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Engineering novel binding proteins from nonimmunoglobulin domains

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Not all adaptive immune systems use the immunoglobulin fold as the basis for specific recognition molecules: sea lampreys, for example, have evolved an adaptive immune system that is based on leucine-rich repeat proteins. Additionally, many other proteins, not necessarily involved in adaptive immunity, mediate specific high-affinity interactions. Such alternatives to immunoglobulins represent attractive starting points for the design of novel binding molecules for research and clinical applications. Indeed, through progress and increased experience in library design and selection technologies, gained not least from working with synthetic antibody libraries, researchers have now exploited many of these novel scaffolds as tailor-made affinity reagents. Significant progress has been made not only in the basic science of generating specific binding molecules, but also in applications of the selected binders in laboratory procedures, proteomics, diagnostics and therapy. Challenges ahead include identifying applications where these novel proteins can not only be an alternative, but can enable approaches so far deemed technically impossible, and delineate those therapeutic applications commensurate with the molecular properties of the respective proteins.

At present, antibodies are indisputably the most successful binding molecules in biomedical science. Part of this success is historic: until about 15 years ago, the immune system was the only source of molecular diversity from which specificity could be selected at will. Today, however, highly specific and tight-binding antibodies can easily be generated by a variety of methods using either the immune system or synthetic libraries. Immunoglobulins also show remarkable diversity in the types of compounds they can bind: proteins, peptides, sugars and diverse small molecules. The extensive investigations, engineering and use of antibodies have allowed researchers to define rules for selecting affinity reagents from synthetic libraries.

However, limitations of immunoglobulins have also been uncovered, based largely on their biophysical properties and their complicated molecular composition. This has inspired many attempts to create alternative binding proteins, based either on scaffolds with the immunoglobulin fold[1] or on completely different protein topologies. We present here an overview of the latter group, describing the molecular scaffold characteristics, the applications of such binders as well as the selection technologies used to isolate such binding molecules from synthetic repertoires (**Tables 1** and **2**). We cite many recent and relevant publications in the text, but we provide a more extensive list of references on the different alternative binding proteins and selection technologies in **Supplementary Notes** online. In this review, the term 'scaffold' is meant to describe a protein framework that can carry altered amino acids or sequence insertions that confer on protein variants different functions, usually for binding specific targets.

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Why not antibodies?

The motivation to create alternative binding molecules derives from a desire to enable new applications where antibodies show some limitations. The IgG molecule, while providing bivalency, fully human composition and a long serum half-life, is a large, multidomain assemblage with disulfide bonds and glycosylation, both essential for immune effector functions. Consequently, IgG molecules are comparatively difficult and expensive to manufacture. Whereas antibodies have been successful in therapy (reviewed by Moroney and A.P. in ref. 2), other molecular formats are needed for additional innovative therapeutic approaches—and for nontherapeutic applications the IgG format is irrelevant. Smaller versions of antibodies, such as scFv, Fab fragments and multivalent miniantibodies can be produced in microbial hosts, but their stability still relies to a significant degree on intradomain disulfide bonds (see Wörn and A.P.[3]), which do not form in reducing intracellular environments. In addition, some antibody fragments tend to aggregate, especially when fused to additional domains added, for example, to achieve therapeutic efficacy, for detection or for immobilization (see below). Some of these fusion proteins need to be expressed in eukaryotic cells[4] because they fold poorly in bacteria. Finally, the success, and consequently the extensive use, of antibodies has led to a complicated patent situation of antibody technologies and applications.

Ideally, an alternative to antibodies should improve on all of the above mentioned limitations, while not compromising target affinity and specificity, although the intended application will determine the relative importance of each molecular feature. Preferably such a specific binding protein will be a small single-chain protein, as this facilitates the application of most selection technologies (see below) and the subsequent construction of fusion proteins. High thermodynamic stability and the absence of disulfide bonds or free cysteines provide a clear advantage, as these properties allow for the expression

Table 1 Scaffolds used for generating protein binders with prescribed specificity

Scaffold name[a]	Fold (size in aa)	Randomization (number of randomized aa)	Target proteins[a,b]	Selection method used	Company; Key ref.[c]
CTLA-4	β-sandwich (136)	Loop (6 aa around RGD motif)	Integrin	Phage display	Evogenix; 127
Tendamistat	β-sandwich (74)	Loop (6 to 8 aa; RGD for integrin)	MAb, integrins	Phage display	DuPont; 133
^{10}FN3 (Fibronectin)	β-sandwich (94)	Different loops (different strategies; RGD for integrin)	TNFα, ubiquitin, integrin, SH3, estrogen receptor domains	Phage display; mRNA display; yeast two-hybrid	Compound Therapeutics; 8, 49
Neocarzinostatin	β-sandwich (113)	Loop grafting; binding cleft randomization (13 aa)	Lysozyme (testosterone)	Phage display	134, 135
CBM4-2	β-sandwich (168)	Binding site (12 aa)	Human IgG4 (sugars: xylan, manan)	Phage display	136
Lipocalins	β-barrel (160–180)	4 loops (16 aa)	Hemoglobin, CTLA-4 streptavidin, (digoxigenin, fluorescein, benzyl butyl phthalate)	Screening; phage display; ribosome display	Pieris Proteolab; 7, 58
T-cell receptor	β-sandwich (250)	5 aa or error-prone PCR or several randomized loops in CDR3	Peptide/MHC complexes	Yeast surface display/FACS; phage display	Avidex; 85, 86, 88
Protein A domain (protein Z)	α_3 (58)	13 aa on flat surface (α-helices 1 and 2)	*Taq* DNA polymerase, insulin, apolipoprotein A1, protein Z, IgA, Factor VIII, RSV protein G fragment, CD28, HER-2/*neu*	Phage display	Affibody; 56
Im9	α_4 (86)	Error-prone PCR	ColE9	*In vitro* compartmentalization	137
Designed AR proteins	α_2/β_2 repeated (67 + n·33)[d]	7 aa, on β-turn and first α-helix of every repeat	MBP, p38, JNK2, APH	Ribosome display	Molecular Partners; 41
Designed TPR proteins	α_3 repeated (18 + n·34)[d]	n.d.	Hsp90 peptide	Rational design	75
Zinc finger	α/β (Zn^{2+}) (26)	5 aa on helix	MAb	Phage display; peptide screening	124
pVIII	Mainly α (50)	6 aa on helix	Fibrinogen	Phage display	138
Avian pancreatic polypeptide	α/ppII helix (36)	5 aa randomized or rational design	CBP KIX, Abl-SH3	Phage display; rational design	139, 140
GCN4	α (33)	19 aa 'grafted'	gp41 N-terminal heptad repeat	Rational design	141
WW domain	β_3 (52)	8 aa	NacWW1 peptide	Phage display	142
Src homology domain 3 (SH3)	Mainly β (~60)	6 or 12 aa	Different peptides (Abl-1, Src, Nef)	Phage display; λ phage display	70, 71
Src homology domain 2 (SH2)	α/β (~100)	5 aa	Phosphorylated peptides	Filter screening	69
PDZ domains	α/β (~100)	Error-prone PCR; n.d.	Peptides with free C terminus	Yeast two-hybrid; phage display; rational design	BioTech Studio; 46, 72, 74
TEM-1 β-lactamase	α/β (265)	3 to 12 aa in two loops	β-galactosidase, streptavidin, ferritin, mAbs	Phage display	26
GFP	β-barrel (238)	Loop randomization (12 or 18 aa); C-terminal peptide fusion (20 aa)	n.d. (functional screening)	Visual screening; FACS	28
Thioredoxin	α/β (108)	20 aa loop insert	mAbs, CDK2, Mdm-2, E2F, E6	Flagellin fusion; phage display; yeast two-hybrid; rational design	Aptanomics; 143
Staphylococcal nuclease	α/β (149)	16 aa loop insert	(Ydr517d)	Functional screening (spindle checkpoint inactivation; pheromone response pathway inhibition)	119
PHD-finger	β-loops (50–100)	5 aa loop insert	CtBP2	Rational design	144
CI-2	α/β (64)	Peptide grafting	Mdm-2	Rational design	145

