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The DARPin encyclopedia: from Basic Research towards Therapeutics

Philipp Wild[1], **Rohan S. Eapen**[1], **Patrik Forrer**[1], **and Christian Jost**[1]

[1] Athebio AG, Grabenstrasse 11a, 8952 Zürich-Schlieren, Switzerland

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Correspondence: *christian.jost@athebio.com*

Introduction

The adaptive immune system in vertebrates comes in two flavors. Historically, adaptive immunity focused on the study of immunoglobulins (also referred to as Ig-based antibodies herein); more recently, jawless vertebrates were found to use an entirely different class of proteins for their adaptive immune system. Specifically, these organisms express variable lymphocyte receptors (so called VLR antibodies) that create diversity based on the rearrangement of leucine-rich repeats.[1] Meanwhile, various, naturally occurring repeat motifs have been used as building blocks for the design of artificial binding scaffolds which, among others, include designed ankyrin repeat proteins (DARPins). Such binding scaffolds are also referred to as non-Ig-based antibodies or non-Ig scaffolds herein). DARPins display several benefits such as a low molecular weight (~15 kDa), high thermal stability, high specificity and affinity, versatility (e.g., in terms of valency and multi-specificity), speedy preclinical development, low production costs and, thus, bear the potential to not only complement existing therapeutic Ig-based antibodies but also open up novel therapeutic strategies. The first generation DARPin therapeutic abicipar pegol has completed two Phase III studies in 2020 while several other DARPin drug candidates are currently undergoing clinical validation. Most recently, the rapid development of tri-specific SARS-CoV-2 DARPin therapeutics showcases the immense potential of recombinant repeat proteins in adopting quickly e.g., new forms of target neutralization that a single Ig-based antibody cannot afford. Here, we highlight the design principles of repeat proteins in general and summarize in detail the continuous advancements of the DARPin scaffold that made it one of the most promising antibody mimetics to date. This review provides an overview of current and emerging applications of DARPins as both a research tool and therapeutic drug that can match or even surpass Ig-based antibody applications.

Background

At the beginning of the 20th century Paul Ehrlich envisioned that Ig-based antibodies could serve as 'magic bullets' for therapeutic purposes based on their ability to bind to a wide variety of target molecules with high specificity and affinity.[2] With currently over 100 (FDA approved) molecules in therapeutic use (and over 500 in clinical development), more than a century later, monoclonal Ig-based antibodies (mAbs) have taken center stage as therapeutics in modern

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medicine.[3] Advances in recombinant DNA technologies and the development of in vitro selection methods were key to facilitate the successful translation of mAbs from bench-to-bedside in 1986 (Muromonab) and since then led to an ever-increasing number of clinically approved mAbs.[4] In addition, immunoglobulins have become valuable tools in research and diagnostic applications. For many applications, however, the complex architecture of immunoglobulin molecules constrains their use. Their high molecular weight (∼150 kDa), their composition of four individual protein chains, cost-intensive manufacturing and the complex patent situation are substantial drawbacks for the development and use of mAbs, especially in a commercial setup. Furthermore, for historical reasons, immunoglobulins applied in research have mostly not been defined by their sequences and produced as recombinant proteins. Instead, researchers have used polyclonal Ig-based antibodies for decades. These are produced by injecting a target (typically a protein) into an animal such as a rabbit and using the resulting serum as a source of Ig-based antibodies. However, only a minor fraction of the Ig-based antibodies in a polyclonal reagent bind to their intended target, and the problem of poorly or not at all defined sequences of Ig-based antibodies is also inherent to most mAbs used for research, which have not gone through thorough characterization and validation like their clinical siblings. These circumstances make it hard for researchers to be sure of the specificity of any particular batch of binding reagent obtained in this way and researchers call for an international collaboration and funding initiative to define all binding reagents according to the sequences that encode them.[5]

While some of these issues have been addressed by smaller Ig-based antibody fragments (e.g., Fabs, scFvs, sdAb), within the last two decades, non-Ig-based antibodies have been developed which not only obviate the limitations of mAbs pointed out above but also pave the way for the development of entirely new therapeutic approaches. In order to engineer such high affinity binding scaffolds without immunization, in vitro selection techniques like phage display or ribosome display have been essential and significantly reduced the production time and costs. Besides designed ankyrin repeat proteins (DARPins), several other non-Ig-based antibodies relying on repeat domains (e.g., leucine-rich repeats, tetratricopeptide repeats, HEAT and armadillo repeats) have been engineered and are being developed. While they have found use in basic research as laboratory reagents and considerable efforts propel their biopharmaceutical development, only DARPins reached the clinical stage so far and are on the verge of becoming the first clinically approved 'magic bullet' based on repeat domains.

Properties of Ig-based Antibody formats

Due to innovative recombinant DNA technologies and advanced selection strategies, increasingly supported by *in silico* modeling, the range of engineered Ig-based antibody formats is virtually only limited by one's imagination and creativity nowadays. However, despite the variety in size, valency, architecture and (multi)specificity that can be conceived by the modular arrangements of variable (and constant) domains, the Ig-based antibody scaffold brings about limitations that are intrinsic to its minimal building unit, the Ig domain, and cannot easily be overcome by protein engineering (**Table 1**).

Regular, full-size mAbs are complex glycoproteins consisting of four polypeptides which are covalently connected by disulfide bonds which precludes their fast and economical production in *Escherichia coli* bacteria. The large size of mAbs (∼150 kDa) affects their pharmacokinetic properties (**Table 1**) resulting in low diffusion rates into peripheral tissues and constrains the scope of diseases that are amenable to therapeutic intervention as, for example, mAbs cannot cross the blood-brain barrier.[6] Moreover, the high molecular weight demands high concentrations to be administered for effective target neutralization which may cause adverse effects. The split composition of the antigen-binding site comprised of the variable regions of both the light chain (LC) and heavy chain (HC) complicates the design and architecture of multi-specific fusion proteins, since in such an immunoglobulin-derived multi-paratopic protein different pairs of "half-paratopes" need to pair in the intended configuration. To overcome this chain pairing problem towards multi-specific immunoglobulins, different strategies have been conceived and implemented, utilizing mutations to define the desired HC:HC and HC:LC interfaces.[7,8] However, some level of mispairing in such assembly of multiple polypeptides is rather the rule than the exception, and leads to e.g., small yet significant fractions of unintended isoforms, that complicate and thereby retard the downstream processing in translation towards the clinic.

The Fc domain of mAbs mediates downstream cytotoxic effector functions through its interaction with Fc-gamma receptors on immune cells and/or the complement system and extends the molecules´ serum half-life by binding to the neonatal Fc receptor which rescues albumin and IgG from degradation following endocytosis and thereby recycles endocytosed mAbs back to the blood stream.[9–11] There are, however, a number of applications in which these features are not required or even unfavorable. For example, in image-based diagnostics, used to monitor disease progression or to evaluate the response to a therapeutic drug, prolonged serum half-lives result in higher background signals and non-specific tissue accumulation (e.g., in the liver) hampering a reliable diagnosis. Furthermore, inadvertent activation of Fc-receptor expressing cells can lead to excessive release of cytokines causing toxic side effects. Lastly, strong intellectual property restrictions and the intricate patent situation has impeded the commercial development of Ig-based antibody formats.

