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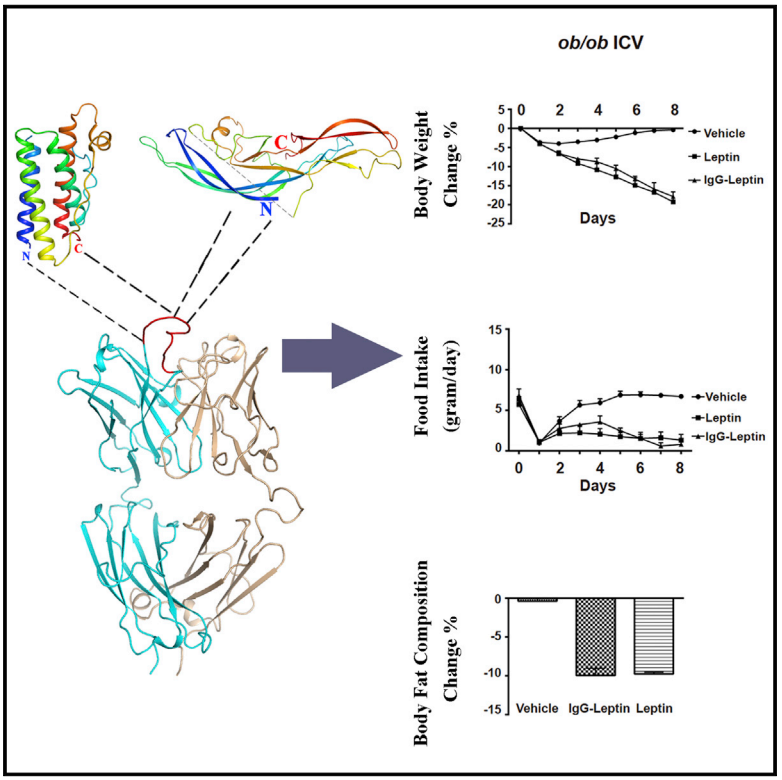
A General Method for Insertion of Functional Proteins within Proteins via Combinatorial Selection of Permissive Junctions

Graphical Abstract



Highlights

- Robust high-throughput screening-based method for inserting proteins into proteins
- Functional Leptin in human IgG with longer serum half-life in vivo than native Leptin
- Single-chain FSH in human IgG with similar activity compared with native FSH

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In Brief

Peng et al. report a general approach based on robust high-throughput screening for inserting functional proteins into proteins. The selected Leptin and single-chain FSH in human IgG were as potent as the native hormones.

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A General Method for Insertion of Functional Proteins within Proteins via Combinatorial Selection of Permissive Junctions

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SUMMARY

A major goal of modern protein chemistry is to create new proteins with different functions. One approach is to amalgamate secondary and tertiary structures from different proteins. This is difficult for several reasons, not the least of which is the fact that the junctions between secondary and tertiary structures are not degenerate and usually affect the function and folding of the entire complex. Here, we offer a solution to this problem by coupling a large combinatorial library of about 10^7 different N- and C-terminal junctions to a powerful system that selects for function. Using this approach, the entire Leptin and follicle-stimulating hormone (FSH) were inserted into an antibody. Complexes with full retention of function in vivo and in vitro, although rare, were found easily by using an autocrine selection system to search for hormonal activity. Such large diversity systems, when coupled to robust selection systems, should enable construction of novel therapeutic proteins.

INTRODUCTION

Structurally, a protein can be considered to be an ensemble of secondary and tertiary folds connected to each other by N- and C-terminal junctions (Gerstein et al., 1994; Pritchard et al., 2003). Sometimes these junctions are simple extended polypeptide chains, whereas in other cases they are part of a more complicated secondary structure. Because of advances in protein structure determination, we have a large and ever-growing menu of secondary and tertiary folds in proteins. Thus, in principle, one should be able to construct new proteins by inserting some of these secondary structure elements (guests) into a new protein partner (host). Indeed, in early pioneering studies, different guests have been successfully inserted into host proteins, including short peptides and cytochrome b_{562} into β-lactamase (Edwards et al., 2008; Mathonet et al., 2006), β-lactamase into maltodextrin-binding protein (Betton et al., 1997), calmodulin into GFP (Baird et al., 1999), 1,4-β-xylanase into 1,3-1,4-β-glucanase (Ay et al., 1998), and, most recently, different hormones into bovine and humanized antibodies (Liu et al., 2015; Zhang et al., 2015; Zhang et al., 2013b; Zhang et al., 2013c). However, these studies were design based, whereas here we present a selection-based method that is arguably much more powerful and general. There were two main problems to be solved in the generation of a method based on selection. The most widely appreciated problem is that function often requires approximation in three dimensions of an array of secondary structure elements. A less appreciated, but perhaps more critical constraint, is that the junctions between secondary structures likely represent a distinct structural element that enables proper folding of the secondary structures to which they are appended. Indeed, recent studies have shown that some of these junctions (β/β, α/β, and β/α motifs) may function according to a defined set of rules (Balasco et al., 2013; Koga et al., 2012).

The problem of defining the functional requirements of junction sequences can be separated into two parts: (1) to experimentally generate a large number of random junctions, and (2) to test which junctions can act in concert with guests to enable function. In principle, the first problem can be approached experimentally using modern combinatorial library methods. Thus secondary structural elements, either singly or in combination, can be linked to the protein scaffold by an arbitrarily large number of different N- and C-terminal junctions so as to assemble new tertiary structures. To solve the selection problem, the junctions that are permissive for function can be selected using autocrine-based screenings. In such autocrine selection systems, millions of potential agonists and their receptor are expressed in cells such that all cells have the same

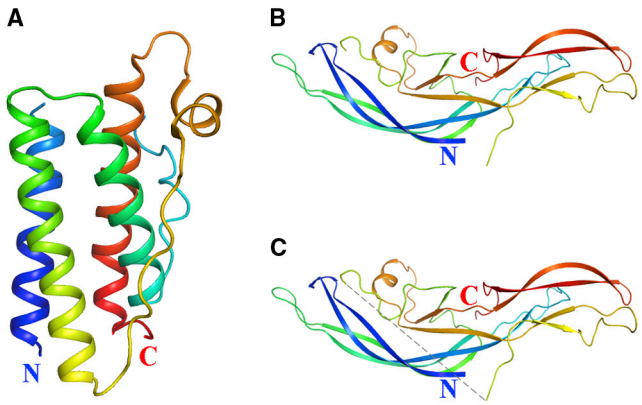

Figure 1. Overall Structures of Guest Molecules

(A) Cartoon representation of human Leptin (PDB: 1AXB) (Zhang et al., 1997).
(B) Cartoon representation of human FSH (PDB: 4AY9) (Jiang et al., 2012).
(C) Cartoon representation of the scFSH. The polypeptide (GSGSNATGSGS NATSGSTS) linking the C terminus of the FSH β chain and N terminus of the FSH α chain is indicated by a gray dashed line.

