Yeast surface display platform for rapid discovery of conformationally selective nanobodies

Conor McMahon¹, Alexander S. Baier*,¹, Roberta Pascolutti¹, Marcin Wegrecki*,², Sanduo Zheng¹, Janice X. Ong¹, Sarah C. Erlandson¹, Daniel Hilger³, Søren G. F. Rasmussen², Aaron M. Ring⁴, Aashish Manglik*⁵,⁶ and Andrew C. Kruse*¹

Camelid single-domain antibody fragments (‘nanobodies’) provide the remarkable specificity of antibodies within a single 15-kDa immunoglobulin VHH domain. This unique feature has enabled applications ranging from use as biochemical tools to therapeutic agents. Nanobodies have emerged as especially useful tools in protein structural biology, facilitating studies of conformationally dynamic proteins such as G-protein-coupled receptors (GPCRs). Nearly all nanobodies available to date have been obtained by animal immunization, a bottleneck restricting many applications of this technology. To solve this problem, we report a fully in vitro platform for nanobody discovery based on yeast surface display. We provide a blueprint for identifying nanobodies, demonstrate the utility of the library by crystallizing a nanobody with its antigen, and most importantly, we utilize the platform to discover conformationally selective nanobodies to two distinct human GPCRs. To facilitate broad deployment of this platform, the library and associated protocols are freely available for nonprofit research.

Antibodies have had a transformative impact on science and medicine because of their exceptional specificity and biochemical versatility, enabling applications in almost every aspect of biomedical inquiry. Conventional antibodies are composed of two heavy chains and two light chains. Each of these components contributes to antigen-binding specificity through a variable domain, termed V-heavy and V-light for the variable domains of the heavy chain and light chain, respectively. A key exception to this general architecture is found in camelids (llamas, camels, alpacas, and their relatives), which possess a parallel antibody repertoire composed solely of heavy chains. Such antibodies bind to their target antigens through a single variable domain, termed VHH, which contains the entire antigen-binding surface. Unlike the antigen-binding fragments (Fabs) of conventional antibodies, isolated VHH domains (also called nanobodies) can be readily expressed in bacteria as the product of a single gene, and in many cases, these fragments can even fold and retain antigen specificity in the reducing environment of the eukaryotic cytosol. Owing to their versatility, nanobodies have found applications in protein structural biology and cell biology and as potential diagnostic and therapeutic agents. Nanobodies have been particularly critical tools in studies of GPCRs, in which conformationally selective nanobodies have been used extensively to stabilize these receptors in defined states for crystallographic studies that would otherwise be impossible.

Despite the growing importance of nanobodies throughout biomedical research, the current methods for creating these powerful tools remain slow, costly, and often unreliable. The majority of nanobodies described to date from animal-derived antibodies are frequently restricted from binding conserved epitopes because of the immunological tolerance of self-antigens. As conserved epitopes often drive key protein functions like protein–protein recognition and allosteric communication, a rapid method to identify nanobodies that target such sites would provide a robust means to interrogate protein function.

Previous efforts have sought to address challenges in nanobody identification by combining phage display with a synthetic library. However, these libraries remain available only through contract work with an expensive commercial provider, thereby limiting their broad utilization. Moreover, with synthetic libraries, it remains particularly challenging to identify nanobodies that not only bind to their target, but also specifically recognize a defined conformation, which represents one of the most important applications of animal-derived nanobodies. Phage-display techniques enable isolation of high-affinity binders, but identification of functional clones (for example, conformationally selective nanobodies) remains challenging, despite select successes. Here, we address these challenges through the development and application of a fully synthetic yeast-display nanobody library, devised using an alignment of structurally characterized nanobodies from the Protein Data Bank (PDB). The use of a cell-based selection scheme enabled by fluorescence-activated cell sorting (FACS) allows straightforward and rapid identification of conformationally selective nanobodies for two human GPCRs. To enable broad utility of this new resource, we have made the library and all associated protocols available to the scientific community.

Results

Library construction and validation. To develop a platform for rapid in vitro nanobody discovery, we first designed a synthetic nanobody library, starting from a consensus framework derived exclusively from conserved epitopes from the Protein Data Bank (PDB). The use of a cell-based selection scheme enabled by fluorescence-activated cell sorting (FACS) allows straightforward and rapid identification of conformationally selective nanobodies from the Protein Data Bank (PDB). The use of a cell-based selection scheme enabled by fluorescence-activated cell sorting (FACS) allows straightforward and rapid identification of conformationally selective nanobodies for two human GPCRs. To enable broad utility of this new resource, we have made the library and all associated protocols available to the scientific community.

1Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA, USA. 2Department of Neuroscience, University of Copenhagen, Copenhagen, Denmark. 3Department of Molecular and Cellular Physiology, Stanford University, Stanford, CA, USA. 4Department of Immunobiology, Yale School of Medicine, New Haven, CT, USA. 5Department of Pharmaceutical Chemistry, University of California San Francisco, San Francisco, CA, USA. 6Department of Anesthesia and Perioperative Care, University of California San Francisco, San Francisco, CA, USA.

*e-mail: Aashish.Manglik@ucsf.edu; Andrew_Kruse@hms.harvard.edu
from llama genes IGHVIS1–IGHVIS1S. This constant framework was combined with designed variation of the complementarity-determining loops (CDRs) that comprise the highly variable antigen-binding interface of the VHH. Although there are many previously described strategies for introducing variation in antibody CDR loops, we postulated that nanobodies of known structure in PDB represent an especially curated set of highly stable, biochemically well-behaved variants, as evidenced by their tractability in crystallographic studies. The entire set of unique nanobodies in PDB (93 sequences at the time of analysis) was analyzed for position-specific variation in the CDRs, and our design aimed to recapitulate this diversity. For the residues immediately adjacent to the CDRs, we introduced partial randomization, allowing only a few possible amino acids guided by the observed frequencies of nanobodies in the PDB. For the highly variable positions in each CDR, we introduced much more thorough randomization reflective of the high diversity at these positions. First, the frequency of each amino acid within CDR3 was determined for all nanobodies in the PDB.
The resulting mixture of amino acids was modified to eliminate cysteine and methionine to avoid chemical reactivity and was introduced into various positions in each CDR in the synthetic library. On the basis of nanobody sequence analysis, we elected to introduce four such highly variable positions in CDR1 and one in CDR2. The longer CDR3 represents a critically important loop for antigen recognition, in which we introduced 7, 11, or 15 consecutive positions of high-diversity mixture to emulate CDR3 length variation seen in the natural repertoire (Fig. 1a–c).