A new generation of biologically engineered immunoglobulin molecules have emerged designed to address the

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Table 1. Comparison of the properties of classical Ig-based antibodies, single-chain Ig-based antibodies and DARPins

	Classical IgG	Nanobody	DARPin
Molecular weight (kDa)	150	15	15
Polypeptide chains	4	1	1
Thermostability (°C)	60-80	~ 90	≥90
Solubility (g/l)	>100	>100	>100
Disulfide bonds	Yes	Yes	No
Engineered specificities	2	3	up to 6
Effector function	Yes	No	No
Binding mode	Flexible loops	Flexible loops	Rigid interaction area
Production	Mammalian cells	*E. coli*	*E. coli*

drawbacks of conventional antibodies. Smaller and less complex antibody fragments harboring only the antigen-binding site (scFv, Fab) are commonly used which can be selected by phage display in a straightforward manner. However, this, comes at the expense of the solubility as these molecules have the propensity to aggregate and thus oftentimes need to be converted back into the IgG format through genetic fusion to the Fc moiety.[12,13] It has been reported that the conversion of single-chain antibody fragments into the full antibody framework can be accompanied by a loss of affinity that to some degree cancels the benefits gained through affinity maturation during repeated rounds of selection (panning).[14] While a variety of strategies attempt to improve the issues related to IgG conversion of recombinant antibody fragments, it represents a cumbersome additional step.[15–17]

Bispecific antibodies with the desired specificities can be obtained by the CrossMab technology introduced by Roche or through the fusion of two additional antigen binding sites at the Fc region, generating tetravalent, bispecific molecules (Fcab).[8,18] Another approach that achieves multi-specificity comes in the form of dual targeting Fabs (DutaFabs) which comprise two spatially separated and independent binding sites within the CDR loops providing two paratopes for two different targets.[19] Arguably the most promising Ig-derived scaffold are nanobodies (single domain antibodies) that consist of a single monomeric variable Ig-based antibody domain and thereby circumvent the above-mentioned problems of chain pairing. Usually obtained from heavy chain-only antibodies found in camelids and sharks, recombinant nanobodies can be selected by phage display. As mentioned before, conventional antibody-derived scaffolds tend to aggregate due to solvent-exposed hydrophobic patches; yet, as an intrinsically monomeric domain, nanobodies are distinctly more soluble than Fabs and scFvs. Their favorable biophysical and biochemical properties (e.g., small size ~16 kDa, high thermostability, resistance against harsh conditions, relatively stable in the absence of disulfide bonds, bulk production in bacteria) make them attractive drug candidates. In addition, the modular combination of nanobodies within a single polypeptide chain enables the targeting of two or more distinct epitopes.[20] It is worth to note that nanobodies, like mAbs, can be generated by passive immunization which is beneficial in case the target molecule is difficult to express as a recombinant protein.

There is no doubt that mAbs – along with their infinite number of derivative formats that aim to address the deficits of the classical antibody molecule while also expanding its features – have come of age as the leading biologic on the pharmaceutical market and will continue to play a chief role in the treatment of human diseases. The methods (e.g., recombinant DNA technologies, phage display) established to overcome the shortcomings of antibodies, however, may make the Ig-based antibody format itself dispensable over time, i.e. other non Ig-derived scaffolds can be used that fulfil the above-mentioned criteria of a robust and versatile binding protein with suitable developability from the get-go. Besides a small and rigid fold, ideal alternative protein scaffolds should provide a conserved framework that grants stability while an exposed variable surface facilitates the structural versatility to form a multitude of tailor-made target binding sites. In addition, bi- or multivalency that ensures longer retention times at the antigen (avidity) and/or multi-specificity, i.e. the ability to interact with different epitopes simultaneously (like bispecific antibodies), would be desired features (**Table 1**). The latter is becoming increasingly important as the inhibition of a single target can be bypassed by the upregulation of compensatory signaling pathways as seen in cancer patients or the development of escape mutations during viral infections.[21–23] Thus, a flexible, additive arrangement ('beads on a string') through combinatorial fusion of the minimal binding domains without affecting the individual antigen-binding properties constitutes a hallmark of suitable non-Ig scaffolds.

Variable lymphocyte receptors: nature's alternative antibody format

Remarkably, nature itself has evolved an alternative to Ig-based antibodies as the adaptive immune system of vertebrates emerged in two flavors. In 2004, a landmark publication by Pancer and colleagues demonstrated that in jawless vertebrates, such as lampreys, the genetic reassembly from a large set of leucine-rich repeat (LRR) cassettes

serves to generate variable lymphocyte receptors (VLRs).[1] Composed of highly-diverse LRRs, antigen-specific VLR antibodies can be created in response to any target like "regular" antibodies, i.e. immunoglobulins.

VLR antibodies evolved approximately 500 million years ago and display a vast degree of diversity with similar affinity and specificity as immunoglobulins. Like Ig-based antibodies, VLR antibodies are generated in lymphocytes by DNA recombination (**Figure 1**). They are assembled by sequential insertion of diverse LRR modules from adjacent genomic cassettes into the germ line incomplete VLR gene.[24,25] In striking resemblance to activation-induced cytidine deaminase (AID) dependent control of secondary Ig-antibody diversification, two lymphocyte-specific AID-APOBEC family members have been identified in lampreys that are implicated to play a role in the assembly and diversification process of VLRs.[26] The combinatorial events of VLR assembly and diversification creates a huge repertoire of receptors, estimated at more than 10^{14} unique VLR antibodies, sufficiently diverse to recognize most, if not all, potential antigens. Following somatic DNA rearrangement and hypermutation, VLRs – in analogous evolution with immunoglobulins - are either expressed on the surface of the lymphocytes or, upon activation, secreted as disulfide-linked tetra- or pentamers. Three types of VLRs, denoted A, B and C, have been identified in jawless vertebrates and are differentially expressed by mutually exclusive lymphocyte populations. VLRB lymphocytes resemble B cells secreting soluble VLRB antibodies, whereas VLRA and VLRC lymphocytes express their receptors as transmembrane proteins much like T cell receptors.

Isolated monoclonal VLRA and VLRB antibodies have been found to bind their cognate ligands with affinities in the same range as immunoglobulins (some VLRs exhibiting K_D values in the picomolar range).[27] Remarkably, comparative crystal structures of a VLRB, camel and shark antibody bound to hen egg lysozyme showed that their epitopes almost overlap entirely: the VLRB molecule binds over the active site with the C-terminal capping LRR module (LRRCT) insert projecting deeply into the carbohydrate-binding cleft. In an analogous manner, the elongated CDR3 loop of both the camel and shark antibodies which consist of a single V_H domain protrudes directly into the catalytic site.[28,29] The VLRB antibody, however, makes additional contacts with the antigen through its concave surface composed of the LRR modules. Other LRR-based receptors usually bind to planar or convex antigen surfaces without participation of their N- or C-terminal capping modules. Thus, VLR antibodies might have evolved to recognize topologically distinct epitopes combining the target binding mechanisms of both V_LV_H (induced fit) and V_H only (lock and key) antibodies. The former is reflected by the large structural adjustments of the LRRCT insert upon binding, while the latter is owed to the structural rigidity of the repeat modules. Interestingly, VLRAs exceed the micromolar affinities of TCRs for peptide MHC ligands by several orders of magnitude and, therefore, may share certain properties of secreted antibodies (nanomolar K_D values, affinity maturation) with other characteristics that pertain to TCRs (surface expression on lymphocytes). Notably, it has been demonstrated that recombinant VLR antibodies ('lampribodies') are amenable to selection by phage, yeast and human cell line display techniques, either from synthetic DNA libraries (designed VLRs) or from cloned VLR cDNAs isolated from lamprey larvae after immunization with an antigen.[30,31]

Apart from their role in the adaptive immune system of jawless vertebrates, LRR motifs were found to play a vital role in the innate immune system of plants, invertebrates and vertebrates as part of pattern recognition receptors (e.g. Toll-like receptors) that are present at the cell surface or in endosomes detecting non-self structures from pathogens (e.g. bacteria, fungi, viruses). Beyond orchestrating immune responses, LRR motifs have been identified in a large number of functionally unrelated proteins. The best-known example is the ribonuclease inhibitor (see chapter 'Leucine-rich repeat proteins' for further details) but other proteins such as the tropomyosin regulator tropomodulin as well as proteins involved in neural development (e.g. SALM, NGL, LGI) also possess LRR motifs.[32] The functional diversity displayed by LRR proteins stems from their ability to efficiently bind a plethora of target proteins. This enormous versatility is based on their special repetitive architecture which will be described in the next section.