cognate receptor while each individual cell expresses only a single unique member of the large repertoire of potential agonists. When combined with modern cell sorting, one can use this approach to explore a large repertoire of combinatorial junctions by inserting a known protein guest agonist in frame into a protein host, such as an antibody, where the guest is separated from the host on either side by random amino acid sequences that may function as permissive junctions. Functional autocrine selection allows determination of which junctions allow proper folding and function of the guest.

As proof of concept, the goal of the present experiment was to test the central idea that if a very large number of different junctions can be tested, we could select those were permissive for proper function of the guest, even if they were rare. We studied whether antibodies could be used as hosts into which hormones could be introduced as guests. The variable amino acids of the complementarity determining region (CDR) H3 loop of an antibody were replaced by two sets of 8–18 amino acids (potential N- and C-terminal junction sequences) between which were cloned the entire 146 and 203 amino acids of the hormones human Leptin and human follicle-stimulating hormone (FSH), respectively. Since it was our intention to investigate a very large number of candidates, the key was to construct a robust system for selection of permissive junctions. Thus, we coupled high junctional diversity to autocrine-based phenotypic screens to select for those junctions that allow function of the embedded Leptin and FSH proteins. Sequencing of the clones that retained agonist activity allowed us to characterize those junctions that permitted proper guest function from a combinatorial diversity system of $\sim 3 \times 10^7$ different members. Only about 0.06% and about 0.18% members were permissive for function of Leptin and FSH, respectively, and the permissive sequences were different for the two guests.

These constructs allowed us to study critical biological issues. Because the embedded Leptin is too large to cross the blood-brain barrier (BBB), the generation of embedded Leptin enabled an analysis of the relative contributions of Leptin signaling at circumventricular organs (CVOs) versus brain parenchyma.

The methods described here also provide a starting point for the generation of new proteins in which the embedded probe can report on proper folding. In this case, the role of the guest is simply to report on proper folding of the complex as a function of changes made elsewhere. When combined with current computational methods (Balasco et al., 2013; Koga et al., 2012), these host-guest complexes could be a new tool to address the protein-folding problem because they allow a high-throughput analysis of a very large number of variant proteins for those that fold properly. This changes the search for proper folding from analysis of a bulk solution or limited number of variants to an analysis of a very large number of single molecules. Also, one does not rely on an intrinsic function of the host that could be perturbed by substitutions even if they are some distance from the active site, thereby confusing the analysis. Finally, this method should help in the generation of new protein therapeutics with distinct properties.

RESULTS

Choice of Guest Molecules

To determine whether the combinatorial process of inserting guests into protein hosts could be general, we studied two very different protein hormones, Leptin and FSH. Because the two hormones have very different structures, their choice allows analysis of whether the method is general as opposed to being limited to only certain folds. As Leptin is folded into a compact four-helix bundle (Figure 1A) (Zhang et al., 1997), one might predict that the favorable binding energy of the helices for each other would make selection of junctions less critical. However, this same rigidity of the guest could interfere with the folding of the entire host-guest complex. FSH folds mostly into β-sheet structures, and is composed of α and β subunits with central cysteine-knot motifs from which three β hairpins extend (Figure 1B) (Jiang et al., 2012). In terms of these experiments, the heterodimer assembly may pose a special problem. If the FSH subunits are treated individually the process becomes a four-linker combinatorial problem, whereas if the monomeric subunits of the heterodimer are covalently linked, the number of linkers is reduced to three; one on either end and one in the middle. Thus, we set out to construct a single-chain FSH (scFSH) by linking the α and β subunits using a GSGSNATGSGSNATSGSTS peptide linker that is permissive for heterodimer formation (Figure 1C) (Trousdale et al., 2007). Indeed, the scFSH covalent dimer was shown to be functional using a reporter cell line expressing the FSH receptor (see below).

Construction of Combinatorial Junctions

The host is a randomly chosen antibody, scFv, from our very large human naive antibody library (Gao et al., 1997) with a CDR H3 containing 19 residues (Kabat numbering [Kabat et al., 1991]) (Figure 2A). To maintain proper folding of the antibody core while maximizing the flexibility of junctions, most of the CDR H3 loop, residues L^{H98}GITKTSTCYT^{H100H} (red), were replaced by members of a combinatorial N- and C-terminal junction library

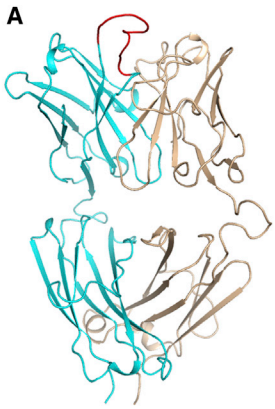

Figure 2. Construction of Combinatorial Junctions

(A) Cartoon representation of the homology model of the antibody scaffold, which was constructed using Modeller (Sali and Blundell, 1993), with structures of 1RZI, 3QOT, 4HJ0, 3NPS, 3G04, 3D69, and 4J4P as templates. The heavy and light chains are colored in cyan and wheat, respectively. The L^{H98}GITKTSTCYT^{H100H} segment in the CDR H3 loop, which was replaced to generate the combinatorial junction library, is colored in red.

(B) Schematic demonstration of combinatorial junction design. The three-residue random linker and one to three copies of GGGGS linker are indicated with XXX and $(GGGGS)_{1-3}$, respectively.

designed to generate a highly diverse array of sequences that attach the Leptin and scFSH guests to the antibody host. This choice of site of insertion was based on examination of the homology model of the parental scaffold (Figure 2A) to determine where a large insert can readily be accommodated: most of the guest would extend into the solvent, with the rest expected to pack against other CDRs of the antibody host.