Primers encompassing the desired sequence diversity were synthesized, with high-diversity regions constructed using a mixed pool of trimer phosphoramidites to match target codon frequency. We then built a pooled DNA library encoding the full nanobody sequence by assembly PCR of these primers (details in

---

**Fig. 2 | Validation of nanobody platform using HSA as the target antigen.**  
- **a**, Histogram of yeast library HSA binding, showing pre-selection (black) and after four rounds of selection (blue). The percentage of HSA-binding cells is indicated. The fraction of the total library composed by Nb.b201 was assessed by deep sequencing, showing progressive enrichment of this clone. **b**, Size-exclusion chromatography analysis confirmed the binding of purified recombinant Nb.b201 to HSA. **c**, Crystal structure of nanobody–HSA complex (PDB 5VNW), including close-up view of the CDR loops interacting with antigen. Hydrogen bonds are shown as dotted lines. **d**, Rotated view of the complex shown in **c**. **e**, Comparison of nanobody structures in the antigen-bound (yellow) and antigen-free state (gray, PDB 5VNV), showing conformational change upon antigen binding. Changes are highlighted with arrows, and CDR loops are colored blue, green, and orange for CDR1, CDR2, and CDR3, respectively (more details in Table 1 and Supplementary Fig. 3).
Methods). A pervasive but often unappreciated drawback of synthetic antibody fragments is poor biochemical behavior. Prior to generating a large yeast display library, we sought to assess whether our library design would reliably produce biochemically tractable clones suitable for structural and cell biological studies. A total of 11 nanobody sequences were chosen at random from the library DNA and tested for biochemical behavior by expression and purification from Escherichia coli. Of these clones, nine were well expressed (> 20 mg/L), could be purified by routine procedures, and showed reasonably monodisperse size-exclusion profiles (Supplementary Fig. 2). For library creation, we chose to use a 649–amino acid tether. In addition, we included an N-terminal engineered mating factor α preprotein that has been reported to enhance antibody expression in yeast\(^1\). On the C terminus, we included a glycosylphosphatidylinositol anchor sequence, which resulted in covalent tethering of the protein to the yeast cell wall\(^1\) (Fig. 1d). On the basis of these results, we linearized the engineered surface display plasmid (pYDS649), which was then transformed into the Saccharomyces cerevisiae protease-deficient strain BJ5465 together with the DNA library, which had been amplified to include flanking sequences homologous to pYD649 for recombination. This resulted in a yield of \(5 \times 10^6\) transformants. While this yield is modest in comparison to some libraries, we show that our approach yields comparable clones to those obtained by animal immunization, attesting to the value of our structure-based library design.

Identification of nanobodies using magnetic cell sorting. We next sought to determine whether our approach could yield nanobody clones targeting diverse antigen classes, including both soluble and membrane proteins. As an initial test, we aimed to identify nanobodies targeting human serum albumin (HSA), the most abundant protein in human plasma. Binding of therapeutic proteins and small molecules to HSA is a key determinant of drug pharmacokinetics; nanobodies targeting HSA may therefore provide a modular tool for enhancing the half-life of biologics\(^1\). In this first test, we exclusively employed magnetic cell sorting (MACS), a low-cost cell separation method that can be performed in even the most basic laboratory environments without specialized equipment\(^2\).

To identify novel HSA-binding nanobodies, we first prepared fluorescently labeled HSA protein and then used this reagent to perform iterative staining and magnetic-bead-based enrichments. In brief, this approach entails incubation of the yeast library with fluorescent HSA antigen, washing off excess antigen, and further staining desired clones with anti-fluorophore magnetic microbeads. Labeled cells are then isolated by magnet-based separation and amplified in standard yeast culture medium (Fig. 1c). As with any library-based selection, it is of paramount importance to ensure that binders recognize the antigen itself, rather than the reagents or fluorescent tags used for separation. To achieve such specificity, yeast with reactivity to beads alone were depleted from the library before each selection step. To decrease the probability of enriching fluorophore binders, the fluorophore tag used was alternated between Alexa Fluor 647 and fluorescein isothiocyanate (FITC) for each consecutive round of magnetic selection. After four rounds of selection (Fig. 2a), single yeast colonies were isolated and stained with fluorescently labeled HSA for validation by analytical flow cytometry. For further study, we selected one clone with strong antigen binding (named Nb.b201) for in-depth characterization.

### Table 1 | Data collection and refinement statistics

|                          | Nb.b201 (PDB 5VNV) | Nb.b201 complex with HSA (PDB 5VNW) |
|--------------------------|---------------------|-------------------------------------|
| **Data collection**      |                     |                                     |
| Space group              | 14 22               | P1                                  |
| Cell dimensions          |                     |                                     |
| \(a, b, c\) (Å)          | 106.8, 106.8, 52.2  | 66.2, 721, 108.5                    |
| \([\alpha, \beta, \gamma]\) (^°) | 90, 90, 90         | 96.2, 104.5, 104.1                  |
| Resolution (Å)          | 46.92-1.40 (1.48-1.40) | 7.70-2.60 (2.76-2.60)               |
| \(R_{\text{free}}\) (%) | 8.4 (98.4)          | 8.7 (140.0)                         |
| \(\bar{i}/\sigma(i)\)   | 11.85 (1.20)        | 8.99 (0.75)                         |
| \(CC_{1/2}\)            | 99.8 (56.0)         | 99.8 (48.7)                         |
| Completeness (%)         | 97.3 (87.0)         | 97.7 (94.4)                         |
| Multiplicity             | 6.4 (5.2)           | 3.5 (3.3)                           |
| **Refinement**           |                     |                                     |
| No. reflections          | 29,221              | 55,812                              |
| \(R_{\text{free}} / R_{\text{free}}\) (%) | 18.3 / 21.4        | 22.1 / 25.6                         |
| No. atoms                | 946                 | 10,994                              |
| Protein                  | 946                 | 10,994                              |
| Ion/other\(^a\)          | 39                  | 77                                  |
| Water                    | 134                 | 69                                  |
| \(B\) factors           |                      |                                     |
| HSA                     | 107.7 (chain A), 125.3 (chain B) |                                     |
| Nb.b201                 | 22.6                | 90.4 (chain C), 125.3 (chain D)     |
| Solvent                 | 39.3                | 91.2                                |
| R.m.s. deviations        | 0.008               | 0.003                               |
| Bond lengths (Å)         | 0.990               | 0.500                               |

\(^a\)Values in parentheses are for highest-resolution shell. PDB 5VNW contains polyethylene glycol and formate ions; PDB 5VNW contains glycerol and bound fatty acids.
Fig. 3 | Structural and functional modulator nanobodies targeting a GPCR. a, β2AR interconverts between ensembles of inactive conformations (red) and active conformations (green). The active state of the receptor can be stabilized by nanobodies (yellow) that bind to the intracellular face of the receptor. b, Results of selection summarized in flow cytometry plots. After FACS selection, a substantial fraction (23.6%) of clones show agonist-specific binding to the β2AR. c, Selection schematic for isolation of active-state stabilizing nanobodies. In MACS rounds (1, 2, and 5), depletion and enrichment steps were performed sequentially. In FACS rounds, two-color sorting was used to enrich agonist-specific clones and deplete antagonist-specific and nonselective clones simultaneously. Cz, carazolol (antagonist); BI, BI167107 (agonist). d, Sequence analysis of β2AR conformationally selective clones, showing highly divergent CDR3 sequences. e, Radioligand competition binding in nanodiscs confirmed that synthetic nanobodies stabilize the active-state conformation β2AR. Data are shown as mean ± s.e.m. for measurements performed in triplicate. f, Single-cycle surface plasmon resonance (SPR) experiment showing measurement of affinity and kinetics for Nb.c200 binding to β2AR-BI167107. Experimental data are in red, and the curve fit is in black. g, Summary of nanobody affinities measured by SPR (Supplementary Fig. 5). h, Nb.c200 immobilized by metal ion affinity chromatography pulled down purified β2AR before crystallographic trials. i, Nb.c200 enabled crystallization of the β2AR using the lipidic cubic phase method. j, cAMP signaling assay to measure β2AR signaling in the presence or absence of synthetic nanobodies. Data are shown as mean ± s.e.m. for transfections performed in triplicate (Supplementary Fig. 4).