Architecture and characteristics of repeat proteins

Repeat motifs are widespread across the tree of life (present in at least 14% of all proteins) and are highly abundant in the human proteome.[33] According to a census of tandem repeat proteins >60% of all human proteins contain repeat motifs ranging from homorepeats to large repetitions containing multiple repeats over 150 amino acids such as in the cytoskeletal protein titin.[34] Their high frequency in the genome translates into a multitude of diverse functions repeat proteins fulfil including cell cycle progression, immune response, cell signaling, transcriptional control, and protein transport. Most repeat proteins are composed of repeating structural motifs of 20–50 amino acids which originated from intragenic duplication and recombination events in the course of protein evolution.[35] The repeat motifs stack together to form curved solenoid structures, creating a larger surface area than present in typical globular proteins, which is instrumental in mediating protein-protein interactions.[36] The shape and the exceptional stability of the elongated structure arises from short-range hydrophobic interactions and hydrogen bonds within each repeat and between adjacent repeats.[37] A characteristic of many repeat domains are special capping repeats at the N- and C-terminus of the protein that have a more hydrophilic surface to shield the hydrophobic core of the domain from the solvent. The modular structure of the repeat proteins provides the basis for the specific engagement with a diverse set of ligands. High binding affinities are favored thermodynam-

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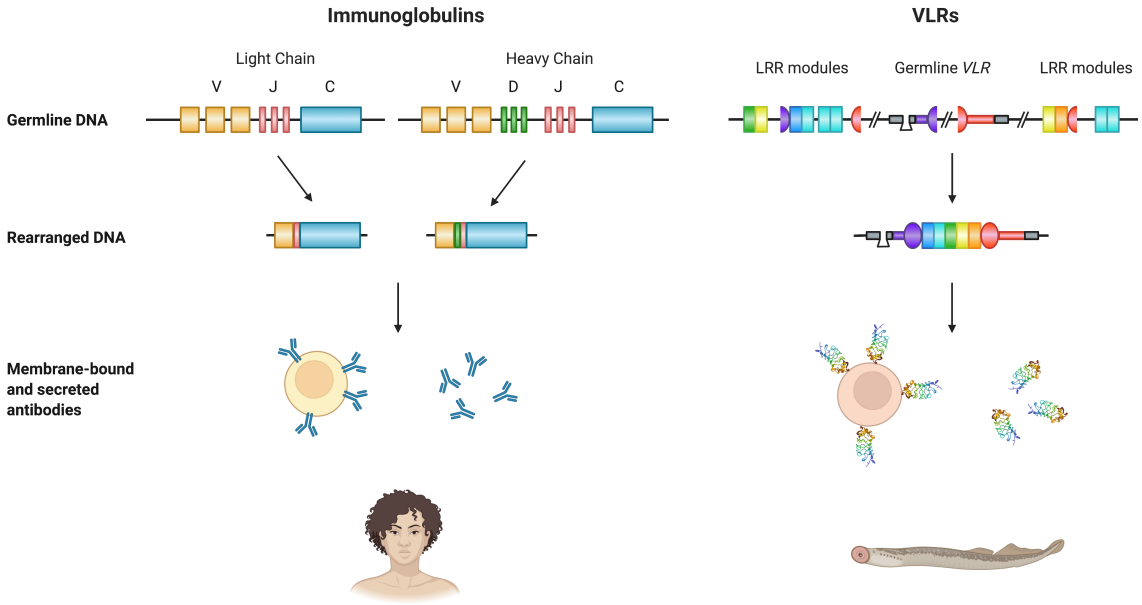

Figure 1. Assembly of immunoglobulin antibodies and variable lymphocyte receptors (VLRs). Jawed vertebrate antibody genes (on the left) are assembled through recombination activating gene (RAG)-promoted joining of immunoglobulin gene fragments including variable (V), diversity (D), joining (J) elements and Ig constant (C) domains. In jawless vertebrates (on the right), VLR antibodies are created by the insertion of LRR cassettes from flanking genomic arrays into the germline incomplete VLR gene. A mature VLR antibody consists of several highly diverse LRR modules delimited by N- and C-terminal capping modules (purple and red circles). The first 18 residue LRR motif (dark blue) is followed by a variable number of 24 residue LRR modules (light blue) and a connecting peptide (orange). The invariant segments of the VLR antibody include a signal peptide, a threonine/proline rich-stalk and a glycosyl phosphatidylinositol membrane anchorage motif which tethers the VLR to the lymphocyte membrane. Both Ig-based and VLR antibodies are expressed on lymphocytes or secreted into the plasma.

ically through the extended rigid surface that minimizes entropy loss upon binding to the target. Due to their extraordinary properties (e.g. large and rigid potential interaction surface, modular architecture with easily exchangeable surface residues) repeat proteins are attractive scaffolds to engineer target-specific, high-affinity binders. In the following sections brief descriptions of the most common repeat motifs will be provided.

Leucine-rich repeat proteins

Leucine-rich repeats (LRRs) are defined by repetitive 20-30 amino acid stretches that possess a disproportionately high number of leucine residues (LxxLxLxxNxL being the overall consensus sequence).[38] Each repeat unit typically consists of a β-strand-turn-α-helix structure which when stacked onto each other gives rise to a horseshoe-shaped tertiary fold with an interior parallel β-sheet and an exterior array of helices. The tightly packed region between the helices and sheets constitutes the protein's hydrophobic core that is shielded by hydrophilic solvent-exposed parts of the β-sheet and one side of the helix array. The robustness of LRR domains stems predominantly from the hydrophobic inner core of the solenoid. The closely packed arrangement of the side chains of the conserved leucine residues (and other aliphatic amino acids) supports van der Waals interactions and, thus, confers stability to the entire LRR domain. The side chains of neighboring repeats fill holes in an interlocking fashion such that the core of the solenoid structure remains compact and devoid of unfavorable cavities. The rigid, intertwined arrangement of adjacent repeat motifs prevents the LRR domain from disintegrating and exposing its hydrophobic core to the solvent. Capping structures (often cysteine-rich domains) flanking both the first and last repeat units similarly protect the hydrophobic residues.[38,39] Apart from structural aspects, the cap motif can additionally serve as a secondary interaction surface.[40]