To construct proteins with functional inserts, a GGGGS linker and similar glycine-serine linkers have been commonly used together with some other more rigid linkers. The GGGGS linker provides tremendous flexibility and has been successfully used in many cases. However, in some cases the GGGGS linker might not the best in terms of protein expression, stability, and extension of the guest. To generalize this method, based on the structure model of antibody, we introduced a random three-residue linker (denoted XXX) between the GGGGS linker and the antibody scaffold to provide extra length and, more importantly, the potential to optimize the linker for higher expression, better stability, and greater extension of domains of host and guest of selected clones (Klein et al., 2014; Reddy Chichili et al., 2013; Silacci et al., 2014; Volkel et al., 2001). The combinatorial junction library was designed to contain junctions of an XXX linker (Figure 2B) and one to three copies of a GGGGS linker denoted $(GGGGS)_{1-3}$ at both the N and C termini of Leptin and scFSH (Figure 2B). The total number of colonies in the Leptin and scFSH libraries were 2.88×10^7 and 1.09×10^7, respectively, for the combination of the N- and C-terminal libraries. While the theoretical diversity of the library is $\sim 9 \times 10^9$ different members, we carried out experiments to ensure that there is not an excessive repetition of only a few unique clones. Thus, we sequenced 20 clones, and no antibody was repeated.

Autocrine-Based Selection of Functional Junctions

Autocrine-based flow cytometry sorting of cells in which the expression of β-lactamase reporter gene is induced by various cell signaling responsive elements has been widely used because of its high accuracy and sensitivity (Qureshi, 2007). When β-lactamase is not expressed in the reporter cells loaded with an engineered fluorescent substrate that covalently links two fluoroprobes (coumarin and fluorescein), the substrate remains intact, and thus excitation of the coumarin results in fluorescence resonance energy transfer (FRET) to the fluorescein and emission of green fluorescence. When the expression of β-lactamase is induced in the reporter cells the substrate is cleaved, and excitation of the now free coumarin functionality results in a blue fluorescence signal. The resulting ratio of blue fluorescence signal to green fluorescence signal provides a normalized measure of the reporter response. The sis-inducible element (SIE) (Bendinelli et al., 2000) and the cyclic AMP-responsive element (CRE) (Heindel et al., 1975) driven β-lactamase-based reporter cell lines were constructed and tested for the human Leptin receptor (*LepR*) (designated LepR SIE-Bla) and the human FSH receptor (FSHR) (designated FSH CRE-Bla), respectively (Figure 3). To isolate functional antibody clones, FRET-based fluorescence-activated cell sorting (FACS) was carried out using stringent gating (Figure 3). For LepR SIE-Bla cells, there was significant enrichment after two rounds of sorting, from only 0.06% positive cells (versus 66.9% positive cells in positive control infected with Leptin lentivirus) in the first round to 3.30% positive cells (versus 6.57% positive cells in positive control infected with Leptin lentivirus) in the second round (Figures 3F and 3G). The reason that the population of positive cells in the positive control in the second round is less than that in the first round is because we used less lentivirus input to minimize transactivation by secreted antibodies. For FSH CRE-Bla cells, one round of sorting yielded 0.18% positive cells (Figure 3H). After each round of sorting, antibody gene fragments were recovered by the PCR using cell lysates as templates, and re-inserted into the lentiviral vector for the next round of sorting and/or clone screening of the clones as necessary. Twenty clones were picked from the second round for LepR SIE-Bla cells, and the first round for FSHR CRE-Bla cells. Plasmids were extracted from these clones and transfected into HEK293T cells to express secreted antibodies. Supernatants were isolated 2 days after transfection and tested to identify clones with agonist activity. Six clones out of 20 were found to be capable of activating LepR SIE-Bla cells, while 3 out of 20 clones were able to activate FSHR CRE-Bla cells. These clones were sequenced. The sequences of permissive junctions were analyzed (Table 1). For scFSH, clone B (Figure S1A) was the most potent. In addition, the sequences of non-permissive junctions that showed no activity in the FRET assay were analyzed (Tables S1 and S2; Figures S1C and S1D). Possible reasons why there were still negative clones in the sorted pool include stimulation of close neighboring cells by secreted antibody (these transactivated clones would not contain the active antibody genes), mutation in the PCR reaction when recovering