Table 1 (continued)

Scaffold name[a]	Fold (size in aa)	Randomization (number of randomized aa)	Target proteins[a,b]	Selection method used	Company; Key ref.[c]
BPTI	α/β (58)	5 aa	Human neutrophil elastase	Phage display	83
APPI	α/β (58)	5 aa	TF-FVIIa, plasma kallikrein	Phage display	146, 147
HPSTI	α/β-loops (56)	6 to 8 aa; n.d.	Chymotrypsin, NS3 protease, leukocyte elastase	Phage display; rational design	148
Ecotin	β-sandwich (142)	20 aa or rational design	uPA, trypsin, plasma kallikrein, MT-SP1, FXIIA	Phage display	81
LACI-D1	α/β (58)	4, 5 or 9 aa	Plasmin, thrombin, plasma kallikrein	Phage display	Dyax; 149
LDTI	α/β (46)	5 aa	Thrombin	Phage display	150
MTI-II	α/β[e] (63)	5 aa	Trypsin, chymotrypsin	Phage display	151
Scorpion toxins	α/β_3 (25-40)	Loop grafting or 4 aa	Acetylcholine receptor, gp120, mAbs	Rational design; screening; phage display	Selecore; 76, 77
Insect defensin A peptide	α/β_2 (29)	7 aa in two loops	TNFα, TNFR, mAb	Phage display	152
EETI-II	Coiled, cys-rich (28)	6 aa; n.d.	Elastases, chymotrypsin, trypsin, carboxypeptidase, antibody epitopes	mRNA display; screening	153
Min-23	β/coiled (23)	10 aa in β-turn	MAbs, AMA-1, Tom70, HIV-1 Nef	Phage display	78
CBD	β_3 (36)	11 aa on loops or 7 aa on flat surface	Alkaline phosphatase, α-amylase (Ni-NTA beads)	Phage display	154, 155
PBP	α/β (variable)	n.d.	(Trinitrotoluene, L-lactate, serotonin, Zn^{2+})	Rational design	Becton-Dickinson, Nomadics; 131
Cytochrome b_{562}	α_4 (106)	9 aa in two loops	(N-methyl-p-nitrobenzylamine)	Phage display	156
Ldl receptor domain A	Coiled, cys-rich (~40)	n.d.	n.d.	n.d. (selection; avidity eng.)	Avidia; 157[c]
γ-crystallin	β-sandwich (~174)	n.d. (8 aa)	n.d.	n.d.	Scil Proteins; 158[c]
Ubiquitin	α/β (76)	n.d. (8 aa)	n.d.	n.d.	Scil Proteins; 159[c]
Transferrin	α/β (~679)	n.d. (constrained peptides)	n.d.	n.d.	Biorexis; 160[c]
C-type lectin-like domain	α/β (129)	n.d.	n.d. (different carbohydrates)	n.d.	Borean; 161[c]

[a]Abbreviations: ^{10}FN3, 10th fibronectin type 3 domain; aa, amino acid; ABL1, Abelson tyrosine kinase; APH, Aminoglycoside phosphotransferase; AMA-1, malarial apical membrane protein 1; APP, avian pancreatic polypeptide; APPI, Alzheimer amyloid β-protein precursor inhibitor; AR, ankyrin repeat; BPTI, bovine pancreatic trypsin inhibitor; CBD, cellulose-binding domain; CBM4-2, carbohydrate-binding module 4 of family 2 of xylanase of *Rhodothermus marinus*; CDK-2, Cyclin-dependent kinase; CI2, chymotrypsin inhibitor 2; CPB, carboxypeptidase B ; CTBP-2, C-terminal binding protein 2 (a transcription corepressor); CTLA-4, cytotoxic T lymphocyte-associated antigen 4; E2F, E2 promoter binding factor: EETI II, *Ecballium elaterium* trypsin inhibitor II; FXIIA, Blood clotting factor XIIa; GCN4, General control nonderepressible (yeast transcription factor); Gp-41, HIV gene product 41; hPSTI, human pancreatic secretory trypsin inhibitor; Hsp, Heat shock protein (molecular chaperone); Im9, immunity protein 9; JNK-2, c-Jun N-terminal kinase 2; CBP KIX, Kinase-inducible domain of CREB-binding protein; LACI-D1, human lipoprotein–associated coagulation inhibitor domain 1; LDL-R, Low-density lipoprotein receptor; LDTI, leech-derived trypsin inhibitor; LMTI II, mustard trypsin inhibitor 2; MBP, maltose binding protein; MDM-2, Murine double minute 2 gene product; MT-SP-1, Membrane-type serine protease 1; NACWWW, N-acetylated WW1 peptide; NEF, HIV negative factor; uPA, Urokinase-Plasminogen Activator; PBP, periplasmic binding proteins; PDZ, PSD-95/Discs-large/ZO-1; PHD finger, plant homeodomain finger protein; PPII helix, poly proline type II helix; pVIII, protein VIII of filamentous bacteriophage; RSV, Respiratory Syncytial Virus; SH2, src homology domain 2; SH3, src homology domain 3; TF-FVIIb, tissue factor-Factor VIIb complex; TNF-α, Tumor necrosis factor α; TNFR, TNF receptor; Tom70, outer membrane translocase receptor of human mitochondria; TPR, tetratricopeptide repeat. [b]Non-protein targets are shown in parentheses. [c]For the bottom group, no refereed publications were available at the time of writing that would have successfully demonstrated the generation of new specificities. [d]The number of repeats contained in repeat proteins can be varied. In the examples, the number of amino acids in the capping repeats is given together with the varying number (*n*) of central repeats of a given number of amino acids. [e]Predicted by homology.