The concave face of the LRR domain contains the ligand-binding site and is composed of a parallel β-sheet to which each repeat contributes one strand. The inner lining of the horseshoe-shaped interaction surface is well-suited to surround protein ligands by a maximum number of protein-protein contacts. Superimposition of ligand-free and bound LRR domains shows no major structural rearrangements indicating little entropic penalty upon binding. The extended binding area in combination with a rigid tertiary structure enables very strong protein-protein interactions. For instance, the dissociation constants of various members of the ribonuclease superfamily with ribonuclease inhibitor, the first LRR protein whose crystal structure had been solved, lie in the femtomolar range, affinities which are among the highest for any known protein-protein interaction.[40,41] However, it should be noted that the strength of the interaction is not only dictated by the fraction of buried surface. As the irregular shape of ligands does not always fit well into the concave binding surface, LRR ligands tend to form

few non-continuous contacts with the curved LRR β-sheet. Crystal structures of ligand-bound ribonuclease inhibitor revealed that electrostatic interactions contribute to these non-continuous contacts resulting in a sufficient number of short-range interactions across the interface that yield high affinity associations with cognate ligands.[40]

Structure determination of different LRR domains disclosed a high degree of structural variability that provides the basis for the enormous functional versatility of this protein family. Therefore, its members fulfil such diverse functions as RNA processing, immune response, pathogen recognition, neuronal development and cell signaling.[1,42–46] Most prominently, LRR domain-containing proteins play important roles in both the innate and adaptive immune response: in the form of toll-like receptors which bind to pathogen- and danger-associated molecular patterns and as antibody-like VLRs where the combinatorial assembly of LRR gene segments generate the structural diversity for antigen recognition. Thus, it seems that LRR domains are particularly well suited for the recognition of molecular determinants from a structurally diverse set of bacterial, viral, fungal and parasite-derived components.

Tetratricopeptide repeat proteins

The tetratricopeptide repeat (TPR) is a structural interaction motif found in a wide variety of proteins and, as the name suggests, consists of repeating stretches of 34 amino acids in length. Tandem arrays ranging from 2 to 20 repeats adopt an open solenoid structure composed of coiled and superhelical elements.[47] Each repeat motif is composed of a pair of antiparallel α-helices linked to one another by a short loop sequence of 4 amino acids. The TPR sequence is characterized by a combination of small and large hydrophobic residues; while there is no strict consensus sequence, strong preferences for certain amino acids exist at various positions: for instance, alanine tends to be more conserved at specific sites (8, 20 and 27) while other positions show a strong bias for either small, bulky or aromatic residues.[48] A typical TPR structure is formed through interactions between helices A and B of the first motif and helix A' of the next repeat. Within a single motif, the first two helices of the TPR motif usually have a packing angle of ∼24 degrees. The right-handed superhelix formed by multiple TPR motifs creates both a concave and a convex face, of which the concave face typically constitutes the ligand binding site. A notable exception represents the binding mode of the TPR protein $p67^{phox}$ to the small GTPase Rac: between motifs TPR3 and TPR4 of $p67^{phox}$ a 20 amino acid insertion forms two anti-parallel β-strands which is part of the Rac-binding site along with the loops that connect TPR1-TPR3.[49] Thus, TPR proteins offer distinct modes of target recognition. TPR domains have the ability to recognize a diverse set of structural motifs ranging from short as well as extended linear peptide motifs to folded, globular domains.[50,51] Short linear peptides usually bind to the concave groove formed by 2-3 TPRs with binding affinities in the low-to mid-micromolar range. While there is a large degree of variability regarding the amino acid composition, the target peptides are typically negatively charged (e.g. MEEVD peptides) and are located at the C-terminus, as the C-terminal carboxylate group forms key contacts with the TPR protein.[52] By contrast, longer peptides (20-40 amino acids) can interact across the span of the solenoid groove and, thus, higher affinities in the nanomolar range are achieved.[53] Interestingly, although TPRs are considered to provide a rigid binding surface like LRR domains, more extensive conformational changes are observed upon binding to very long peptides, indicating an induced-fit mechanism. Emerging data demonstrates that TPR proteins are also able to interact with globular domains across different stretches of the solenoid structure. For example, one of PP5's two TPR domains masks the globular catalytic region of the phosphatase in an auto-inhibitory fashion while the 14-repeat containing TTC7 protein supports complex assembly through the interaction with different folded domains of three members of the phosphatidylinositol 4-kinase complex.[50,54] While the binding affinities of TPR domains can span over six orders of magnitude, TPRs seem to be ideally suited to perform bi-functional tasks and mediate multiple interactions during complex formation rather than providing high affinity ligand recognition. Members of the TPR protein family include several subunits of the anaphase-promoting complex – an E3 ubiquitin ligase that regulates chromosome segregation during cell division –, co-chaperones of Hsp90 complexes (e.g. HOP, FKBP51, FKBP38) and kinesin light chains which bind cargo proteins through their TPR motif. TPR proteins participate in numerous cellular processes such as cell cycle progression, transcription, protein translocation, protein degradation and host defence against invading pathogens.

Armadillo repeat proteins

Armadillo (ARM)-repeat proteins possess a repeating amino acid sequence of ∼40 residues in length constituting a motif of three α-helices. The first helix is shorter and lies perpendicular to the other two helices that stack against each other. Multiple copies of ARM-repeat units fold together as a superhelix in a linear solenoid structure, forming a versatile platform for interactions with various binding partners. The elongated shape of the ARM domain forms a slight curvature such that an outer and inner surface is generated. The concave side provides a rigid binding surface where the ligand is bound to the ARM-repeat in an antiparallel arrangement.[55] The ARM repeat motif was initially identified in β-catenin (a functional homologue of the Drosophila gene armadillo), in which its 12 tandem repeats form a charged inner groove that facilitates the interaction with acidic surfaces of β-catenin binding partners. Although ARM-repeat proteins exhibit a relatively poor sequence conservation among each other, they adopt a similar tertiary fold.[56] The degenerate nature of a consensus ARM-repeat sequence hampers the detection of ARM-repeat proteins, many of which being identified by solution of their crystal structure. Nonetheless, designed ARM-repeat proteins (dArmRPs) have been successfully engineered and selected to bind any short peptides.[57,58]

The first known ARM-repeat protein β-catenin serves

fundamentally different cellular functions. Initially characterized as a cytoplasmic anchor of cadherins which mediate cell-cell adhesion, β-catenin also has a vital role in developmental processes (e.g., Wnt signaling) acting as a transcriptional coactivator through the interaction with transcription factors of the TCF/LEF family. Another prominent function of ARM-repeat proteins is during nuclear import of proteins through the nuclear pore complex, which is catalyzed by the ARM-repeat protein importin α. Remarkably, to fulfil its function, importin α forms a heterodimer with importin β, a member of the structurally related HEAT protein family.

HEAT repeat proteins

The HEAT (Huntingtin, elongation factor 3 (F3), protein phosphatase 2A (PP2A), PI3-kinase TOR1) repeat motif – structurally related to ARM-repeats – is composed of two antiparallel α-helices connected by a short loop.[59] Despite having one helix less, the structure of the HEAT motif is quite similar to that of the ARM motif. Its strongly bent first helix corresponds to the first and second helices of the ARM-repeats while the C-terminal helices of HEAT and ARM-repeats are also equivalent. In HEAT-repeat proteins, neighboring repeats are stacked together into a single domain as a continuous hydrophobic core which forms an elongated superhelix.[60] Analogous to the C-terminal helices of ARM-repeats generating a curved interaction area, the second helices of HEAT repeat motifs form a concave binding surface as found in the prototypical HEAT proteins importin β1 and β2. Given the structural similarities as well as a series of conserved residues that form the repeats' hydrophobic cores, a common phylogenetic origin of ARM and HEAT repeats has been proposed subsequently having diverged into different structural families.[61,62] HEAT repeat sequences display an even higher variability in length, amino acid composition and tertiary structure than ARM-repeats making their identification by sequence alignments difficult. HEAT repeat proteins can function in protein degradation (e.g. Cand1), transcriptional and translational regulation (e.g. TAF6, eEF3), DNA repair (e.g. DNA-PK, FANCF), chromosome biology (e.g. different subunits of cohesion and condensin complexes) and cell proliferation (e.g. TOR kinase).

