration calorimetry data for GBP7.17 based proteins that contain mutations found in GBP7.15. Stated K_D 's are the mean of three independent experiments, with standard deviation error. (H) Co-purification of His₆-gankyrin and GBP7.19 from *E. coli* cell lysate.

Figure 6.

(A) S6 ATPase (red) superimposed on the C-terminal ATPase domain of FtsH (grey, PDB: 1LV7). (B) Isothermal titration calorimetry data for an experiment that involves titrating FtsH-S6 ATPase into gankyrin. The dissociation constant (K_D) for this complex is ~67 nM. (C) Isothermal titration calorimetry data for an experiment that involves titrating FtsH-S6 ATPase into a pre-formed GBP7.19/gankyrin complex. (D) and (E) Levels of native p53 following treatment with a ubiquitination cocktail. Lane 1: p53 that was treated with the ubiquitination cocktail that lacks ubiquitin (no ubiquitination occurs); lane 2: native p53 levels following ubiquitination under normal conditions; lane 3: native p53 levels following ubiquitination with 1 equivalent of gankyrin; lane 4: native p53 levels following ubiquitination with 1 equivalent of gankyrin and 0.25 equivalent of GBP7.19; lane 5: native p53 levels following ubiquitination with 1 equivalent of gankyrin and 0.5 equivalent of GBP7.19; lane 6: native p53 levels following ubiquitination with 1 equivalent of gankyrin and 1.0 equivalent of GBP7.19. Bars represent the average of three independent experiments. (E) Errors bars represent S.D.; statistical analysis: unpaired parametric t-test: *P<0.05; ***P<0.0005