of functional molecules in the reducing environment of the bacterial cytoplasm, which usually gives higher yields than periplasmic expression, and is more convenient and economical than refolding *in vitro*, or eukaryotic expression.

Applications of alternative binding proteins

Alternative binding molecules are gradually entering the arenas of classical antibody applications and are currently being evaluated.

Therapeutic applications of binding proteins. An ever-increasing number of therapeutic antibodies are reaching the clinic[2], most of them as IgG molecules. Their therapeutic effect is obtained either by blocking a target, or by exerting effector functions residing in the Fc region to activate the complement system or cytotoxic cells. Alternative binding proteins do not have effector functions, and, at first sight, their therapeutic mode of action would appear to be limited to target neutralization. Approaches with this aim in mind have indeed been used in combination with different molecular designs[5–8]. Targets reported thus far include the coreceptor of T-cell activation CD28 (inhibiting the interaction with CD80)[6], the cytokine tumor necrosis factor-α (inhibiting the receptor interaction)[8] and the tumor antigen HER2 (only binding was reported)[5].

However, to confer effector functions on these proteins, one can also fuse those scaffolds that fold well to the Fc region of antibodies, an

Table 2 Selection methods for libraries of alternative binding proteins

Selection Technologies used[a]	*In vitro*[b]	No antigen purification[c]	Disulfide compatibility[d]	References[e]
Filamentous phage display	–	–	+	56
λ-phage display	–	–	–	70
Yeast surface display	–	–	+	162
Bacterial surface display[f]	–	–	±	106–109
Flagellin display	–	–	+	99
Yeast two-hybrid	–	+	–	72
DHFR PCA	–	+	–	113
Ribosome display	+	–	+	41
mRNA display	+	–	+	8
In vitro compartmentalization	+	–	+	137
Potential selection technologies[a,g]	***In vitro*[b]**	**No antigen purification[c]**	**Disulfide compatibility[d]**	**References[g]**
T7 phage display	–	–	–	Novagen
β-lactamase PCA	–	+	–	115
Split ubiquitin	–	+	–	116
GFP PCA	–	+	–	114
YFP PCA	–	+	–	163
Luciferase PCA	–	+	–	164
Bacterial two-hybrid	–	+	–	117
Mammalian two-hybrid	–	+	–	118
DNA display	+	–	+	110–112

[a]Abbreviations used: DHFR, dihydrofolate reductase; GFP, green fluorescent protein; PCA, protein fragment complementation assay; YFP, yellow fluorescent protein. [b]As *in vitro* selection systems do not require transformation steps, highly diverse libraries are obtained with less effort. [c]Antigens do not have to be purified for *in vivo* selection systems. [d]Theoretically, according to environment. *In vitro* technologies can be used together with disulfide isomerase. [e]These references illustrate the use of the respective selection system in combination with alternative binding molecules. Additional references on the selection technologies can be found in the **Supplementary Notes** online. [f]Bacterial surface display is a family of technologies using different bacterial hosts, different membrane anchors with different biosynthetic routes and thus different compatibilities to disulfides. Also, the application of the screening to diverse libraries has only been reported for some systems. For details, see the cited reviews. [g]No library selections with diverse libraries of alternative binding molecules reported yet.

example of which is a protein Z:Fc fusion produced in *Escherichia coli*[9]. The production of molecules containing a glycosylated Fc fragment, which is required for Fc-receptor binding functionality[10], is laborious because they must be produced in mammalian cells. The use of 'normal' IgG molecules then becomes an obvious alternative. In contrast, other fusions, for example, with cytokines[11,12] or toxins[13], which are more difficult to produce with antibodies, may be well suited to equip alternative binding proteins with effector functions. Cytokine fusions may, for example, activate the function of effector cells at the desired site, such as a tumor, whereas toxin fusions would exert a direct killing effect on the targeted cells, thus expanding future therapeutic opportunities. Cysteine-free scaffolds offer the additional advantage that unique cysteines can be introduced by protein engineering, allowing convenient site-directed coupling of effector compounds, for example, small-molecule toxins, possibly even in a combinatorial manner[14]. This should also make binding proteins amenable to site-directed radioactive labeling, to facilitate their use in radiotherapy.

Usually, the IgG format is beneficial in therapy, because bivalency increases the functional affinity to surface-bound antigens and the half-life is increased by the special endosome recycling mechanism mediated by the Fc region[15]. Bi- or oligovalency can also be achieved in other binding molecules, either by making an oligomer genetically as a head-to-tail fusion protein[16], by Fc-fusions[9] or by fusing other oligomerization domains to the protein[17]. In general, very high affinity is desirable both for *in vitro* and *in vivo* applications. This can be achieved by a high intrinsic monovalent affinity of each binding site and/or by oligomerization. Yet, in some circumstances, oligomerization may be undesirable, such as in antagonistic molecules that should not dimerize a receptor. *In vitro*, affinity influences the sensitivity of most assays, and *in vivo*, the efficacy of target blocking is also directly related to affinity. Only in tumor or organ targeting, where more complicated relationships apply, do affinities better than 10^{-9} to 10^{-10} M seem to bring no further gain in localization, as discussed elsewhere[18].

Few or even no data exist on serum half-life, tissue penetration, tissue-to-blood ratios or immunogenicity of most alternative binding molecules. However, several alternative binding proteins are now under preclinical investigation (e.g. affibodies, fibronectins, DARPins and anticalins) or even in clinical trials[19] (http://www.dyax.com/). Due to their small size, most alternative binding proteins will probably exhibit good tissue penetration but short serum half-lives. Modulation of serum half-life by different strategies is well established, such as by site-directed PEGylation[20] on free cysteines, by the use of immunoglobulin or albumin-binding peptides or domains as fusion constructs, or by oligomerization of the protein, which at the same time increases the size and the valency of the binder.