Ankyrin repeat proteins

Ankyrins are a large protein family that bind with high specificity to a large range of different proteins. For instance, the eponymous protein ankyrin anchors integral membrane proteins to the spectrin-actin based membrane cytoskeleton. This linkage is instrumental in the maintenance of the plasma membrane integrity and e.g., provides structural resistance to shear stress experienced by erythrocytes in circulation.[63,64] The immobilization and 'anchoring' of membrane ion channels and transporters gave rise to the name derived from the Greek word ankyra ("anchor"). Though less common, interactions with small molecules and nucleic acid have been reported. In mammals, ankyrins are encoded by three genes which contribute through alternative splicing to the large variety of ankyrin proteins (**Figure 2A**). However, as in the case of VLR antibodies, the remarkable mix of diversity and specificity is mainly accomplished by consecutive copies of tightly packed repeats which can be shuffled without affecting the tertiary structure of the domain.[65] Arrays of up to 29 tandem repeat copies have been found in proteins. Each ankyrin repeat motif comprises 33-amino acid residues and folds into two antiparallel α-helices followed by a β-turn (**Figure 2B**).[66]

The ankyrin repeat motif was first discovered in the human erythrocyte protein ankyrin.[67] Ankyrin repeat domains are typically composed of 4-6 repeats, which give rise to a right-handed solenoid structure with a continuous hydrophobic core and a large solvent accessible surface. Tandem arrays of tightly packed repeats adopt an elongated, curved shape that results in a groove-like binding surface.[68] As illustrated in the following chapter ankyrin repeats are present in a large number of protein families, including transcription factors, cytoskeletal proteins and development regulators. Ankyrin repeat proteins can be found in all kingdoms of life yet are most common in eukaryotes. Interestingly, ankyrin repeat proteins also appear in the genome of poxviruses. Most poxviral ankyrin repeat proteins are large (400-800 residues) and possess a C-terminal F-box-like motif. F-box proteins direct the degradation of proteins via the ubiquitin-proteasome system. It is believed that poxviral ankyrin repeat proteins hijack the host cell's SCF ubiquitin ligase complex via their F-box-like motif and target different cellular substrates via their ankyrin repeat domains to subvert the NF-κB coordinated anti-viral response.[69] Notably, the precursors of the NF-κB transcription factors as well as its inhibitory proteins of the IκB protein family contain a central ankyrin repeat domain. The ankyrin repeats of the IκB inhibitor form an extensive protein interaction interface to bind to NF-κB and mask its nuclear localization, keeping NF-κB inactive in the cytoplasm.[70] All members of the INK4 family of cyclin-dependent kinase inhibitors contain multiple ankyrin repeats.[71] INK4 proteins are tumor suppressors that bind to CDK4 and CDK6, two kinases involved in the regulation of cell cycle progression. INK4 proteins form stable complexes with CDK4/6, thereby blocking the association with cyclins which renders them inactive.

Taken together, repeat proteins are the most versatile class of binding proteins that can adapt to many different environments and perform a wider variety of cellular functions than immunoglobulins. However, although repeat proteins are nature's most versatile binding scaffolds, they have been widely overlooked to build antibody mimetics. Currently, the most promising of these alternative scaffolds are DARPins, whose design principles and exceptional properties are illuminated in the following chapters.

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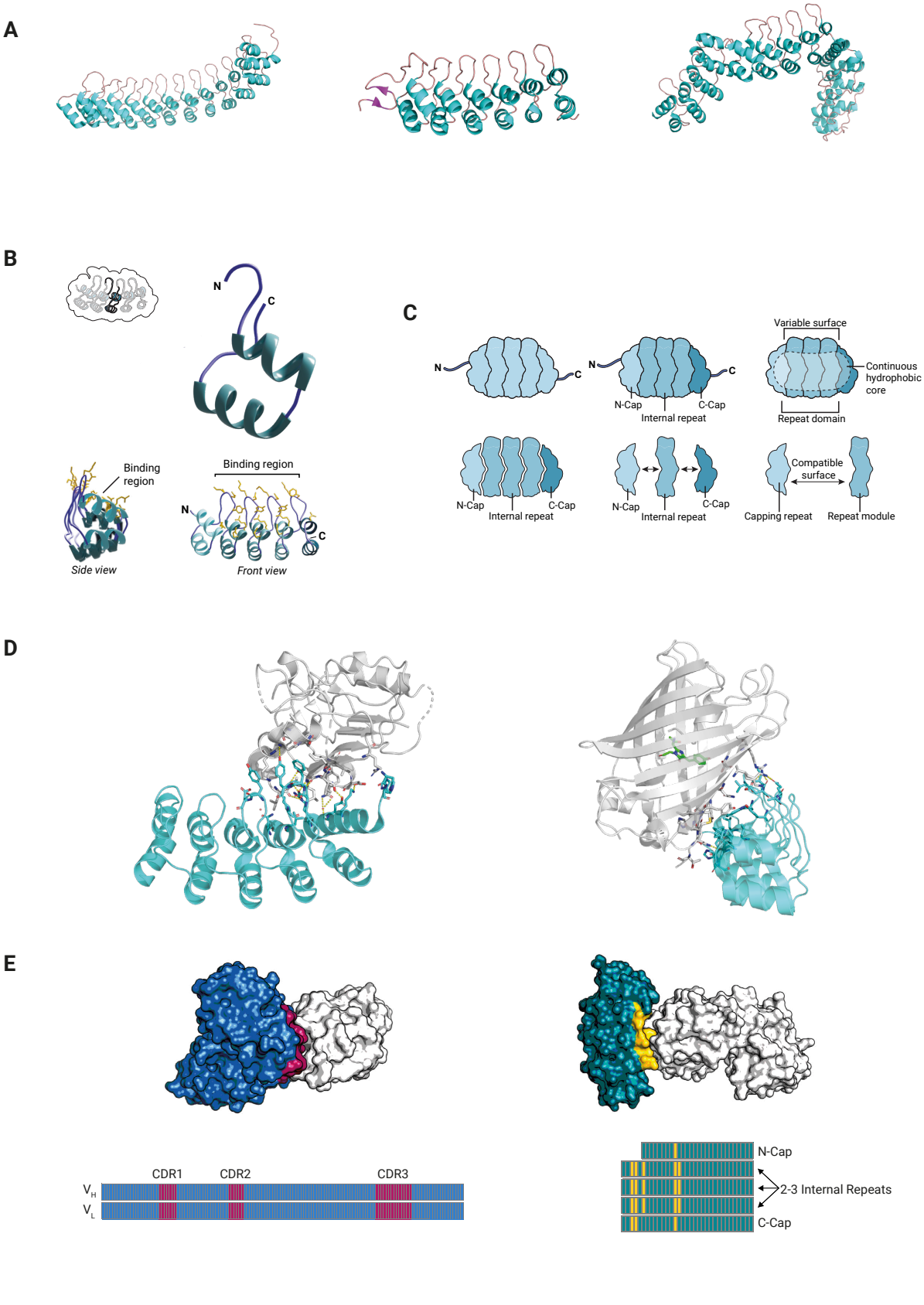

