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Massachusetts Institute of Technology

Exploring cellular biochemistry with nanobodies

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Ross W. Cheloha[1,2], **Thibault J. Harmand**[1,2], **Charlotte Wijne**[1,2], **Thomas U. Schwartz**[3], **and Hidde L. Ploegh**[1,2,*]

From the [1]Program in Cellular and Molecular Medicine, Boston Children's Hospital, Boston, Massachusetts, USA, [2]Harvard Medical School, Boston, Massachusetts, USA, and the [3]Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts, USA

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Reagents that bind tightly and specifically to biomolecules of interest remain essential in the exploration of biology and in their ultimate application to medicine. Besides ligands for receptors of known specificity, agents commonly used for this purpose are monoclonal antibodies derived from mice, rabbits, and other animals. However, such antibodies can be expensive to produce, challenging to engineer, and are not necessarily stable in the context of the cellular cytoplasm, a reducing environment. Heavy chain–only antibodies, discovered in camelids, have been truncated to yield single-domain antibody fragments (VHHs or nanobodies) that overcome many of these shortcomings. Whereas they are known as crystallization chaperones for membrane proteins or as simple alternatives to conventional antibodies, nanobodies have been applied in settings where the use of standard antibodies or their derivatives would be impractical or impossible. We review recent examples in which the unique properties of nanobodies have been combined with complementary methods, such as chemical functionalization, to provide tools with unique and useful properties.

Tools to detect, visualize, and modulate the properties of proteins are essential to understand the function of the targets recognized and the biology that follows. Introduction of exogenous expression vectors and CRISPR/Cas gene-editing tools provide an unprecedented ability to introduce, alter, or eliminate proteins of choice in cells or intact organisms. These approaches are designed to modify biological processes of interest. Introduction of expression vectors allows production of proteins of choice, WT or mutant, including versions fused with fluorescent proteins or other tags for visualization. Expression of proteins from nonnative loci, as in exogenous expression vectors, or as fusion proteins with tags often alters expression levels, subcellular localization, and biological function. The development of antibody fragments that can interact with and perturb endogenous proteins in cells and organisms without the need for genomic modification would be useful. Nanobodies have unique qualities that make them well-suited for this goal.

Nanobodies, like full-size conventional antibodies, show the affinity and antigen specificity required for specific targeting of molecules of interest, even though they comprise only a single variable region. Nanobodies have several useful features not regularly found in conventional antibodies. These include their small size, the capacity to bind and stabilize specific receptor conformations, and their availability in high yield from bacterial expression systems. Nanobodies have been widely used to target soluble protein antigens or those found at the surface of cells (*e.g.* for structural studies and imaging applications (for reviews see Refs. 1 and 2). Similar to full-sized antibodies, nanobodies are suitable for flow cytometry, immunoprecipitation, affinity purification, and microscopy (3–7). Although nanobodies are often applied in settings that could just as well use standard, full-size antibodies, we emphasize scenarios where the use of a nanobody provides advantages. In this review, we cover topics including methods for the identification of target-specific nanobodies, functionalization of nanobodies using chemical and enzymatic methods, and the use of nanobodies that engage targets inside or at the surface of the cell as well as viral targets. We cover the development of nanobody-epitope tag pairs and the use of nanobodies in synthetic biology. This review may serve as an accessible resource for scientists looking to identify nanobodies useful for their system of interest. We focus on areas such as nanobody functionalization and synthetic biology, in which methods and use of nanobodies are rapidly evolving.

Screening platforms

Conventional antibodies (Igs) consist of two identical heavy (H) and light (L) chains that pair to form a stably folded protein, with an antigen-binding site to which the two variable (V) domains, V_H and V_L, contribute. Both interchain and intrachain disulfides and *N*-linked glycosylation are needed for effective assembly of Igs. These requirements preclude the proper assembly of full-size antibodies in the reducing environment of the cytoplasm. Single-chain variable fragments (scFvs) consist of the variable domains from the heavy and light chains, connected by a linker. Although some scFvs can function in the cytoplasm, many scFvs require intrachain disulfides to afford stability and appropriate heavy-light chain pairing. Heavy chain–only antibodies from camelids fold and function in the absence of light chains. These camelid immunoglobulin heavy chains can be shrunk to just their variable domains (Fig. 1) to yield VHHs or nanobodies, which can retain antigen binding in the absence of disulfide bond formation. They can thus be used in the cytosol of live cells, as discussed below. This feature of nanobodies is one of the signature advantages of their application, relative to more conventional alternatives, as discussed below.

* For correspondence: Hidde L. Ploegh, hidde.ploegh@childrens.harvard.edu.

Present address for Ross W. Cheloha: Laboratory of Bioorganic Chemistry, NIDDK, National Institutes of Health, Bethesda, Maryland, USA.

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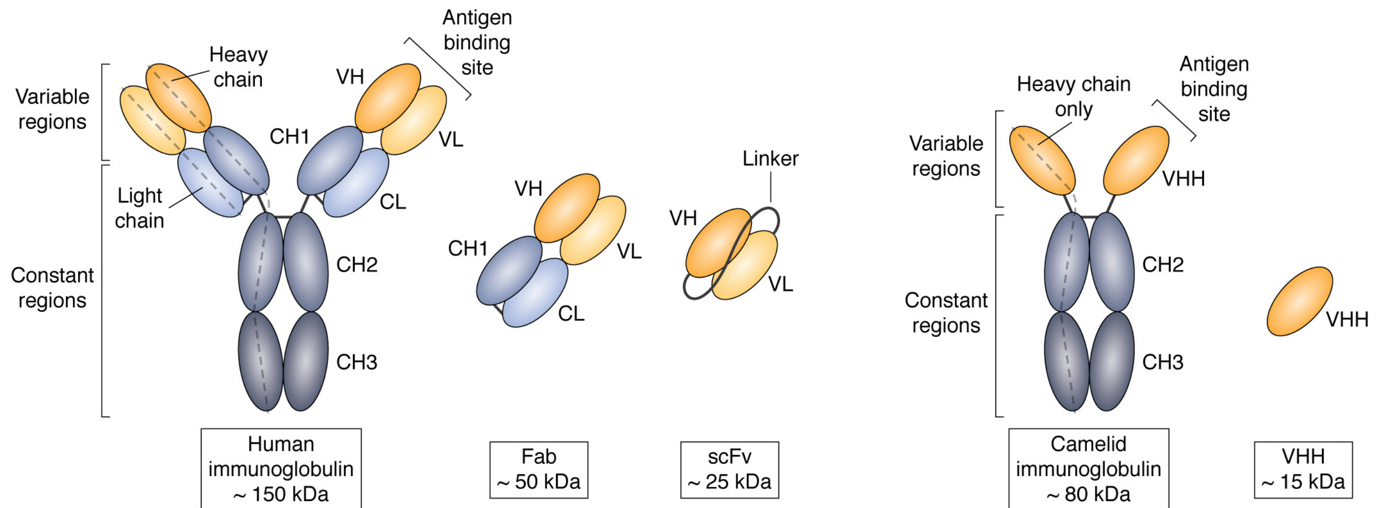

Figure 1. Structures of human and camelid Igs and fragments. Conventional human Igs (*i.e.* IgG) have been truncated to provide functional fragments (Fab and scFv) that contain variable regions from the light and heavy chains. In the case of the scFv, a linker is required to facilitate appropriate pairing of heavy- and light-chain variable regions. A subset of antibodies from camelids consists of only the heavy chains. Expression of the isolated variable region from heavy chain–only antibodies provides functional single-domain antibodies (VHHs/nanobodies).

Methods to identify nanobodies that bind to targets of interest are essential for their effective deployment (8). Target-binding nanobody clones are usually isolated from screening highly diverse pools of nanobodies. Such pools must be sufficiently large to contain appropriately specific nanobodies, a suitable screening method must be at hand to identify specific binders, and such binders should retain their properties in the relevant contexts, as in the case of cytoplasmic expression or when dealing with membrane proteins. Screening methods that provide nanobodies with desirable functional properties (receptor antagonism, agonism), selectivity for specific target conformations (structural studies, biosensors), and functionality in different subcellular localization (cytoplasmic, cell surface) are in short supply and constitute an area of emphasis for future exploration. Both immunization and screening strategies ought to be designed with the final application(s) of the resulting nanobodies in mind. For example, immunization with unfolded, denatured proteins is more likely to yield reagents that are useful in immunoblotting or immunohistochemistry on fixed samples.

For library construction, B cells from naive or immunized camelids can serve as the point of departure, as can cultured camelid B cells exposed to antigens of interest (9). Purified proteins (10), cells or cell lysates containing antigens of interest (11, 12), or recombinant DNA to induce antigen expression in the host (13, 14) can serve as immunogens. DNA-based immunization has been particularly valuable for the generation of nanobodies against properly folded membrane proteins (15, 16). The diversity of nanobody sequences available in a given pool can be further expanded through mutagenesis. Both natural diversity mutagenesis, in which residues at positions in a nanobody with high diversity in naturally occurring collections of nanobodies are varied (17), and virus-mediated directed evolution (18) can increase diversity and identify novel nanobodies. Important features in the screening approach include the source of the nanobody pool (synthetic *versus* naive *versus* immunized library) (8, 19), the mechanism by which nanobody proteins are produced and displayed (phage display *versus* yeast display *versus* bacterial display *versus* ribosome display *versus* DNA/RNA display) (20), and the method by which antigens of interest are presented for selection (peptide or protein immobilization on solid support *versus* display of antigens on the cell surface *versus* labeled soluble antigen) (21). Given the importance of identifying nanobodies that bind to membrane proteins, a variety of approaches have yielded nanobodies that bind to intact, properly folded membrane targets (22–24) and that either block or induce activation (25).

The defining feature of a display method is the mechanism by which the biochemical properties of the nanobodies are linked to the genetic information encoding the nanobodies. The type of display method used also dictates the diversity of the library of nanobody sequences used. Phage display, in which nanobodies are fused in frame with viral proteins for display on the surface of phages—typically an M13 derivative—that encapsulate the relevant DNA sequence, is commonly used to pan for nanobodies (26, 27). Phage display libraries with a diversity of 10^7 to 10^8 clones are common. Display-based approaches can also be applied using model single-cell organisms, such as *Escherichia coli* (20), *Staphylococcus* sp. (28, 29), and yeast (30, 31).

Yeast display platforms have succeeded in the identification of nanobodies that bind to specific conformations of cell surface proteins, such as G protein–coupled receptors (GPCRs) (30, 31). Bacterial and yeast display platforms of a complexity comparable with that of phage libraries have the advantage that antigen-binding clones can be detected and enriched by flow cytometry (20).

Ribosome display relies on a covalent bond between the nanobody and the encoding RNA chain. Both the translated nanobody sequence and the RNA that encodes it remain tethered to the ribosome when the mRNA lacks a stop codon. Nanobodies that bind to cell membrane proteins in specific conformations were thus obtained (32–34). An approach called RNA display or cDNA display relies on the antibiotic puromycin

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applied *in vitro* to enter the ribosomal active site and form a cross-linking covalent bond with the nascent nanobody polypeptide and the encoding RNA sequence to enable selection (35, 36). Library complexity (up to 10^{12} unique clones) used in *in vitro* selection techniques can exceed by far those used in phage, bacterial, and yeast display, but screening then requires multiple rounds of selection to arrive at individual high-affinity binders (33).

Alternative methods of screening have been developed to identify nanobodies that function in their intended environment. In one such method, nanobody-coding sequences (minus the signal peptide) were inserted into lentiviral vectors for expression in the cytoplasm of mammalian cells. Nanobodies that protected cells from a lytic infection with influenza A virus or vesicular stomatitis virus were then identified through enrichment of surviving cells and recovery by PCR of the protective nanobody sequences (37). A similar approach was used to identify a nanobody that protected against porcine reproductive and respiratory syndrome virus (38). The use of a functional readout, like cell survival, ensured that the nanobodies were functional in the cytosol. The size of the library tested in the lentivirus-based approach is similar to that used in phage display ($\sim 10^7$ clones). Another method to identify nanobodies that are functional in the cytosol involves a yeast two-hybrid system, in which propagation of the yeast is contingent on the interaction of a nanobody clone with a target antigen of interest (39). This approach yielded nanobodies that bind HIV VPR and capsid proteins and the hemagglutinin-neuraminidase protein of the Newcastle disease virus (40, 41).

Chemical and enzymatic functionalization

Conventional recombinant expression in bacteria produces nanobodies in high yields, providing ample material for chemical functionalization. Conjugation of nanobodies with fluorescent dyes, small-molecule drugs, oligonucleotides, and other moieties allows complex yet controlled functionalization of nanobodies to extend their application to a wide range of areas, including imaging, therapeutics, and detection, and as delivery agents. Early examples of nanobody functionalization mostly relied on reactivity of cysteine and lysine residues using maleimide (42) and *N*-hydroxysuccinimide ester–based chemistry (43, 44). Nanobodies typically require the introduction of an unpaired Cys and disulfide reduction prior to labeling. *N*-Hydroxysuccinimide ester–based labeling lacks selectivity, resulting in heterogeneous mixtures of labeled proteins. Excessive labeling of nanobodies can cause loss of antigen recognition and specificity and result in altered pharmacokinetic properties (45–47). Chemoenzymatic labeling methods, incorporation of unnatural amino acids, and expressed protein ligation are therefore attractive alternatives for the bioconjugation of nanobodies, as will be summarized below (Fig. 2). These methods enable the conjugation of nanobodies with a virtually unlimited selection of chemical cargoes. Even with these advances, it remains difficult to use nanobody conjugates prepared *in vitro* to address biology inside of live cells because of their membrane impermeability. The development of robust methods for delivery of nanobodies across the cell membrane (48–50) or labeling in cells with minimal background will empower new and powerful applications with conjugates.

Oldenlandia affinis asparaginyl endopeptidase (*Oa*AEP1)

Asparaginyl endopeptidases (AEPs) are an increasingly attractive class of enzymes for protein modification. AEPs are nominally Cys proteases that recognize a tripeptide motif, Asn/Asp-Xaa-Yaa, and generate a thioester intermediate C-terminally of the Asn or Asp residue. The thioester can be then attacked by a suitable nucleophile: a dipeptide Gly/Ala-Zaa, where Zaa is a hydrophobic amino acid residue. AEPs of plant origin, such as *Oa*AEP1 from *O. affinis*, catalyze head-to-tail cyclization of peptides and have been used to prepare cyclic peptides (51–53). The slow kinetics of *Oa*AEP1 limited its application for protein labeling. Substitution of cysteine residue 247 by an alanine residue enhances *Oa*AEP1's catalytic efficiency, making it an efficient tool for protein modification (54). The mutant *Oa*AEP1 has been applied to the modification of nanobodies. By screening different nucleophiles, a Gly-Val dipeptide was identified that readily served as a nucleophile in the ligation reaction, but the product of that reaction (Asn-Gly-Val) was poorly recognized by the enzyme. This yields a ligation product resistant to the reverse reaction, a common shortcoming of enzymatic labeling methods such as sortagging (see "Sortase A") (55). The use of *Oa*AEP1 with Asn-Gly-Leu–based modified tripeptides allowed efficient modification of the N terminus of a nanobody with the Gly-Val sequence at the N terminus. The use of *Oa*AEP1 thus enabled conjugation of nanobodies with a broad range of different molecules: dyes, lipids, biotin, tetrazine, azide, cyclooctene, small-molecule drugs, PEG oligomers, D-amino acids, and β amino acids (55).

The resistance of the Asn-Gly-Val sequence—the ligation product—to attack by *Oa*AEP1 allowed efficient site-specific modification of a nanobody both at the C and N terminus using the same enzyme, making possible the preparation of doubly functionalized nanobodies (55), as follows. A C-terminal NGL sequence is enzymatically modified first with a Gly-Val modified peptide, yielding a cleavage-resistant product. The N-terminal modification required transient protection of the future N-terminal Gly-Val nucleophile by a TEV protease recognition sequence. Its removal freed up the N terminus for a second *Oa*AEP reaction. Obviously, this method is not limited to modification of nanobodies and can be applied to other proteins of interest, and it might be particularly useful for single-molecule studies. It is also worth noting that, although not observed during nanobody modification, unwanted cleavage within the protein of interest is a side reaction that can occur while using enzyme from the AEP family.

Tub-tag

Tubulin tyrosine ligase (TTL) modifies the C terminus of a protein through conjugation of an unnatural tyrosine residue. This modified tyrosine can be equipped with a wide range of chemical substituents and thus serves to introduce into the newly modified protein important functionalities, such as azides, aldehydes, iodides, alkynes, and dyes. The TTL enzyme recognizes a glutamic acid–rich 14-amino acid sequence, also

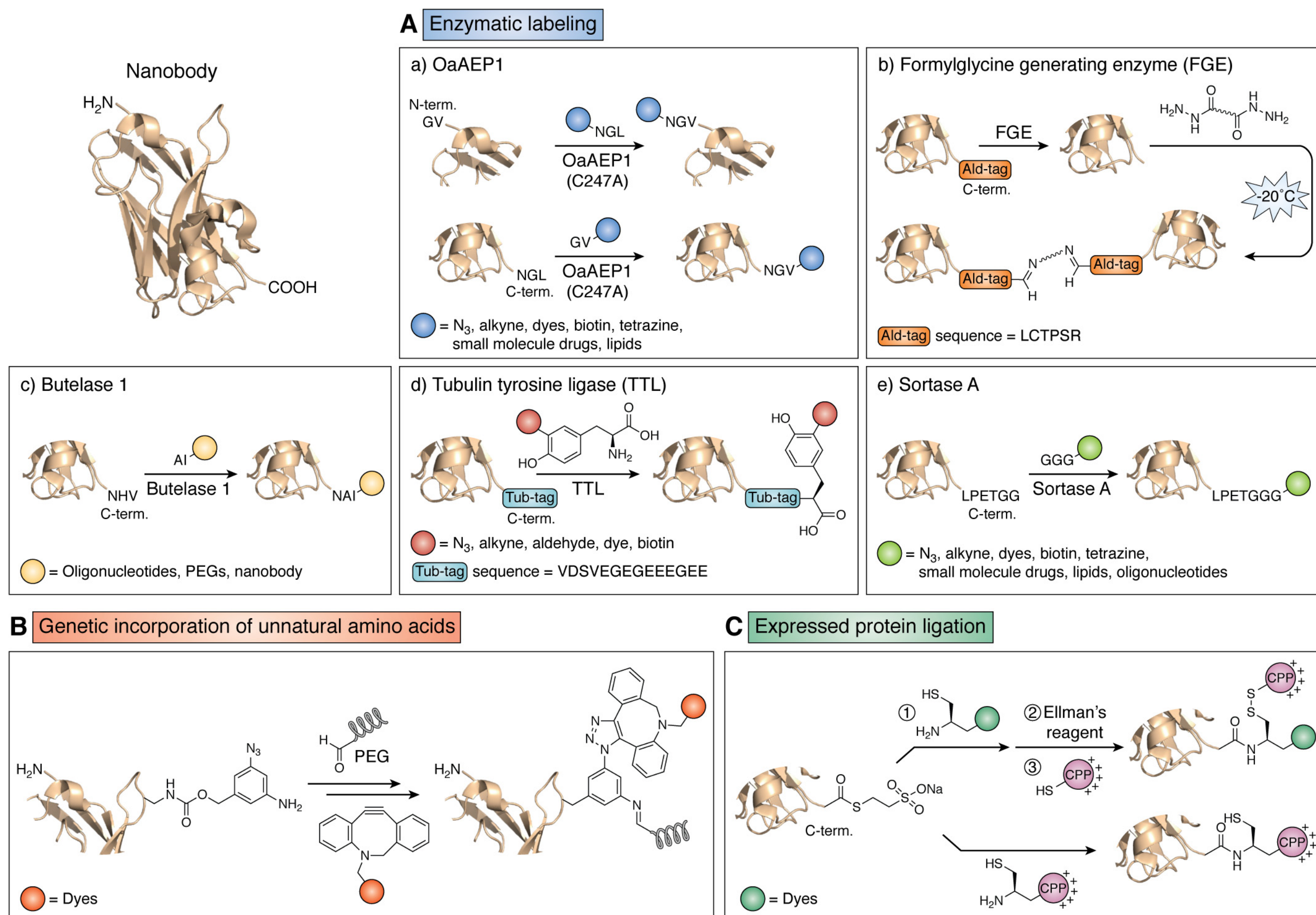

Figure 2. Recent examples of nanobody bioconjugation. *A*, enzymatic approaches, including *Oa*AEP1 (*a*), formylglycine-generating enzyme (*b*), tubulin Butelase and tubulin tyrosine ligase need to be switched in the figure legend (Butelase is panel *c*, Tubulin tyrosine ligase is panel *d*) and sortase A (*e*). *B*, incorporation of unnatural amino acids by stop codon suppression. *C*, expressed protein ligation. See the Chemical and Enzymatic Functionalization section for a discussion of strengths and drawbacks of these approaches and associated references.

called Tub-tag (VDSVEGEGEEEGEE), which must be placed at the C terminus of the protein of interest. Labeling with TTL and Tub-tag has been used to modify nanobodies (56) with fluorescent coumarin and biotin derivatives. Recombinantly produced nanobodies equipped with a Tub-tag sequence were likewise used to prepare nanobody-based immunoprecipitation tools and superresolution probes (56, 57).