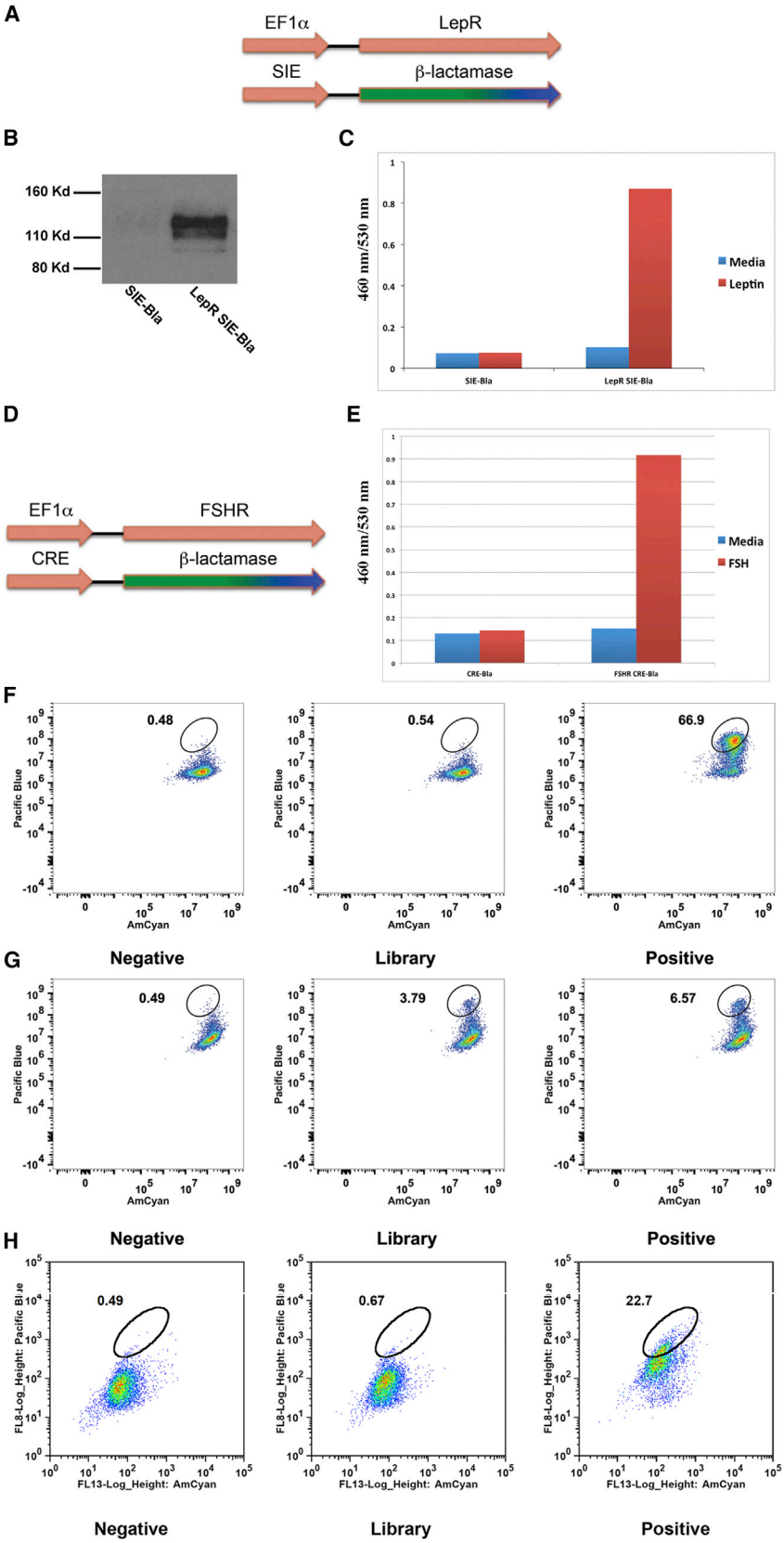

Figure 3. Construction of Autocrine-Based Stable Cell Lines and Autocrine Selection of Functional Junctions

(A) Schematic diagram of elements used for autocrine cell construction of LepR SIE-Bla cells. Once LepR on the surface of the LepR SIE-Bla cells is stimulated upon binding of agonist, the downstream SIE signaling pathway is turned on and the cells start to express β-lactamase, which can then cleave the LiveBLAzer-FRET B/G substrate (CCF4-AM) and change the FRET signal.

(B) Expression of HA-tagged LepR in LepR SIE-Bla cells. One million of the parental SIE-Bla cells and LepR SIE-Bla cells were lysed in 100 μl of 1× SDS-PAGE loading buffer. The expression of HA-tagged LepR was detected with α-HA antibody.

(C) Response of LepR SIE-Bla cells to 1 ng/ml Leptin.

(D) Schematic diagram of elements used for autocrine cell construction of FSHR CRE-Bla cells.

(E) Response of FSHR CRE-Bla cells to 1 ng/ml FSH.

(F and G) First and second round of sorting for Leptin activity. The negative and positive controls for sorting are non-infected LepR SIE-Bla cells and LepR SIE-Bla cells infected with Leptin lentiviruses, respectively. Cells with the highest fluorescence ratio in the Pacific Blue channel to the AmCyan channel were collected using a MoFlo Astrios Flow Cytometry Sorter.

(H) One-round sorting for FSH activity. The negative and positive controls for sorting are non-infected FSHR CRE-Bla cells and FSHR CRE-Bla cells infected with scFSH lentiviruses, respectively. The x axis is the green signal of uncleaved substrate and the y axis is the blue signal of substrate after cleavage by β-lactamase.

Table 1. Sequences of Active Clones

Clones	N Linker	C Linker
Active Clones for LepR		
A	HLTGGGGS	GSGGSGGGGSDPS
B	PWAGGGGSGGGGSGGGGS	GGGGSQPP
C	LGVGGGGS	GGGGSERT
D	KTSGGGGSGGGGS	GGGGSSDE
E	LGVGGGGS	GGGGSERT
F	KVTGGGGS	GGGGSQLE
Active Clones for FSHR		
A	RSHGGGGS	GGGGSVNP
B	QRVGGGGS	GGGGSGGGGSRSA
C	RVLGGGGS	GGGGSGGGGSQSS

the gene from the lysates of sorted cells, and recombination or deletion of DNA in cells.

Improved Selection of Junctions

While the first selection yielded functional proteins, the yield of the host-guest proteins was not optimal. To improve protein production, another selection was carried out whereby a protein yield parameter was incorporated. To accomplish this, a new vector was used in which the gene encoding mCherry was fused to the C terminus of the scFv gene (Shaner et al., 2004). This allowed selection for high-expressing clones by using an additional gate for a high mCherry signal prior to the FRET gate. Three libraries were studied. One was the original library. In the other two libraries, either the N- or the C-terminal linkers identified in the previous experiment were held constant and the other linkers comprised the same random sequences that were used previously. For Leptin, these studies yielded a new clone (clone 2 in Figure S1B) with an RHMGGGGS at the N terminus and GGGGSGGGGSTDT at the C terminus. This protein expressed much better than the previously selected clone 1 with LGVGGGGS at the N terminus and GGGGSERT at the C terminus while still retaining full Leptin activity (Figure S1B).

The most active scFv clone of the antibody containing the Leptin guest with the highest yield was converted to a Fab format (Fab-Leptin) or full immunoglobulin G (IgG) format (IgG-Leptin). The unaltered host, Fab-Leptin, and IgG-Leptin were cloned in pFUSE-based vectors (InvivoGen) and expressed in FreeStyle 293-F cells (Life Technologies). After a one-step affinity purification, all antibodies were purified to ~95% (Figure S2). The production yield was 6–10 mg from 1 l of cell culture for all antibodies.