Figure 2. Sequence and structural characteristics of the DARPin scaffold. A) Natural ankyrin repeat motifs are found in a large number of proteins with pleiotropic functions: AnkyrinR (24 repeats) connects integral membrane proteins to the cytoskeleton; NOTCH1 (6 repeats) is a transmembrane receptor that plays a major role in the regulation of embryonic development; Tankyrase (21 repeats) acts as a poly-ADP-ribosyltransferase involved in various processes such as Wnt signaling, vesicle trafficking and telomere length. B) From structure and sequence alignments of natural ankyrin repeats a consensus ankyrin repeat motif has been designed that comprises 33-amino acid residues and folds into two antiparallel α-helices followed by a β-turn (left panel). Sequential stacking of multiple consensus repeats forms a repeat domain which contains fixed framework residues and randomized interaction residues (shown in yellow stick mode; right panel). C) Schematic structure of an ankyrin repeat domain. Various ankyrin repeat modules (three depicted) are cloned between N- and C-terminal capping repeats thus forming a continuous hydrophobic core. Using this strategy, combinatorial libraries of DARPins of varying repeat numbers can be generated. The randomized positions on several adjacent repeats create an extended interaction surface presented on a rigid ankyrin repeat scaffold. D) X-ray structures of DARPin molecules (blue) in complex with a target protein (white; here: Her2 and GFP, respectively). The target protein is bound by the randomized ligand interaction surface of the DARPin (stick representation), resembling the natural target-binding mode indistinguishable from natural ankyrin repeat proteins. E) Structural differences in antigen recognition between DARPins and antibodies. While the antigen binding surface (composed of the complementarity-determining regions, CDRs) of Ig-based antibodies (antigen binding surface in red and the rest of the antibody in blue) may undergo conformational rearrangements to accommodate the target epitope following a "lock-and-key" or "handshake" (induced fit) mechanism, the rigid interaction surface of DARPins (interaction surface in yellow and the rest of the DARPin in green) affords high affinities due to minimal entropy loss upon binding to the target.

Designed Ankyrin Repeat Proteins (DARPins)

Designed ankyrin repeat proteins (DARPins) emulate the tandem repeat structure of natural ankyrins. The basic repeating unit comprises 33 amino acid residues. A variable number of ankyrin repeats stack on each other to yield an extended core, concave-shaped scaffold with an expansive solvent-exposed paratope. Notably, this fold entirely originates from short range interactions - stabilizing contacts formed solely between residues close in sequence either within repeats or at the interface between two neighboring motifs. In quantitative terms this equates to a distance of 6-9 residues between contacts in repeat proteins whereas in globular proteins contacts are separated by 10-30 residues in sequence.[72] The absence of long-range interactions that characterize the complex topologies of globular proteins simplifies the design and development of repeat proteins. The molecular weight of the most common DARPins with two or three internal ankyrin repeats between the capping repeats is 14 and 18 kDa, respectively, only about one tenth of the size of conventional mAbs or about one third of the size of Fab fragments. The small size of DARPins has several benefits over mAbs in terms of their pharmacokinetic profile, tissue penetration and the possibility of different administration routes that require large amounts of stable protein.

Consensus design ensures a robust framework

An important step in the development of the ankyrin repeat motif as a robust basic unit for DARPins was the consensus design strategy that led to the identification of a prototypical repeat unit that can be freely assembled (shuffled, added and inserted) without affecting the stability or expression of the molecule. The sequential arrangements of multiple consensus repeats form a repeat domain which contains fixed framework residues and randomized interaction residues (**Figure 2B**).[73–77] This approach is based on the assumption that within one protein family, the positions of highly conserved residues reflect their importance for maintaining the fold and, therefore, scaffold residues can be identified easily by sequence alignment. Conversely, the positions of an individual family member that participate in the binding to a specific target will not be conserved. Remarkably, this strategy led to self-compatible modules that can be stacked on each other without additional design of the interfaces between juxtaposed repeats, indicating that the favorable inter-repeat interactions are an inherent part of the consensus sequence. Several hundreds of natural ankyrin repeat protein sequences combined with structure-based design were used to define a library of idealized DARPin molecules which exhibit improved biophysical properties such as high solubility, increased stability and high expression rates (200 mg purified protein per 1 l shake flask bacterial culture, more than 10 g/l with high cell-density fermentation).[73,77] Although the resulting consensus sequence determined by this approach is artificial, the designed library modules display a high degree of similarity to human ankyrin sequences ($\sim$67% identity) which constitutes an important aspect for therapeutic applications. The strategy of using sequence and structural analyses – building on eons of protein evolution – not only helped to define a consensus framework with fixed positions vital for structural robustness but also highlighted variable surface residues that are ideally suited for interactions with target proteins. The initial libraries of DARPin molecules consist of a minimal conserved scaffold of 33 residues, of which 6 are variable for all amino acids except cysteine, glycine or proline, and therefore amenable to randomization for creating target specificity.[73] Such a library offers a theoretical variability of 7.2×10^7 per repeat module which steadily increases by the addition of further modules (10^{14} for two repeats, 10^{21} for three repeats etc.). The huge diversity that is afforded by this system resembles the high variability Ig-based antibodies achieve through V(D)J recombination and somatic hypermutation.

For the construction of the initial DARPin libraries, trinucleotide building blocks were used to avoid certain undesirable amino acids: Cysteine residues to both preclude dimerization via disulfide formation and maintain the freedom to site-specifically introduce cysteine residues for conjugation to effector molecules (e.g., toxins, fluorophores, enzymes) as well as structurally unfavorable proline and glycine residues.[78] Usually, DARPin libraries are made up of molecules consisting of 2-3 repeat motifs flanked by the N- and C-Cap repeats. The composition of a DARPin molecule is typically formulated as NxC, in which x indicates the

number of internal repeats while the caps are denoted as N and C (**Figure 2C**). Notably, it has been shown that both N- and C-terminal capping repeats are oftentimes involved in target binding. Randomization of residues in the capping repeats can thus further increase variability of the DARPin library.[79,80] The large binding area and the fact that DARPins bind their targets through rigid-body interactions (i.e., the DARPin backbone does not undergo conformational changes), resulting in only a minimal loss of entropy, gives rise to very potent target binding with affinities in the low nano- or even picomolar range as well as high specificity.[78,81] In summary, the consensus-design approach yielded a versatile scaffold characterized by high stability and solubility which enables the cost-efficient expression and straightforward purification from microbial host systems. At the same time, these features expedite the drug discovery and development process. The high yield requires only small-scale cultures and in conjunction with the fast production in *E. coli* cells allows to screen a large number of evolved DARPin molecules for initial characterization in various assays.

Capping of repeat domains

A key factor for the successful design of DARPins was the addition of capping repeats that flank the internal ankyrin repeat(s).[74] These N- and C-terminally located capping units are crucial to shield the hydrophobic patches of the most N- and C-terminal internal repeats from the surrounding solvent.[82] The capping repeats have undergone various iterations during successive efforts to improve the thermostability and solubility of the consensus framework of DARPins. In the initial design both the N- and C-Cap sequence were derived from natural human guanine-adenine-binding protein, GABP.[73] In an effort to improve the stability of the C-terminal capping repeat, seven point mutations were introduced, five of which being located in the interface to the preceding internal repeat and constituting additional hydrogen bonds, thereby increasing the overall melting temperature (T_m) by about 17°C.[82] The gain in thermal stability is attributed to better packing as revealed by X-ray crystallography.[83] It has now further been demonstrated that the substitution of an aspartate residue to leucine, isoleucine or valine at position 17 (see Figure 2 in Schilling *et al.*[80]) of the N-Cap yields a significant increase in thermal stability too.[80] Notably, the mutation resulted in generically improved T_m-values of the DARPin domain of up 10°C as determined by equilibrium unfolding experiments. The benefit of this altered N-Cap is further evidenced by the design of ensovibep[84], a tri-specific anti-COVID-19 DARPin developed by Molecular Partners and partnered with Novartis, currently requesting Emergency Use Authorization from the U.S. FDA. Ensovibep harbors the same mutation described by Schilling *et al.* in three of its five DARPin domains. The thermal stabilities of DARPin domains (typically in the range of 66°C to 95°C) strongly surpass the melting temperatures of natural ankyrin repeat proteins and increase even more with additional repeats.[85] The exceptional high thermal stability of DARPins may overcome cold-chain requirements during shipment and storage of corresponding drugs.