As expected, Nb.b201 was expressed and purified from E. coli using standard methods, with a yield of 26 mg of purified protein per liter of culture. We assessed HSA binding in vitro by analytical size-exclusion chromatography (Fig. 2b) and more quantitatively by surface plasmon resonance (SPR, Supplementary Fig. 3). Both techniques confirmed specific binding of Nb.b201 to HSA. Although the binding affinity was modest at 430 nM, Nb.b201 was highly specific, demonstrating no binding to the closely related mouse serum albumin (MSA, Supplementary Fig. 3), and formed a stable complex with HSA, as assessed by size-exclusion chromatography (Fig. 2b).

A major goal of our approach presented here was to facilitate structural biology efforts; thus, we assessed the structural basis...
for Nb.b201 binding to HSA. Nb.b201 crystallized readily, both in isolation and in complex with HSA. X-ray data collection yielded datasets to a resolution of 1.4 Å and 2.6 Å for the free nanobody and HSA complex, respectively (Table 1). These crystal structures revealed that Nb.b201 adopts the typical V-set immunoglobulin fold previously observed for animal-derived nanobodies (Fig. 2c–e). Comparison of the two structures provides an opportunity to compare bound and free states of the same antibody fragment. Remarkably, Nb.b201 undergoes large-scale conformational changes upon antigen binding, resulting in the reorientation of the CDR3 backbone and amino acid side chains (Fig. 2e). The mode of antigen recognition involves Nb.b201 binding its target largely through its CDR3 loop, with relatively few direct contacts between HSA and CDR1 or CDR2. Interestingly, the epitope recognized by Nb.b201 is a convex protrusion on the surface of HSA, in contrast to most nanobody epitopes, which are more typically concave. The Nb.b201 is a convex protrusion on the surface of HSA, in contrast to most nanobody epitopes, which are more typically concave. The Nb.b201 is a convex protrusion on the surface of HSA, in contrast to most nanobody epitopes, which are more typically concave.

To assess the versatility of our yeast-based nanobody-discovery platform, we sought to develop methods enabling the use of unpurified antigens as selection reagents.

We focused our efforts on the metabolic hormone adiponectin, a complex protein with many post-translational modifications, which can only be expressed in eukaryotic cells, typically at low yields. Human adiponectin tagged with an amino-terminal FLAG epitope was expressed as a secreted protein in HEK293 cells, and the resulting medium containing a complex mixture of proteins was used as the selection antigen (Supplementary Fig. 4a,b). Nanobody-expressing yeast were incubated with adiponectin-containing conditioned medium, washed, and then stained with fluorescently labeled anti-FLAG M1 antibody. Adiponectin binding was confirmed for three unique clones by SPR and isolated after three rounds of MACS, followed by FACS (Supplementary Fig. 4c). Approaches like these may enable rapid reagent development for the large fraction of the human proteome that remains poorly characterized by biochemical methods.

**Conformationally selective GPCR-binding nanobodies.** While nanobodies have contributed to a broad range of biological fields, they have had a uniquely transformative impact on our understanding of GPCR biology. GPCRs are the largest family of transmembrane receptors in humans, playing essential roles in every aspect of physiology ranging from function of the central nervous system to regulation of metabolism and cardiovascular biology. Nanobodies have proven to be invaluable tools for detailed structural and biochemical characterization of GPCRs, enabling profound insights into receptor structure and function. As an example, the llama-derived nanobody Nb80, which stabilizes the active conformation of the β2 adrenergic receptor (β2AR), enabled determination of the first

**Nanobodies targeting unpurified antigen.** Selection of yeast or phage surface displayed libraries is usually performed with purified and labeled protein samples. Owing to its idiosyncratic nature, protein purification remains a critical bottleneck for protein engineering by surface-display methods. This barrier proves especially steep for poorly expressed proteins, such as membrane proteins and extensively post-translationally modified protein hormones.
X-ray crystal structure of the active conformation of a hormone-activated GPCR. The conformational selectivity of Nb80 was also subsequently used to probe the localization of active β2AR in live cells, thereby revealing a new paradigm in intracellular GPCR signaling. Another nanobody, Nb35, was instrumental in examining the structure and signaling of the heterotrimeric G protein Gs in complex with β2AR, enabling determination of the first structure of a GPCR–G protein heterotrimer complex. In recent years, the GPCR-binding nanobody repertoire has broadened to include a range of GPCRs. However, all GPCR-targeting nanobodies reported to date trace their origins to animal immunization, a technique that is slow, expensive, and often unsuccessful.

Given the broad utility of conformationally selective antibodies in GPCR research, any in vitro platform aiming to supplant immunization-based methods must be able to generate conformationally selective GPCR binders. Hence, we sought to identify nanobodies that selectively bind the active conformation of β2AR using our synthetic nanobody library platform (Fig. 3). Nanobody-displaying yeast were first selected for binding to purified, FLAG-tagged β2AR bound to the agonist BI167107 by MACS. We subsequently introduced a counterselection strategy to deplete undesired clones, which included nanobodies that bind the M1 antibody itself, the Alexa Fluor 647 fluorophore, conformationally invariant epitopes, or the inactive β2AR conformation. For example, in the third MACS round, we first depleted clones that bound to β2AR occupied by the high-affinity antagonist carazolol before enriching clones that bound agonist-occupied receptor. To enrich for the desired agonist-bound β2AR-specific nanobodies, we performed two rounds of FACS. Here, the yeast library was incubated simultaneously with both BI167107- and carazolol-occupied β2AR, with each receptor–ligand complex labeled with a specific Alexa fluorophore (Fig. 3a–c). This specialized selection using FACS requires a cell-based display system. As an indication that the selected clones bind the desired epitope, we observed that a large fraction were competitive with Nb6B9, a previously affinity-matured variant of Nb80 that binds with high affinity to the intracellular side of β2AR. Finally, we performed a round of MACS with a decreased concentration of agonist-bound β2AR to enrich the highest-affinity clones (Fig. 3c).