In Vitro Activity

Circular dichroism (CD) spectroscopy (Greenfield, 2006) and differential amide hydrogen-deuterium exchange mass spectrometry (HDX-MS) (Chalmers et al., 2011; Pascal et al., 2012) studies showed that the antibody scaffold folded properly after the insertion of Leptin and that the embedded Leptin in the antibody scaffold was essentially the same as the native Leptin (see Supplemental Information and Figure S3). The activity of purified host-guest complexes for the LepR was tested in two different assays. First, the FRET assay using the β-lactamase-based LepR SIE-Bla cell line was used. The half-maximal effective concentration (EC_{50}) of Fab-Leptin, IgG-Leptin, and native Leptin were 118.3 ± 17.2, 59.7 ± 21.2, and 313.4 ± 27.7 pM, respectively (Figure 4A). The activity of native Leptin was similar to values reported in the literature, EC_{50} of ~420–550 pM, while the activity of Fab-Leptin and IgG-Leptin were significantly higher (Ceccarini et al., 2009). We also used an orthogonal proliferation assay using a Baf3 LepR cell line, which is dependent on Leptin or interleukin-3 for survival. The activity was similar to that measured using the FRET assay: EC_{50} of 131.9 ± 6.5 pM for Fab-Leptin, 37.2 ± 3.9 pM for IgG-Leptin, and 309.6 ± 3.7 pM for native Leptin (Figure 4B).

The most active scFSH clone was converted to IgG-scFSH as for IgG-Leptin, and also cloned into the pFUSE-based vector and produced as for IgG-scFSH. Highly pure scFSH and IgG-scFSH were obtained after one-step affinity purification (Figure S2). The EC_{50} of IgG-scFSH, scFSH, and native FSH were 5.5 ± 0.5, 5.7 ± 0.5, and 4.6 ± 0.5 pM, respectively (Figure 4C) in a FRET assay using the β-lactamase-based FSHR CRE-Bla cell line.

In Vivo Activity

We selected the Leptin construct for comprehensive in vivo studies because, rather than simply confirming the in vitro studies, we could also address some critical issues in the field. Initially we showed that, similar to Leptin, IgG-Leptin is stable at core body temperature, making it possible to administer it chronically. Moreover, since antibodies generally exhibit a long half-life in circulation, we reasoned that embedding Leptin into the antibody scaffold might increase the half-life of the hormone, which is normally rapidly cleared by the kidneys and other peripheral organs (Ceccarini et al., 2009). Indeed, IgG-Leptin had a significantly increased half-life in plasma, with over 50% of the protein remaining at 4 hr after administration (Figure 5A). This decreased clearance is likely a result of the fact that the embedded Leptin is 187.2 kDa, which is greater than the exclusion limit for glomerular filtration (Ceccarini et al., 2009).

We next tested the bioactivity of IgG-Leptin in vivo whereby the studies were designed to assess the relative contributions of Leptin action on either side of the BBB. Leptin can act on neurons that project dendrites to CVOs in the median eminence in front of the BBB and can also cross the BBB at these sites after uptake by tanycytes and transport to the cerebrospinal fluid (Balland et al., 2014; Langlet et al., 2013). Because the embedded Leptin is of a larger size than the exclusion limit for uptake at CVOs, any effects of the embedded Leptin would by necessity be a result of effects in front of the BBB. Thus, we were able to assess efficiently the relative importance of Leptin action at CVOs versus brain parenchyma. We first compared neuronal activation in the hypothalamus in response to central treatment with IgG-Leptin versus native Leptin. IgG-Leptin was injected intracerebroventricularly (i.c.v.) by delivering the protein directly into the ventricles. Robust phosphorylation of STAT3 in multiple regions in the hypothalamus was observed. STAT3 activation in neurons adjacent to the ventricles was comparable with that observed with the i.c.v. injection of an equal molar amount of Leptin, consistent with the in vitro data that IgG-Leptin can activate signal transduction with similar or even greater efficacy than native Leptin (Figure 5B). Somewhat lower levels of STAT3

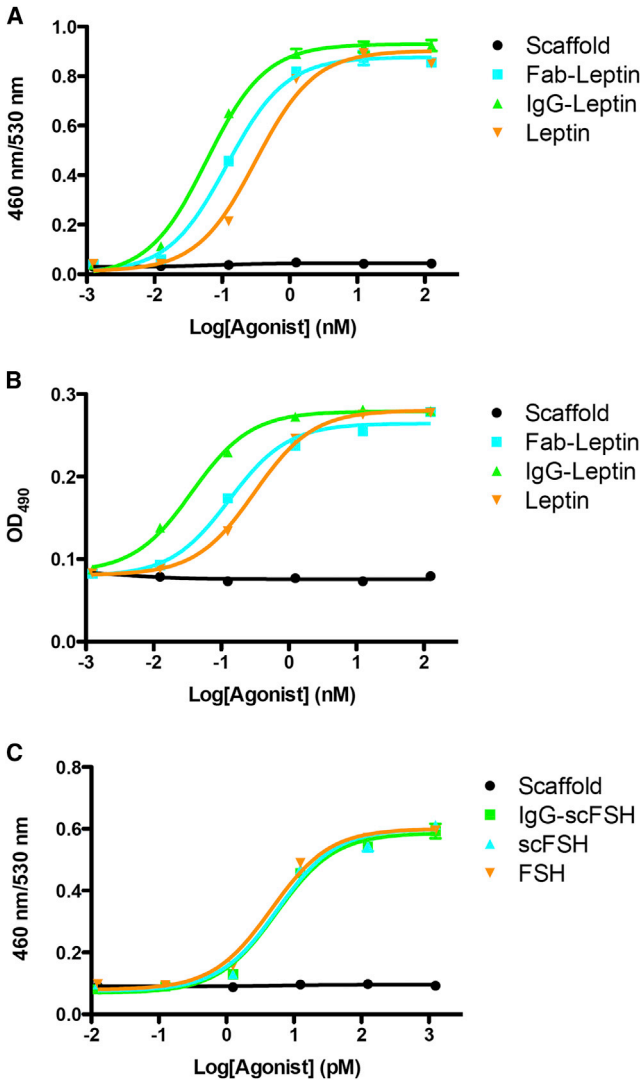

Figure 4. In Vitro Activity

(A) FRET assay using LepR SIE-Bla cells to measure the Leptin EC_{50} of the selected embedded Leptin clone. The EC_{50} of Fab, IgG, and native Leptin were 118.3 ± 17.2, 59.7 ± 21.2, and 313.4 ± 27.7 pM, respectively.

(B) Proliferation assay using Baf3 LepR cells to measure the EC_{50} of the selected embedded Leptin clone. The EC_{50} of Fab, IgG, and native Leptin were 131.9 ± 6.5, 37.2 ± 3.9, and 309.6 ± 3.7 pM, respectively.