Immunogenicity should be carefully assessed for all the scaffolds intended for therapy, as the final molecule will be a nonhuman protein, and/or the framework may have been altered by engineering. Importantly, all of them carry altered binding sites introduced by the respective diversification strategy, potentially introducing novel T-cell epitopes (**Fig. 1**). Immunogenicity can potentially affect efficacy and dosing, and it can also cause adverse side reactions, such as hypersensitivity and allergic reactions, thrombocytopenia, anemia and others. It would be especially problematic if a therapeutic protein were to elicit an immune response against one of the body's own proteins. Thus, the use of a 'human scaffold' is not necessarily advantageous. However, different strategies are now emerging for rational reduction of protein immunogenicity, including PEGylation[20] as well as T-cell epitope engineering[21–23]. Nevertheless, these methods are still experimental in nature, and the final answer can only be obtained in a clinical trial. One

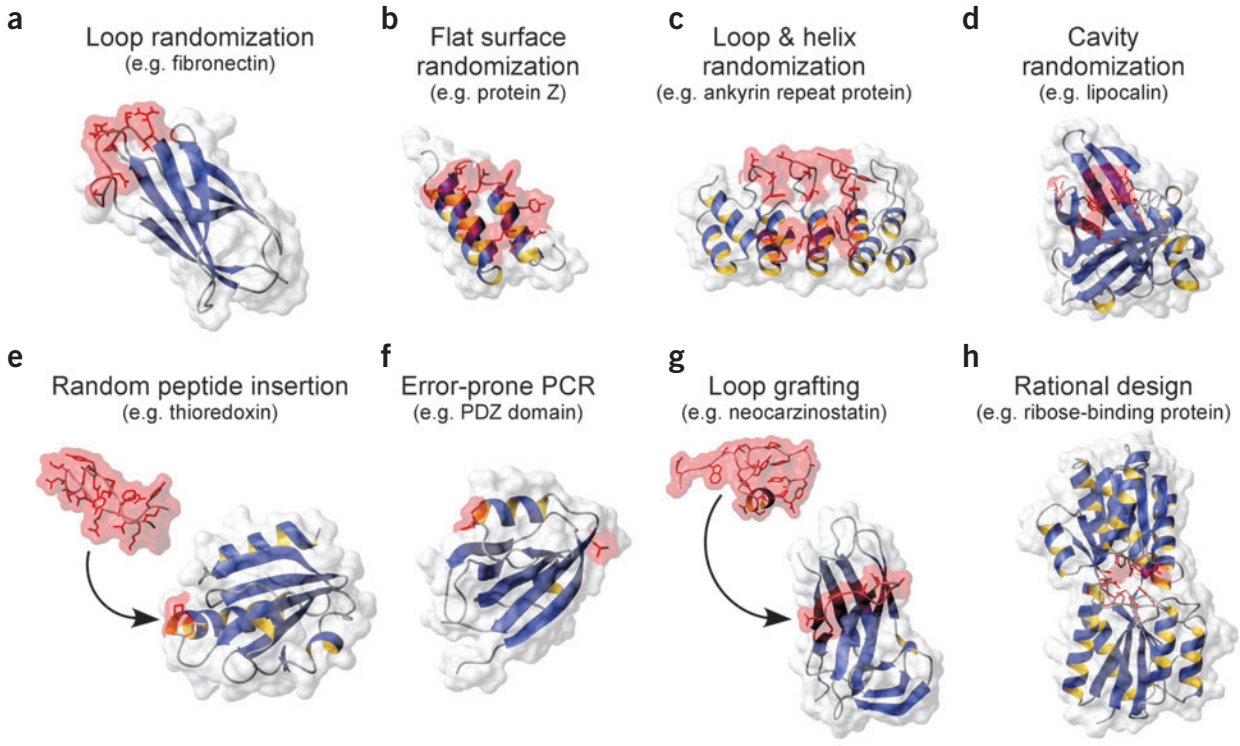

Figure 1 Binding-site engineering strategies used with different alternative scaffolds. (**a**–**h**) In combinatorial engineering approaches, sequences of a scaffold can be diversified at specified positions by means of defined randomized codons (e.g., in loops (**a**), flat surfaces (**b**), combinations of loops and helices (**c**), or cavities (**d**)), or a random peptide sequence is inserted into the scaffold (**e**), usually at a loop, or the scaffold sequence is randomized at undefined positions by error-prone PCR (**f**). Target-binding variants of the resulting libraries are subsequently isolated using selection or screening technologies (see **Tables 1** and **2**). In rational engineering approaches, preexisting binding sequences (e.g. loops) have been grafted onto a novel scaffold (**g**), or binding sites have been engineered *de novo* into a suitable scaffold (**h**). The different engineering possibilities are illustrated by alternative binding molecules where the engineering in question has been applied: loop randomization (fibronectin)[49], flat surface randomization (protein Z)[56], loop and helix randomization (ankyrin repeat protein)[41], cavity randomization (lipocalin)[58], random peptide insertion (thioredoxin)[143], error-prone PCR (PDZ domain)[72], loop grafting (neocarzinostatin)[135] and rational design (ribose-binding protein)[131]. Many other permutations of randomization strategies and scaffolds are conceivable; this figure illustrates each strategy with one published example. This figure has been prepared with MolMol[165].

should also be aware that most therapeutic proteins, including fully human antibodies, elicit some degree of immune response[23].

Diagnostic applications of binding proteins. The high specificity and affinity of antibody-antigen interactions have led to applications in many diagnostic platforms. The most popular formats are sandwich enzyme-linked immosorbent assay, flow cytometry and immunohistochemistry[24], with new miniaturized and massively parallel chip formats likely to play an ever-increasing role in the future. Alternative binding proteins will be measured against the specificity and sensitivity achievable with current reagents, still mostly dominated by monoclonal antibodies made from mouse hybridomas. For diagnostics, long shelf-life and high-level expression in bacteria, translating to low production costs, are of additional interest. Different alternative scaffolds have been successfully tested in experimental diagnostic set-ups[8,25,26], but further work is required to meet clinical standards. For cysteine-free binding molecules, again the introduction of a unique cysteine allows facile site-directed immobilization on surfaces, which might prove beneficial for protein chip applications or for coupling a fluorophore or other detectors to the protein[27]. Also, new diagnostic formats may become accessible in the form of fusion proteins to fluorescent proteins or enzymes[25]. Alternatively, scaffolds that comprise intrinsic[26,28] or engineered[29] detection means (described below) could be used as well. Finally, binding proteins other than antibodies can be advantageous in diagnostic experiments, where heterophilic anti-animal immunoglobulin antibodies can lead to false-positive signals[30].