Sequencing individual clones revealed that we isolated 13 unique nanobodies that bound agonist-occupied β2AR (Fig. 3d and Supplementary Fig. 5). We further prioritized clones with on-yeast titrations, which demonstrated that the isolated nanobodies have a range of affinities for the β2AR–BI167107 complex in the low-to-midnanomolar range. We anticipate that conformationally selective nanobodies identified from this library will find utility in biochemical, structural, and cell biological applications. Therefore, we next validated the active-state-specific β2AR nanobodies in pharmacological, crystallographic, and cellular signaling experiments. Four clones were selected for further characterization and were expressed and purified from E. coli. Each of the four nanobodies increased agonist affinity of β2AR, as assessed by a competition radioligand binding assay (Fig. 3e). This gold-standard pharmacological assay provides definitive evidence that each nanobody stabilizes the active conformation of β2AR. Next, we determined binding affinities of each nanobody for BI167107-occupied β2AR by SPR using single-cycle kinetics (Fig. 3f,g). Measured affinities ranged from 44 nM to 151 nM, comparing favorably to the most extensively studied llama-derived GPCR-targeting nanobody, Nb80, which binds with roughly 140 nM affinity.

We next tested whether this conformational stabilization is sufficient to enable crystallization of an agonist-bound β2AR. Likely owing to conformational dynamics in the agonist-bound state, all previous attempts to crystallize the β2AR–BI167107 complex have failed in the absence of an intracellular binding nanobody or the heterotrimeric G protein. Because of the low throughput of crystallography experiments, we selected one nanobody, Nb.c200, for crystallization trials. The nanobody readily copurified with β2AR (Fig. 3h) and crystallized easily by the lipidic cubic-phase method (Fig. 3i). Of note, Nb.c200 enabled crystallization in a range of crystallization conditions, rather than just a small subset.

Additionally, we assessed the potential of our synthetic nanobodies to modulate GPCR signaling in live cells, a key application of llama-derived nanobodies. As with llama-derived nanobodies, we found that our synthetic nanobodies could substantially impair β2AR signaling in response to adrenaline. Among the nanobodies tested, we found that Nb.c203 was the most effective, reducing adrenaline Emax by 45% (Fig. 3j). The observed variability in maximal signaling inhibition is probably reflective of both nanobody affinity and stability in the reducing environment of the cytosol. The effectiveness of Nb.c203, despite its lower-affinity binding, highlights the importance of testing multiple unique clones for intrabody applications. Notably, the insurmountable inhibition observed in the presence of these nanobodies is consistent with a G-protein-competitive binding mode for the nanobodies. Unlike an orthosteric inhibitor, adding excess agonist cannot overcome nanobody inhibition, because the site of binding is distinct.

In order to further examine the versatility of our nanobody library, we sought to identify conformationally selective nanobodies recognizing another GPCR. As a test case, we chose the human A3A adenosine receptor (A3AR). This receptor has long been known to play important roles in the central nervous system and has recently emerged as a target for cancer immunotherapy drug development. Importantly, there are no active-state-selective nanobodies targeting A3AR, although an inactive-state-specific Fab has been reported. To generate active-state-specific nanobody clones, we performed two rounds of MACS to generate a library of A3AR-binding nanobodies followed by one round of FACS to enrich conformationally selective binders (Fig. 4a). From this pool, two clones called Nb.AD101 and Nb.AD102 were chosen for further characterization. In a flow cytometry–based binding assay, each of these clones showed a strong preference for agonist-bound A3AR (Fig. 4b). To further validate these clones, we expressed and purified them and measured binding to receptor in an in vitro pulldown assay. Consistent with cell-based staining experiments, these nanobodies showed binding exclusively to agonist-occupied receptor (Fig. 4c), confirming the generalizability of the approach.

Taken together, these data show that conformationally selective nanobodies identified by our in vitro platform recapitulate all key features of llama-derived clones. In addition, the large diversity of conformationally selective clones identified from the synthetic library may enable a larger repertoire of applications; some may perform better as crystallographic chaperones, others may be more stable in the reducing intracellular environment or have increased specificity required for identifying a specific protein in a complex milieu.

Discussion

Antibodies and their minimal binding fragments have transformed biomedical research. Nanobodies, derived from camelid heavy chain antibodies, have emerged as particularly powerful research tools in large part because of their ability to detect and stabilize specific conformations for GPCRs and other biochemically challenging proteins. However, despite their broad utility, nanobodies have only been exploited for a subset of potential applications, owing to challenges associated with nanobody discovery. Foremost among these challenges is the requirement for camel, llama, or alpaca immunization, a slow and expensive process requiring large quantities of purified protein and access to large-animal husbandry and veterinary facilities. Moreover, this process frequently yields only small numbers of nanobody clones, many or all of which may not possess the desired activity. Our approach reported here addresses these challenges by providing a fully in vitro platform for nanobody discovery based on an engineered library displayed on S. cerevisiae.
To assess the utility of our library, we selected nanobodies targeting a diverse array of antigens: soluble proteins, nonpurified proteins, and two human GPCRs. In each case, our platform enabled identification of nanobody clones capable of binding to the target antigen with the desired activity. To date, all nanobody-selection efforts conducted with this library have led to the successful isolation of clones with submicromolar affinity, with the exception of an effort targeting FLAG peptide, a short unstructured sequence. Strikingly, with the addition of FACs enrichment to our selection methodology, the affinities of our synthetic β2AR-binding clones were comparable to or better than that of the most extensively studied GPCR-targeting nanobody, the llama-derived Nb80. Where there are many potential selection strategies that enable nanobody identification, we demonstrate that a combination of MACS and FACs enrichments provides a rapid approach to the identification of nanobodies selective for a particular target or to a specific conformation. Early MACS rounds enable depletion of large pools of non-binding clones, rapidly reducing library diversity by two to three orders of magnitude. However, MACS-based counter-selection strategies suffer from poor precision, and identification of clones specific for a target or conformational states often requires many iterations for sufficient enrichments. Given the throughput of modern FACS instruments, we opt to leverage the precision afforded by fluorescence-based selections as early in the selection scheme as possible, usually after two rounds of MACS to reduce library diversity to a manageable level. Though it is possible that this approach may miss the single highest-affinity clone in the library, nanobodies are readily amenable to affinity maturation to improve affinity, even for difficult targets such as GPCRs5. Importantly, our library generates nanobody binders that are amenable to crystallization and subsequent structural determination, and thus, it demonstrates considerable promise as a tool to aid structural studies.

From soluble protein binders to conformationally selective GPCR stabilizers, our platform allows straightforward, rapid, and low-cost isolation of nanobodies, even with highly specialized functionalities. To facilitate broad deployment of this resource, we have made the library publicly available and free of charge for nonprofit use. Detailed protocols for nanobody-binder selection are included in the Supplementary Note and can be adapted or modified to enable more diverse applications. We envision that rapid and simple nanobody discovery will be an enabling resource for applications throughout biomedical research.