Formylglycine-generating enzyme

Formylglycine-generating enzymes allow the post-translational modification of cysteine or serine residues within distinct consensus motifs ((C/S)*X*P*X*R) to produce formylglycine. Such aldehydes are of particular interest as they can selectively react with hydrazides and amino-oxy moieties for site-specific modification of proteins (58, 59). This approach was used to install an aldehyde motif on two nanobodies that recognize different epitopes on human β_2-microglobulin (60). This method allowed the preparation of C-to-C–linked homodimer nanobodies using an unusual method: aqueous solutions of formylglycine-containing nanobodies and bivalent hydrazide or aminoxy linkers were frozen at −20 °C. This reduction in temperature and freezing drastically increased the rate of dimer formation. Using a sequential approach in which one nanobody was reacted with an excess of the bivalent linker, followed by the addition of the second nanobody, yielded heterodimeric bivalent C-to-C–linked conjugates that were superior in antigen binding relative to C-to-N–linked dimers provided by simple genetic fusion.

Butelase 1

Butelase 1 is also a cysteine protease of the AEP family found in the seed pods of *Clitoria ternatea*. It recognizes a C-terminal Asn/Asp-containing tripeptide motif, Asn/Asp-His-Val, to form an Asn/Asp-Xaa-Yaa peptide bond, where Xaa can be any amino acid and Yaa is a hydrophobic residue. Butelase 1 is more than 10,000 times faster than other known ligases, with catalytic efficiencies of up to 1,340,000 $\text{M}^{-1}\ \text{s}^{-1}$ (61, 62). This unique characteristic has made butelase 1 a powerful tool for the preparation of cyclic peptides and proteins or for the direct labeling of a protein (61, 63). Our group used this enzyme together with sortase A to prepare homodimeric and heterodimeric nanobody conjugates connected via DNA linkers (64). The use of dsDNA as linker between two nanobodies imparts rigidity on the linkage and is a straightforward method to

control the length of spacing. Such control can be precious for biophysical studies (65). Although butelase 1 is an attractive enzyme for protein engineering, a major drawback remains its availability. Despite new approaches to produce it in bacteria (66, 67), the main source of butelase 1 remains its extraction from seed pods of *Clitoria ternatea* (62).

Sortase A

Sortase A is an enzyme from *Staphylococcus aureus* that recognizes the amino acid sequence LP*X*TGG with high specificity (68). This tag can be placed at the C terminus of the protein of interest but also internal to its sequence, as long as the recognition tag remains accessible (69). After recognition, sortase A cleaves between the threonine and glycine residue to form a thioacyl intermediate. An N-terminal polyglycine equipped with a payload of choice can attack this intermediate and form a new peptide bond (70). We have used this approach to prepare diverse nanobody conjugates: nanobody dimers through C-to-C fusion (71), bispecific nanobodies against GFP and mouse class II major histocompatibility complex (71), nanobodies labeled with radionuclides for PET imaging *in vivo* (72, 73), nanobody-drug conjugates against B-cell lymphoma (74), and many fluorescently labeled versions (73). The orthogonality of sortase A and butelase 1 allowed the preparation, in a one-pot reaction, of C-C fusion nanobody dimers linked together with PEG and oligonucleotide linkers (64). Proteins equipped with a suitably exposed stretch of Gly residues at the N terminus can be labeled with LP*X*TG-based peptides in a similar way (75).

Native chemical and expressed protein ligation

Native chemical ligation links unprotected polypeptides through an amide bond that relies on the reaction of a C-terminal thioester with an N-terminal Cys. Expressed protein ligation is based on the naturally occurring splicing of proteins, which proceeds via formation of a thioester intermediate (76). The protein of interest is expressed as a fusion with a mutant version of an intein. Activation with a thiol-containing small molecule, such as 2-mercaptoethanol, generates a C-terminal thioester on the protein of interest, such as a nanobody. Using this thioester, native chemical ligation can be used to attach the desired Cys-containing moiety to the nanobody. The expressed protein ligation approach was used to install on nanobodies two distinct arginine-rich cell-penetrating peptides (CPPs) for comparison. These synthetic CPPs contained D-amino acids, are cyclized, and are therefore impossible to introduce by standard genetic means (48). After attachment of the CPPs to an anti-GFP nanobody, these conjugates were delivered to the interior of the cell with an efficiency of up to 95% of cells, in different cell lines, and at relatively low concentrations (10 μM). Nucleolar localization caused by CPP-nucleic acid interactions could be avoided by attaching the CPP via a disulfide linkage.

Unnatural amino acid incorporation using stop codon suppression

Introduction of an unnatural bio-orthogonally functionalized amino acid can be achieved by using the cellular translational machinery and reassignment of a stop codon (77, 78). This new functional group then enables conjugation of a payload of choice in a site-selective manner. For example, a bifunctional unnatural amino acid derivative was introduced into an anti EGFR nanobody. The modified amino acid (AmAzZLys) contains an aryl amine and an azido group, which allows the conjugation of two different probes in orthogonal and selective fashion. Moreover, the azide moiety was used to perform a photoinduced cross-linking reaction to EGFR upon antigen binding (79). Although attractive in principle, codon reassignment remains technically demanding and often suffers from reduced yields compared with conventional production methods.

Targeting membrane proteins

The high stability, propensity to bind and stabilize specific receptor conformations, and ease of production by recombinant expression make nanobodies well-suited for studying membrane proteins. These characteristics have inspired investigators from a variety of disciplines to use nanobodies. Immense efforts have gone into the identification of membrane protein–binding nanobodies. These efforts have positioned nanobodies as useful reagents for structural studies of membrane proteins using cryo-EM (80, 81) in a way analogous to past work with X-ray crystallography (10) (see also Fig. 3).

Visualization of the trafficking of membrane proteins using monovalent nanobodies avoids cross-linking–induced artifacts that can arise from the use of bivalent antibodies (82, 83). Nanobodies that bind Igs from mice (84), rabbits (84), pigs (85), and humans (86) can facilitate their use in place of conventional secondary antibodies (87). Nanobodies have also proven useful for affinity purification of delicate membrane protein complexes (6, 88), but these are properties they share with conventional antibodies of similar specificity.

Structural biologists are well-aware of the ability of nanobodies to facilitate crystallization of otherwise difficult-to-crystallize proteins. Nanobodies frequently contribute to crystal-packing contacts that facilitate structure determination (Fig. 3*A*). Structures where even two nanobodies bind to a single polypeptide have been produced (Fig. 3*B*). The binding of nanobodies to discontinuous epitopes that span more than one protein can also facilitate crystallization of the nanobody-bound complex (Fig. 3*C*). The single-domain nature of nanobodies implies that the universe of epitopes they can sample overlaps with, but is distinct from, that of conventional Igs. These unconventional modes of nanobody-antigen interaction should inspire new modes of application in biological settings, such as nanobody-induced target heterodimerization and target-induced nanobody dimerization. Moreover, such nanobodies can stabilize a receptor in a particular, functionally relevant conformation, as shown for various GPCRs and bacterial proteins. Structural studies have benefited from the use of nanobodies as chaperones, most notably for membrane proteins such as GPCRs (89–91). In most cases, GPCR-binding nanobodies generated for structural studies bind the cytoplasmic face of the protein (89, 92, 93) and have visualized different conformers of the GPCRs to which they bind. These features

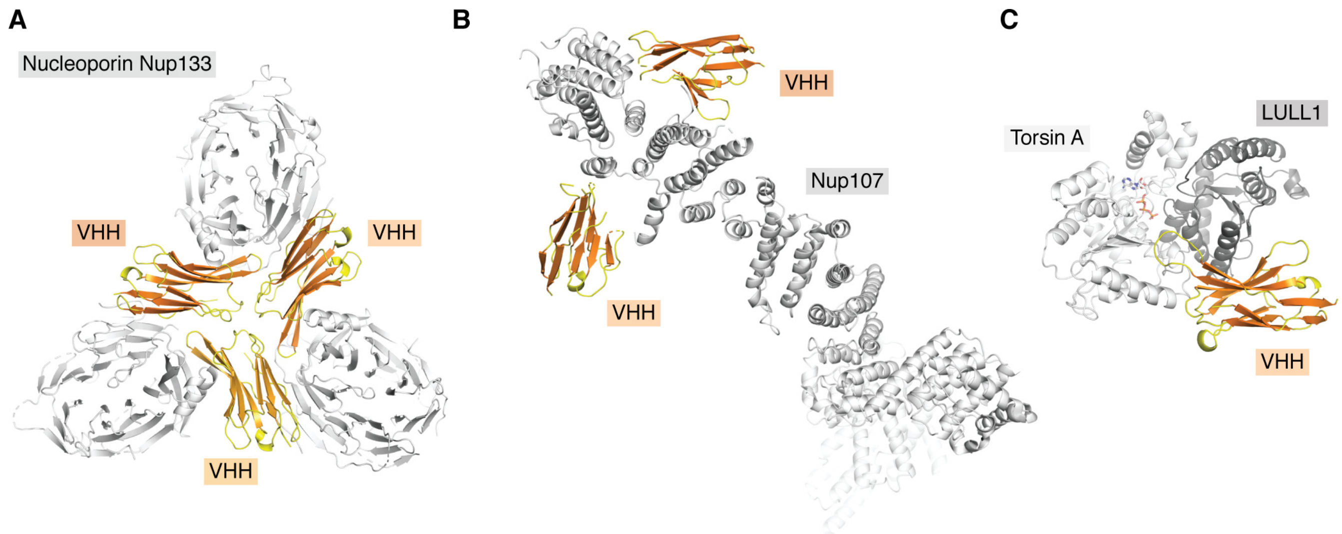

Figure 3. Three examples of nanobodies used as crystallization chaperones. *A*, in the structure of nucleoporin Nup133 from *S. cerevisiae*, three nanobodies (*shades* of *orange*) generated the critical packing interface necessary to build up the crystal lattice (PDB code 6X04). *B*, in the structure of the nucleoporin complex of Nup107 and Nup133 from *H. sapiens* two different nanobodies that bind the Nup107 moiety in separate locations were co-crystallized (PDB code 6X03). *C*, in the TorsinA-LULL1 complex structure, the nanobody recognizes both binding partners and binds neither TorsinA (*white*) nor LULL1 (*gray*) individually (PDB codes 5J1S and 5J1T).

have been exploited by intracellular expression of GPCR-binding nanobodies, as will be discussed below. Nanobodies that bind to the extracellular domain of GPCRs facilitated crystallization of metabolic glutamate receptor-2 (mGluR2) (94) and of the apelin receptor (95). The latter enabled the first identification of nanobodies that activate a GPCR (25, 95). Bacterial membrane proteins have also been trapped by conformation-specific nanobodies, both for structural characterization (96, 97) and for inhibition of their activity (98). Several accounts and reviews cover the development of nanobodies as reagents for structural studies (10) and their use as chaperones (2, 90). A comprehensive overview of structures containing nanobodies was published (99), and a regularly updated database is available online (100).

Conformation-specific nanobodies that bind the cytoplasmic face of GPCRs have been valuable for structural studies of GPCRs in distinct conformations (101–105). When expressed in cells, they can serve as sensors to report on the localization of active receptors. Several mGluR2-binding nanobodies that bind the receptor ectodomain act as positive allosteric modulators and sensitize the receptors to respond to subthreshold levels of glutamate (94, 106). A nanobody that binds the extracellular face of CXCR4 reports on conformational changes induced by small-molecule allosteric modulators (107). The use of nanobodies that lock β_2-adrenergic receptor (β2AR) into active or inactive receptor conformations allowed identification of small-molecule agonists, antagonists, and inverse agonists using binding assays (108). The high yield of nanobodies from recombinant expression systems and the variety of chemical functionalization methods available have enabled the synthesis of conjugates between nanobodies and other complex molecules. A small-molecule ligand for mGlu2R was tethered to the receptor through a GFP-specific nanobody that recognized GFP grafted onto the receptor(109). The use of a photoactivatable ligand provided reversible photocontrol of receptor activation on a time scale of seconds. In another approach, using a combination of enzymatic labeling and click chemistry, a truncated peptide ligand (PTH_{1-11}) for the parathyroid hormone receptor (PTHR) was conjugated to a PTHR-specific nanobody (Fig. 4) (110). Conjugation of a suboptimal peptide ligand to the nanobody enhanced the potency of the peptide by >100-fold in some cases and improved selectivity for one PTHR subtype over another. In a separate set of studies, the nanobodies that recognize surface proteins on antigen-presenting cells were conjugated to weakly immunogenic peptides, including those with nonnatural amino acids. These conjugates showed dramatically enhanced immunogenicity relative to free peptides *in vitro* and *in vivo* (111, 112). It was thus possible to generate antibodies against the otherwise poorly immunogenic cyclotides, heavily disulfide-bonded circular peptides found in plants (113).

Targeting extracellular proteins

Many nanobodies were developed to target soluble extracellular proteins. The list of such targets continues to expand at a rapid pace. The only nanobody currently approved for clinical use targets the secreted protein von Willebrand factor to treat a blood-clotting disorder (114). More nanobodies will find application as therapeutics and diagnostics, as evidenced by a wealth of preclinical data, some of which are summarized below. Nanobodies' small size endows them with a short circulatory $t_{1/2}$ and superior tissue penetration. These two features in combination distinguish nanobodies from full-size conventional Igs and will determine the investigative and therapeutic areas to which they can be applied. One ongoing challenge is to identify targets, either extracellular or otherwise, for which nanobody application has advantages over conventional antibodies.

Nanobodies can be used to block a variety of biological processes, such as placental growth factor-induced angiogenesis in cancer (115); the action of inflammatory proteins, such

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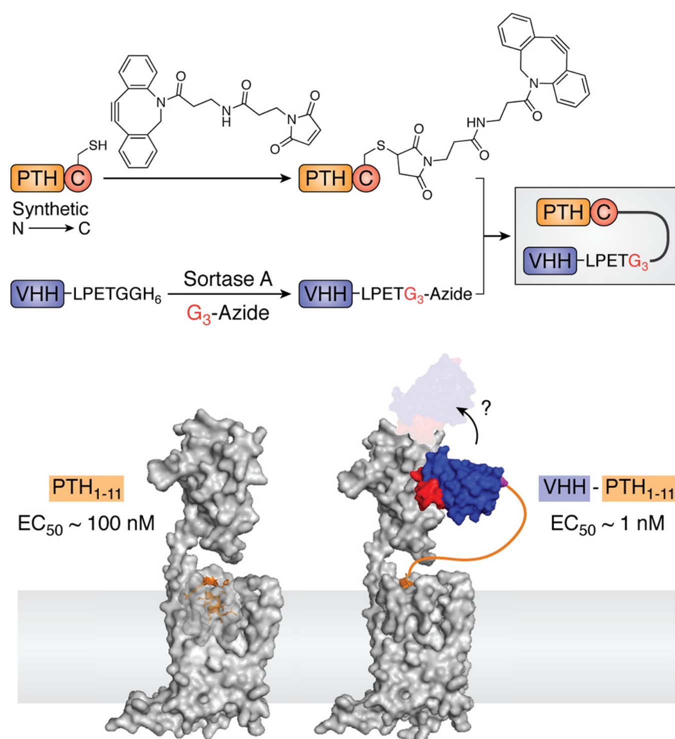

Figure 4. Nanobody-ligand conjugates to target a G protein–coupled receptor. Synthetic fragments of parathyroid hormone were site-specifically linked to nanobodies to provide conjugates (*bottom right*) with biological activity (EC_{50}) superior to the free ligand (*bottom left*). Structures are based on human parathyroid hormone receptor (*gray*) in complex with PTH (*orange*) (PDB code 6FJ3) and a generic VHH (*blue*) with complementarity-determining regions highlighted (*red*) (PDB code 3K1K). The binding of the nanobody to PTHR1 (*bottom right*) is shown in two possible orientations as the actual site of binding is unknown.

as tumor necrosis factor, interleukin-23, granulocyte colony–stimulating factor, and macrophage migration inhibitory factor (116–120); and the action of various toxins and venoms (121–123). A chimeric heavy chain–only antibody consisting of a proprotein convertase subtilisin/kexin type 9–binding nanobody and a portion of the human immunoglobulin heavy chain lowers low-density lipoprotein levels when administered in transgenic rats (124). Nanobodies against neuronal tau (125), human prion protein (126), and α-synuclein (127) provided insight into structural transitions that lead to amyloid formation (126) and served as sensors to differentiate between fibrils at characteristically different stages (127). Nanobodies raised against β_2-microglobulin, a protein for which mutations frequently lead to amyloidosis, have illuminated structural features of aggregation intermediates in mutant versions of β_2-microglobulin (128, 129). They can prevent amyloid formation (130) and remove β_2-microglobulin from blood to treat dialysis-related amyloidosis (131). Gelsolin, a protein for which mutations lead to aberrant proteolytic processing and the formation of amyloidogenic fragments, has likewise been targeted with nanobodies. Nanobodies that bind gelsolin prevent proteolysis, either extracellularly or in the secretory pathway and reduce amyloidosis (132, 133). *In vivo* delivery of gelsolin-binding nanobodies using a viral vector reduces the amyloid burden in a mouse model (134). Anti-gelsolin nanobodies have also been used to visualize gelsolin amyloid deposits by SPECT/CT (135).