Intracellular applications. Many drug targets are located in the cytoplasm of the cell. In principle, proteins binding to these targets would be well suited for research on such drug targets and, eventually, for intracellular therapy. Although efficient delivery of therapeutic proteins to specific cells in patients remains difficult despite extensive research on viral or other protein delivery systems[31,32], intracellular target validation in the laboratory is possible already, offering a complementary method to RNA interference or gene-knockout strategies for independent verification or when problems are encountered with these systems[33]. However, the stability of antibody fragments and several alternative binding proteins relies on disulfide bonds, which do not form in the reducing intracellular milieu[34]. This leads to limited stability and poor intracellular activity, even though extensive engineering efforts have now been able to alleviate this problem for antibodies (A.P. and colleagues[35]). For intracellular applications, a better option is alternative scaffolds that fold efficiently and have no disulfide bonds as these remain fully functional under reducing conditions, enabling the efficient binding of target proteins. Koide and colleagues[36] demonstrated this using a specific binder from a fibronectin library, which could discriminate between the

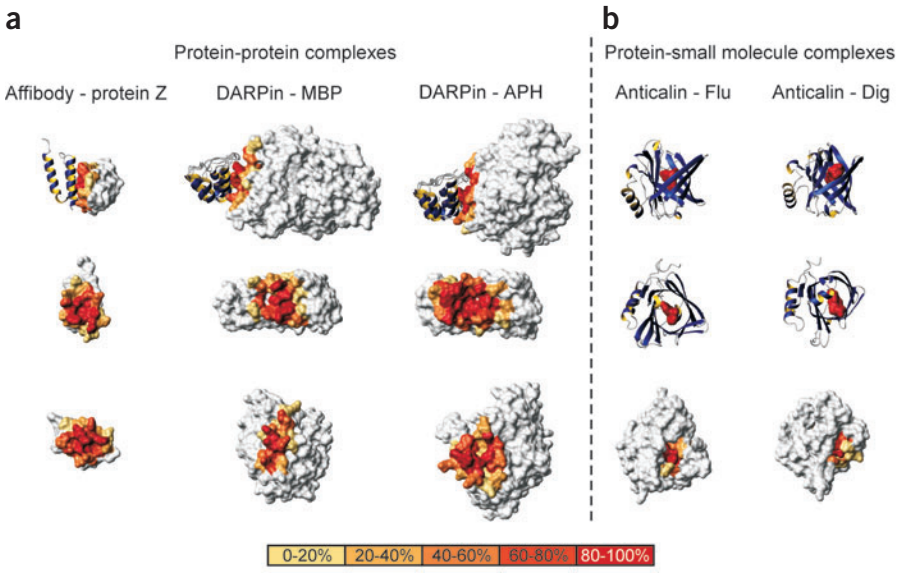

Figure 2 Structures of affinity-selected binding molecules interacting with their protein or small molecule targets. (**a**) The crystal structures of three protein-protein complexes have been determined: an 'anti-idiotypic' affibody in complex with protein Z[40] and two different DARPins in complex with either maltose-binding protein (MBP)[41] or aminoglycoside-phosphotransferase (APH)[42]. The complexes are represented in the top row with the target in solid-surface representation and the binder in ribbon representation. The open sandwich representation (opening of the complex such that both interaction interfaces are now facing the reader) of the complexes illustrates the interacting residues of the binder (middle row) and the target (bottom row). Interacting residues are colored according to the percentage of change in solvent accessible surface area upon interaction (see panel in figure). The interacting residues of the binders overlap well with the randomized potential target interaction residues of the scaffolds in all three cases. (**b**) Two crystal structures of designed lipocalins in complex with the small molecules fluorescein[95] (Flu) or digoxigenin[96] (Dig) have been determined. To illustrate the binding mode, we show two perpendicular views of the lipocalin-target complexes, either looking onto the 'side' of the β-barrel (top row) or on the 'top' of the barrel and the bound compound (middle row), with the bottom row illustrating the sequestration of the small molecule in the same orientation. The accessible surfaces have been assessed using NACCESS (http://wolf.bms.umist.ac.uk/naccess/), and the figure has been prepared using MolMol[165].

ligand-bound and unbound form of the human estrogen receptor in the cell nucleus; more recently, work in our laboratory[37] has shown that a designed ankyrin-repeat protein ('DARPin'), which binds and inhibits an intracellular kinase, leads to a kinase knockout phenotype.

Cocrystallization. High-resolution crystal structures of proteins not only contribute to the understanding of biological processes but are also pivotal for rational drug design. Some proteins do not yield crystals that diffract well, owing to, for example, intrinsic flexibility, often seen in kinases, or because of the presence of only a small hydrophilic portion, a classic problem of membrane proteins[38]. In both cases, specific binders might support crystallization, either by restricting the flexible protein in one conformation by providing a rigid surface for crystal contacts, or by increasing the hydrophilic portion. Additionally, such binders might be used in molecular replacement, helping to determine the phases in a diffraction pattern of proteins with unknown fold.

Full-size antibodies are not ideal for such tasks, as their multidomain architecture contains flexible hinges linking the domains. Both antibody Fab and Fv fragments, in contrast, have been successfully applied in membrane protein crystallography[38,39], but usually require running a fermenter to obtain the amounts necessary for structure determination. Two alternative scaffolds have been used for cocrystallization with macromolecular targets, protein Z ('affibody')[40], derived from a domain of *Staphylococcus aureus* protein A, and DARPins from our laboratory[41,42]. In these cases, previously determined structures of closely related affinity molecules were used in the molecular replacement. For cocrystallization in general, the binding molecule should be large enough to mediate enough crystal contacts and to allow molecular replacement, and be rigid.