Methods
Methods, including statements of data availability and any associated accession codes and references, are available at https://doi.org/10.1038/s41594-018-0028-6.

Received: 1 November 2017; Accepted: 5 January 2018;
Published online: 12 February 2018

References
1. Hamers-Casterman, C. et al. Naturally occurring antibodies devoid of light chains. Nature 363, 446–448 (1993).
2. Muyldeurnans, S. Nanobodies: natural single-domain antibodies. Annu. Rev. Biophys. 42, 775–797 (2013).
3. Iroannejad, R. et al. Conformational biosensors reveal GPCR signalling from endosomes. Nature 495, 534–538 (2013).
4. Rasmussen, S. G. et al. Structure of a nanobody-stabilized active state of the β(2) adrenergic receptor. Nature 469, 173–180 (2011).
5. Stauss, D. P. et al. Allosteric nanobodies reveal the dynamic range and diverse mechanisms of G-protein-coupled receptor activation. Nature 535, 448–452 (2016).
6. Manglik, A., Kobikla, B. K. & Steyaert, J. Nanobodies to study G protein-coupled receptor structure and function. Annu. Rev. Pharmacol. Toxicol. 57, 19–37 (2017).
7. Moutel, S. et al. NaLH1: A universal synthetic library of humanized nanobodies providing highly functional antibodies and intrabodies. elife 5, e16228 (2016).
8. Gao, J., Sidhu, S. S. & Wells, J. A. Two-state selection of conformation-specific antibodies. Proc. Natl. Acad. Sci. USA 106, 3071–3076 (2009).
9. Rizk, S. S. et al. Allosteric control of ligand-binding affinity using engineered conformation-specific effector proteins. Nat. Struct. Mol. Biol. 18, 437–442 (2011).
10. Adams, J. J. & Sidhu, S. S. Synthetic antibody technologies. Curr. Opin. Struct. Biol. 24, 1–9 (2014).
11. Kayushin, A., Korostelova, M. & Miroshnikov, A. Large-scale solid-phase preparation of 3′-unprotected trinucleotide phosphothioesters–precursors for synthesis of trinucleotide phosphoraminides. Nucleosides Nucleotides Nucleic Acids 19, 1967–1976 (2000).
12. Kayushin, A. L. et al. A convenient approach to the synthesis of trinucleotide phosphoraminides–synthons for the generation of oligonucleotide/peptide libraries. Nucleic Acids Res 24, 3748–3755 (1996).
13. Boden, T., E. & Wittrup, K. D. Yeast surface display for screening combinatorial polypeptide libraries. Nat. Biotechnol. 15, 553–557 (1997).
14. Kruse, A. C. et al. Activation and allosteric modulation of a muscarinic acetylcholine receptor. Nature 504, 101–106 (2013).
15. Raketaew, A. A., Sazinsky, S. L., Patesi, A., Antipov, E. & Wittrup, K. D. Directed evolution of a secretory leader for the improved expression of heterologous proteins and full-length antibodies in Saccharomyces cerevisiae. Biotechnol. Bioeng. 103, 1192–1201 (2009).
16. Oreile, P. Architecture and biosynthesis of the Saccharomyces cerevisiae cell wall. Genetics 192, 775–818 (2012).
17. Maleide, S. C. et al. Extended in vivo half-life of human soluble complement receptor type 1 fused to a serum albumin-binding receptor. J. Pharmacol. Exp. Ther. 277, 534–542 (1996).
18. Van Roy, M. et al. The preclinical pharmacology of the high affinity anti-IL-6R Nanobody ALX-0061 supports its clinical development in rheumatoid arthritis. Arthritis Res. Ther. 17, 135 (2015).
19. Tijink, B. M. et al. Improved tumor targeting of anti-epidermal growth factor receptor Nanobodies through albumin binding: taking advantage of modular Nanobody technology. Mol. Cancer Ther. 7, 2288–2297 (2008).
20. Kim, C. C., Wilson, E. B. & DeRisi, J. L. Improved methods for magnetic purification of malaria parasites and haemotoin. Malar. J. 9, 17 (2010).
21. Rasmussen, S. G. et al. Crystal structure of the β(1) adrenergic receptor-Gs protein complex. Nature 477, 549–555 (2011).
22. Ring, A. M. et al. Adrenaline-activated structure of β(2)-adrenergoc receptor stabilized by an engineered nanobody. Nature 502, 575–579 (2013).
23. Manglik, A. & Kobikla, B. The role of protein dynamics in GPCR function: insights from the β(2)AR and rhodopsin. Curr. Opin. Cell Biol. 27, 136–143 (2014).
24. Rosenbaum, D. M. et al. Structure and function of an irreversible agonist-β(2) adrenergoc receptor complex. Nature 469, 236–240 (2011).
25. Stauss, D. P. et al. Regulation of β2-adrenergoc receptor function by conformationally selective single-domain intrabodies. Mol. Pharmacol. 85, 472–481 (2014).
26. Vijayan, D., Young, A., Teng, M. W. L. & Smyth, M. J. Targeting immunosupressive adenosine in cancer. Nat. Rev. Cancer 17, 709–724 (2017).
27. Hino, T. et al. G-protein-coupled receptor inactivation by an allosteric inverse-agonist antibody. Nature 482, 237–240 (2012).

Acknowledgements
Financial support for this work was provided by the Valle Foundation (A.C.K.), the Smith Family Foundation (A.C.K.), National Institutes of Health grants 5DP5OD013545 (A.C.K.), 1DP5OD023048 (A.M.), and 1DP5OD023888 (A.M.R.), the Lundbeck Foundation (grant no. R37-A3457 to S.G.F.R.), and the Danish Independent Research Council (grant no. 0602-02407B to S.G.F.R.).

Author contributions
A.C.K., A.M., and C.M. designed and generated the nanobody library. C.M., A.S.B., and A.C.K. performed quality control of the library. C.M., R.P., S.Z., J.X.O., D.H., and A.M. prepared antigens, performed selections, and isolated nanobody binders. C.M., R.P., S.Z., J.X.O., D.H., and A.M. prepared the yeast display system and associated expression vectors. M.W. and S.G.F.R. purified the A2A adenosine receptor. C.M., A.M., and A.C.K. wrote the manuscript with assistance and input from all coauthors.

Competing interests
The authors declare no competing financial interests.

Additional information
Supplementary information is available for this paper at https://doi.org/10.1038/s41594-018-0028-6. Reprints and permissions information is available at www.nature.com/reprints. Correspondence and requests for materials should be addressed to A.M. or A.C.K.