Early evaluation of nanobodies raised against carbonic anhydrase and amylase demonstrated inhibitory activity for several of them (136), encouraging further experiments to deploy nanobodies to modulate enzyme activity. Nanobodies that bind to and inhibit the protease urokinase-type plasminogen activator (uPA) (137–139), which can contribute to cancer metastasis, may find clinical application. Crystallization of complexes between uPA and nanobodies shows how substrate binds and reveals the conformational equilibria that contribute (137, 139). An inhibitory nanobody against matrix metalloprotease-8, one of ~25 matrix metalloprotease family members in mammals that contribute to inflammatory responses, provides protection against pathological inflammation induced by lipopolysaccharide (140). Nanobodies that bind β-secretase affect enzyme function, with two nanobodies increasing and one inhibiting activity (141). Injection of the inhibitory nanobody directly into the cerebrospinal fluid decreased deposition of β-amyloid as a result of β-secretase inhibition in a mouse model of Alzheimer's disease (141). γ-Secretase, also relevant for Alzheimer's disease, has likewise been targeted for inhibition by nanobodies (22). Nanobodies raised against plasminogen activator inhibitor-1 induced a profibrinolytic effect through stimulation of protease activity via neutralization of the protease inhibitor (142, 143). Nanobodies that bind thrombin-activatable fibrinolysis inhibitor (procarboxypeptidase U) block protease activation and thereby promote fibrinolysis (144). Combined, these examples demonstrate the versatility of the various nanobody platforms in their application to extracellular space.

Targeting intracellular proteins

Many nanobodies require neither glycosylation nor disulfide bond formation to retain their antigen-binding properties. They can thus be expressed as targeting reagents in the reducing environment of the cytosol. Conventional antibodies and their fragments mostly rely on their entry into the endoplasmic reticulum for assembly and glycosylation. The cytosol precludes association of immunoglobulin heavy and light chains, thus compromising their intracellular assembly into a functional unit. Intracellular nanobodies are typically introduced through transfection of DNA. This allows expression of nanobodies in either a constitutive or an inducible manner. Nanobodies can be expressed as monomeric units to modulate the activity of their targets upon binding or as fusions with fluorescent proteins or taggable protein domains for visualization of targets. Nanobodies, their variants, and fusions have also been used to redirect protein localization, induce protein degradation, and serve as biological sensors of protein conformation, abundance, and localization. Nanobodies that target nuclear proteins are often equipped with a nuclear localization sequence, although this is not always required (145). One of the main bottlenecks restricting the deployment of nanobodies in cells is the paucity of intracellular target-specific nanobodies. We provide summary of many relevant examples below without making claims as to completeness of the list provided (Table 1).

Transfection-based approaches do not allow direct installation on nanobodies of bright organic fluorophores, which

Table 1
Summary of nanobodies used to target intracellular proteins
Note that this table does not include nanobodies used primarily for structural studies or those that target viral proteins and secreted proteins. The use of GFP-targeting nanobodies in this context is discussed in the text.

Intracellular target (function)	Species[a]	Application and biological impact	Reference
Cytoplasmic proteins			
STAT3 (transcription factor)	H	Slows breast cancer growth *in vitro* and *in vivo*	151
TUT4 (uridyltransferase)	H, M	Blocks microRNA uridylation and degradation	152
Calcium-dependent kinase	T	Inhibits kinase activity, crystallization chaperone	153
CapG (actin-capping enzyme)	H	Blocks actin binding, inhibits cancer metastasis	154
HypE (AMPylation)	H	Inhibits or activates enzyme, cellular imaging	155
SpvB (ADP-ribosylation)	S	Inhibits enzyme, blocks cytoskeletal changes	156
ASC (inflammasome adaptor)	H	Interrupts assembly, cellular imaging	160
Roco (GTPase)	B	Destabilizes dimer, enhances GTP hydrolysis	157
RhoA (GTPase)	H	Either inhibits RhoA or tracks localization	19, 161
RhoB (GTPase)	H	Targeted degradation of GTP-bound RhoB	190
$G\beta/\gamma$ (GTPase subunit)	H	Blockade of signaling following GPCR activation	163
L-plastin (actin-bundling protein)	H	Inhibits enzyme function, defective immune synapse formation	164, 165
p53 (tumor suppressor protein)	H	Relocalization to mitochondria or protection from proteasomal degradation	175, 176
H2A/H2B (histone)	Y, M, H	Directs ubiquitination to induce DNA damage signaling, imaging	189
UBC6e (E2 enzyme)	M, H	Enhances enzyme function *in vitro*	158
Dynamin (GTPase)	H	Binds GTP-bound enzyme, visualization of localization	162
Intracellular face of plasma membrane protein			
VGLUT (Glu transporter)	R	Inhibits glutamate transport, visualization	166
P-glycoprotein (transporter)	M	Inhibits function *in vitro*	167
β2AR (GPCR)	H	Binds and stabilizes the receptor active or inactive states for structural studies and visualization	194, 195
Muscarinic acetylcholine receptor (GPCR)	H	Binds and stabilizes the receptor active state for structural studies	92
κ- and μ-opioid receptors (GPCR)	H	Binds and stabilizes the receptor active or inactive states for structural studies and visualization	103, 198, 199
Ca_V1/Ca_V2 (high voltage–activated calcium channel)	H, M, G	Expressed as E3 fusion to ubiquitinate, redirect localization, inhibit function	188

[a]Species of the antigen bound by the referenced nanobody. H, human; M, murine; B, bacterial; S, *Salmonella*; Y, yeast; R, rat; G, guinea pig.

mostly requires chemical methods applied to purified nanobodies. Delivery of labeled nanobodies and other antibody derivatives across the plasma membrane (146) has relied on appending cell-penetrating peptides (48, 147, 148), mutagenesis of nanobody surface residues to increase their positive charge (149), complexation with cell-penetrating mesoporous silica nanoparticles (150), and the use of a microfluidics-based cell permeabilization platform (49). Cell-penetrating nanobodies have been used for imaging (48, 49), to inhibit EGFR function from the cytoplasmic side of the membrane in lung cancer cells (147), and to evaluate the importance of subcellular localization in photosensitizer-induced cell killing (148). The design of methods to deliver proteins such as nanobodies across the plasma membrane is an area of active research, but accumulation of most such exogenously added proteins in the endocytic compartment is difficult to avoid and remains a major confounding factor.

Nanobodies that bind intracellular proteins enable characterization and modulation of proteins of interest and can avoid the need for genetic modification of the target protein. A summary of several nanobodies that target intracellular proteins is shown in Table 1. Targets include mammalian and bacterial proteins; soluble cytoplasmic proteins and those embedded in membranes; and proteins in the nucleus, endoplasmic reticulum membrane, and inner leaflet of the plasma membrane. Nanobodies can inhibit intracellular signaling proteins, such as the transcription factor STAT3 (151), the uridyltransferase TUT4 (152), apicomplexan calcium-dependent kinase (153), the actin-capping protein CapG (154), the AMPylation enzyme HypeE (155) and the *Salmonella* ADP-ribosylation enzyme SpvB (156). A nanobody that targets an allosteric site on a bacterial LRRK2 GTPase homologue modulates oligomerization and enzymatic activity (157). The ER-localized E2 enzyme UBC6e is targeted by a nanobody that augments enzymatic activity without obvious biological consequences (158). A biological sensing platform that relies on the expression of two nanobodies that bind to different epitopes on GFP, each linked to one part of a transcription factor, converts the production of intracellular GFP to a transcriptional output (159).

Intracellular proteins that require oligomerization and those trafficked through the endoplasmic reticulum have also been probed using nanobodies. Co-translational delivery of a nanobody to the endoplasmic reticulum prevents aberrant processing and aggregation of a variant of the secreted form of gelsolin (132). Expression of a nanobody that recognizes ASC, an adaptor protein important in inflammasome assembly, enabled visualization of inflammasome assembly in cells and altered the morphology of assembled inflammasomes (160). Nanobodies raised against the active (GTP-bound) forms of the GTPases RhoA and dynamin enabled tracking of active enzymes in living cells (161, 162). Using these tools, active RhoA was detected at the inner plasma membrane upon overt activation; active dynamin was formed in stochastic bursts associated with membrane fission. A nanobody that binds and inhibits the function of β/γ-subunit complex of the heterotrimeric G proteins showed that blockade of β/γ-subunit function has minimal impact on $G\alpha$ function (163). The expression of nanobodies that inhibit the actin-bundling protein L-plastin uncovered a role for this protein and the T cell integrin LFA-1 in facilitating the formation of the immune synapse (164, 165). Two proteins found in the plasma membrane, VGLUT and P-glycoprotein, are targeted at their cytoplasmic faces by nanobodies that inhibit their function (166, 167).

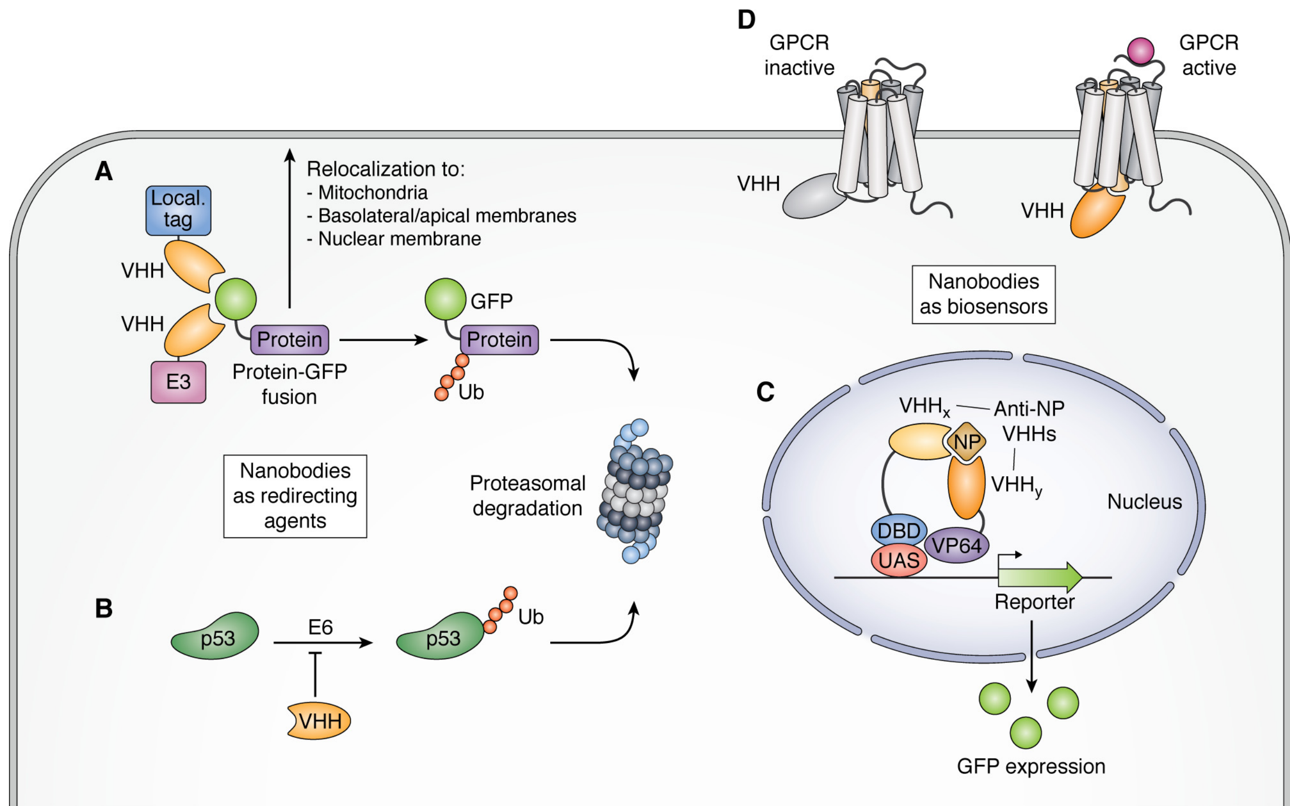

Figure 5. Nanobodies as redirecting and sensing agents in live cells. *A*, use of GFP-binding nanobodies to redirect tagged proteins to subcellular locations or for degradation. *B*, use of a p53-binding nanobody to block HPV E6-mediated ubiquitination and degradation. *C*, use of orthogonal anti-NP nanobodies as biosensors coupled to a transcriptional output (214). The DNA-binding domain (DBD) and VP64 activation domain are separately fused to anti-NP nanobodies (VHHs). *UAS*, upstream activator sequence that binds DBD. Transcription of the reporter gene produces GFP. *D*, use of nanobodies as biosensors to detect active and inactive states of GPCRs.

Intracellular nanobody constructs can be adapted to yield fusion proteins that enable the relocalization, destruction, or enzymatic modification of nanobody-bound targets. The fusion of a nanobody to *O*-GlcNAc transferase enabled directed glycosylation of proteins targeted by the nanobody fusion (168), a post-translational modification that is otherwise widespread. Nanobodies, expressed as fusions with tags that dictate a particular subcellular localization, can redirect the localization of proteins of interest and serve to control protein diffusion. GFP-binding nanobodies routed to subcellular sites have been used to assess the impact of redirecting GFP-tagged targets in living multicellular organisms (Fig. 5*A*) (169–171). Forced mislocalization of the drosophila regulatory myosin light chain, tagged with GFP, to either the basolateral or apical membrane, caused an alteration in the shape of wing cells or aberrant sibling cell asymmetry in neural cells (169, 170). Membrane anchoring of a protein essential for the development of cellular polarity in *Caenorhabditis elegans* revealed the importance of clustering for asymmetry (171). This approach has been used to redirect mRNAs engineered to contain the GFP-binding binding sequence MS2, which showed that forced mRNA relocalization also caused protein relocalization (172). In a variation on this approach, a secreted protein was tethered to the cell surface, with rates of diffusion controlled by the strength of the nanobody-epitope interaction employed, to assess the importance of local and distal action of secreted proteins (173, 174). This approach showed that diffusion of a secreted morphogenic protein in *Drosophila* is essential for proper wing patterning (173). A p53-binding nanobody with a mitochondrial localization tag showed that mitochondrial mislocalization led to loss of cell viability in some cases (Fig. 5*B*) (175). A p53-binding nanobody that blocked the degradation of p53 mediated by human papillomavirus E6 protein failed to promote apoptosis (176).

The targeting function of nanobodies can be exploited to link particular substrates to the degradation machinery. The modular nature of nanobodies thus enabled their use in a variety of fusion proteins for targeted degradation of proteins of interest (177). This approach typically involves genetic fusion of a nanobody with a (fragment of a) ubiquitin ligase (E3), which recruits the endogenous ubiquitination machinery to tag nanobody-targeted proteins for degradation by the proteasome. One widely deployed version of this approach relies on a fusion of a nanobody that binds GFP/YFP with an F-box protein domain from the Skp cullin F-box E3 complex, which can degrade GFP/YFP-tagged proteins (Fig. 5*A*) (178, 179). An alternative version uses von Hippel–Lindau (VHL)-nanobody fusions to degrade GFP-tagged proteins (180, 181). Targeted degradation using this approach, in combination with expression under tissue-specific promoters, has enabled evaluation of the role of targeted proteins in specific tissues in *Drosophila* development (182, 183). Tissue-specific degradation of myosin-II showed it is not essential for tracheal elongation or the closure of the dorsal opening

during development of *Drosophila*. Alternative versions of nanobody-mediated target degradation, relying on other E3 fragments, have also been developed for application in zebrafish (184, 185) and *C. elegans* (186). The use of nanobody-E3 fragment fusions to target cell surface ion channels, either as a YFP fusion or as the WT protein, showed that ubiquitylation can have divergent consequences for the trafficking and function of cell surface proteins (187, 188). An E3 fragment–nanobody fusion that bound directly to the histone H2A-H2B protein dimer enabled targeted ubiquitylation of histones and caused signaling associated with DNA damage (189). A nanobody that selectively bound to the active (GTP-bound) form of RhoB GTPase was applied as an F-box fusion to knock down active RhoB. It showed that the GTP-bound fraction of RhoB mediates its role in cell invasion (190). Control of the properties of the E3 fragment–nanobody produced in cells, such as expression level and degradation rate, allows quantitative control of cellular protein levels (191).

Specialized, targeted approaches have produced nanobodies that bind to the target only when found in a specific conformation, with a particular emphasis on membrane proteins. These approaches rely on screening with antigens locked into the desired conformation: amyloidogenic protein variants at various stages of self-assembly, complexes formed by protein-protein interactions, and receptors bound to ligands constitute some of the targets of conformation-specific nanobodies (30). They have served as biosensors to visualize the distribution of proteins in a specific conformation in living cells (Fig. 5*D*) (192, 193), with a particular emphasis on GPCRs. A nanobody raised against the β2AR, used as a chaperone to facilitate its crystallization (89), binds to the cytoplasmic face of β2AR and stabilizes its active state, much like a G protein would. This same nanobody, when expressed as a fluorescent fusion protein served as a biosensor to visualize ligand-bound β2AR in its active state (194). Surprisingly, activated β2AR was found both at the plasma membrane and in early endosomes. Further characterization of other β2AR-binding nanobodies identified one that bound to and stabilized the inactive form of β2AR (101, 195). This assembly of nanobodies enabled the classification of several β2AR ligands as agonists, antagonists, or inverse agonists (101, 108). Certain β-adrenergic receptor ligands affect the conformation of receptor molecules found in the Golgi, suggesting that receptors *en route* to the cell surface can be activated by cell-permeable ligands (196). This possibility is of interest also in view of the exclusive Golgi localization of GPCRs such as GPR107 (197). Conformation-specific nanobodies have been applied for similar applications to the muscarinic acetylcholine receptor (92) and for the κ- and μ-opioid receptors (103, 198, 199). Although these conformation-specific nanobodies are restricted to the indicated receptors, study of a nanobody specific for the κ-opioid receptor in its active state showed that the intracellular loop fragment responsible for nanobody binding could be grafted onto other GPCRs with retention of binding, highlighting the exciting possibility of designing receptor chimeras bound by conformation-specific nanobodies (103). This select set of examples shows that intracellular expression of nanobodies directed against cytoplasmic targets clearly is a feasible approach to modulate cellular functions. However, considerable efforts must be expended to identify nanobodies with the desired properties. As methods for the production of large and completely synthetic nanobody libraries improve, the need for deliberate immunization of animals is reduced. Synthetic libraries may also make it possible to obtain nanobodies against proteins that are not immunogenic in camelids.

Targeting viral proteins

Nanobodies have been used to target viral proteins. Inhibition of viral entry by nanobodies is well-documented (200, 201). The recent description of camelid-derived nanobodies capable of neutralizing SARS-CoV-2, the coronavirus responsible for the COVID-19 pandemic, is one such example (202, 203). There are fewer examples of nanobodies that target cytoplasmic viral proteins. Because infected cells produce cytoplasmic proteins required for proper virus replication, assembly, and release, nanobody-mediated interference with intracellular viral proteins requires cytoplasmic expression as well. Inhibition of viral polymerases by a cytoplasmically expressed nanobody inhibits influenza propagation (204). A nanobody that blocks multimerization of HIV REV causes defects in viral RNA transport and inhibits virus production (205). HIV NEF is a nonstructural protein that alters protein trafficking in infected cells, and a nanobody directed against NEF blocks these effects (206). Nanobodies that target viral nucleoproteins (NPs) inhibit viral fitness by several mechanisms, including indirect inhibition of polymerase function (207), inhibition of nuclear import of viral ribonucleoproteins (208), and disruption of virus assembly by NP cross-linking with dimeric nanobodies (209). A nanobody that binds grapevine fanleaf virus capsid protein provides resistance to the virus when expressed in plant cells (210, 211) These few examples show that cytoplasmically expressed viral proteins can serve as excellent nanobody targets to explore aspects of viral replication that might be more challenging to study otherwise. For example, the many essential roles of influenza NP have made a dissection of its various functions difficult by standard mutagenesis. The use of NP-specific nanobodies has uncovered novel aspects of nuclear localization (208).