Chromatography. Despite its conceptual elegance, immunoaffinity purification[43] is rarely used in the downstream processing of proteins. As the affinity ligand is usually needed in large amounts, the expense and effort to make such a column is a major consideration if one uses conventional monoclonal antibodies because of the reagent cost. Alternative binding ligands must offer the possibility of low-cost production, and the protein must be chemically robust enough to allow simple elution of the purified protein by pH-shift, and to allow for harsh cleaning in place (e.g., 1M NaOH for several hours)[44]. For several alternative binding molecules, such affinity chromatography applications have been reported[45,46] with the best known example being affibodies, which work in a similar manner to their progenitor, protein A, a binder used in commercial immunoglobulin purification (see **Supplementary Notes** online for detailed referencing). An economically attractive solution might be to use an approach developed in our laboratory[47] in which extracts of bacteria expressing recombinant binding molecules are bound to inexpensive column material by selective noncovalent immobilization.

Protein scaffolds

The diversity of potential applications has led to the investigation of numerous alternative scaffolds (**Table 1** and **Fig. 1**). Whereas the binding proteins appear to be very diverse in size, topology, mode of interaction and applicability, some rational justification can be given for the choice of almost any scaffold. For example, fibronectin, which is one of the most frequent mediators of protein-protein interactions in humans[48], has an antibody-like structure and displays complementarity-determining region (CDR)-like loops (**Fig. 1**)[8,49]. But in contrast to antibodies, the fold does not rely on disulfide bonds, yet still displays high thermodynamic stability[49]. Fibronectin may therefore fulfill some of the binding functions of an antibody; stability combined with the absence of disulfides also enables intracellular applications[36].

Affibodies (**Figs. 1** and **2**) are derived from an immunoglobulin-binding domain of *Staphylococcus aureus* protein A[50]. They possess no disulfide bonds, consist of three α-helices and display reversible folding. Numerous laboratory applications of affibodies have been described for purification, detection and targeting, also suggesting the therapeutic and diagnostic potential of this protein scaffold[5,6,16,45,51–56].

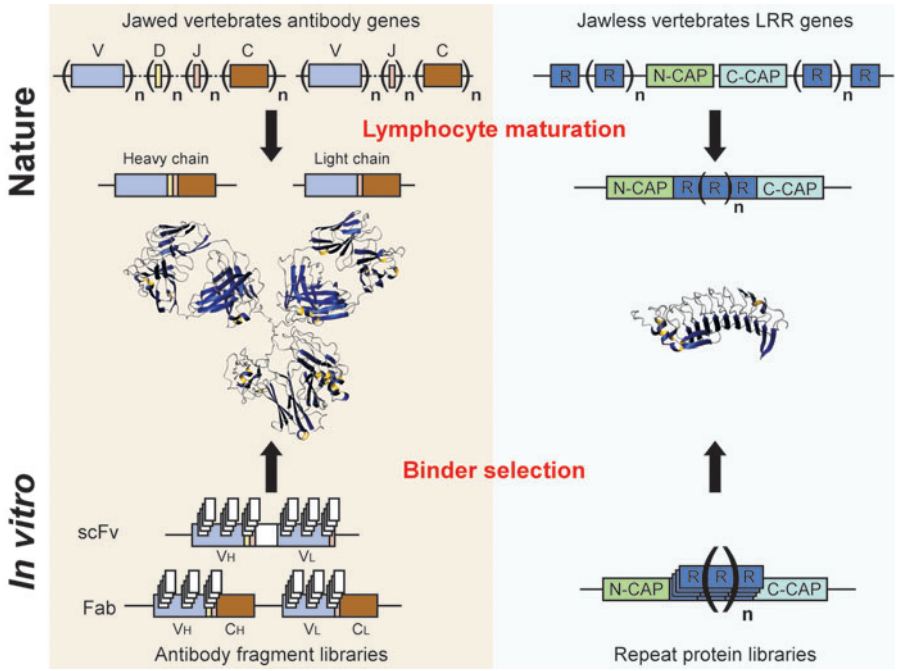

Figure 3 The generation of diversity in nature and in the test tube. The adaptive immune response of jawed vertebrates is based on antibodies. The genes of antibodies are split in several subgenes (V, D, J, C), which are present in numerous copies. The recombination of these genes during B-cell maturation allows the generation of innumerable recombined antibody genes (one heavy- and one light-chain gene), which together code for an antibody with a binding specificity for a nonself molecule. The adaptive immune response of sea lampreys (jawless vertebrates) works similarly but involves leucine-rich repeat genes[68]. Their genome contains numerous copies of leucine-rich repeat genes (R, N-CAP, C-CAP), which recombine during lamprey-lymphocyte maturation, yielding genes coding for leucine-rich repeat proteins. Both of these natural immune systems have been rebuilt *in vitro*. Synthetic antibody fragment libraries (e.g., see refs. 89,166,167) as well as repeat protein libraries[37,41] have successfully been used for the generation of binding molecules using powerful selection technologies (see **Table 2**). Additionally, several different single-chain (scFv) or Fab antibody fragment libraries have been generated (e.g., see refs. 1,2) either by extracting the corresponding genes from lymphocyte-derived genomic libraries or by generating synthetic antibody libraries with diversified CDRs (represented as stacks of rectangles above the gene fragments). Designed repeat protein libraries of varying repeat numbers have also been generated *in vitro* and high-affinity binding molecules could be isolated[41]. In comparison to antibody libraries, designed repeat protein libraries offer one additional degree of randomization. The number of repeats, and hence the size of the interacting surface, can be modulated by the addition or deletions of repeats.

Lipocalins ('anticalins'; **Figs. 1** and **2**) are a diverse β-barrel protein family, most of whose members contain disulfide bonds. Their natural function is usually to bind small molecules in their barrel/loop region[7,57]. Libraries could be made to generate analogous binders for a variety of small molecules such as fluorescein[58], benzyl butyl phthalate[59] as well as the toxic digoxigenin[60] for which the selected binder might represent a therapeutic antidote[7]. Moreover, randomization of the loop sequences connecting the strands of the barrel also allows the generation of protein binders[7,61], some with high affinity and specificity.

Repeat proteins—proteins that contain consecutive copies of small (about 20–40 amino acid residues) structural units (repeats) that stack together to form contiguous domains—constitute abundant classes of protein-protein interaction mediators in nature, and their use appears particularly interesting, since they feature large and modular target interaction surfaces. A unique feature of such surfaces is that they can be adapted to the target size by adjusting the number of repeats in the protein (**Fig. 3**; see A.P., H.K.B and colleagues[62]). Designed ankyrin-repeat proteins (DARPins) (**Figs. 1** and **2**), which we have successfully used to generate high-affinity binders against a variety of targets[37,41], also feature high thermodynamic stability and reversible folding behavior. As our work has shown[63,64], these DARPins show some of the highest expression levels reported for soluble, functional proteins in *E. coli*. Because of the absence of cysteines and low aggregation tendencies, they seem optimally suited not only for novel fusions and conjugates and extracellular targeting use, but also for intracellular applications[37].