(C) FRET assay using FSHR CRE-Bla cells to measure EC_{50} of FSH in the selected embedded FSH clone. The EC_{50} of IgG-scFSH, scFSH, and native FSH were 5.5 ± 0.5, 5.7 ± 0.5, and 4.6 ± 0.5 pM, respectively.

All data are plotted as means ± SEM (error bars).

activation were noted at some sites distant from the ventricle, suggesting that the rate of diffusion of IgG-Leptin through the tissue might be reduced. While similar levels of STAT3 activation were seen after i.c.v. administration (Kastin and Pan, 2000), peripheral administration of Leptin resulted in a different pattern of STAT3 phosphorylation versus wild-type Leptin, was confined to the neurons in the arcuate nucleus (ARC), and was otherwise absent from other neuronal populations in the hypothalamus that were activated by peripheral infusion of native Leptin and central administration of IgG-Leptin. These data suggest that peripherally administered IgG-Leptin can activate dendrites from ARC neurons that project to the median eminence but cannot cross the BBB and activate other populations. Both pro-opiomelanocortin and neuropeptide Y/agouti-related protein neurons in the ARC are known to project in close proximity to the median eminence and can directly sense circulating molecules in plasma, and are thus able to modulate neural activity without transport across the BBB (Morita and Miyata, 2013; Mullier et al., 2010).

We next examined whether activation of the ARC neurons by IgG-Leptin was sufficient to elicit a metabolic effect by infusing IgG-Leptin and native Leptin into obese mice via subcutaneous osmotic pumps. While IgG-Leptin significantly reduced food intake as well as body weight following the administration, the effect was ~50% lower than that of native Leptin (Figure 5C, left). Similarly, the ability of IgG-Leptin to decrease food intake, body weight, and fat composition in wild-type mice was reduced (Figure 5C, middle). Thus despite the fact that the IgG-Leptin is more stable and has greater efficacy in vitro, in vivo it is less potent than native Leptin, suggesting that approximately one-half of the effects of Leptin to reduce food intake and body weight are mediated by actions in front of the BBB while a complete response requires transport across the BBB. To confirm this notion, we measured the effects of native Leptin and IgG-Leptin after administering them directly into brains of *ob/ob* mice via an osmotic infusion pump. As predicted, both native Leptin and IgG-Leptin had equivalent effects in reducing food intake and body weight (Figure 5C right panel). Consistent with prior reports, neither native Leptin nor IgG-Leptin reduced food intake or body weight in diet-induced-obese (DIO) mice (Figure S4, left) or *db/db* mice (Figure S4, right), thus excluding the possibility that the anorectic and weight-reducing effect of IgG-Leptin might be due to non-specific effects.

DISCUSSION

Here we provide evidence that proteins can fold normally and retain bioactivity when embedded within an antibody host, provided that suitable N- and C-terminal junctions to the protein are found. Although the loops of antibodies are designed to allow significant sequence variation, our experiments and those of others show that not all, indeed few, sequences are transferable directly (Jones et al., 1986). While the modified immunoglobulin appears to retain its normal structure, we do note that our autocrine selections are in some ways similar to the real-time evolution of functional antibody molecules after the repertoire is exposed to antigen, in that changes in the antibody CDRs are selected for function.

The ability to embed a functional protein in an antibody could depend on at least two key features of the system. The first is that the binding energy of the four helices of Leptin or the α and β subunits of FSH for each other is high enough to overcome any distortion caused by the insertion of defined junction sequences. The other is that while permissive junctions are rare, the use of an autocrine method for selecting suitable junctions was powerful enough to yield junctions that were compatible with the proper folding and integration of the guest molecules. Further studies will reveal whether similar or different junction sequences are required for the retention of bioactivity of other agonists inserted

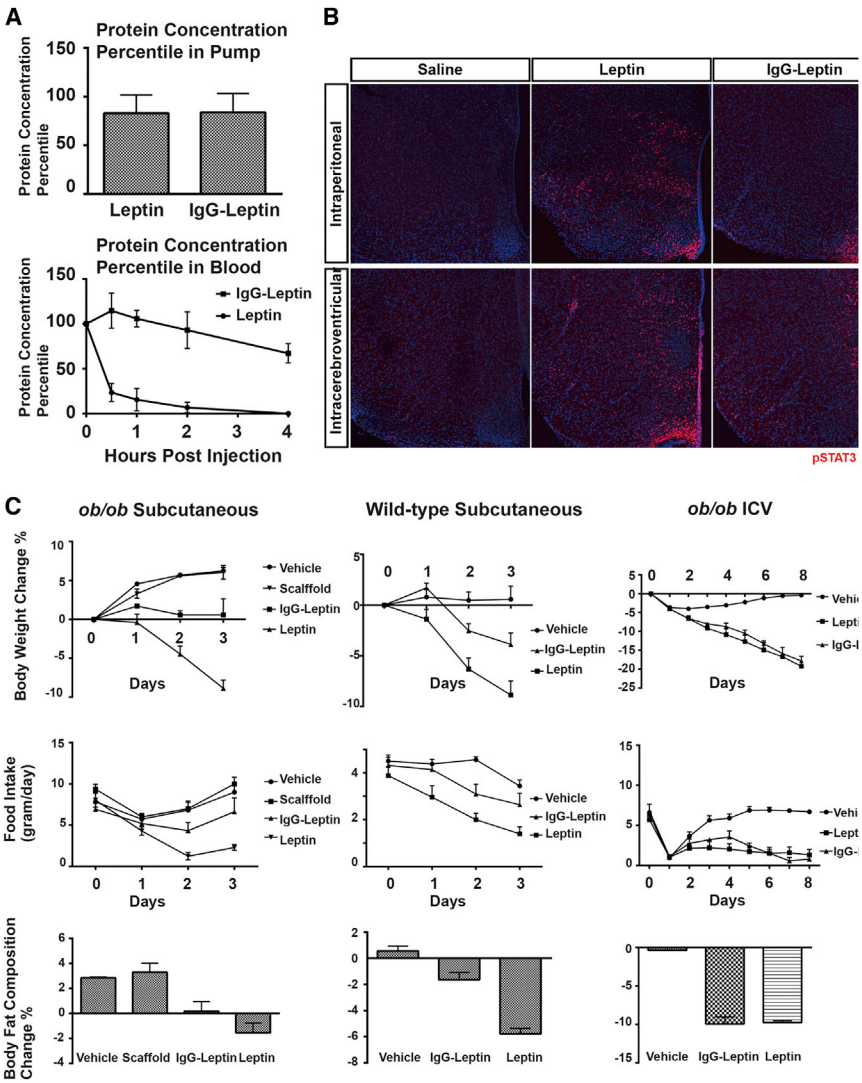

Figure 5. IgG-Leptin Regulates Metabolism In Vivo

(A) IgG-Leptin is stable at body temperature with increased half-life in circulation. (Upper) Leptin or IgG-Leptin was loaded into osmotic pumps, which were implanted subcutaneously. The concentration of Leptin and IgG-Leptin inside of the pumps was measured by ELISA at 3 days after the implantation. (Lower) A single dose of Leptin or IgG-Leptin was administered via the jugular vein, and protein concentration in plasma was determined through orbital bleeding at various time points.