Nanobodies that target viral proteins can be turned into tunable biological tools and sensors. A nanobody-fluorescent protein fusion that recognized the HIV capsid protein p21 was deliberately destabilized by introducing mutations that resulted in rapid degradation of the fusion protein (212). This fusion was stabilized upon binding of p21, thus providing a fluorescent reporter of HIV infection (212). Intracellular expression of influenza NP-specific nanobodies enabled the tracking of viral distribution (213). Use of a pair of orthogonal anti-influenza NP nanobodies linked to different domains of a transcription factor, which must be brought into proximity to function, provided a method in which transcription of a fluorescent protein reporter is controlled by the presence of NP (Fig. 5*C*) (214). A nanobody that blocks nuclear import of influenza ribonucleoproteins through occlusion of interactions of the viral nuclear localization sequence with its host receptor was repurposed as a way to control viral infectivity: engraftment of the nuclear

Table 2
Summary of nanobody-epitope tag pairs and applications
Listed affinities were determined using a variety of methods as described in the listed references.

Name	Affinity	Sequence/applications	Notes	References
	nM			
EPEA	190	EPEA/affinity purification, flow cytometry	Tag must be at C terminus, cross-reacts with α-synuclein, marketed as C-tag	127, 168, 218
myc	ND	EQKLISEEDL/enzyme-linked immunoassay	Not extensively characterized	219
BC2	2–6	PDRVRAVSHWSS/affinity purification, immunoblotting, (superresolution) microscopy	Cross-reacts with endogenous β-catenin, marketed as Spot-tag	4, 220
6E tag	1	QADQEAKELARQIS/affinity purification, immunoblotting, cell surface tethering of GPCR ligand	Reacts with endogenous UBC6e	110, 158
Alfa	0.02	SRLEEELRRRLTE/affinity purification, immunoblotting, superresolution microscopy, target relocalization in live cells	Tag adopts α-helical structure	6, 221, 223
Moon	30	KNEQELLELDKWASL/live-cell microscopy	Tag derived from HIV gp41	221
PepTag	0.6	AVERYLKDQQLLGIW/immunoprecipitation, live-cell microscopy	Tag derived from HIV gp41, helical, previously named VHH 2E7	239, 240

localization sequence onto the nanobody itself restored infectivity (215).

Epitope tags

Antibodies that target peptide fragments of less than 20 amino acids, commonly called epitope tags, have been widely applied to target tagged proteins of interest in a variety of assays, including microscopy, immunohistochemistry, immunoblotting, and immunoprecipitation experiments (216). Such epitope tags are valuable when high-quality antibodies or nanobodies against the protein portion of the tagged protein are lacking (7) and to avoid cross-reactions with related proteins. Small epitope tags can be preferable to the use of GFP, as the presence of a GFP moiety can impact protein behavior (217). Only a handful of nanobodies that recognize short peptides suitable as epitope tags have been reported thus far. The reason for the scarcity of nanobodies that recognize epitope tags in a variety of settings is unknown but most likely relates to the often convex nature of their antigen-binding portion (paratope) and the need to achieve the necessary buried surface area upon binding of antigen through interaction with a single variable region, instead of the conventional V_H-V_L pair. The reported nanobody-peptide epitope pairs are summarized in Table 2.

A nanobody (NbSyn2) raised against α-synuclein binds to the extreme C terminus of the protein, involving the four terminal amino acids (EPEA) (127, 218). Although the tetrapeptide epitope tag is the smallest nanobody tag reported, the NbSyn2-EPEA interaction is of lower affinity (K_d ~190 nM) than other nanobody-epitope tag pairs and requires that EPEA be placed at the C terminus (127). There is a report of nanobodies that recognize the myc tag (219) derived from the c-Myc protein, but the affinities of these nanobodies have not been reported, and they have not been widely tested. A different nanobody-epitope tag pair resulted from characterization of nanobodies raised against β-catenin (BC2 nanobody) (4, 5). The BC2 nanobody binds to a 12-residue epitope with high affinity (K_d ~2-6 nM). This tag can be placed at the N or C terminus of the protein of interest with retention of binding (4) and has been used in proteomics and superresolution microscopy studies. Optimal results in the latter application required the use of a homodimeric nanobody construct to increase avidity (4, 220). A nanobody that recognizes a 14-mer peptide with high affinity (K_d ~1 nM), derived from the endoplasmic reticulum-localized protein UBC6e (6E tag) is nanobody VHH05 (158). Incorporation of the 6E tag into the extracellular portion of a GPCR enabled efficient delivery of ligands linked to VHH05 to a tagged receptor, resulting in potent activation (110). Most nanobody-recognized epitopes are derived from endogenous cellular proteins and can be used in a heterologous setting but may cause high background in applications where the endogenous protein is present. This complication was circumvented by generating a nanobody (NbAlfa) that binds with very high affinity (K_d ~20 pM) to a synthetic 14-mer helical peptide (Alfa tag) not found in nature (6). NbAlfa was used for affinity purification, high-sensitivity immunoblotting, and superresolution microscopy (6). Whether the introduction of a tag with a strong propensity toward helical structure and book-ended by proline residues disrupts the structure and function of proteins engineered to contain it must be determined on a case-by-case basis.

In a system designed to evaluate translation kinetics in live cells, efforts to identify nanobody-epitope tag pairs that function in live cells evaluated seven different candidate nanobody-epitope tag pairs. Only a single nanobody-epitope tag pair was found to function in cells (221). This pair (dubbed "moon tag") consists of a nanobody raised against the HIV envelope protein gp41 and a 15-mer peptide that interact with an affinity of ~30 nM (222). The moon tag was used along with an orthogonal epitope recognition pair based on an scFv antibody (named "sun tag") to demonstrate a high degree of stochasticity and variation in translation kinetics in live cells (221). Both the moon tag and the alfa tag system can be used to deliver a nanobody-fluorescent protein fusion to a subcellular site of choice (nucleus, mitochondria, cell membrane) when the nanobodies carry the appropriate targeting signals (223). The orthogonality of the moon tag and alfa tag suggests the potential for multiplexing (223). The VHH05-6E pair can also efficiently redirect subcellular protein localization.[4] An important consideration for the selection of tag-specific nanobodies is a lack of cross-reactivity with the species of origin in which these nanobodies will be used. Nanobodies against pathogen-specific antigens may provide a possible source of such reagents.

[4] N. McCaul, J. Ling, and H. L. Ploegh *et al.*, unpublished observation.

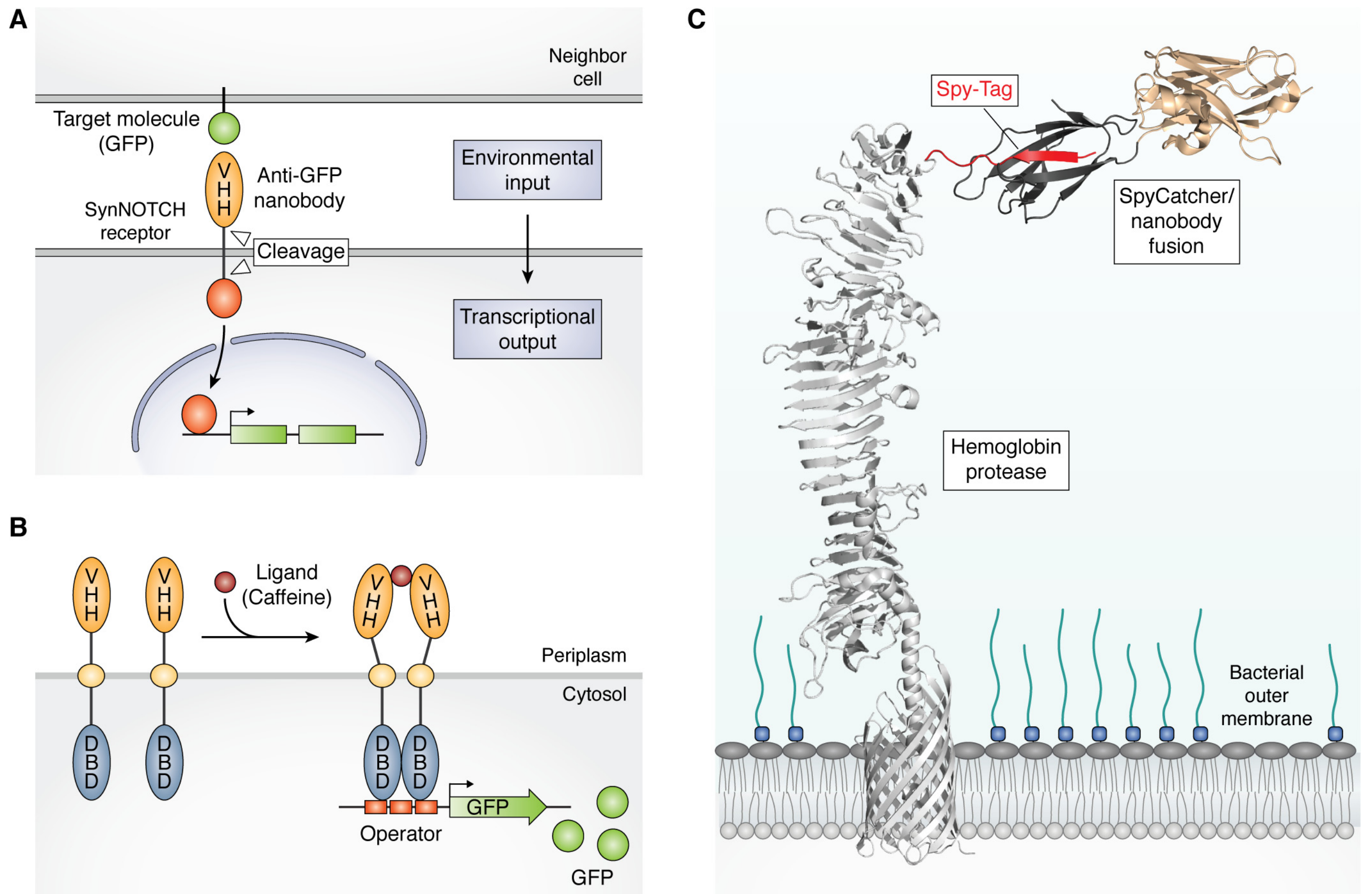

Figure 6. Application of nanobodies in synthetic biology. *A*, use of nanobodies as recognition elements in programmable synNotch constructs (225). Upon ligand binding, sometimes found on the surface of neighbor cells, synNotch receptors undergo a conformational change that promotes cleavage and release of an intracellularly linked transcription factor. *B*, nanobodies as recognition elements in bacterial constructs for sensing of extracellular ligands (226). The addition of caffeine causes clustering of extracellular single-domain antibodies and subsequent assembly of split DBDs and a transcriptional output. *C*, scheme for attachment of nanobodies to bacterial outer membranes or OMVs using SpyCatcher/SpyTag (229). An outer membrane protease, hemoglobin protease (*gray*), is linked to SpyTag (*red*). A SpyCatcher/nanobody fusion (*black*/*gold*) covalently labels the hemoglobin protease-SpyTag fusion.

Synthetic biology with nanobodies

The adaptable and modular nature of nanobodies makes it possible to reprogram, or report on, cell function. Two widely deployed examples of this approach are the targeted degradation of cellular proteins tagged with GFP (discussed above) and the expression of nanobody-containing chimeric antigen receptors in T cells (CAR-Ts) (reviewed recently (224)). Many of the applications of nanobodies in synthetic biology described below rely on widely deployed clones that target GFP or viral proteins. Extension of these approaches to epitope tag–targeting nanobodies will expand their adaptability.

One goal of synthetic biology is to allow cells to bind or respond to molecules for which they do not have a natural receptor. This goal can be achieved through expression of cell surface protein fusions that contain nanobodies that bind to a molecule of interest. Adaptation of the NOTCH signaling pathway through introduction of a nanobody-sensing element enabled initiation of transcriptional responses in mammalian cells in the presence of extracellular GFP (Fig. 6*A*) (225). An analogous platform developed in bacteria used a nanobody fused to a dimerizable DNA-binding domain, which then enabled caffeine-stimulated induction of transcription (Fig. 6*B*) (226). Modification of a bacterial flagellar protein by insertion of a GFP-binding nanobody yielded bacteria that bound GFP with retention of flagellar structure (Fig. 6*C*) (227). Other bacterial outer membrane proteins could also be expressed as fusions with a GFP-binding nanobody (228). Modification of the bacterial hemoglobin protease gene yielded membrane vesicles (OMVs) equipped with a recognition tag for SpyCatcher that allowed production of nanobody-modified OMVs (229). The ability to equip bacteria or their outer membrane vesicles with nanobodies sets the stage for delivery to desired biological sites through nanobody targeting. A glycosylphosphatidylinositol-anchored version of a nanobody that binds to HIV gp41 protects susceptible cells from HIV infection, even where the free nanobody itself fails to protect (230). Nanobodies localized at the cell surface can trap secreted proteins near their site of secretion (169, 173). Variation in the strength of the interaction between cell surface nanobody and secreted protein can then be used to control protein diffusivity (174).

Nanobody variants that are unstable in mammalian cells unless bound to their target can report on relative protein abundance (212). Several nanobody constructs whose function or localization can be controlled by light or small molecules have been reported. This approach can achieve improved spatial (localized illumination) and temporal

control of cellular function relative to processes that require transcription and translation, which take much longer. The fusion of nanobodies with proteins that engage in light-stimulated interactions with binding partners enables light-directed routing of nanobodies or nanobody-bound targets to large clusters formed by protein hetero-oligomerization (231) or to specific subcellular compartments (232). In these examples, illumination controls only the nanobodies' localization and therefore access to targets with restricted subcellular localization. An alternative strategy hinges on coupling illumination with nanobody function directly. Proteins that heterodimerize upon illumination have been fused with nanobody fragments to enable light-stimulated binding and inhibition of the nanobody targets by light-induced reassembly of nanobodies inside cells (233). Introduction of a photoresponsive protein into the nanobody framework regions also enabled light-induced or light-inhibited nanobody function, although many constructs retained target binding to some extent even in the absence of illumination (234). To enable small-molecule control of nanobodies, a dihydrofolate reductase variant, which folds only upon ligand binding, was inserted into a nanobody complementarity-determining region (235), which allowed small-molecule control of nanobody binding. A different strategy for generating photoresponsive nanobodies relies on stop codon suppression to incorporate a nonnatural photocaged tyrosine residue into a nanobody complementarity-determining region (236, 237). Nanobodies with an appropriately placed caged tyrosine, produced through recombinant expression in bacteria, failed to bind to targets expressed on the cell surface, but upon irradiation, binding was restored.

Concluding remarks

Properties unique to nanobodies make them useful tools, with features that differentiate them from conventional antibodies and synthetic recognition modules. Nanobodies have proven their worth as chaperones for structural studies, particularly in the study of membrane proteins such as GPCRs (10). Expanding efforts to apply nanobodies as intracellular reagents and to equip nanobodies with synthetic moieties using chemical and enzymatic methods are opening up exciting new possibilities.

Nanobodies appear particularly well-suited to report on cellular events that happen on a rapid timescale, where reporters based on transcription and translation may be too sluggish. The specialized properties of nanobodies have allowed insight at high temporal resolution into complex and stochastic events, such as the dynamics of protein translation (221), endomembrane trafficking (162), and receptor conformational changes (103). Designs that turn on or off the function of nanobodies with light (233) or small molecules (235) will allow unprecedented temporal control over processes such as protein degradation (191), subcellular relocalization (175), and even post-translational modifications (168). This strategy relies on the availability of nanobodies that target proteins of interest in the context of the cytoplasm, of which there is a substantial but still limited selection. New screening methods to identify nanobodies against new targets will allow extension to other targets. The emergence of nanobodies that bind epitope tags in the cytoplasm will help to bridge this gap (223).

Chemical and enzymatic labeling strategies applied to nanobodies provide semisynthetic conjugates with properties not achievable without this combination. The use of nanobodies chemically modified with moieties for visualization as *in vivo* imaging agents is an example (1). The conjugation of nanobodies with synthetic ligands for cell surface receptors provides a path toward conjugates with improved receptor selectivity (110) and light-responsiveness (109). The ability to site-specifically modify nanobodies at more than one position should enable further elaboration (55). The ability to prepare nanobodies through chemical synthesis would permit the ultimate level of control for nanobody chemical functionality (238). The unique properties of nanobodies have firmly secured their place in the biochemist's toolbox, with yet more exciting and original applications to emerge.

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Abbreviations—The abbreviations used are: scFv, single-chain variable fragment; GPCR, G protein–coupled receptor; AEP, asparaginyl endopeptidase; *Oa*AEP1, *Oldenlandia affinis* asparaginyl endopeptidase; TTL, tubulin tyrosine ligase; CPP, cell-penetrating peptide; EGFR, epidermal growth factor receptor; mGluR2, metabolic glutamate receptor-2; PTHR, PTH receptor; uPA, urokinase-type plasminogen activator; YFP, yellow fluorescent protein; β2AR, β_2-adrenergic receptor; NP, nucleoprotein; OMV, outer membrane vesicle; PDB, Protein Data Bank; DBD, DNA-binding domain.