Display selection techniques allow rapid sampling of DARPin libraries

Using synthetic DARPin libraries, binders can be obtained essentially for any target of interest (**Figure 3**). A major challenge, however, originates from physically sampling the enormous combinatorial complexity of such libraries. To sample the available sequence space, putative binders of synthetic DARPin libraries can be selected by various, well-established high-throughput techniques of *in vitro* selection such as phage display, yeast surface display or ribosome display.[86–88] While each selection method offers other advantages, the most frequently used strategy for DARPin selection is based on the cell-free ribosome display approach.

A prerequisite for the selection of specific binders from DARPin libraries (as for any *in vitro* selection set-up) is the coupling of phenotype (the actual DARPin capable of binding the desired target) to genotype (the coding sequence encoding that DARPin as DNA or RNA, respectively). In ribosome display, this link is achieved by stabilizing the ternary complex consisting of the ribosome, the mRNA without stop codon that encodes the DARPin and the freshly translated DARPin during *in vitro* translation. In the following step, these ribosomal complexes are exposed to the target of interest, immobilized to wells or beads. Undesired ternary complexes containing unspecific, "sticky" DARPin molecules (and their respective coding sequence) are then washed away and the retained mRNAs of complexes displaying binding-competent DARPins are recovered by RT-PCR to yield the sequences encoding target-specific binders.

A decisive advantage of this type of selection is the intrinsic PCR step which allows to conduct the selection procedure as "evolution in the test tube", i.e. with concurrent introduction of minor genetic changes (e.g., by error-prone PCR) and selection rounds with increasing stringency to maturate the affinity of the selected binders.[89] The coding regions of defined (or a large pool of) binders are thereby diversified by error-prone PCR or site-direct mutagenesis of relevant amino acids in a controlled fashion (i.e. directed evolution) and refined in subsequent selection rounds.[90,91] It has been demonstrated that this technique appears to be exceptionally well suited for the stable fold of DARPins which tolerates the randomization process to a greater extent than recombinant scFv antibody fragments and, thus, requires fewer selection rounds to evolve high affinity binders.[89] Ribosome display generated a variety of highly specific DARPins with subnanomolar affinities binding to a wide range of different proteins, including kinases, growth factors and proteases.[92–95] Due to their extraordinary biophysical properties, DARPins are being used in basic research in a variety of experimental applications, e.g., as biosensors visualizing the localization, trafficking, interac-

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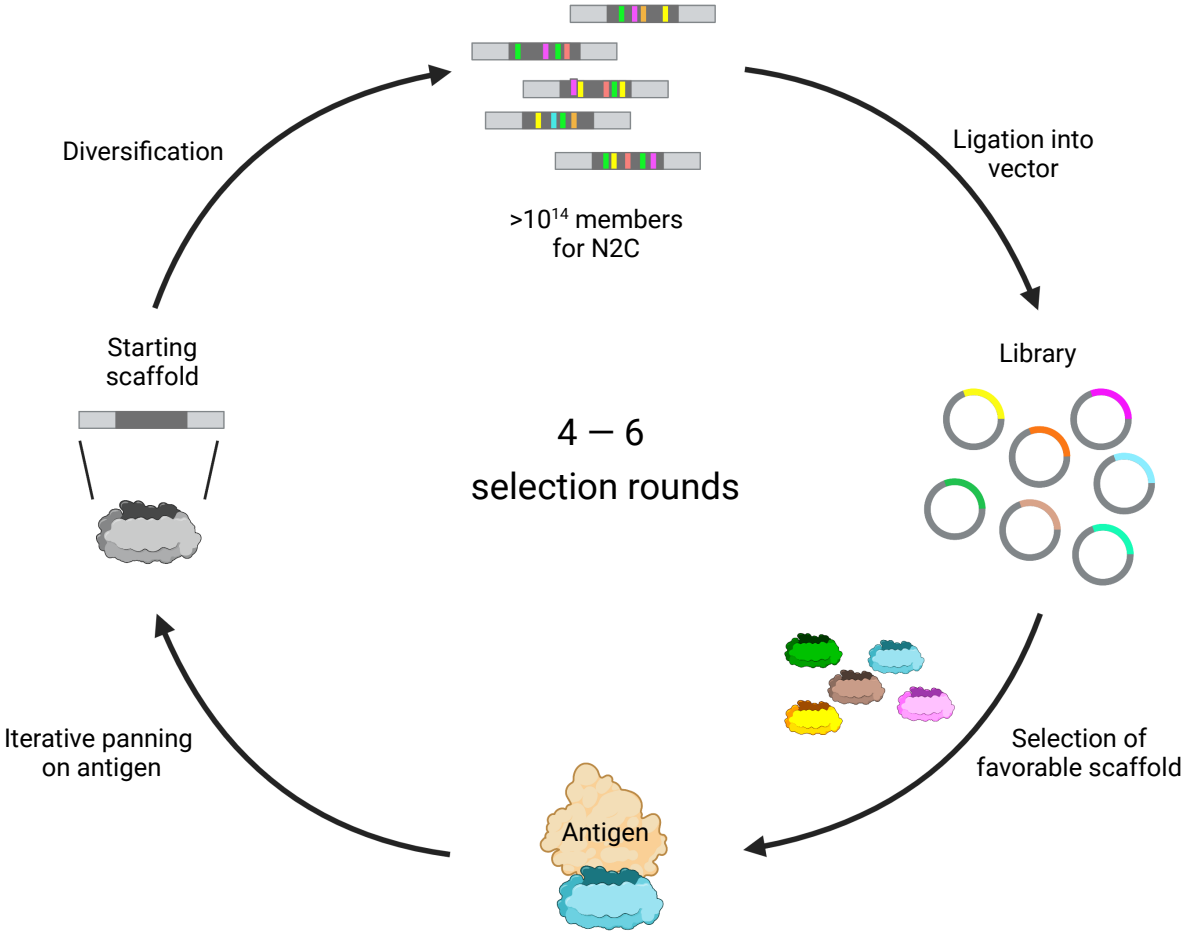

Figure 3. Generation of synthetic DNA libraries and selection of high affinity binders. The basic steps that yield highly diverse and high affinity Ig- and VLR-antibodies have been emulated by in vitro techniques. To construct a synthetic DNA library, variation throughout the gene coding for the binding molecule (e.g. DARPin coding sequence) can be introduced randomly by either error-prone PCR and/or DNA shuffling to recombine parts of similar genes together. Alternatively, mutations can be restricted to specific codons during de novo synthesis or saturation mutagenesis to obtain mutants of a gene in a controlled manner. This results in a mixture of double stranded DNA molecules which represent variants of the original gene. Upon ligation into a suitable vector backbone, the expressed proteins (e.g. DARPins) from these libraries can then be screened by phage or ribosome display for variants which exhibit favorable (biochemical) properties such as stability, binding affinity, solubility, expression level, unique paratope architecture. This can be repeated in cycles of creating gene variants and screening the expression products in a directed evolution process (panning).