(B) IgG-Leptin activates STAT3 in the hypothalamus. Leptin or IgG-Leptin was administered peripherally or centrally, and neuronal activation of STAT3 in the hypothalamus was examined by immunohistochemistry. Magnification, 10×.

(C) IgG-Leptin exerts a metabolic function in vivo. Leptin or IgG-Leptin was administered peripherally into *ob/ob* mice (left), peripherally into wild-type mice (middle), or centrally into *ob/ob* mice (right). Body weight and food intake were recorded at indicated time points before and after the treatment. Body fat composition was measured at beginning and end points of treatment.

All data are plotted as means ± SEM (error bars).

into the antibody host using the method we have developed. While one cannot easily deconvolute the contribution of each parameter needed for bioactive incorporation of an agonist, the fact that only a very small percentage of the junctional combinations were allowed strongly suggests that the binding energy of the guest secondary structures for each other was not sufficient to overcome the presence of non-permissive junctions. In a certain sense, the junctions that are non-permissive are as interesting as those that are. The main question is where does the problem with non-permissive junctions arise? We speculate that the main difficulty occurs during the folding of the host-guest complex because non-permissive junctions do not allow either the host, the inserted guests, or both to properly fold. To determine the advantage gained by using the random three-residue linker in combination with the GGGGS linker, we compared the efficacy of the simple glycine-serine linker to one where it is connected to the random three-residue linker at both the N and C termini. In the Leptin system, absence of the random three-residue linker greatly decreased the activity of the most active clone (Figure S5A). To ensure that the process of selection was important, we also randomly picked 15 clones from the original libraries and tested the activity of these clones using an in vitro FRET assay. None of these unselected clones showed activity that was comparable with the most active selected clone, again emphasizing the importance of the process of selection (Figures S5B and S5C). The sequences of these nonfunctional clones are shown in Tables S3 and S4.

Furthermore, the successful construction of host-guest complex also depends on the position in the host chosen for substitution. The key issue concerns choosing a position in the protein where substitution is allowed. Thus, we carried out a comprehensive study by substituting a randomized four-residue peptide at five different positions in the most active antibody clone that contained embedded Leptin. In this case the embedded Leptin serves as a probe to monitor protein folding. Here, no linker at the N or C terminus of the substituted segments was used. The DNA fragments coding the randomized four-residue peptides were designed using the NNK codon strategy (N = A/C/G/T, K = G/T), and overlapped with the antibody scaffold that contained embedded Leptin. Substitutions were made at segments H6–H9 (A), H23–H26 (B), H36–H39 (C), H47–H50 (D), and H64–H67 (E) (Kabat numbering [Kabat et al., 1991]) (Figure S6A). Lentiviruses of these five libraries were produced and LepR SIE-Bla cells were infected. Autocrine-based flow cytometry sorting was carried out (Figures S6B and S6C). Compared with a 0.50% positive population in negative control (no infection) and ~32% in the positive control (infected with lentivirus containing the most active previously selected clone), the positive

population was 4.22%, 18.91%, 1.43%, 1.43%, and 2.22% in libraries for segments A, B, C, D, and E, respectively. This result showed significant difference in terms of tolerance of substitution at different antibody regions. Segment B showed the highest tolerance of substitution, which is consistent with the fact that it is structurally adjacent to CDRH1. Other segments also showed different levels of tolerance of substitution, shedding light on potential positions where one can insert functional guests into the antibody scaffold.

Our study demonstrates that when properly incorporated into the antibody host, Leptin can retain its biological function. Indeed, the fact that IgG-Leptin exhibits an enhanced potency for downstream signaling in vitro confirms that the method is robust and, together with the scFSH data, suggests that our method for selecting functional junctions can be used for other agonists. Recent evidence has shown that Leptin can act directly on dendrites from neurons that project to the median eminence (a CVO) and that it can also enter the median nucleus through capillary fenestrations. After entering the median eminence, Leptin is taken up by tanycytes and transported into the ventricles, and enters the brain parenchyma from the ventricular system and in turn activates other brain regions (Balland et al., 2014). We found that while IgG-Leptin activated STAT3 normally in brain and had full bioactivity when administered i.c.v., it only activated arcuate neurons in proximity to the median eminence and had ~50% reduction of bioactivity when administered peripherally. This was despite the fact the IgG-Leptin was more stable and had a lower EC_{50} than native Leptin. Although the current version of IgG-Leptin showed a reduced ability to cross the BBB, its BBB transport could possibly be enhanced by further modifying the antibody structure. For instance, it has recently been shown that a bispecific antibody that binds with low affinity to the transferrin receptor, but with high affinity to the target, efficiently penetrates the BBB through transcytosis (Yu et al., 2011).

Overall, the generation of IgG-Leptin enabled us to establish that full activity of Leptin requires BBB transport and that activation of ARC neurons that project dendrites to CVOs accounts for half of the effect of Leptin on food intake and body weight. Leptin also regulates of thermogenesis, immunity, and reproductive function. Thus, this approach will allow us to assess the effects of peripherally versus centrally administered IgG-Leptin on these other physiologic readouts.

Since the antibody molecule has six loops, the system we have developed may also have the potential for approximating a variety of secondary structure elements in a relatively confined three-dimensional space. While their functional synergy may be rare, the power of autocrine-based phenotypic screens may be sufficient to isolate those rare molecules where the guests operate in concert. Moreover, if only one antibody loop is used for the guest, it may be possible to isolate molecules in which the binding function of the antibody is preserved so that the appended new functionality can be targeted to only certain cells.