References

1. Chanier, T., and Chames, P. (2019) Nanobody engineering: toward next generation immunotherapies and immunoimaging of cancer. *Antibodies (Basel)* **8,** 13 CrossRef Medline
2. Manglik, A., Kobilka, B. K., and Steyaert, J. (2017) Nanobodies to study G protein-coupled receptor structure and function. *Annu. Rev. Pharmacol. Toxicol.* **57,** 19–37 CrossRef Medline
3. Beghein, E., and Gettemans, J. (2017) Nanobody technology: a versatile toolkit for microscopic imaging, protein-protein interaction analysis, and protein function exploration. *Front. Immunol.* **8,** 771 CrossRef Medline
4. Braun, M. B., Traenkle, B., Koch, P. A., Emele, F., Weiss, F., Poetz, O., Stehle, T., and Rothbauer, U. (2016) Peptides in headlock–a novel high-affinity and versatile peptide-binding nanobody for proteomics and microscopy. *Sci. Rep.* **6,** 19211 CrossRef Medline
5. Traenkle, B., Emele, F., Anton, R., Poetz, O., Haeussler, R. S., Maier, J., Kaiser, P. D., Scholz, A. M., Nueske, S., Buchfellner, A., Romer, T., and

Rothbauer, U. (2015) Monitoring interactions and dynamics of endogenous beta-catenin with intracellular nanobodies in living cells. *Mol. Cell. Proteomics* **14,** 707–723 CrossRef Medline

6. Götzke, H., Kilisch, M., Martínez-Carranza, M., Sograte-Idrissi, S., Rajavel, A., Schlichthaerle, T., Engels, N., Jungmann, R., Stenmark, P., Opazo, F., and Frey, S. (2019) The ALFA-tag is a highly versatile tool for nanobody-based bioscience applications. *Nat. Commun.* **10,** 4403 CrossRef Medline
7. Bruce, V. J., and McNaughton, B. R. (2017) Evaluation of nanobody conjugates and protein fusions as bioanalytical reagents. *Anal. Chem.* **89,** 3819–3823 CrossRef
8. Liu, W., Song, H., Chen, Q., Yu, J., Xian, M., Nian, R., and Feng, D. (2018) Recent advances in the selection and identification of antigen-specific nanobodies. *Mol. Immunol.* **96,** 37–47 CrossRef Medline
9. Comor, L., Dolinska, S., Bhide, K., Pulzova, L., Jiménez-Munguía, I., Bencurova, E., Flachbartova, Z., Potocnakova, L., Kanova, E., and Bhide, M. (2017) Joining the *in vitro* immunization of alpaca lymphocytes and phage display: rapid and cost effective pipeline for sdAb synthesis. *Microb. Cell Fact.* **16,** 13 CrossRef Medline
10. Pardon, E., Laeremans, T., Triest, S., Rasmussen, S. G. F., Wohlkönig, A., Ruf, A., Muyldermans, S., Hol, W. G. J., Kobilka, B. K., and Steyaert, J. (2014) A general protocol for the generation of Nanobodies for structural biology. *Nat. Protoc.* **9,** 674–693 CrossRef Medline
11. Jähnichen, S., Blanchetot, C., Maussang, D., Gonzalez-Pajuelo, M., Chow, K. Y., Bosch, L., De Vrieze, S., Serruys, B., Ulrichts, H., Vandevelde, W., Saunders, M., De Haard, H. J., Schols, D., Leurs, R., Vanlandschoot, P., *et al.* (2010) CXCR4 nanobodies (VHH-based single variable domains) potently inhibit chemotaxis and HIV-1 replication and mobilize stem cells. *Proc. Natl. Acad. Sci. U. S. A.* **107,** 20565–20570 CrossRef Medline
12. Itoh, K., Reis, A. H., Hayhurst, A., and Sokol, S. Y. (2019) Isolation of nanobodies against *Xenopus* embryonic antigens using immune and non-immune phage display libraries. *PLoS ONE* **14,** e0216083 CrossRef Medline
13. Koch-Nolte, F., Reyelt, J., Schössow, B., Schwarz, N., Scheuplein, F., Rothenburg, S., Haag, F., Alzogaray, V., Cauerhff, A., and Goldbaum, F. A. (2007) Single domain antibodies from llama effectively and specifically block T cell ecto-ADP-ribosyltransferase ART2.2 *in vivo*. *FASEB J.* **21,** 3490–3498 CrossRef Medline
14. Rossotti, M. A., Henry, K. A., van Faassen, H., Tanha, J., Callaghan, D., Hussack, G., Arbabi-Ghahroudi, M., and MacKenzie, C. R. (2019) Camelid single-domain antibodies raised by DNA immunization are potent inhibitors of EGFR signaling. *Biochem. J.* **476,** 39–50 CrossRef Medline
15. Eden, T., Menzel, S., Wesolowski, J., Bergmann, P., Nissen, M., Dubberke, G., Seyfried, F., Albrecht, B., Haag, F., and Koch-Nolte, F. (2017) A cDNA immunization strategy to generate nanobodies against membrane proteins in native conformation. *Front. Immunol.* **8,** 1989 CrossRef Medline
16. Peyrassol, X., Laeremans, T., Gouwy, M., Lahura, V., Debulpaep, M., Van Damme, J., Steyaert, J., Parmentier, M., and Langer, I. (2016) Development by genetic immunization of monovalent antibodies (nanobodies) behaving as antagonists of the human ChemR23 receptor. *J. Immunol.* **196,** 2893–2901 CrossRef Medline
17. Tiller, K. E., Chowdhury, R., Li, T., Ludwig, S. D., Sen, S., Maranas, C. D., and Tessier, P. M. (2017) Facile affinity maturation of antibody variable domains using natural diversity mutagenesis. *Front. Immunol.* **8,** 986 CrossRef Medline
18. English, J. G., Olsen, R. H. J., Lansu, K., Patel, M., White, K., Cockrell, A. S., Singh, D., Strachan, R. T., Wacker, D., and Roth, B. L. (2019) VEGAS as a platform for facile directed evolution in mammalian cells. *Cell* **178,** 748–761.e17 CrossRef Medline
19. Moutel, S., Bery, N., Bernard, V., Keller, L., Lemesre, E., de Marco, A., Ligat, L., Rain, J.-C., Favre, G., Olichon, A., and Perez, F. (2016) NaLi-H1: a universal synthetic library of humanized nanobodies providing highly functional antibodies and intrabodies. *Elife* **5,** e16228 CrossRef Medline
20. Salema, V., and Fernández, L. Á. (2017) *Escherichia coli* surface display for the selection of nanobodies. *Microb Biotechnol.* **10,** 1468–1484 CrossRef Medline
21. Kavousipour, S., Mokarram, P., Gargari, S. L. M., Mostafavi-Pour, Z., Barazesh, M., Ramezani, A., Ashktorab, H., Mohammadi, S., and Ghavami, S. (2018) A comparison between cell, protein and peptide-based approaches for selection of nanobodies against CD44 from a synthetic library. *Protein Pept. Lett.* **25,** 580–588 CrossRef Medline
22. Veugelen, S., Dewilde, M., De Strooper, B., and Chávez-Gutiérrez, L. (2017) Screening and characterization strategies for nanobodies targeting membrane proteins. *Methods Enzymol.* **584,** 59–97 CrossRef Medline
23. Doshi, R., Chen, B. R., Vibat, C. R. T., Huang, N., Lee, C.-W., and Chang, G. (2014) *In vitro* nanobody discovery for integral membrane protein targets. *Sci. Rep.* **4,** 6760 CrossRef Medline
24. Crepin, R., Veggiani, G., Djender, S., Beugnet, A., Planeix, F., Pichon, C., Moutel, S., Amigorena, S., Perez, F., Ghinea, N., and de Marco, A. (2017) Whole-cell biopanning with a synthetic phage display library of nanobodies enabled the recovery of follicle-stimulating hormone receptor inhibitors. *Biochem. Biophys. Res. Commun.* **493,** 1567–1572 CrossRef Medline
25. Ren, H., Li, J., Zhang, N., Hu, L. A., Ma, Y., Tagari, P., Xu, J., and Zhang, M.-Y. (2020) Function-based high-throughput screening for antibody antagonists and agonists against G protein-coupled receptors. *Commun. Biol.* **3,** 146 CrossRef Medline
26. Reader, R. H., Workman, R. G., Maddison, B. C., and Gough, K. C. (2019) Advances in the production and batch reformatting of phage antibody libraries. *Mol. Biotechnol.* **61,** 801–815 CrossRef Medline
27. Romao, E., Morales-Yanez, F., Hu, Y., Crauwels, M., De Pauw, P., Hassanzadeh, G. G., Devoogdt, N., Ackaert, C., Vincke, C., and Muyldermans, S. (2016) Identification of useful nanobodies by phage display of immune single domain libraries derived from camelid heavy chain antibodies. *Curr. Pharm. Des.* **22,** 6500–6518 CrossRef Medline
28. Cavallari, M. (2017) Rapid and direct VHH and target identification by staphylococcal surface display libraries. *Int. J. Mol. Sci.* **18,** 1507 CrossRef Medline
29. Fleetwood, F., Devoogdt, N., Pellis, M., Wernery, U., Muyldermans, S., Ståhl, S., and Löfblom, J. (2013) Surface display of a single-domain antibody library on Gram-positive bacteria. *Cell. Mol. Life Sci.* **70,** 1081–1093 CrossRef Medline
30. Uchański, T., Pardon, E., and Steyaert, J. (2020) Nanobodies to study protein conformational states. *Curr. Opin. Struct. Biol.* **60,** 117–123 CrossRef Medline
31. McMahon, C., Baier, A. S., Pascolutti, R., Wegrecki, M., Zheng, S., Ong, J. X., Erlandson, S. C., Hilger, D., Rasmussen, S. G. F., Ring, A. M., Manglik, A., and Kruse, A. C. (2018) Yeast surface display platform for rapid discovery of conformationally selective nanobodies. *Nat. Struct. Mol. Biol.* **25,** 289–296 CrossRef Medline
32. Hutter, C. A. J., Timachi, M. H., Hürlimann, L. M., Zimmermann, I., Egloff, P., Göddeke, H., Kucher, S., Štefanić, S., Karttunen, M., Schäfer, L. V., Bordignon, E., and Seeger, M. A. (2019) The extracellular gate shapes the energy profile of an ABC exporter. *Nat. Commun.* **10,** 2260 CrossRef Medline
33. Zimmermann, I., Egloff, P., Hutter, C. A., Arnold, F. M., Stohler, P., Bocquet, N., Hug, M. N., Huber, S., Siegrist, M., Hetemann, L., Gera, J., Gmür, S., Spies, P., Gygax, D., Geertsma, E. R., Dawson, R. J., and Seeger, M. A. (2018) Synthetic single domain antibodies for the conformational trapping of membrane proteins. *Elife* **7,** e34317 CrossRef Medline
34. Ferrari, D., Garrapa, V., Locatelli, M., and Bolchi, A. (2020) A novel nanobody scaffold optimized for bacterial expression and suitable for the construction of ribosome display libraries. *Mol. Biotechnol.* **62,** 43–55 CrossRef Medline
35. Suzuki, T., Mochizuki, Y., Kimura, S., Akazawa-Ogawa, Y., Hagihara, Y., and Nemoto, N. (2018) Anti-survivin single-domain antibodies derived from an artificial library including three synthetic random regions by *in vitro* selection using cDNA display. *Biochem. Biophys. Res. Commun.* **503,** 2054–2060 CrossRef Medline
36. Takahashi, K., Sunohara, M., Terai, T., Kumachi, S., and Nemoto, N. (2017) Enhanced mRNA-protein fusion efficiency of a single-domain antibody by selection of mRNA display with additional random sequences in the terminal translated regions. *Biophys. Physicobiol.* **14,** 23–28 CrossRef Medline
37. Schmidt, F. I., Hanke, L., Morin, B., Brewer, R., Brusic, V., Whelan, S. P. J., and Ploegh, H. L. (2016) Phenotypic lentivirus screens to identify

ASBMB

functional single domain antibodies. *Nat. Microbiol.* **1,** 16080 CrossRef Medline
38. Liu, Z.-H., Lei, K.-X., Han, G.-W., Xu, H.-L., and He, F. (2020) Novel lentivirus-based method for rapid selection of inhibitory nanobody against PRRSV. *Viruses* **12,** 229 CrossRef Medline
39. Visintin, M., Tse, E., Axelson, H., Rabbitts, T. H., and Cattaneo, A. (1999) Selection of antibodies for intracellular function using a two-hybrid in vivo system. *Proc. Natl. Acad. Sci. U.S.A.* **96,** 11723–11728 CrossRef Medline
40. Matz, J., Hérate, C., Bouchet, J., Dusetti, N., Gayet, O., Baty, D., Benichou, S., and Chames, P. (2014) Selection of intracellular single-domain antibodies targeting the HIV-1 Vpr protein by cytoplasmic yeast two-hybrid system. *PLoS ONE* **9,** e113729 CrossRef Medline
41. Gao, X., Hu, X., Tong, L., Liu, D., Chang, X., Wang, H., Dang, R., Wang, X., Xiao, S., Du, E., and Yang, Z. (2016) Construction of a camelid VHH yeast two-hybrid library and the selection of VHH against haemagglutinin-neuraminidase protein of the Newcastle disease virus. *BMC Vet. Res.* **12,** 39 CrossRef Medline
42. Massa, S., Xavier, C., De Vos, J., Caveliers, V., Lahoutte, T., Muyldermans, S., and Devoogdt, N. (2014) Site-specific labeling of cysteine-tagged camelid single-domain antibody-fragments for use in molecular imaging. *Bioconjug. Chem.* **25,** 979–988 CrossRef Medline
43. Ries, J., Kaplan, C., Platonova, E., Eghlidi, H., and Ewers, H. (2012) A simple, versatile method for GFP-based super-resolution microscopy via nanobodies. *Nat. Methods* **9,** 582–584 CrossRef Medline
44. Nevoltris, D., Lombard, B., Dupuis, E., Mathis, G., Chames, P., and Baty, D. (2015) Conformational nanobodies reveal tethered epidermal growth factor receptor involved in EGFR/ErbB2 predimers. *ACS Nano* **9,** 1388–1399 CrossRef Medline
45. Pleiner, T., Bates, M., Trakhanov, S., Lee, C.-T., Schliep, J. E., Chug, H., Böhning, M., Stark, H., Urlaub, H., and Görlich, D. (2015) Nanobodies: site-specific labeling for super-resolution imaging, rapid epitope-mapping and native protein complex isolation. *Elife* **4,** e11349 CrossRef Medline
46. Agarwal, P., and Bertozzi, C. R. (2015) Site-specific antibody-drug conjugates: the nexus of bioorthogonal chemistry, protein engineering, and drug development. *Bioconjug. Chem.* **26,** 176–192 CrossRef Medline
47. Massa, S., Xavier, C., Muyldermans, S., and Devoogdt, N. (2016) Emerging site-specific bioconjugation strategies for radioimmunotracer development. *Expert Opin. Drug Deliv.* **13,** 1149–1163 CrossRef Medline
48. Herce, H. D., Schumacher, D., Schneider, A. F. L., Ludwig, A. K., Mann, F. A., Fillies, M., Kasper, M.-A., Reinke, S., Krause, E., Leonhardt, H., Cardoso, M. C., and Hackenberger, C. P. R. (2017) Cell-permeable nanobodies for targeted immunolabelling and antigen manipulation in living cells. *Nat. Chem.* **9,** 762–771 CrossRef Medline
49. Klein, A., Hank, S., Raulf, A., Joest, E. F., Tissen, F., Heilemann, M., Wieneke, R., and Tampé, R. (2018) Live-cell labeling of endogenous proteins with nanometer precision by transduced nanobodies. *Chem. Sci.* **9,** 7835–7842 CrossRef Medline
50. Teng, K. W., Ishitsuka, Y., Ren, P., Youn, Y., Deng, X., Ge, P., Lee, S. H., Belmont, A. S., and Selvin, P. R. (2016) Labeling proteins inside living cells using external fluorophores for microscopy. *eLife* **5,** e20378 CrossRef Medline
51. Mylne, J. S., Chan, L. Y., Chanson, A. H., Daly, N. L., Schaefer, H., Bailey, T. L., Nguyencong, P., Cascales, L., and Craik, D. J. (2012) Cyclic peptides arising by evolutionary parallelism via asparaginyl-endopeptidase-mediated biosynthesis. *Plant Cell* **24,** 2765–2778 CrossRef Medline
52. Jackson, M. A., Gilding, E. K., Shafee, T., Harris, K. S., Kaas, Q., Poon, S., Yap, K., Jia, H., Guarino, R., Chan, L. Y., Durek, T., Anderson, M. A., and Craik, D. J. (2018) Molecular basis for the production of cyclic peptides by plant asparaginyl endopeptidases. *Nat. Commun.* **9,** 2411 CrossRef Medline
53. Craik, D. J., and Malik, U. (2013) Cyclotide biosynthesis. *Curr. Opin. Chem. Biol.* **17,** 546–554 CrossRef Medline
54. Yang, R., Wong, Y. H., Nguyen, G. K. T., Tam, J. P., Lescar, J., and Wu, B. (2017) Engineering a catalytically efficient recombinant protein ligase. *J. Am. Chem. Soc.* **139,** 5351–5358 CrossRef Medline
55. Rehm, F. B. H., Harmand, T. J., Yap, K., Durek, T., Craik, D. J., and Ploegh, H. L. (2019) Site-specific sequential protein labeling catalyzed by a single recombinant ligase. *J. Am. Chem. Soc.* **141,** 17388–17393 CrossRef Medline
56. Schumacher, D., Lemke, O., Helma, J., Gerszonowicz, L., Waller, V., Stoschek, T., Durkin, P. M., Budisa, N., Leonhardt, H., Keller, B. G., and Hackenberger, C. P. R. (2017) Broad substrate tolerance of tubulin tyrosine ligase enables one-step site-specific enzymatic protein labeling. *Chem. Sci.* **8,** 3471–3478 CrossRef Medline
57. Schumacher, D., Helma, J., Mann, F. A., Pichler, G., Natale, F., Krause, E., Cardoso, M. C., Hackenberger, C. P. R., and Leonhardt, H. (2015) Versatile and efficient site-specific protein functionalization by tubulin tyrosine ligase. *Angew. Chem. Int. Ed. Engl.* **54,** 13787–13791 CrossRef Medline
58. Frese, M.-A., and Dierks, T. (2009) Formylglycine aldehyde tag—protein engineering through a novel post-translational modification. *Chembiochem* **10,** 425–427 CrossRef Medline
59. Krüger, T., Dierks, T., and Sewald, N. (2019) Formylglycine-generating enzymes for site-specific bioconjugation. *Biol. Chem.* **400,** 289–297 CrossRef Medline
60. Zang, B., Ren, J., Li, D., Huang, C., Ma, H., Peng, Q., Ji, F., Han, L., and Jia, L. (2019) Freezing-assisted synthesis of covalent C-C linked bivalent and bispecific nanobodies. *Org. Biomol. Chem.* **17,** 257–263 CrossRef Medline
61. Nguyen, G. K. T., Wang, S., Qiu, Y., Hemu, X., Lian, Y., and Tam, J. P. (2014) Butelase 1 is an Asx-specific ligase enabling peptide macrocyclization and synthesis. *Nat. Chem. Biol.* **10,** 732–738 CrossRef Medline
62. Nguyen, G. K. T., Qiu, Y., Cao, Y., Hemu, X., Liu, C.-F., and Tam, J. P. (2016) Butelase-mediated cyclization and ligation of peptides and proteins. *Nat. Protoc.* **11,** 1977–1988 CrossRef Medline
63. Hemu, X., Zhang, X., Bi, X., Liu, C.-F., and Tam, J. P. (2019) Butelase 1-mediated ligation of peptides and proteins. *Methods Mol. Biol.* **2012,** 83–109 CrossRef Medline
64. Harmand, T. J., Bousbaine, D., Chan, A., Zhang, X., Liu, D. R., Tam, J. P., and Ploegh, H. L. (2018) One-pot dual labeling of IgG 1 and preparation of C-to-C fusion proteins through a combination of sortase A and butelase 1. *Bioconjug. Chem.* **29,** 3245–3249 CrossRef Medline
65. Galimidi, R. P., Klein, J. S., Politzer, M. S., Bai, S., Seaman, M. S., Nussenzweig, M. C., West, A. P., and Bjorkman, P. J. (2015) Intra-spike crosslinking overcomes antibody evasion by HIV-1. *Cell* **160,** 433–446 CrossRef Medline
66. James, A. M., Haywood, J., Leroux, J., Ignasiak, K., Elliott, A. G., Schmidberger, J. W., Fisher, M. F., Nonis, S. G., Fenske, R., Bond, C. S., and Mylne, J. S. (2019) The macrocyclizing protease butelase 1 remains autocatalytic and reveals the structural basis for ligase activity. *Plant J.* **98,** 988–999 CrossRef Medline
67. Pi, N., Gao, M., Cheng, X., Liu, H., Kuang, Z., Yang, Z., Yang, J., Zhang, B., Chen, Y., Liu, S., Huang, Y., and Su, Z. (2019) Recombinant butelase-mediated cyclization of the p53-binding domain of the oncoprotein MdmX-stabilized protein conformation as a promising model for structural investigation. *Biochemistry* **58,** 3005–3015 CrossRef Medline
68. Mao, H., Hart, S. A., Schink, A., and Pollok, B. A. (2004) Sortase-mediated protein ligation: a new method for protein engineering. *J. Am. Chem. Soc.* **126,** 2670–2671 CrossRef Medline
69. Guimaraes, C. P., Witte, M. D., Theile, C. S., Bozkurt, G., Kundrat, L., Blom, A. E. M., and Ploegh, H. L. (2013) Site-specific C-terminal and internal loop labeling of proteins using sortase-mediated reactions. *Nat. Protoc.* **8,** 1787–1799 CrossRef Medline
70. Popp, M. W.-L., and Ploegh, H. L. (2011) Making and breaking peptide bonds: protein engineering using sortase. *Angew. Chem. Int. Ed. Engl.* **50,** 5024–5032 CrossRef Medline
71. Witte, M. D., Cragnolini, J. J., Dougan, S. K., Yoder, N. C., Popp, M. W., and Ploegh, H. L. (2012) Preparation of unnatural N-to-N and C-to-C protein fusions. *Proc. Natl. Acad. Sci. U. S. A.* **109,** 11993–11998 CrossRef Medline
72. Rashidian, M., Keliher, E. J., Bilate, A. M., Duarte, J. N., Wojtkiewicz, G. R., Jacobsen, J. T., Cragnolini, J., Swee, L. K., Victora, G. D., Weissleder,