Repeat proteins appear even more interesting considering that they are often found in nature; many plant genes mediating pathogen resistance rely on repeat proteins[65] and many components of the vertebrate innate immune system are composed of repeat proteins[66,67]. Moreover, Pancer and colleagues recently discovered that the adaptive immune response of the sea lamprey is based on leucine-rich repeats, for the first time demonstrating a natural 'library' and 'selection system' not based on immunoglobulins (**Fig. 3**)[68].

Src homology domains 2 and 3 (SH2 and SH3 domains) and PSD-95/Discs-large/ZO-1-domains (PDZ domains) (**Table 1**), also found abundantly in nature[48], are particularly interesting as they mediate specific protein-peptide interactions. SH2 domains have been used to find binders for phosphorylated peptides[69], SH3 domains have been used to detect proline-rich peptides containing a poly-proline II helix conformation[70,71], and PDZ domains (**Fig. 1**) were used to select binders for peptides with a free C terminus[46,72–74]. Together with proteins containing the tetratricopeptide repeat motif[75], these scaffolds are the only peptide-binding alternatives to antibodies.

Enzymes such as β-lactamase[26] or, more generally, proteins with intrinsic detection means such as green fluorescent protein (GFP)[28] may permit new homogeneous assays, provided the signal changes as a function of binding[26]. This would give those scaffolds a clear advantage over classical fusion proteins, which connect a binding domain with a reporter domain, and which require a separation step for detection.

Small disulfide-bonded scaffolds such as the scorpion toxins[76,77] exhibit very high thermodynamic stabilities. In addition to acting as stable binding proteins[76,78,79], these proteins may also be used as vaccine delivery vehicles[77]. Because of their small size and disulfide-bond constraints, they may be especially suited to expose hairpin loops that need to interact in a cavity.

A last group of scaffolds, different protease inhibitors (**Table 1**; summarized in ref. 80), have been used to generate high-affinity protease inhibitors with new or improved binding specificities (see e.g., ref. 81). Typically, the targeted proteases are of great pharmaceutical importance[19], highlighting the clinical potential of the modified inhibitors.

Scaffold and library design

Engineering scaffolds and designing libraries are mutually interdependent processes. Different protein engineering methods have been used to improve a given protein backbone for solubility, high-level recombinant expression, thermodynamic stability, resistance to aggregation or suitability for a certain selection system (discussed for individual cases below). Similarly, the library design, that is, the diversification strategy, has been tackled in different ways (**Fig. 1**). Additionally, some novel binders have been obtained by rational design, rather than by creating a library with subsequent selection.

Scaffold engineering. As alternative scaffolds need to be engineered for library generation (see below), scaffold optimization to improve chemical robustness, thermodynamic stability or recombinant expression yields is typically introduced early on in the process. Point mutation strategies[44,76,82,83] can efficiently be used for this task, illustrated, for example, by protein Z, where improved resistance against chemical side-reactions (typically hydrolysis and oxidation of side chains and the backbone) was achieved[44,82]. C-terminal peptide extensions were used to increase the solubility of fibronectin[49], and they have also been tested for protein stability improvements in other cases[84].

In the case of T-cell receptors (TCRs)[85,86] the use of the yeast surface display selection system was facilitated by converting the two-domain protein into a single-chain construct, in analogy to antibody scFv fragments. In this case, point mutants were additionally selected that led to more robustly folding TCRs[86,87], which are normally aggregation prone. Recently, it became possible to use TCRs in combination with phage display; by using a different molecular format, a two-chain construct of the TCR with an unnatural interchain-disulfide bond between the constant domains[88] was created.

For repeat proteins, consensus design has been used[62], an engineering strategy that had already proven useful in antibody engineering approaches[89]. The underlying idea of consensus design is that structurally important residues are more conserved than other residues in families of homologous proteins. The design of a protein based on a protein family consensus sequence should hence lead to an 'idealized' protein. As it is a statistical approach, consensus design is particularly well suited for protein scaffolds derived from protein families with many homologous members. In the case of repeat proteins (data have so far been reported for ankyrin, leucine-rich and tetratricopeptide repeats), consensus repeat sequences were derived from the alignment of numerous repeats, which were then used to generate proteins of varying repeat numbers, which both showed high thermodynamic stability and were expressed well in *E. coli*[62,63,90–92].

In principle, one might also think of using completely designed proteins as scaffolds for library generation[93]. However, it is not yet clear how frequently such a design will be successful for creating combinatorial libraries. Also, by using completely synthetic sequences, one might find the problem of immunogenicity of these proteins more pronounced than in the case of randomized natural frameworks.

Scaffold diversification. To obtain specific binders one must generate a combinatorial library of the scaffold. This is done at the DNA level by randomizing the codons at appropriate amino acid positions, by using either degenerate codons or trinucleotides[94]. But which are the 'appropriate' amino acids to be randomized? Here, the knowledge of the amino acid positions that are usually involved when a particular binding protein interacts with its target—revealed by a crystal structure of the complex between the parental protein and its target—facilitates the residue choice (e.g., see ref. 50 or ref. 63). This was the case for most of the scaffolds investigated. Several recent crystal structures of binders selected from libraries of alternative binding proteins (affibodies, DARPins and anticalins) in complex with their respective targets (**Fig. 2**) show the success of this strategy[40–42,95,96]. In other cases, library design was carried out by analogy. For example, fibronectin has an architecture related to immunoglobulins, and hence a randomization of residues in the CDR-like loops appears attractive (**Fig. 1**)[8,49]. However, the transposition of the CDR-loop concept to scaffolds with unrelated architectures may be delicate, as the example of GFP shows, which appears not to tolerate highly diverse β-strand connecting loops[28].

Although in general, antibody-like scaffolds have proven to work well as specific binders, it is not compulsory to stick strictly to the paradigm of a rigid scaffold that displays CDR-like loops (**Fig. 1**). Affibodies[56] and DARPins[41], for example, show that a flat surface or a combination of a large flat surface plus rigid loops are well suited to create high-affinity binders (**Figs. 1** and **2**). In the case of DARPins, the high affinities to the targets might at least partially be caused by the rigid body interaction, leading to a low loss of entropy upon binding. Nevertheless, the way each of the binding proteins is randomized may also restrict the target epitopes for which one can obtain binders. If, for example, a flat surface is randomized, binding to folded proteins, which again expose flat surfaces, may clearly be favored over binding to extended peptides or small molecules, which require a pocket or groove to bind to.