Finally, our experimental approach should complement the ongoing theoretical studies of protein folding in a number of ways (Xie et al., 2013; Yea et al., 2013; Zhang et al., 2012; Zhang et al., 2013a). First, since current theoretical studies investigate the database of natural proteins, they uncover what has happened in evolution, whereas we can define the universe of elements that can work rather than only those that *have* worked. Second, discovery of new permissive junctions can give additional data points for refinement of theoretical and computational studies on the nature of junctions in proteins. These approaches, if successful, could be extended to the general problem of protein folding whereby one could study which new sequences inserted anywhere in a protein allow the folding pathway to proceed along the natural or even a new route. Finally, the identification of junction sequences that preserve function could have practical implications insofar as there are potential pharmacokinetic and functional benefits of generating recombinant proteins that do not have an exposed amino or C terminus.

SIGNIFICANCE

We describe here a powerful selection system for inserting functional proteins into other proteins. This system is different from previous work in that instead of being design based, it is high-throughput selection based. This method should be useful for constructing new proteins and as a tool to help solve the problem of protein folding.

EXPERIMENTAL PROCEDURES

Reagents

Rabbit anti-phospho-STAT3 was from Cell Signaling. Human Leptin ELISA kit was from R&D Systems. Mouse recombinant Leptin was from Amgen. Osmotic pumps and brain infusion kits were from Alzet.

Construction of Combinatorial Antibody Libraries

BamHI and XmaI restriction sites were introduced into the CDR H3 loop in the scaffold. Leptin was amplified with combination of three forward primers (LF1, LF2, LF3) and three reverse primers (LR1, LR2, LR3) (Table S5) and cloned into the scaffold using BamHI and XmaI. scFSH was constructed by linking the 3′ of a gene encoding the β chain and the 5′ of gene encoding the α chain with GGATCAGGATCGAACGCGACGGGGTCAGGTTCTAATGCAACTTCAGGATC GACTAGT. Then scFSH was amplified using a strategy similar to that used for the Leptin constructs (Table S6).

Construction of Stable Cell Lines

The human *LepR* gene fused with C-terminal hemagglutinin epitope (HA) tag was cloned into the pLV2-based vector in which the expression of the gene of interest was driven by the elongation factor 1α promoter. The CellSensor SIE-Bla HEK293T cells were transduced and stimulated with 1 ng/ml recombinant human Leptin protein (R&D Systems). Leptin-responsive cells were sorted using the LiveBLAzer-FRET B/G Loading Kit (Life Technology) with an excitation wavelength of 409 nm and emission wavelengths of 460 nm and 530 nm.

Construction of the human FSHR cell line followed the same strategy as for the human LepR, except that cells were co-transduced with lentiviruses expressing β-lactamase controlled by CRE.

To construct the Baf3 LepR cell line, the human *LepR* was cloned into pMSCVpuro vector, which was then transfected into Phoenix cells to generate retrovirus. Interleukin-3-dependent Baf3 cells were infected with the retrovirus and cells were selected with 10 μg/ml puromycin. The puromycin-resistant cells were subsequently cultured in media containing 10 ng/ml Leptin.

Autocrine-Based Cell Sorting

LepR SIE-Bla and FSHR CRE-Bla cells were transduced with the corresponding lentiviral combinatorial junction libraries at an MOI of approximately 2. Lentiviruses were removed 5 hr after infection, and methylcellulose media was placed on top of cells to limit diffusion of secreted antibodies to neighboring cells. Twenty hours after lentivirus infection, the methylcellulose media were washed away with media and cells were trypsinized, loaded with

LiveBLAzer-FRET B/G substrate, incubated at room temperature for 2 hr with agitation, washed with FACS buffer, and sorted with a MoFlo Astrios sorter. Positive cells were gated based on the highest ratio of fluorescence in the Pacific Blue channel to that in the AmCyan channel.

Animal Procedures

All surgical procedures in mice were performed in compliance with the protocols approved by the Institutional Animal Care and Use Committee of Rockefeller University. Wild-type C57Bl6/J, *ob/ob*, *db/db*, and DIO mice were from the Jackson Laboratory.

To characterize their in vivo function by peripheral administration, proteins were delivered via osmotic pumps implanted subcutaneously, at 600 ng/hr for mouse Leptin, 3,600 ng/hr for IgG-Leptin, or 2,920 ng/hr for scaffold control. For central administration, proteins were delivered to lateral ventricle via osmotic brain infusion pumps, at 50 ng/hr for Leptin and 300 ng/hr for IgG-Leptin. Food intake and body weight for each mouse were measured daily during indicated experimental periods. Fat composition was measured using an EchoMRI Body Composition Analyzer.

To determine the rate of protein clearance, a single dose of Leptin (4.5 μg) or IgG-Leptin (27 μg) was delivered into mice via the jugular vein, and blood was then collected at indicated time points. The plasma concentration of mouse and human Leptin was measured using Mouse/Rat Leptin Quantikine ELISA Kit (R&D Systems) and Human Leptin Quantikine ELISA Kit (R&D Systems), respectively.

To examine neuronal activation in vivo, *ob/ob* mice were treated with Leptin (2.5 μg/g body weight) or IgG-Leptin (15 μg/g body weight) via intraperitoneal injection, or with Leptin (100 ng) or IgG-Leptin (600 ng) via i.c.v. injection. One hour after the treatment, mice were transcardially perfused with PBS followed by 4% paraformaldehyde. Brain samples were then dissected, post-fixed with 4% in the hypothalamus and determined by free-floating immunostaining as previously described (Knight et al., 2010).

The methods of cell culture, production of lentiviruses, proliferation assay, CD spectroscopy measurement, differential HDX-MS, and peptide identification and HDX data processing are detailed in Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes the structure and dynamics of the Leptin host-guest construct, Supplemental Experimental Procedures, six figures, and six tables and can be found with this article online at http://dx.doi.org/10.1016/j.chembiol.2015.07.011.

AUTHOR CONTRIBUTIONS

R.A.L. conceived the project. Y.P., W.Z., H.Y., K.H.H., V.D., and S.N. performed the experiments. Y.P., W.Z., V.D., S.N., I.A.W., P.R.G., J.M.F., and R.A.L. analyzed and interpreted the data. R.A.L. wrote the manuscript. All authors revised the manuscript.

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