R., and Ploegh, H. L. (2015) Noninvasive imaging of immune responses. *Proc. Natl. Acad. Sci. U. S. A.* **112,** 6146–6151 CrossRef Medline

73. Rashidian, M., Wang, L., Edens, J. G., Jacobsen, J. T., Hossain, I., Wang, Q., Victora, G. D., Vasdev, N., Ploegh, H., and Liang, S. H. (2016) Enzyme-mediated modification of single-domain antibodies for imaging modalities with different characteristics. *Angew. Chem. Int. Ed. Engl.* **55,** 528–533 CrossRef Medline
74. Fang, T., Van Elssen, C. H. M. J., Duarte, J. N., Guzman, J. S., Chahal, J. S., Ling, J., and Ploegh, H. L. (2017) Targeted antigen delivery by an anti-class II MHC VHH elicits focused αMUC1(Tn) immunity. *Chem. Sci.* **8,** 5591–5597 CrossRef Medline
75. Theile, C. S., Witte, M. D., Blom, A. E. M., Kundrat, L., Ploegh, H. L., and Guimaraes, C. P. (2013) Site-specific N-terminal labeling of proteins using sortase-mediated reactions. *Nat. Protoc.* **8,** 1800–1807 CrossRef Medline
76. Muir, T. W., Sondhi, D., and Cole, P. A. (1998) Expressed protein ligation: a general method for protein engineering. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 6705–6710 CrossRef Medline
77. Neumann-Staubitz, P., and Neumann, H. (2016) The use of unnatural amino acids to study and engineer protein function. *Curr. Opin. Struct. Biol.* **38,** 119–128 CrossRef Medline
78. Lee, K. J., Kang, D., and Park, H.-S. (2019) Site-specific labeling of proteins using unnatural amino acids. *Mol. Cells* **42,** 386–396 CrossRef Medline
79. Yamaguchi, A., Matsuda, T., Ohtake, K., Yanagisawa, T., Yokoyama, S., Fujiwara, Y., Watanabe, T., Hohsaka, T., and Sakamoto, K. (2016) Incorporation of a doubly functionalized synthetic amino acid into proteins for creating chemical and light-induced conjugates. *Bioconjug. Chem.* **27,** 198–206 CrossRef Medline
80. Mukherjee, S., Erramilli, S. K., Ammirati, M., Alvarez, F. J. D., Fennell, K. F., Purdy, M. D., Skrobek, B. M., Radziwon, K., Coukos, J., Kang, Y., Dutka, P., Gao, X., Qiu, X., Yeager, M., Eric Xu, H., *et al.* (2020) Synthetic antibodies against BRIL as universal fiducial marks for single-particle cryoEM structure determination of membrane proteins. *Nat. Commun.* **11,** 1598 CrossRef Medline
81. Uchański, T., Masiulis, S., Fischer, B., Kalichuk, V., Wohlkönig, A., Zögg, T., Remaut, H., Vranken, W., Aricescu, A. R., Pardon, E., and Steyaert, J. (2019) Megabodies expand the nanobody toolkit for protein structure determination by single-particle cryo-EM. *bioRxiv* CrossRef
82. Cheloha, R. W., Li, Z., Bousbaine, D., Woodham, A. W., Perrin, P., Volarić, J., and Ploegh, H. L. (2019) Internalization of influenza virus and cell surface proteins monitored by site-specific conjugation of protease-sensitive probes. *ACS Chem. Biol.* **14,** 1836–1844 CrossRef Medline
83. Buser, D. P., Schleicher, K. D., Prescianotto-Baschong, C., and Spiess, M. (2018) A versatile nanobody-based toolkit to analyze retrograde transport from the cell surface. *Proc. Natl. Acad. Sci. U. S. A.* **115,** E6227–E6236 CrossRef Medline
84. Pleiner, T., Bates, M., and Görlich, D. (2018) A toolbox of anti-mouse and anti-rabbit IgG secondary nanobodies. *J. Cell Biol.* **217,** 1143–1154 CrossRef Medline
85. Harmsen, M. M., Van Solt, C. B., Fijten, H. P. D., and Van Setten, M. C. (2005) Prolonged in vivo residence times of llama single-domain antibody fragments in pigs by binding to porcine immunoglobulins. *Vaccine* **23,** 4926–4934 CrossRef Medline
86. Ereño-Orbea, J., Sicard, T., Cui, H., Carson, J., Hermans, P., and Julien, J.-P. (2018) Structural basis of enhanced crystallizability induced by a molecular chaperone for antibody antigen-binding fragments. *J. Mol. Biol.* **430,** 322–336 CrossRef Medline
87. Sograte-Idrissi, S., Schlichthaerle, T., Duque-Afonso, C. J., Alevra, M., Strauss, S., Moser, T., Jungmann, R., Rizzoli, S. O., and Opazo, F. (2020) Circumvention of common labelling artefacts using secondary nanobodies. *Nanoscale* **12,** 10226–10239 CrossRef Medline
88. Thomsen, A. R. B., Plouffe, B., Cahill, T. J., Shukla, A. K., Tarrasch, J. T., Dosey, A. M., Kahsai, A. W., Strachan, R. T., Pani, B., Mahoney, J. P., Huang, L., Breton, B., Heydenreich, F. M., Sunahara, R. K., Skiniotis, G., *et al.* (2016) GPCR-G protein-β-arrestin super-complex mediates sustained G protein signaling. *Cell* **166,** 907–919 CrossRef Medline
89. Rasmussen, S. G. F., Choi, H.-J., Fung, J. J., Pardon, E., Casarosa, P., Chae, P. S., Devree, B. T., Rosenbaum, D. M., Thian, F. S., Kobilka, T. S., Schnapp, A., Konetzki, I., Sunahara, R. K., Gellman, S. H., Pautsch, A., *et al.* (2011) Structure of a nanobody-stabilized active state of the β_2 adrenoceptor. *Nature* **469,** 175–180 CrossRef Medline
90. Shukla, A. K., Gupta, C., Srivastava, A., and Jaiman, D. (2015) Antibody fragments for stabilization and crystallization of G protein-coupled receptors and their signaling complexes. *Methods Enzymol.* **557,** 247–258 CrossRef Medline
91. De Groof, T. W. M., Bobkov, V., Heukers, R., and Smit, M. J. (2019) Nanobodies: new avenues for imaging, stabilizing and modulating GPCRs. *Mol. Cell. Endocrinol.* **484,** 15–24 CrossRef Medline
92. Kruse, A. C., Ring, A. M., Manglik, A., Hu, J., Hu, K., Eitel, K., Hübner, H., Pardon, E., Valant, C., Sexton, P. M., Christopoulos, A., Felder, C. C., Gmeiner, P., Steyaert, J., Weis, W. I., *et al.* (2013) Activation and allosteric modulation of a muscarinic acetylcholine receptor. *Nature* **504,** 101–106 CrossRef Medline
93. Burg, J. S., Ingram, J. R., Venkatakrishnan, A. J., Jude, K. M., Dukkipati, A., Feinberg, E. N., Angelini, A., Waghray, D., Dror, R. O., Ploegh, H. L., and Garcia, K. C. (2015) Structural biology: structural basis for chemokine recognition and activation of a viral G protein-coupled receptor. *Science* **347,** 1113–1117 CrossRef Medline
94. Koehl, A., Hu, H., Feng, D., Sun, B., Zhang, Y., Robertson, M. J., Chu, M., Kobilka, T. S., Laeremans, T., Steyaert, J., Tarrasch, J., Dutta, S., Fonseca, R., Weis, W. I., Mathiesen, J. M., *et al.* (2019) Structural insights into the activation of metabotropic glutamate receptors. *Nature* **566,** 79–84 CrossRef Medline
95. Ma, Y., Ding, Y., Song, X., Ma, X., Li, X., Zhang, N., Song, Y., Sun, Y., Shen, Y., Zhong, W., Hu, L. A., Ma, Y., and Zhang, M.-Y. (2020) Structure-guided discovery of a single-domain antibody agonist against human apelin receptor. *Sci. Adv.* **6,** eaax7379 CrossRef Medline
96. Smirnova, I., Kasho, V., Jiang, X., Pardon, E., Steyaert, J., and Kaback, H. R. (2015) Transient conformers of LacY are trapped by nanobodies. *Proc. Natl. Acad. Sci. U. S. A.* **112,** 13839–13844 CrossRef Medline
97. Kumar, H., Finer-Moore, J. S., Jiang, X., Smirnova, I., Kasho, V., Pardon, E., Steyaert, J., Kaback, H. R., and Stroud, R. M. (2018) Crystal structure of a ligand-bound LacY-nanobody complex. *Proc. Natl. Acad. Sci. U. S. A.* **115,** 8769–8774 CrossRef Medline
98. Perez, C., Köhler, M., Janser, D., Pardon, E., Steyaert, J., Zenobi, R., and Locher, K. P. (2017) Structural basis of inhibition of lipid-linked oligosaccharide flippase PglK by a conformational nanobody. *Sci. Rep.* **7,** 46641 CrossRef Medline
99. Zavrtanik, U., Lukan, J., Loris, R., Lah, J., and Hadži, S. (2018) Structural basis of epitope recognition by heavy-chain camelid antibodies. *J. Mol. Biol.* **430,** 4369–4386 CrossRef Medline
100. Dunbar, J., Krawczyk, K., Leem, J., Baker, T., Fuchs, A., Georges, G., Shi, J., and Deane, C. M. (2014) SAbDab: the structural antibody database. *Nucleic Acids Res.* **42,** D1140–D1146 CrossRef Medline
101. Staus, D. P., Strachan, R. T., Manglik, A., Pani, B., Kahsai, A. W., Kim, T. H., Wingler, L. M., Ahn, S., Chatterjee, A., Masoudi, A., Kruse, A. C., Pardon, E., Steyaert, J., Weis, W. I., Prosser, R. S., *et al.* (2016) Allosteric nanobodies reveal the dynamic range and diverse mechanisms of G-protein-coupled receptor activation. *Nature* **535,** 448–452 CrossRef Medline
102. Ring, A. M., Manglik, A., Kruse, A. C., Enos, M. D., Weis, W. I., Garcia, K. C., and Kobilka, B. K. (2013) Adrenaline-activated structure of β2-adrenoceptor stabilized by an engineered nanobody. *Nature* **502,** 575–579 CrossRef Medline
103. Che, T., English, J., Krumm, B. E., Kim, K., Pardon, E., Olsen, R. H. J., Wang, S., Zhang, S., Diberto, J. F., Sciaky, N., Carroll, F. I., Steyaert, J., Wacker, D., and Roth, B. L. (2020) Nanobody-enabled monitoring of κ opioid receptor states. *Nat. Commun.* **11,** 1145 CrossRef Medline
104. Wingler, L. M., McMahon, C., Staus, D. P., Lefkowitz, R. J., and Kruse, A. C. (2019) Distinctive activation mechanism for angiotensin receptor revealed by a synthetic nanobody. *Cell* **176,** 479–490.e12 CrossRef Medline
105. Che, T., Majumdar, S., Zaidi, S. A., Ondachi, P., McCorvy, J. D., Wang, S., Mosier, P. D., Uprety, R., Vardy, E., Krumm, B. E., Han, G. W., Lee, M.-Y., Pardon, E., Steyaert, J., Huang, X.-P., *et al.* (2018) Structure of the

nanobody-stabilized active state of the κ opioid receptor. *Cell* **172,** 55–67.e15 CrossRef Medline
106. Scholler, P., Nevoltris, D., de Bundel, D., Bossi, S., Moreno-Delgado, D., Rovira, X., Møller, T. C., El Moustaine, D., Mathieu, M., Blanc, E., McLean, H., Dupuis, E., Mathis, G., Trinquet, E., Daniel, H., *et al.* (2017) Allosteric nanobodies uncover a role of hippocampal mGlu2 receptor homodimers in contextual fear consolidation. *Nat. Commun.* **8,** 1967 CrossRef Medline
107. Soave, M., Heukers, R., Kellam, B., Woolard, J., Smit, M. J., Briddon, S. J., and Hill, S. J. (2020) Monitoring ligand-induced changes in receptor conformation with NanoBiT conjugated nanobodies. *bioRxiv* CrossRef
108. Pardon, E., Betti, C., Laeremans, T., Chevillard, F., Guillemyn, K., Kolb, P., Ballet, S., and Steyaert, J. (2018) Nanobody-enabled reverse pharmacology on G-protein-coupled receptors. *Angew. Chem. Int. Ed. Engl.* **57,** 5292–5295 CrossRef Medline
109. Farrants, H., Gutzeit, V. A., Acosta-Ruiz, A., Trauner, D., Johnsson, K., Levitz, J., and Broichhagen, J. (2018) SNAP-tagged nanobodies enable reversible optical control of a G protein-coupled receptor via a remotely tethered photoswitchable ligand. *ACS Chem. Biol*. **13,** 2682–2688 CrossRef Medline
110. Cheloha, R. W., Fischer, F. A., Woodham, A. W., Daley, E., Suminski, N., Gardella, T. J., and Ploegh, H. L. (2020) Improved GPCR ligands from nanobody tethering. *Nat. Commun*. **11,** 2087 CrossRef Medline
111. Duarte, J. N., Cragnolini, J. J., Swee, L. K., Bilate, A. M., Bader, J., Ingram, J. R., Rashidfarrokhi, A., Fang, T., Schiepers, A., Hanke, L., and Ploegh, H. L. (2016) Generation of immunity against pathogens via single-domain antibody-antigen constructs. *J. Immunol*. **197,** 4838–4847 CrossRef Medline
112. Cheloha, R. W., Woodham, A. W., Bousbaine, D., Wang, T., Liu, S., Sidney, J., Sette, A., Gellman, S. H., and Ploegh, H. L. (2019) Recognition of class II MHC peptide ligands that contain β-amino acids. *J. Immunol.* **203,** 1619–1628 CrossRef Medline
113. Kwon, S., Duarte, J. N., Li, Z., Ling, J. J., Cheneval, O., Durek, T., Schroeder, C. I., Craik, D. J., and Ploegh, H. L. (2018) Targeted delivery of cyclotides via conjugation to a nanobody. *ACS Chem. Biol.* **13,** 2973–2980 CrossRef Medline
114. Scully, M., Cataland, S. R., Peyvandi, F., Coppo, P., Knöbl, P., Kremer Hovinga, J. A., Metjian, A., de la Rubia, J., Pavenski, K., Callewaert, F., Biswas, D., De Winter, H., Zeldin, R. K., and HERCULES Investigators (2019) Caplacizumab treatment for acquired thrombotic thrombocytopenic purpura. *N. Engl. J. Med*. **380,** 335–346 CrossRef Medline
115. Arezumand, R., Mahdian, R., Zeinali, S., Hassanzadeh-Ghassabeh, G., Mansouri, K., Khanahmad, H., Namvar-Asl, N., Rahimi, H., Behdani, M., Cohan, R. A., Eavazalipour, M., Ramazani, A., and Muyldermans, S. (2016) Identification and characterization of a novel nanobody against human placental growth factor to modulate angiogenesis. *Mol. Immunol.* **78,** 183–192 CrossRef Medline
116. Desmyter, A., Spinelli, S., Boutton, C., Saunders, M., Blachetot, C., de Haard, H., Denecker, G., Van Roy, M., Cambillau, C., and Rommelaere, H. (2017) Neutralization of human interleukin 23 by multivalent nanobodies explained by the structure of cytokine-nanobody complex. *Front. Immunol*. **8,** 884 CrossRef Medline
117. Gorshkova, E. N., Efimov, G. A., Ermakova, K. D., Vasilenko, E. A., Yuzhakova, D. V., Shirmanova, M. V., Mokhonov, V. V., Tillib, S. V., Nedospasov, S. A., and Astrakhantseva, I. V. (2018) Properties of fluorescent far-red anti-TNF nanobodies. *Antibodies (Basel)* **7,** 43 CrossRef Medline
118. Nosenko, M. A., Atretkhany, K.-S. N., Mokhonov, V. V., Efimov, G. A., Kruglov, A. A., Tillib, S. V., Drutskaya, M. S., and Nedospasov, S. A. (2017) VHH-based bispecific antibodies targeting cytokine production. *Front. Immunol*. **8,** 1073 CrossRef Medline
119. Bakherad, H., Gargari, S. L. M., Sepehrizadeh, Z., Aghamollaei, H., Taheri, R. A., Torshabi, M., Yazdi, M. T., Ebrahimizadeh, W., and Setayesh, N. (2017) Identification and *in vitro* characterization of novel nanobodies against human granulocyte colony-stimulating factor receptor to provide inhibition of G-CSF function. *Biomed. Pharmacother.* **93,** 245–254 CrossRef Medline
120. Sparkes, A., De Baetselier, P., Brys, L., Cabrito, I., Sterckx, Y. G.-J., Schoonooghe, S., Muyldermans, S., Raes, G., Bucala, R., Vanlandschoot, P., Van Ginderachter, J. A., and Stijlemans, B. (2018) Novel half-life extended anti-MIF nanobodies protect against endotoxic shock. *FASEB J.* **32,** 3411–3422 CrossRef Medline
121. Kaur, M., Dubey, A., Khatri, M., and Sehrawat, S. (2019) Secretory PLA2 specific single domain antibody neutralizes Russell viper venom induced cellular and organismal toxicity. *Toxicon* **172,** 15–18 CrossRef Medline
122. Vasylieva, N., Kitamura, S., Dong, J., Barnych, B., Hvorecny, K. L., Madden, D. R., Gee, S. J., Wolan, D. W., Morisseau, C., and Hammock, B. D. (2019) Nanobody-based binding assay for the discovery of potent inhibitors of CFTR inhibitory factor (Cif). *Anal. Chim. Acta* **1057,** 106–113 CrossRef Medline
123. Alirahimi, E., Kazemi-Lomedasht, F., Shahbazzadeh, D., Habibi-Anbouhi, M., Hosseininejad Chafi, M., Sotoudeh, N., Ghaderi, H., Muyldermans, S., and Behdani, M. (2018) Nanobodies as novel therapeutic agents in envenomation. *Biochim. Biophys. Acta Gen. Subj*. **1862,** 2955–2965 CrossRef Medline
124. Li, X., Wang, M., Zhang, X., Liu, C., Xiang, H., Huang, M., Ma, Y., Gao, X., Jiang, L., Liu, X., Li, B., Hou, Y., Zhang, X., Yang, S., and Yang, N. (2020) The novel llama-human chimeric antibody has potent effect in lowering LDL-c levels in hPCSK9 transgenic rats. *Clin. Transl. Med*. **9,** 16 CrossRef Medline
125. Dupré, E., Danis, C., Arrial, A., Hanoulle, X., Homa, M., Cantrelle, F.-X., Merzougui, H., Colin, M., Rain, J.-C., Buée, L., and Landrieu, I. (2019) Single domain antibody fragments as new tools for the detection of neuronal tau protein in cells and in mice studies. *ACS Chem Neurosci*. **10,** 3997–4006 CrossRef Medline
126. Abskharon, R. N. N., Giachin, G., Wohlkonig, A., Soror, S. H., Pardon, E., Legname, G., and Steyaert, J. (2014) Probing the N-terminal β-sheet conversion in the crystal structure of the human prion protein bound to a nanobody. *J. Am. Chem. Soc*. **136,** 937–944 CrossRef Medline
127. Guilliams, T., El-Turk, F., Buell, A. K., O'Day, E. M., Aprile, F. A., Esbjörner, E. K., Vendruscolo, M., Cremades, N., Pardon, E., Wyns, L., Welland, M. E., Steyaert, J., Christodoulou, J., Dobson, C. M., and De Genst, E. (2013) Nanobodies raised against monomeric α-synuclein distinguish between fibrils at different maturation stages. *J. Mol. Biol*. **425,** 2397–2411 CrossRef Medline
128. Domanska, K., Vanderhaegen, S., Srinivasan, V., Pardon, E., Dupeux, F., Marquez, J. A., Giorgetti, S., Stoppini, M., Wyns, L., Bellotti, V., and Steyaert, J. (2011) Atomic structure of a nanobody-trapped domain-swapped dimer of an amyloidogenic β2-microglobulin variant. *Proc. Natl. Acad. Sci. U. S. A*. **108,** 1314–1319 CrossRef Medline
129. Vanderhaegen, S., Fislage, M., Domanska, K., Versées, W., Pardon, E., Bellotti, V., and Steyaert, J. (2013) Structure of an early native-like intermediate of β2-microglobulin amyloidogenesis. *Protein Sci*. **22,** 1349–1357 CrossRef Medline
130. Raimondi, S., Porcari, R., Mangione, P. P., Verona, G., Marcoux, J., Giorgetti, S., Taylor, G. W., Ellmerich, S., Ballico, M., Zanini, S., Pardon, E., Al-Shawi, R., Simons, J. P., Corazza, A., Fogolari, F., *et al.* (2017) A specific nanobody prevents amyloidogenesis of D76N β2-microglobulin *in vitro* and modifies its tissue distribution *in vivo*. *Sci. Rep*. **7,** 46711 CrossRef Medline
131. Zhang, L., Zang, B., Huang, C., Ren, J., and Jia, L. (2019) One-step preparation of a VHH-based immunoadsorbent for the extracorporeal removal of β2-microglobulin. *Molecules* **24,** 2119 CrossRef Medline
132. Van Overbeke, W., Wongsantichon, J., Everaert, I., Verhelle, A., Zwaenepoel, O., Loonchanta, A., Burtnick, L. D., De Ganck, A., Hochepied, T., Haigh, J., Cuvelier, C., Derave, W., Robinson, R. C., and Gettemans, J. (2015) An ER-directed gelsolin nanobody targets the first step in amyloid formation in a gelsolin amyloidosis mouse model. *Hum. Mol. Genet*. **24,** 2492–2507 CrossRef Medline
133. Van Overbeke, W., Verhelle, A., Everaert, I., Zwaenepoel, O., Vandekerckhove, J., Cuvelier, C., Derave, W., and Gettemans, J. (2014) Chaperone nanobodies protect gelsolin against MT1-MMP degradation and alleviate amyloid burden in the gelsolin amyloidosis mouse model. *Mol. Ther*. **22,** 1768–1778 CrossRef Medline
134. Verhelle, A., Nair, N., Everaert, I., Van Overbeke, W., Supply, L., Zwaenepoel, O., Peleman, C., Van Dorpe, J., Lahoutte, T., Devoogdt, N., Derave, W., Chuah, M. K., VandenDriessche, T., and Gettemans, J. (2017) AAV9