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.
For the first round of selection, 5 x 10^6 induced yeast were washed and incubated in buffer (20 mM HEPEs, pH 7.5, 150 mM sodium chloride, 2 mM CaCl2, 0.1% BSA, 1.8% maltose) with Alexa647-labeled anti-FLAG M1 and anti-647 microbeads. Clones binding nonspecifically to the staining reagents were removed by passage through an LD column, and the remaining yeast from the flow-through were incubated with unpurified FLAG-adiponectin at ~500 nM for 30 min at 4°C. Cells were then washed with anti-FLAG-647 and anti-647 microbeads. Binding clones were enriched via magnetic selection in an LS column and cultured overnight in Trp medium at 30°C. Rounds 2 and 3 of selection were performed similarly with 3 x 10^6 induced yeast and cells washed, stained with 647- or FITC-labeled M1, and labeled with anti-Alexa Fluor 647 or anti-FITC microbeads before magnetic separation during rounds 2 and 3, respectively.

Following round 3, induced cells incubated with ~500 nM unpurified adiponectin and stained simultaneously with M1-647 and M1-FITC to differentiate binding and adiponectin clones. FACS analyses were performed for cells that exhibited adiponectin-dependent 647 and FITC staining. Nanobodies from individual clones were sequenced and purified, and adiponectin binding was confirmed and characterized by SPR with purified adiponectin.

Human βAR. To isolate agonist-specific βAR-binding nanobodies, two rounds of MACS were performed with 1 μM purified, FLAG-tagged βAR bound to the high-affinity agonist BI167107 and FITC- or Alexa Fluor 647-labeled anti-FLAG antibody in a selection buffer containing 20 mM HEPEs, pH 7.5, 100 mM NaCl, 0.1% dodecyl maltoside (DDM, Anatrace), 0.01% CHS, 0.5% BSA, 5 mM CaCl2, and 1 mM EDTA. For round one of selection, the buffer was the same as detailed above, with anti-FLAG antibody labeled with Alexa Fluor 647 to deplete antibody or fluorophore binders. Round 3 used MACS again, but reverse selection was performed against βAR bound to the high-affinity antagonist carazolol to deplete nanobody clones that bind conformationally invariant or inactive-state epitopes. In subsequent rounds, we used FACS to more specifically select nanobodies that selectively bind the active form of the receptor. In the active state, βAR was simultaneously incubated with 1 μM βAR, BI167107 complex labeled with Alexa Fluor 647 NHS ester and 1 μM βAR-A2AR–carazolol complex labeled with Alexa Fluor 488 NHS ester. Yeast displaying selectivity for agonist (high Alexa Fluor 647 signal and low Alexa Fluor 488 signal) were collected, expanded in growth media, incubated, and subjected to another round of FACS. The conformational selection procedure was repeated as in round 4; however, the fluorophores coupled to agonist and antagonist was switched to deplete nonspecific clones. A final MACS round was performed at 30 nM βAR–BI167107 to isolate the highest affinity nanobodies. Nanobodies from individual clones were subsequently sequenced and purified for biochemical and biophysical studies.

Human adenosine A2A receptor. Two rounds of MACS were performed with 1.5 μM of FLAG-tagged A2AR bound to the agonist 5′-N-ethylcarboxamidoadenosine (NECA) and M1 anti-FLAG antibody labeled with FITC or Alexa Fluor 647 in a selection buffer containing 20 mM HEPEs, pH 7.5, 150 mM NaCl, 0.1% CHS, 2 mM CaCl2, and 0.2% maltose. Each round began with a preclearing step as detailed above. To isolate conformationally selective binders, the second-round yeast were stained for 20 min simultaneously with 500 nM each of A2AR bound to the agonist UK 432097 and A2AR bound to the antagonist ZM 241385, which were labeled with secondary anti-FLAG M1 488 and anti-FLAG M1 647, respectively. Yeast that selectively bound receptor in conformation 2AR was isolated by FACS. Yeast from individual clones were sequenced and stained with both receptor populations to confirm binding selectivity and on-yeast binding titrations were performed to approximate binding affinities. Nanobodies were then purified, and binding was confirmed in vitro with an anti-FLAG pulldown.

**Protein expression and purification.** The ADIPQQ gene was cloned into a pTARGET vector so that an HA signal peptide directs secretion of N-terminally FLAG-tagged adiponectin into the medium. Cells from the lower chamber of the bioreactor were harvested by centrifugation at 4,000 g for 10 min. The supernatant was diluted 1:1 with buffer (20 mM HEPEs, pH 7.5, 150 mM sodium chloride, 2 mM CaCl2, 2 mg/ml FITC resin). FLAG resin was washed with 20 mM HEPEs, pH 7.5, 300 mM NaCl, 2 mM CaCl2, and then with 20 mM HEPEs, pH 7.5, 150 mM NaCl, and 2 mM CaCl2. Adiponectin was eluted with buffer comprised of 150 mM NaCl, 20 mM HEPEs, pH 7.5, 0.2 mg/ml FLAG peptide, and 5 mM EDTA and then dialyzed overnight into 20 mM HEPEs, pH 7.5, and 150 mM sodium chloride.

Human βAR fused to an amino-terminal hemagglutinin signal peptide and FLAG-tag as well as a carboxy-terminal 1D4 tag was purified from infected S9 cells. Cells were collected 64 h after baculovirus infection and stored at −80°C. For receptor preparations used during nanobody selections, purification was performed using ligand-affinity chromatography as described previously. For biochemical assays, we used a solid-phase purification method omitting ligand-affinity chromatography. Cells were lysed in a buffer comprised of 20 mM HEPEs, pH 7.5, 2 mM MgCl2, 2 mM benzamide, and 1 μM IC1-118,551. The resulting lysate was centrifuged, and the pellets were resuspended in solubilization buffer containing 20 mM HEPEs, pH 7.5, 200 mM NaCl, 10% glycerol, 1% MNG, 0.1% CHS, and 1 μM IC1-118,551, then incubated at 4°C for 2 h. The insoluble fraction was
separated by centrifugation at 39,000 × g for 20 min, and the supernatant was loaded over 5 mL of homemade anti-FLAG M1 resin. FLAG resin was subsequently washed with 20 mM HEPES, pH 7.5, 200 mM NaCl, 10% glycerol, 0.1% MCE, and 0.1% CHS. β2AR was eluted in the same buffer but with a lower concentration of detergent and CHS (0.01% and 0.001%, respectively) supplemented with 5 mM EDTA and 0.2 mg/mL FLAG peptide. Receptor was further purified by size-exclusion chromatography in the presence of agonist B1671707. For crystallographic experiments, β2AR fused to an amino-terminal T4 lysozyme was similarly purified.