Selection technologies

Combinatorial scaffold libraries must be subjected to a selection or screening procedure to obtain molecules with the desired characteristics, usually defined as specific binding or inhibition. Most selection technologies were first developed for antibody fragments. The prerequisite of these systems is that they have to couple phenotype (protein) and genotype (corresponding nucleic acid). This can be achieved by surface display on a cell or a virus (e.g., a bacteriophage) that carries the genotype, but it can also happen inside the cell (*in vivo*), or in a cell-free system during translation (on the ribosome) or by means of artificial compartments (water in oil emulsions; **Table 2**) (see also **Supplementary Notes** for additional references).

Phage display[97] is the most widely used selection technology for antibody fragments and was also the basis for many successful selections with alternative binding proteins (see **Table 1**; for example ref. 56 or ref. 88). In phage display, the library of interest is fused to bacteriophage coat proteins and thereby displayed on the phage surface; the pool of phages is mixed with an immobilized target (e.g., a purified protein or a whole cell) to isolate specific binders. Most frequently, filamentous phages, which assemble from the bacterial periplasm and are thus compatible with the display of disulfide-containing proteins are used, but icosahedral λ-phages that assemble intracellularly[70] have also been used for displaying proteins folding well in the cytoplasm. Yeast surface display[85,98] was successfully used in combination with fluorescence-activated cell sorting (FACS), and a flagellin display system[99] was reported for peptide display on thioredoxin as a scaffold (**Table 2**).

Ribosome display and mRNA display work entirely *in vitro*. They couple the protein with the mRNA noncovalently via the ribosome (ribosome display)[100–102] or, after translation in additional steps, covalently via puromycin (mRNA display)[103] (for a comparison of both methods, see paper by Lipovsek and A.P[104].). Both have been used in several selections from complex libraries[8,37,41,105].

Bacterial surface display exists in many permutations, using a variety of hosts and surface proteins for membrane anchoring[106–109], and they have been tested with libraries to a different extent. Several other library display technologies are available, but they have not yet been used in combination with complex library selections of nonantibody proteins (**Table 2**). Examples are T7 phage display (Novagen), and different forms of DNA display[110–112].

In vivo selection systems usually rely on the reconstitution of a protein activity upon binder-target interaction (e.g., enzyme activity, fluorescence or transcriptional activity). The yeast two-hybrid system (e.g., ref. 72) and a dihydrofolate reductase protein-fragment complementation assay (PCA[113]; P.A., Koch, H.K.B. and A.P., unpublished data) have been used for selections of novel binding proteins. However, other PCA and two-hybrid systems have not yet been investigated with complex libraries of binding proteins (**Table 2**)[114–118]. As most such systems work in the cytoplasm, they provide additional screening and selection options for those scaffolds not relying on disulfides for stability. For such proteins, functional screens *in vivo* become possible, such as screening for inhibition of signaling or enzymatic activity, which was illustrated with libraries based on staphylococcal nuclease[119] or DARPins[37].

Which selection technology is best suited for a given binding protein library depends on different parameters: the library diversity, the properties of the scaffold or the intended applications. Combinatorial libraries of maximal functional diversity are generally believed to have a greater chance for harboring molecules with a desired function than smaller libraries[120–122]. Here, the *in vitro* systems of mRNA and ribosome display have a clear advantage, as their library size is not restricted by transformation efficiency[100–104], making library sizes of 10^{11} to 10^{13} possible, depending on the scale of the *in vitro* translation used (**Table 2**). Disulfide-bonded scaffolds should be used in selection systems that allow the correct formation of the disulfide bond (e.g., filamentous phage display or ribosome display in the presence of disulfide isomerase[100]). Selection systems that work in the cytoplasm are preferably used with scaffolds whose stability is independent of disulfide bonds. Because of the lower library diversity achievable (limited by the transformation of cells; **Table 2**) and because of a lower selective pressure for affinity, as sub-nanomolar interactions are not 'rewarded' in the selection at the high intracellular protein concentrations, intracellular selection systems are most attractive when an intracellular application is the aim of the project. However, *in vivo* selections can also be combined with a prior selection with an *in vitro* selection system to reduce the library diversity and enrich binders[37,123]. In any case, the compatibility of a given class of binding proteins with a particular selection technology should be checked experimentally (e.g., with a known interaction pair).

Combinatorial protein libraries can also be screened, rather than selected, for functional members[69,124], which requires, however, that the library diversity is reasonably small or has been preselected by some other means. Several examples exist of rational design of functional alternative binding proteins, including PDZ domains[46], protease inhibitors[125] and tetratricopeptide repeat variants[75], and some other frameworks in early stages of development[77,126–129].

One family of proteins that has been extensively investigated *in silico* and rationally designed is the bacterial periplasmic binding protein family, which includes maltose- or ribose-binding proteins. What made these attractive targets is a conformational closing upon ligand binding, which could be detected upon attaching environmentally sensitive fluorophores[130]. Binding proteins specific for small molecules[131] and metal ions[132] were rationally designed leading to the corresponding binding-sensors[130].

Conclusions

Several scaffolds unrelated to immunoglobulins have now been subjected to important proof-of-principle experiments. The future application potential in research, diagnostics and therapy is readily apparent, and some of these applications have already been demonstrated successfully. For therapeutic applications, however, the potential of alternative binding proteins has yet to be proven, and the scaffolds may be confronted with issues such as immunogenicity or lack of effector functions. Nevertheless, several alternative binding proteins are currently in preclinical studies, and the lack of effector functions may be compensated for by superior functionality or overcome by novel effector-fusion proteins.

"If you can think of it, nature has already tried it a long time ago" is an old saying in molecular biology. The use of alternatives to antibodies was recently supported by the finding that the adaptive immune system of the jawless sea lamprey relies on leucine-rich repeats[69] rather than on immunoglobulins (**Fig. 3**). Hence, nature itself uses topologies other than the immunoglobulin fold for generating diversity. Why shouldn't we?

Note: Supplementary information is available on the Nature Biotechnology website.

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The authors declare competing financial interests (see the *Nature Biotechnology* website for details).

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