delivered bispecific nanobody attenuates amyloid burden in the gelsolin amyloidosis mouse model. *Hum. Mol. Genet.* **26,** 1353–1364 CrossRef Medline

135. Verhelle, A., Van Overbeke, W., Peleman, C., De Smet, R., Zwaenepoel, O., Lahoutte, T., Van Dorpe, J., Devoogdt, N., and Gettemans, J. (2016) Non-invasive imaging of amyloid deposits in a mouse model of AGel using 99mTc-modified nanobodies and SPECT/CT. *Mol. Imaging Biol.* **18,** 887–897 CrossRef Medline
136. Lauwereys, M., Arbabi Ghahroudi, M., Desmyter, A., Kinne, J., Hölzer, W., De Genst, E., Wyns, L., and Muyldermans, S. (1998) Potent enzyme inhibitors derived from dromedary heavy-chain antibodies. *EMBO J.* **17,** 3512–3520 CrossRef Medline
137. Kromann-Hansen, T., Oldenburg, E., Yung, K. W. Y., Ghassabeh, G. H., Muyldermans, S., Declerck, P. J., Huang, M., Andreasen, P. A., and Ngo, J. C. K. (2016) A camelid-derived antibody fragment targeting the active site of a serine protease balances between inhibitor and substrate behavior. *J. Biol. Chem.* **291,** 15156–15168 CrossRef Medline
138. Kaczmarek, J. Z., and Skottrup, P. D. (2015) Selection and characterization of camelid nanobodies towards urokinase-type plasminogen activator. *Mol. Immunol.* **65,** 384–390 CrossRef Medline
139. Kromann-Hansen, T., Louise Lange, E., Peter Sørensen, H., Hassanzadeh-Ghassabeh, G., Huang, M., Jensen, J. K., Muyldermans, S., Declerck, P. J., Komives, E. A., and Andreasen, P. A. (2017) Discovery of a novel conformational equilibrium in urokinase-type plasminogen activator. *Sci. Rep.* **7,** 3385 CrossRef Medline
140. Demeestere, D., Dejonckheere, E., Steeland, S., Hulpiau, P., Haustraete, J., Devoogdt, N., Wichert, R., Becker-Pauly, C., Van Wonterghem, E., Dewaele, S., Van Imschoot, G., Aerts, J., Arckens, L., Saeys, Y., Libert, C., *et al.* (2016) Development and validation of a small single-domain antibody that effectively inhibits matrix metalloproteinase 8. *Mol. Ther.* **24,** 890–902 CrossRef Medline
141. Dorresteijn, B., Rotman, M., Faber, D., Schravesande, R., Suidgeest, E., van der Weerd, L., van der Maarel, S. M., Verrips, C. T., and El Khattabi, M. (2015) Camelid heavy chain only antibody fragment domain against β-site of amyloid precursor protein cleaving enzyme 1 inhibits β-secretase activity *in vitro* and *in vivo*. *FEBS J.* **282,** 3618–3631 CrossRef Medline
142. Zhou, X., Hendrickx, M. L. V., Hassanzadeh-Ghassabeh, G., Muyldermans, S., and Declerck, P. J. (2016) Generation and *in vitro* characterisation of inhibitory nanobodies towards plasminogen activator inhibitor 1. *Thromb. Haemost.* **116,** 1032–1040 CrossRef Medline
143. Sillen, M., Weeks, S. D., Zhou, X., Komissarov, A. A., Florova, G., Idell, S., Strelkov, S. V., and Declerck, P. J. (2020) Molecular mechanism of two nanobodies that inhibit PAI-1 activity reveals a modulation at distinct stages of the PAI-1/plasminogen activator interaction. *J. Thromb. Haemost.* **18,** 681–692 CrossRef Medline
144. Zhou, X., Weeks, S. D., Ameloot, P., Callewaert, N., Strelkov, S. V., and Declerck, P. J. (2016) Elucidation of the molecular mechanisms of two nanobodies that inhibit thrombin-activatable fibrinolysis inhibitor activation and activated thrombin-activatable fibrinolysis inhibitor activity. *J. Thromb. Haemost.* **14,** 1629–1638 CrossRef Medline
145. Ju Shin, Y., Kyun Park, S., Jung Jung, Y., Na Kim, Y., Sung Kim, K., Kyu Park, O., Kwon, S.-H., Ho Jeon, S., Trinh, L. A., Fraser, S. E., Kee, Y., and Joon Hwang, B. (2015) Nanobody-targeted E3-ubiquitin ligase complex degrades nuclear proteins. *Sci. Rep.* **5,** 14269 CrossRef Medline
146. Slastnikova, T. A., Ulasov, A. V., Rosenkranz, A. A., and Sobolev, A. S. (2018) Targeted intracellular delivery of antibodies: the state of the art. *Front. Pharmacol.* **9,** 1208 CrossRef Medline
147. Tabtimmai, L., Suphakun, P., Srisook, P., Kiriwan, D., Phanthong, S., Kiatwuthinon, P., Chaicumpa, W., and Choowongkomon, K. (2019) Cell-penetrable nanobodies (transbodies) that inhibit the tyrosine kinase activity of EGFR leading to the impediment of human lung adenocarcinoma cell motility and survival. *J. Cell. Biochem.* **120,** 18077–18087 CrossRef Medline
148. van Lith, S. A. M., van den Brand, D., Wallbrecher, R., Wübbeke, L., van Duijnhoven, S. M. J., Mäkinen, P. I., Hoogstad-van Evert, J. S., Massuger, L., Ylä-Herttuala, S., Brock, R., and Leenders, W. P. J. (2018) The effect of subcellular localization on the efficiency of EGFR-targeted VHH photosensitizer conjugates. *Eur. J. Pharm. Biopharm.* **124,** 63–72 CrossRef Medline
149. Bruce, V. J., Lopez-Islas, M., and McNaughton, B. R. (2016) Resurfaced cell-penetrating nanobodies: a potentially general scaffold for intracellularly targeted protein discovery. *Protein Sci.* **25,** 1129–1137 CrossRef Medline
150. Chiu, H.-Y., Deng, W., Engelke, H., Helma, J., Leonhardt, H., and Bein, T. (2016) Intracellular chromobody delivery by mesoporous silica nanoparticles for antigen targeting and visualization in real time. *Sci. Rep.* **6,** 25019 CrossRef Medline
151. Singh, S., Murillo, G., Chen, D., Parihar, A. S., and Mehta, R. G. (2018) Suppression of breast cancer cell proliferation by selective single-domain antibody for intracellular STAT3. *Breast Cancer (Auckl.)* **12,** 1178223417750858 CrossRef Medline
152. Yu, C., Wang, L., Rowe, R. G., Han, A., Ji, W., McMahon, C., Baier, A. S., Huang, Y.-C., Marion, W., Pearson, D. S., Kruse, A. C., Daley, G. Q., Wu, H., and Sliz, P. (2020) A nanobody targeting the LIN28:let-7 interaction fragment of TUT4 blocks uridylation of let-7. *Proc. Natl. Acad. Sci. U. S. A.* **117,** 4653–4663 CrossRef Medline
153. Ingram, J. R., Knockenhauer, K. E., Markus, B. M., Mandelbaum, J., Ramek, A., Shan, Y., Shaw, D. E., Schwartz, T. U., Ploegh, H. L., and Lourido, S. (2015) Allosteric activation of apicomplexan calcium-dependent protein kinases. *Proc. Natl. Acad. Sci. U. S. A.* **112,** E4975–E4984 CrossRef Medline
154. Van Impe, K., Bethuyne, J., Cool, S., Impens, F., Ruano-Gallego, D., De Wever, O., Vanloo, B., Van Troys, M., Lambein, K., Boucherie, C., Martens, E., Zwaenepoel, O., Hassanzadeh-Ghassabeh, G., Vandekerckhove, J., Gevaert, K., *et al.* (2013) A nanobody targeting the F-actin capping protein CapG restrains breast cancer metastasis. *Breast Cancer Res.* **15,** R116 CrossRef Medline
155. Truttmann, M. C., Wu, Q., Stiegeler, S., Duarte, J. N., Ingram, J., and Ploegh, H. L. (2015) HypE-specific nanobodies as tools to modulate HypE-mediated target AMPylation. *J. Biol. Chem.* **290,** 9087–9100 CrossRef Medline
156. Alzogaray, V., Danquah, W., Aguirre, A., Urrutia, M., Berguer, P., García Véscovi, E., Haag, F., Koch-Nolte, F., and Goldbaum, F. A. (2011) Single-domain llama antibodies as specific intracellular inhibitors of SpvB, the actin ADP-ribosylating toxin of *Salmonella typhimurium*. *FASEB J.* **25,** 526–534 CrossRef Medline
157. Leemans, M., Galicia, C., Deyaert, E., Daems, E., Krause, L., Paesmans, J., Pardon, E., Steyaert, J., Kortholt, A., Sobott, F., Klostermeier, D., and Versées, W. (2020) Allosteric modulation of the GTPase activity of a bacterial LRRK2 homolog by conformation-specific Nanobodies. *Biochem. J.* **477,** 1203–1218 CrossRef Medline
158. Ling, J., Cheloha, R. W., McCaul, N., Sun, Z.-Y. J., Wagner, G., and Ploegh, H. L. (2019) A nanobody that recognizes a 14-residue peptide epitope in the E2 ubiquitin-conjugating enzyme UBC6e modulates its activity. *Mol. Immunol.* **114,** 513–523 CrossRef Medline
159. Tang, J. C. Y., Szikra, T., Kozorovitskiy, Y., Teixiera, M., Sabatini, B. L., Roska, B., and Cepko, C. L. (2013) A nanobody-based system using fluorescent proteins as scaffolds for cell-specific gene manipulation. *Cell* **154,** 928–939 CrossRef Medline
160. Schmidt, F. I., Lu, A., Chen, J. W., Ruan, J., Tang, C., Wu, H., and Ploegh, H. L. (2016) A single domain antibody fragment that recognizes the adaptor ASC defines the role of ASC domains in inflammasome assembly. *J. Exp. Med.* **213,** 771–790 CrossRef Medline
161. Keller, L., Bery, N., Tardy, C., Ligat, L., Favre, G., Rabbitts, T. H., and Olichon, A. (2019) Selection and characterization of a nanobody biosensor of GTP-bound RHO activities. *Antibodies (Basel)* **8,** 8 CrossRef Medline
162. Galli, V., Sebastian, R., Moutel, S., Ecard, J., Perez, F., and Roux, A. (2017) Uncoupling of dynamin polymerization and GTPase activity revealed by the conformation-specific nanobody dynab. *Elife* CrossRef Medline
163. Gulati, S., Jin, H., Masuho, I., Orban, T., Cai, Y., Pardon, E., Martemyanov, K. A., Kiser, P. D., Stewart, P. L., Ford, C. P., Steyaert, J., and Palczewski, K. (2018) Targeting G protein-coupled receptor signaling at the G protein level with a selective nanobody inhibitor. *Nat. Commun.* **9,** 1996 CrossRef Medline