Isolated nanobody genes were cloned into the plasmid expression vector pET28b containing a C-terminal 6 histidine tag and expressed from BL21 (DE3) E. coli. For each purification, E. coli were grown in Terrific Broth (Research Products International) and induced after reaching OD600 0.6–0.8 with 1 mM IPTG (Gold Biotechnology) at 25 °C for about 15 h. Induced cells were pelletted and resuspended in 1% (w/v) buffer consisting of 0.5 M sucrose, 0.2 M Tris, pH 8, 0.5 mM EDTA and osmotically shocked by adding 200–0.8 mL of water with 45 min of stirring, releasing periplasmic nanobody. The lysate was brought to a concentration of 150 mM NaCl, 2 mM MgCl2, and 20 mM imidazole and then centrifuged at 20,000 × g for 20 min at 4 °C. The supernatant was applied to a gravity column with 3 mL of Ni Sepharose 6 Fast Flow (GE Healthcare). The resin was washed with a high-salt buffer (20 mM HEPES, pH 7.5, 500 mM NaCl, 20 mM Imidazole) and then washed with NH4Cl buffer (20 mM HEPES, pH 7.5, 100 mM NaCl) + 20 mM imidazole, and nanobodies were eluted with NH4Cl + 200 mM NaCl, 100 mM imidazole. Nanobodies used for crystallography were treated to carboxypeptidase A (Sigma) to remove the histidine tag, dialyzed into HBS buffer (10 mM HEPES, pH 7.5, 800 mM NaCl, 10% glycerol, 4 mM theophylline, 5 mM EDTA and 0.2 mg/mL FLAG peptide). Receptor was further purified as described previously31 and solubilized in 1% DDM, 0.1% CHS, 20 mM HEPES, pH 7.4, 150 mM NaCl, and copurified with their respective binding partners over an S200 size-exclusion column (GE Healthcare).

Human A2AR was fused at the N terminus to an HA signal sequence followed by a FLAG epitope, and at the C terminus, it was truncated at Ala317 with the addition of a 9× histidine tag. The receptor was expressed in S9 insect cells following generation of Pichia pastoris [Baculovirus (Expression Systems) mediated by the pVL1393 vector. Membranes from three 1-l cultures were prepared essentially as described previously32 and solubilized in 1% DDm, 0.1% CHS, 20 mM HEPES, pH 7.5, 800 mM NaCl, 10% glycerol, 4 mM theophylline, 5 μg/mL leupeptin, 5 μg/mL benzamidine, 2 mg/mL iodoacetamide, and 100 μM TCEP. Following centrifugation, the supernatant was incubated overnight with Ni2+ chelating Sepharose under rotation at 4 °C. The resin was transferred to a column and washed in five column volumes of ice-cold wash buffer (0.1% DDm, 0.1% CHS, 20 mM HEPES, pH 7.5, 100 mM NaCl, 10% glycerol, 4 mM theophylline, 5 μg/mL leupeptin, 5 μg/mL benzamidine) containing 25 mM imidazole and repeated with 50 μM Ni2+.

Dilutions of Nb.b201 in running buffer (10 mM HEPES, pH 7.5, 150 mM NaCl, 0.03% Tween) were made and the sample was injected at a flow rate of 30 μL/minute with a contact time of 120 s and dissociation time of 300 s. For SPR, a 20 mM EDTA buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 0.05% Tween) were made and the sample was injected at a flow rate of 30 μL/minute with a contact time of 120 s and dissociation time of 300 s. For SEC-MALS, a 1:1 molar mix of Nb.b201 with β2AR was labeled with NHS-PEG4-Biotin and immobilized on an Agilent HP515 SEC CAP (GE Healthcare). Dilutions of the Nb.b201 sample were made in running buffer (10 mM HEPES, pH 7.5, 150 mM NaCl, 0.01% CHS, 0.001% CHS, 20 mM B161707), and the sample was injected at a flow rate of 30 μL/minute with a contact time of 240 s and dissociation time of 700 s.

For SPR of Nb.AQ103, EZ-Link NHS-PEG4-Biotin-labeled (Thermo Fisher Scientific) immobilized on a Sensor Chip CAP (GE Healthcare). Dilutions of the Nb.b201 sample were made in running buffer (10 mM HEPES, pH 7.5, 150 mM NaCl, 0.01% CHS, 0.001% CHS, 20 mM B161707), and the sample was injected at a flow rate of 30 μL/minute with a contact time of 120 s and dissociation time of 300 s. For SEC-MALS, a 1:1 molar mix of Nb.b201 with β2AR was labeled with NHS-PEG4-Biotin and immobilized on an Agilent 1260 Infinity 3D SEC (GE Healthcare). Dilutions of the Nb.b201 sample were made in running buffer (10 mM HEPES, pH 7.5, 150 mM NaCl, 0.01% CHS, 0.001% CHS, 20 mM B161707), and the sample was injected at a flow rate of 30 μL/minute with a contact time of 240 s and dissociation time of 700 s.

β2AR cAMP signaling assay. β2AR signaling was measured using a transcriptional CRE-SEAP (secreted embryonic alkaline phosphatase) reporter to indirectly measure cAMP production. Briefly, HEK293T cells were seeded at 3.3 × 105 cells/well in 96-well plates the day before transfection in 200 μL/ well of DMEM. The following day, medium was aspirated from the cells and replaced with 50 μL of standard DMEM/F12. After 15 h, the medium was aspirated from the cells, and 200 μL of the indicated DMEM was added with the indicated final concentrations of adrenaline. The cells were incubated at 37 °C for 48 h, then at 70 °C for 2 h. To determine SEAP activity, the substrate 4-methylumbelliferyl phosphate (Sigma Aldrich) was prepared at 1.2 mM in 2 M diethanolamine bicinephate pH 10 and mixed with an equal volume of cell supernatant overnight. For 10 min, fluorescence was measured on an EnVision 2103 Multilabel Reader (Perkin Elmer) with an excitation wavelength of 360 nm and an emission wavelength of 449 nm. For each condition, the baseline fluorescence value was determined from cells not treated with adrenaline and was subtracted from every value in the dataset. β2AR signaling was calculated as a fraction of the maximum observed response (pEGFP-N1 empty vector) and plotted using GraphPad Prism.

Protein crystallization. Nb.b201 for crystallographic study was purified from E. coli as described above. The Nb.b201 complex with HSA was prepared by mixing HSA (Sigma) with Nb.b201 at a ratio of 1:1.15, then subjected to size-exclusion chromatography purification. Purified complex was concentrated to 120 mg/mL and mixed in 200 μL + 200 mL drops with Morpheus HT-96 screen ( Molecular Dimensions). Crystals were grown in a sitting-drop format and were obtained directly from the screen without further optimization. The precipitant solution consisted of 0.2 M each of 1.6 hexanesol, 1-butanol, 2-propanol, 1,4-dioxane, 2% w/v polyethylene glycol (PEG) 5000, 10% w/v PEG 2000. Crystals of Nb.b201 alone were obtained when the complex was mixed in a 1:1 drop with a solution comprised of 4.0 M potassium formate, 0.1 M Bis-Tris propane, pH 9.0, 2% w/v polyethylene glycol monomethyl ether 2,000. Crystals were soaked with 20% glycerol as a cryoprotectant before flash freezing in liquid nitrogen.

For β2AR-Nb.b201 crystallization, the two-syringe mixing method was used to reconstitute the sample in lipidic cubic phase phase. Crystals were obtained from MemMeso LCP screen ( Molecular Dimensions) conditions consisting of 100 mM MES, pH 6, 1% NaCl, 200 mM MgCl2, and 40% PEG 200. Crystals were also obtained in a variety of additional conditions.

Data collection and structure refinement. Data collection was performed at Advanced Photon Source GM/CA beamlines 23ID-B and 23ID-D. Diffraction data were collected at an energy of 12 keV with a 0.2 s exposure per frame. Each frame covered a 0.2° oscillation, and beam intensity was attenuated 100–1,000 fold depending on crystal size. The structure of Nb.b201 in isolation was solved by molecular replacement in Phaser using the structure of the β2AR-binding nanobody Nb8B0 as a search model (PDB 3P0G). The structure of the HSA–Nb.b201 complex (PDB ID 5VNV) was solved by molecular replacement using the structure of Nb.b201 (PDB 5VNV) and the structure of HSA (PDB 3JRY) as search models. Structural refinement for Nb.b201 in isolation was performed by alternate manual building in COOT and reciprocal space refinement in PHENIX. In the final stages, TLS refinement was used to model anisotropic B factors. Refinement for the HSA–Nb.b201 complex was performed similarly, with the additional inclusion of noncrystallographic symmetry restraints in the first two rounds of refinement. As in the case of Nb.b201 in isolation, TLS refinement was used in final stages of refinement.
refinement. Crystallographic data analysis was performed with xds and phenix. refine, using standard metrics to assess structure quality. In the final Ramachandran plots, the favored and allowed residues were 97.5% and 2.5%, respectively, for Nb.b201 in isolation and 97.4% and 2.6%, respectively, for the HSA–Nb.b201 complex. Full details of crystallographic statistics are summarized in Table 1.

Life Sciences Reporting Summary. Further information on experimental design is available in the Life Sciences Reporting Summary.

Data availability. Atomic coordinates and structure factors for the nanobody structures are deposited in the Protein Data Bank under the accession codes PDB 5VNV (Nb.b201 alone) and PDB 5VNW (Nb.b201 in complex with human serum albumin). All other data are available upon reasonable request.

References
28. Weiskopf, K. et al. Engineered SIRPα variants as immunotherapeutic adjuvants to anticancer antibodies. Science 341, 88–91 (2013).
29. Manglik, A. et al. Structural insights into the dynamic process of β2-adrenergic receptor signaling. Cell 161, 1101–1111 (2015).
30. Zou, Y., Weis, W. I. & Kobilka, B. K. N-terminal T4 lysozyme fusion facilitates crystallization of a G protein coupled receptor. PLoS One 7, e66039 (2012).
31. Jaakola, V. P. et al. The 2.6 angstrom crystal structure of a human A2A adenosine receptor bound to an antagonist. Science 322, 1211–1217 (2008).
32. Whorton, M. R. et al. A monomeric G protein-coupled receptor isolated in a high-density lipoprotein particle efficiently activates its G protein. Proc. Natl. Acad. Sci. USA 104, 7682–7687 (2007).
33. Liberles, S. D. & Buck, L. B. A second class of chemosensory receptors in the olfactory epithelium. Nature 442, 645–650 (2006).
34. Caffrey, M. & Cherezov, V. Crystallizing membrane proteins using lipidic mesophases. Nat. Protoc. 4, 706–731 (2009).
35. Hein, K. L. et al. Crystallographic analysis reveals a unique lidocaine binding site on human serum albumin. J. Struct. Biol. 171, 353–360 (2010).
36. Emsley, P. & Cowtan, K. Coot: model-building tools for molecular graphics. Acta Crystallogr. D Biol. Crystallogr 60, 2126–2132 (2004).
37. Adams, P. D. et al. PHENIX: a comprehensive Python-based system for macromolecular structure solution. Acta Crystallogr. D Biol. Crystallogr 66, 213–221 (2010).
Life Sciences Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form is intended for publication with all accepted life science papers and provides structure for consistency and transparency in reporting. Every life science submission will use this form; some list items might not apply to an individual manuscript, but all fields must be completed for clarity.

For further information on the points included in this form, see Reporting Life Sciences Research. For further information on Nature Research policies, including our data availability policy, see Authors & Referees and the Editorial Policy Checklist.

Experimental design

1. Sample size
   Describe how sample size was determined.
   Described in Methods where applicable.

2. Data exclusions
   Describe any data exclusions.
   No data were excluded

3. Replication
   Describe whether the experimental findings were reliably reproduced.
   All attempts at replication have been successful

4. Randomization
   Describe how samples/organisms/participants were allocated into experimental groups.
   Not applicable

5. Blinding
   Describe whether the investigators were blinded to group allocation during data collection and/or analysis.
   Experiments were not blinded

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

6. Statistical parameters
   For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

| n/a | Confirmed |
|-----|-----------|
| ☑   | The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.) |
| ☑   | A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly |
| ☑   | A statement indicating how many times each experiment was replicated |
| ☑   | The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section) |
| ☑   | A description of any assumptions or corrections, such as an adjustment for multiple comparisons |
| ☑   | The test results (e.g. P values) given as exact values whenever possible and with confidence intervals noted |
| ☑   | A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range) |
| ☑   | Clearly defined error bars |

See the web collection on statistics for biologists for further resources and guidance.
Software

Policy information about availability of computer code

7. Software

Describe the software used to analyze the data in this study.

Software used is described in methods section along with references to original publications describing the software.

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

Materials and reagents

Policy information about availability of materials

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

All materials are available upon request contingent on completion of materials transfer agreement.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

Anti-adiponectin and anti-His antibodies were validated using antigen-non expressing cells to confirm minimal crossreactivity in experimental conditions. Synthetic nanobody characterization is described in detail in the manuscript itself.

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

This information is in the methods section. All cell lines are from ATCC unless otherwise noted.

b. Describe the method of cell line authentication used.

Yeast cell lines were validated by assessment of reported auxotrophic characteristics. Insect and mammalian cell lines used for protein production were not authenticated since these were used only for production of proteins and were not experimental subjects per se.

c. Report whether the cell lines were tested for mycoplasma contamination.

Cell lines were not tested.

d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.

Not applicable

Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.

No animal studies in this work.

Policy information about studies involving human research participants

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

No human subjects.
Flow Cytometry Reporting Summary

Form fields will expand as needed. Please do not leave fields blank.

- **Data presentation**

  For all flow cytometry data, confirm that:

  - 1. The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
  - 2. The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
  - 3. All plots are contour plots with outliers or pseudocolor plots.
  - 4. A numerical value for number of cells or percentage (with statistics) is provided.

- **Methodological details**

  5. Describe the sample preparation. See methods.

  6. Identify the instrument used for data collection. See methods.

  7. Describe the software used to collect and analyze the flow cytometry data. See methods.

  8. Describe the abundance of the relevant cell populations within post-sort fractions. See methods.

  9. Describe the gating strategy used. Standard gating was used. Yeast form a single population in an FSC/SSC plot and were gated accordingly. Cells were further gated on an FSC-A/FSC-H plot to exclude doublets. A representative gating figure is included in the Supplementary Note.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information. ✗