164. De Clercq, S., Zwaenepoel, O., Martens, E., Vandekerckhove, J., Guillabert, A., and Gettemans, J. (2013) Nanobody-induced perturbation of LFA-1/L-plastin phosphorylation impairs MTOC docking, immune synapse formation and T cell activation. *Cell. Mol. Life Sci.* **70,** 909–922 CrossRef Medline
165. Delanote, V., Vanloo, B., Catillon, M., Friederich, E., Vandekerckhove, J., and Gettemans, J. (2010) An alpaca single-domain antibody blocks filopodia formation by obstructing L-plastin-mediated F-actin bundling. *FASEB J.* **24,** 105–118 CrossRef Medline
166. Schenck, S., Kunz, L., Sahlender, D., Pardon, E., Geertsma, E. R., Savtchouk, I., Suzuki, T., Neldner, Y., Štefanić, S., Steyaert, J., Volterra, A., and Dutzler, R. (2017) Generation and characterization of anti-VGLUT nanobodies acting as inhibitors of transport. *Biochemistry* **56,** 3962–3971 CrossRef Medline
167. Ward, A. B., Szewczyk, P., Grimard, V., Lee, C.-W., Martinez, L., Doshi, R., Caya, A., Villaluz, M., Pardon, E., Cregger, C., Swartz, D. J., Falson, P. G., Urbatsch, I. L., Govaerts, C., Steyaert, J., *et al.* (2013) Structures of P-glycoprotein reveal its conformational flexibility and an epitope on the nucleotide-binding domain. *Proc. Natl. Acad. Sci. U. S. A.* **110,** 13386–13391 CrossRef Medline
168. Ramirez, D. H., Aonbangkhen, C., Wu, H.-Y., Naftaly, J. A., Tang, S., O'Meara, T. R., and Woo, C. M. (2020) Engineering a proximity-directed *O*-GlcNAc transferase for selective protein *O*-GlcNAcylation in cells. *ACS Chem. Biol.* **15,** 1059–1066 CrossRef Medline
169. Harmansa, S., Alborelli, I., Bieli, D., Caussinus, E., and Affolter, M. (2017) A nanobody-based toolset to investigate the role of protein localization and dispersal in *Drosophila*. *Elife* **6,** e22549 CrossRef Medline
170. Roubinet, C., Tsankova, A., Pham, T. T., Monnard, A., Caussinus, E., Affolter, M., and Cabernard, C. (2017) Spatio-temporally separated cortical flows and spindle geometry establish physical asymmetry in fly neural stem cells. *Nat. Commun.* **8,** 1383 CrossRef Medline
171. Rodriguez, J., Peglion, F., Martin, J., Hubatsch, L., Reich, J., Hirani, N., Gubieda, A. G., Roffey, J., Fernandes, A. R., St Johnston, D., Ahringer, J., and Goehring, N. W. (2017) aPKC cycles between functionally distinct PAR protein assemblies to drive cell polarity. *Dev. Cell* **42,** 400–415.e9 CrossRef Medline
172. Ramat, A., Hannaford, M., and Januschke, J. (2017) Maintenance of Miranda localization in *Drosophila* neuroblasts involves interaction with the cognate mRNA. *Curr. Biol.* **27,** 2101–2111.e5 CrossRef Medline
173. Harmansa, S., Hamaratoglu, F., Affolter, M., and Caussinus, E. (2015) Dpp spreading is required for medial but not for lateral wing disc growth. *Nature* **527,** 317–322 CrossRef Medline
174. Mörsdorf, D., and Müller, P. (2019) Tuning protein diffusivity with membrane tethers. *Biochemistry* **58,** 177–181 CrossRef Medline
175. Steels, A., Verhelle, A., Zwaenepoel, O., and Gettemans, J. (2018) Intracellular displacement of p53 using transactivation domain (p53 TAD) specific nanobodies. *MAbs* **10,** 1045–1059 CrossRef Medline
176. Steels, A., Vannevel, L., Zwaenepoel, O., and Gettemans, J. (2019) Nb-induced stabilisation of p53 in HPV-infected cells. *Sci. Rep.* **9,** 12680 CrossRef Medline
177. Böldicke, T. (2017) Single domain antibodies for the knockdown of cytosolic and nuclear proteins. *Protein Sci.* **26,** 925–945 CrossRef Medline
178. Caussinus, E., Kanca, O., and Affolter, M. (2011) Fluorescent fusion protein knockout mediated by anti-GFP nanobody. *Nat. Struct. Mol. Biol.* **19,** 117–121 CrossRef Medline
179. Caussinus, E., and Affolter, M. (2016) deGradFP: a system to knockdown GFP-tagged proteins. *Methods Mol. Biol.* **1478,** 177–187 CrossRef Medline
180. Fulcher, L. J., Hutchinson, L. D., Macartney, T. J., Turnbull, C., and Sapkota, G. P. (2017) Targeting endogenous proteins for degradation through the affinity-directed protein missile system. *Open Biol.* **7,** 170066 CrossRef Medline
181. Fulcher, L. J., Macartney, T., Bozatzi, P., Hornberger, A., Rojas-Fernandez, A., and Sapkota, G. P. (2016) An affinity-directed protein missile system for targeted proteolysis. *Open Biol.* **6,** 160255 CrossRef Medline
182. Ochoa-Espinosa, A., Harmansa, S., Caussinus, E., and Affolter, M. (2017) Myosin II is not required for *Drosophila* tracheal branch elongation and cell intercalation. *Development* **144,** 2961–2968 CrossRef Medline
183. Pasakarnis, L., Frei, E., Caussinus, E., Affolter, M., and Brunner, D. (2016) Amnioserosa cell constriction but not epidermal actin cable tension autonomously drives dorsal closure. *Nat. Cell Biol.* **18,** 1161–1172 CrossRef Medline
184. Yamaguchi, N., Colak-Champollion, T., and Knaut, H. (2019) zGrad is a nanobody-based degron system that inactivates proteins in zebrafish. *Elife*. CrossRef
185. Daniel, K., Icha, J., Horenburg, C., Müller, D., Norden, C., and Mansfeld, J. (2018) Conditional control of fluorescent protein degradation by an auxin-dependent nanobody. *Nat. Commun.* **9,** 3297 CrossRef Medline
186. Wang, S., Tang, N. H., Lara-Gonzalez, P., Zhao, Z., Cheerambathur, D. K., Prevo, B., Chisholm, A. D., Desai, A., and Oegema, K. (2017) A toolkit for GFP-mediated tissue-specific protein degradation in *C. elegans*. *Development* **144,** 2694–2701 CrossRef Medline
187. Kanner, S. A., Morgenstern, T., and Colecraft, H. M. (2017) Sculpting ion channel functional expression with engineered ubiquitin ligases. *Elife* **6,** e29744 CrossRef Medline
188. Morgenstern, T. J., Park, J., Fan, Q. R., and Colecraft, H. M. (2019) A potent voltage-gated calcium channel inhibitor engineered from a nanobody targeted to auxiliary CaVβ subunits. *Elife* **8,** e49253 CrossRef Medline
189. Jullien, D., Vignard, J., Fedor, Y., Béry, N., Olichon, A., Crozatier, M., Erard, M., Cassard, H., Ducommun, B., Salles, B., and Mirey, G. (2016) Chromatibody, a novel non-invasive molecular tool to explore and manipulate chromatin in living cells. *J. Cell Sci.* **129,** 2673–2683 CrossRef Medline
190. Bery, N., Keller, L., Soulié, M., Gence, R., Iscache, A.-L., Cherier, J., Cabantous, S., Sordet, O., Lajoie-Mazenc, I., Pedelacq, J.-D., Favre, G., and Olichon, A. (2019) A targeted protein degradation cell-based screening for nanobodies selective toward the cellular RHOB GTP-bound conformation. *Cell Chem Biol.* **26,** 1544–1558.e6 CrossRef Medline
191. Zhao, W., Pferdehirt, L., and Segatori, L. (2018) Quantitatively predictable control of cellular protein levels through proteasomal degradation. *ACS Synth Biol.* **7,** 540–552 CrossRef Medline
192. Ortiz Zacarías, N. V., Lenselink, E. B., IJzerman, A. P., Handel, T. M., and Heitman, L. H. (2018) Intracellular receptor modulation: novel approach to target GPCRs. *Trends Pharmacol. Sci.* **39,** 547–559 CrossRef Medline
193. Heukers, R., De Groof, T. W. M., and Smit, M. J. (2019) Nanobodies detecting and modulating GPCRs outside in and inside out. *Curr. Opin. Cell Biol.* **57,** 115–122 CrossRef Medline
194. Irannejad, R., Tomshine, J. C., Tomshine, J. R., Chevalier, M., Mahoney, J. P., Steyaert, J., Rasmussen, S. G. F., Sunahara, R. K., El-Samad, H., Huang, B., and von Zastrow, M. (2013) Conformational biosensors reveal GPCR signalling from endosomes. *Nature* **495,** 534–538 CrossRef Medline
195. Staus, D. P., Wingler, L. M., Strachan, R. T., Rasmussen, S. G. F., Pardon, E., Ahn, S., Steyaert, J., Kobilka, B. K., and Lefkowitz, R. J. (2014) Regulation of β_2-adrenergic receptor function by conformationally selective single-domain intrabodies. *Mol. Pharmacol.* **85,** 472–481 CrossRef Medline
196. Irannejad, R., Pessino, V., Mika, D., Huang, B., Wedegaertner, P. B., Conti, M., and von Zastrow, M. (2017) Functional selectivity of GPCR-directed drug action through location bias. *Nat. Chem. Biol.* **13,** 799–806 CrossRef Medline
197. Tafesse, F. G., Guimaraes, C. P., Maruyama, T., Carette, J. E., Lory, S., Brummelkamp, T. R., and Ploegh, H. L. (2014) GPR107, a G-protein-coupled receptor essential for intoxication by *Pseudomonas aeruginosa* exotoxin A, localizes to the Golgi and is cleaved by furin. *J. Biol. Chem.* **289,** 24005–24018 CrossRef Medline
198. Stoeber, M., Jullié, D., Lobingier, B. T., Laeremans, T., Steyaert, J., Schiller, P. W., Manglik, A., and von Zastrow, M. (2018) A genetically encoded biosensor reveals location bias of opioid drug action. *Neuron* **98,** 963–976.e5 CrossRef Medline
199. Livingston, K. E., Mahoney, J. P., Manglik, A., Sunahara, R. K., and Traynor, J. R. (2018) Measuring ligand efficacy at the μ-opioid receptor using a conformational biosensor. *Elife* **7,** e32499 CrossRef Medline
200. De Vlieger, D., Ballegeer, M., Rossey, I., Schepens, B., and Saelens, X. (2018) Single-domain antibodies and their formatting to combat viral infections. *Antibodies (Basel)* **8,** 1 CrossRef Medline

201. Laursen, N. S., Friesen, R. H. E., Zhu, X., Jongeneelen, M., Blokland, S., Vermond, J., van Eijgen, A., Tang, C., van Diepen, H., Obmolova, G., van der Neut Kolfschoten, M., Zuijdgeest, D., Straetemans, R., Hoffman, R. M. B., Nieusma, T., *et al.* (2018) Universal protection against influenza infection by a multidomain antibody to influenza hemagglutinin. *Science* **362,** 598–602 CrossRef Medline
202. Wrapp, D., De Vlieger, D., Corbett, K. S., Torres, G. M., Wang, N., Van Breedam, W., Roose, K., van Schie, L., Response Team, V.-C. C.-1., Hoffmann, M., Pöhlmann, S., Graham, B. S., Callewaert, N., Schepens, B., Saelens, X., *et al.* (2020) Structural basis for potent neutralization of betacoronaviruses by single-domain camelid antibodies. *Cell* **181,** 1436–1441 CrossRef Medline
203. Hanke, L., Perez, L. V., Sheward, D. J., Das, H., Schulte, T., Morro, A. M., Corcoran, M., Achour, A., Hedestam, G. K., Hällberg, B. M., Murrell, B., and McInerney, G. M. (2020) An alpaca nanobody neutralizes SARS-CoV-2 by blocking receptor interaction. *bioRxiv* CrossRef
204. Fan, H., Walker, A. P., Carrique, L., Keown, J. R., Serna Martin, I., Karia, D., Sharps, J., Hengrung, N., Pardon, E., Steyaert, J., Grimes, J. M., and Fodor, E. (2019) Structures of influenza A virus RNA polymerase offer insight into viral genome replication. *Nature* **573,** 287–290 CrossRef Medline
205. Vercruysse, T., Pardon, E., Vanstreels, E., Steyaert, J., and Daelemans, D. (2010) An intrabody based on a llama single-domain antibody targeting the N-terminal α-helical multimerization domain of HIV-1 rev prevents viral production. *J. Biol. Chem.* **285,** 21768–21780 CrossRef Medline
206. Bouchet, J., Basmaciogullari, S. E., Chrobak, P., Stolp, B., Bouchard, N., Fackler, O. T., Chames, P., Jolicoeur, P., Benichou, S., and Baty, D. (2011) Inhibition of the Nef regulatory protein of HIV-1 by a single-domain antibody. *Blood* **117,** 3559–3568 CrossRef Medline
207. Hanke, L., Schmidt, F. I., Knockenhauer, K. E., Morin, B., Whelan, S. P., Schwartz, T. U., and Ploegh, H. L. (2017) Vesicular stomatitis virus N protein-specific single-domain antibody fragments inhibit replication. *EMBO Rep.* **18,** 1027–1037 CrossRef Medline
208. Hanke, L., Knockenhauer, K. E., Brewer, R. C., van Diest, E., Schmidt, F. I., Schwartz, T. U., and Ploegh, H. L. (2016) The antiviral mechanism of an influenza A virus nucleoprotein-specific single-domain antibody fragment. *mBio* **7,** e01569-16 CrossRef Medline
209. Darling, T. L., Sherwood, L. J., and Hayhurst, A. (2017) Intracellular crosslinking of filoviral nucleoproteins with Xintrabodies restricts viral packaging. *Front. Immunol.* **8,** 1197 CrossRef Medline
210. Hemmer, C., Djennane, S., Ackerer, L., Hleibieh, K., Marmonier, A., Gersch, S., Garcia, S., Vigne, E., Komar, V., Perrin, M., Gertz, C., Belval, L., Berthold, F., Monsion, B., Schmitt-Keichinger, C., *et al.* (2018) Nanobody-mediated resistance to grapevine fanleaf virus in plants. *Plant Biotechnol. J.* **16,** 660–671 CrossRef Medline
211. Orlov, I., Hemmer, C., Ackerer, L., Lorber, B., Ghannam, A., Poignavent, V., Hleibieh, K., Sauter, C., Schmitt-Keichinger, C., Belval, L., Hily, J.-M., Marmonier, A., Komar, V., Gersch, S., Schellenberger, P., *et al.* (2020) Structural basis of nanobody recognition of grapevine fanleaf virus and of virus resistance loss. *Proc. Natl. Acad. Sci. U.S.A.* **117,** 10848–10855 CrossRef Medline
212. Tang, J. C., Drokhlyansky, E., Etemad, B., Rudolph, S., Guo, B., Wang, S., Ellis, E. G., Li, J. Z., and Cepko, C. L. (2016) Detection and manipulation of live antigen-expressing cells using conditionally stable nanobodies. *Elife* **5,** e15312 CrossRef Medline
213. Ashour, J., Schmidt, F. I., Hanke, L., Cragnolini, J., Cavallari, M., Altenburg, A., Brewer, R., Ingram, J., Shoemaker, C., and Ploegh, H. L. (2015) Intracellular expression of camelid single-domain antibodies specific for influenza virus nucleoprotein uncovers distinct features of its nuclear localization. *J. Virol.* **89,** 2792–2800 CrossRef Medline
214. Cao, J., Zhong, N., Wang, G., Wang, M., Zhang, B., Fu, B., Wang, Y., Zhang, T., Zhang, Y., Yang, K., Chen, Y., Yuan, Q., and Xia, N. (2019) Nanobody-based sandwich reporter system for living cell sensing influenza A virus infection. *Sci. Rep.* **9,** 15899 CrossRef Medline
215. Tome-Amat, J., Ramos, I., Amanor, F., Fernández-Sesma, A., and Ashour, J. (2019) Influenza A virus utilizes low-affinity, high-avidity interactions with the nuclear import machinery to ensure infection and immune evasion. *J. Virol.* **93,** e01046-18 CrossRef Medline
216. Waugh, D. S. (2005) Making the most of affinity tags. *Trends Biotechnol.* **23,** 316–320 CrossRef Medline
217. Küey, C., Larocque, G., Clarke, N. I., and Royle, S. J. (2019) Unintended perturbation of protein function using GFP nanobodies in human cells. *J. Cell Sci.* **132,** jcs234955 CrossRef Medline
218. De Genst, E. J., Guilliams, T., Wellens, J., O'Day, E. M., Waudby, C. A., Meehan, S., Dumoulin, M., Hsu, S.-T. D., Cremades, N., Verschueren, K. H. G., Pardon, E., Wyns, L., Steyaert, J., Christodoulou, J., and Dobson, C. M. (2010) Structure and properties of a complex of α-synuclein and a single-domain camelid antibody. *J. Mol. Biol.* **402,** 326–343 CrossRef Medline
219. Jin-Jing Li, F. X. (2018) Biopanning of anti c-Myc-tag nanobodies and its application for bioimaging. *China Biotechnol.* **38,** 61–67
220. Virant, D., Traenkle, B., Maier, J., Kaiser, P. D., Bodenhöfer, M., Schmees, C., Vojnovic, I., Pisak-Lukáts, B., Endesfelder, U., and Rothbauer, U. (2018) A peptide tag-specific nanobody enables high-quality labeling for dSTORM imaging. *Nat. Commun.* **9,** 930 CrossRef Medline
221. Boersma, S., Khuperkar, D., Verhagen, B. M. P., Sonneveld, S., Grimm, J. B., Lavis, L. D., and Tanenbaum, M. E. (2019) Multi-color single-molecule imaging uncovers extensive heterogeneity in mRNA decoding. *Cell* **178,** 458–472.e19 CrossRef Medline
222. Lutje Hulsik, D., Liu, Y., Strokappe, N. M., Battella, S., El Khattabi, M., McCoy, L. E., Sabin, C., Hinz, A., Hock, M., Macheboeuf, P., Bonvin, A. M. J. J., Langedijk, J. P. M., Davis, D., Forsman Quigley, A., Aasa-Chapman, M. M. I., *et al.* (2013) A gp41 MPER-specific llama VHH requires a hydrophobic CDR3 for neutralization but not for antigen recognition. *PLoS Pathog.* **9,** e1003202 CrossRef Medline
223. Vigano, M. A., Ell, C.-M., Kustermann, M. M., Aguilar, G., Matsuda, S., Zhao, N., Stasevich, T. J., Pyrowolakis, G., and Affolter, M. (2020) Protein manipulation using single copies of short peptide tags in cultured cells and in *Drosophila melanogaster*. *bioRxiv* CrossRef
224. Rahbarizadeh, F., Ahmadvand, D., and Moghimi, S. M. (2019) CAR T-cell bioengineering: single variable domain of heavy chain antibody targeted CARs. *Adv. Drug Deliv. Rev.* **141,** 41–46 CrossRef Medline
225. Morsut, L., Roybal, K. T., Xiong, X., Gordley, R. M., Coyle, S. M., Thomson, M., and Lim, W. A. (2016) Engineering customized cell sensing and response behaviors using synthetic Notch receptors. *Cell* **164,** 780–791 CrossRef Medline
226. Chang, H.-J., Mayonove, P., Zavala, A., De Visch, A., Minard, P., Cohen-Gonsaud, M., and Bonnet, J. (2018) A modular receptor platform to expand the sensing repertoire of bacteria. *ACS Synth. Biol.* **7,** 166–175 CrossRef Medline
227. Klein, Á., Kovács, M., Muskotál, A., Jankovics, H., Tóth, B., Pósfai, M., and Vonderviszt, F. (2018) Nanobody-displaying flagellar nanotubes. *Sci. Rep.* **8,** 3584 CrossRef Medline
228. Wendel, S., Fischer, E. C., Martínez, V., Seppälä, S., and Nørholm, M. H. H. (2016) A nanobody:GFP bacterial platform that enables functional enzyme display and easy quantification of display capacity. *Microb. Cell Fact.* **15,** 71 CrossRef Medline
229. van den Berg van Saparoea, H. B., Houben, D., de Jonge, M. I., Jong, W. S. P., and Luirink, J. (2018) Display of recombinant proteins on bacterial outer membrane vesicles by using protein ligation. *Appl. Environ. Microbiol.* **84,** e02567-17 CrossRef Medline
230. Liu, L., Wang, W., Matz, J., Ye, C., Bracq, L., Delon, J., Kimata, J. T., Chen, Z., Benichou, S., and Zhou, P. (2016) The glycosylphosphatidylinositol-anchored variable region of llama heavy chain-only antibody JM4 efficiently blocks both cell-free and T cell-T cell transmission of human immunodeficiency virus type 1. *J. Virol.* **90,** 10642–10659 CrossRef Medline
231. Lee, S., Park, H., Kyung, T., Kim, N. Y., Kim, S., Kim, J., and Heo, W. D. (2014) Reversible protein inactivation by optogenetic trapping in cells. *Nat. Methods* **11,** 633–636 CrossRef Medline
232. Redchuk, T. A., Karasev, M. M., Verkhusha, P. V., Donnelly, S. K., Hülsemann, M., Virtanen, J., Moore, H. M., Vartiainen, M. K., Hodgson, L., and Verkhusha, V. V. (2020) Optogenetic regulation of endogenous proteins. *Nat. Commun.* **11,** 605 CrossRef Medline
233. Yu, D., Lee, H., Hong, J., Jung, H., Jo, Y., Oh, B.-H., Park, B. O., and Heo, W. D. (2019) Optogenetic activation of intracellular antibodies

for direct modulation of endogenous proteins. *Nat. Methods* **16,** 1095–1100 CrossRef Medline

234. Gil, A. A., Zhao, E. M., Wilson, M. Z., Goglia, A. G., Carrasco-Lopez, C., Avalos, J. L., and Toettcher, J. E. (2019) Optogenetic control of protein binding using light-switchable nanobodies. *bioRxiv* CrossRef
235. Farrants, H., Tarnawski, M., Müller, T. G., Otsuka, S., Hiblot, J., Koch, B., Kueblbeck, M., Kräusslich, H.-G., Ellenberg, J., and Johnsson, K. (2020) Chemogenetic control of nanobodies. *Nat. Methods* **17,** 279–282 CrossRef Medline
236. Jedlitzke, B., Yilmaz, Z., Dörner, W., and Mootz, H. D. (2020) Photobodies: light-activatable single-domain antibody fragments. *Angew. Chem. Int. Ed. Engl.* **59,** 1506–1510 CrossRef Medline
237. Bridge, T., Shaikh, S. A., Thomas, P., Botta, J., McCormick, P. J., and Sachdeva, A. (2019) Site-specific encoding of photoactivity in antibodies enables light-mediated antibody-antigen binding on live cells. *Angew. Chem. Int. Ed. Engl.* **58,** 17986–17993 CrossRef Medline
238. Hartmann, L., Botzanowski, T., Galibert, M., Jullian, M., Chabrol, E., Zeder-Lutz, G., Kugler, V., Stojko, J., Strub, J.-M., Ferry, G., Frankiewicz, L., Puget, K., Wagner, R., Cianférani, S., and Boutin, J. A. (2019) VHH characterization: comparison of recombinant with chemically synthesized anti-HER2 VHH. *Protein Sci.* **28,** 1865–1879 CrossRef Medline
239. Traenkle, B., Segan, S., Fagbadebo, F. O., Kaiser, P. D., and Rothbauer, U. (2020) A novel epitope tagging system to visualize and monitor antigens in live cells with chromobodies. *Sci. Rep.* **10,** 14267 CrossRef Medline
240. Strokappe, N. M., Hock, M., Rutten, L., Mccoy, L. E., Back, J. W., Caillat, C., Haffke, M., Weiss, R. A., Weissenhorn, W., and Verrips, T. (2019) Super potent bispecific llama VHH antibodies neutralize HIV via a combination of gp41 and gp120 epitopes. *Antibodies (Basel)* **8,** 38 CrossRef Medline