tions and modification status of a protein of interest or as crystallization chaperones enabling the structure determination of membrane proteins and transient conformational states.[88,96–98] Since this review focuses on the current stage of DARPins as an upcoming new class of therapeutics, the readership interested in DARPins as research tools are referred to excellent, previous reviews.[99–102]

Properties of the DARPin scaffold

Repeat modules provide a stable framework while allowing structural plasticity in form of variable residues that can be exploited to create high specificity and affinity to target molecules (**Figure 2D**). Another layer of versatility is afforded by the successive arrangements of multiple repeat motifs. On the one hand, it enables the generation of an elongated interaction surface and, consequently, supports a gain in affinity not attainable by globular proteins[36]; on the other hand, the combination of variable repeats with interchangeable surface residues brings about a similar or even higher degree of variability of the antigen binding site than that achieved by mAbs. Furthermore, in comparison to the long and partly unstructured CDRs of Ig scaffolds the rigid body of stacked repeat proteins favors high affinities, as upon binding to the target epitope only minimal entropy is lost (**Figure 2E**). In addition, the compact fold guarantees robust folding and high thermostability. In combination with the flexible, yet still relatively small size of repeat proteins and the absence of any disulfide bonds or posttranslational modifications, repeat proteins are ideally suited for fast, low-cost production in simple expression systems such as *E. coli* or yeast cells – an important aspect not only from a point of view of pharmaceutical development but also from

an protein engineering perspective as it offers the potential for high-throughput screening of a large number of evolved binding molecules.

In contrast to recombinant single chain Ig-based antibody fragments which require rational protein design to simplify the complex structure of mAbs, repeat protein scaffolds naturally avoid complex molecular compositions (e.g., only one polypeptide chain, absence of intra- or intermolecular disulfide bonds and posttranslational modifications) and theoretically infinite copies of repeat domains can be assembled in a single polypeptide chain to create multi-specific scaffolds in a plug and play fashion without the tendency to aggregate. The latter property is not only a prerequisite for therapeutic applications in patients but also facilitates an efficient selection process. The absence of disulfide bonds bears two noteworthy advantages: first, it enables large scale production in the cytoplasm of bacterial host cells and, importantly, allows the functional expression within the reducing environment of eukaryotic cells. This feature significantly expands the potential therapeutic space of repeat proteins which is not easily accessible to mAbs. Second, the deliberate site-specific insertion of a cysteine makes it possible to further diversify the use of the scaffold by the chemical coupling to e.g., PEG, radionuclides, toxins, enzymes or fluorescent dyes. The combinatorial arrangement of repeat domains – like beads on a string – provides multivalency allowing both to crosslink different epitopes and increase avidity. As a consequence of the high folding stability of each individual repeat domain, multimeric assemblies have the same expression yield as monomers. While conventional mAbs are constrained in their binding geometries, variations of the length and the composition of the linker sequence connecting neighboring repeats provides vast spatial flexibility over the mode of interaction and can be easily realized.

For clinical use, an additional aspect has to be taken into consideration which relates to the potential immunogenicity of the engineered DARPin scaffold. Since its framework originates from natural ankyrin repeat sequences the likelihood of triggering an immune response is predicted to be minimal. In line with this notion are the results from the DARPin drug candidate MP0250 which demonstrated low immunogenicity.[103,104] Notably, the consensus DARPin scaffolds show no tendency for aggregation. However, the detection of antidrug antibodies lowering the therapeutic efficacy in patients treated with fully human antibodies demonstrates that immunogenicity constitutes an issue that cannot be fully assessed in the absence of long-term studies in clinical patients.[105] This being said, accumulating clinical data (outlined below in further detail) demonstrate the favorable safety profile of the first DARPin molecules tested in patients.

DARPins in basic research

Because of their exceptional biophysical and biochemical properties, DARPins are taken advantage of as versatile research tools to study protein behavior and function. For instance, posttranslational modifications (PTMs) constitute an important means to regulate activities, localization, interactions and half-lives of the cellular proteome in a dynamic manner and are thereby a field of huge interest and study in basic research. Due to the cysteine-free design of the DARPin framework, DARPins are fully functional when expressed inside the cell and thus are ideally suited to act as biosensors for PTMs in living cells. Using ribosome display, DARPins have been selected against the mitogen-activated protein kinase ERK2 that were able to discriminate between the inactive (unphosphorylated) and active (phosphorylated) state.[96] X-ray crystallography revealed that both types of DARPins bind to the same region of the kinase, yet only the phospho-ERK2 specific DARPin molecule recognizes a conformational change within the activation loop induced upon phosphorylation. These activation-state-specific sensors were able to precipitate the cognate form of the kinase selectively from cell lysates and bound their target specifically inside intact cells as demonstrated by bioluminescence resonance energy transfer. To visualize activated ERK2 in cells, the phospho-ERK2 specific DARPin binder was subsequently modified with the solvatochromic dye merocyanine, which fluorescence increases in a more hydrophobic environment upon target binding.[106] This biosensor detected endogenous, phospho-ERK2 in the nucleus in a selective manner, i.e. it did not recognize other mitogen-activated protein kinases or inactive ERK2. Such PTM specific biosensors will be valuable tools to study biological processes in a spatiotemporal fashion in real-time. The ability of DARPins to function as allosteric kinase inhibitors (in contrast to small compounds blocking exclusively the active site) allows for higher specificity towards inactive conformations of pharmaceutically relevant enzymes. Functional screening of DARPins selected to bind aminoglycoside phospho-transferase (3')-IIIa (APH) – a bacterial kinase that confers resistance to aminoglycoside antibiotics such as kanamycin – revealed APH-specific DARPin inhibitors that, when co-expressed with APH, attenuated bacterial growth on kanamycin-containing agar plates.[92] The co-crystal structure showed that the DARPin molecule does not block the catalytic site of the kinase; rather, APH is trapped in an inactive conformation, stabilized by binding of the DARPin inhibitor to three α helices located in the C-terminal region necessary for substrate binding.[107]

Another powerful strategy to interrogate a protein's function is to study the effects upon its depletions (loss of function approach). Genetic methods such as CRISPR/Cas9 or RNA interference are accurate and versatile but not very dynamic, which may over time trigger cellular adaptations and complicate functional analyses. On the other hand, manipulating protein abundance directly adds the benefit of also targeting protein conformations, PTMs, splice variants and different functional epitopes. Recently, the concept of targeted proteolysis was applied in the form of DARPin-based ubiquibodies (uAbs). uAbs are made of an

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E3 ubiquitin ligase genetically fused to synthetic binding proteins to facilitate the proteasome-dependent degradation of proteins of interest. Replacing the substrate-binding domain of the human ubiquitin ligase CHIP with DARPins of different specificities towards ERK1/ERK2 phosphorylation status enabled the targeted depletion of all subpopulations of endogenous ERK protein kinase.[108] Fusion of a DARPin recognizing both unphosphorylated and doubly phosphorylated ERK1 and ERK2 yielded a pan-ERK1/2 degrader while tethering of DARPins that discriminated between modified (DARPin pE59) and unmodified (DARPin E40) ERK1 and ERK2 makes it possible to study subpopulations of ERK kinases, i.e. the active or inactive fraction or irrespectively the entire pool. Customizable proteome editing technology such as uAbs hold great potential as a research tool for dissecting or – as therapeutics – to manipulate cellular signaling networks allowing to selectively degrade protein subpopulations (e.g., posttranslationally modified/unmodified, conformation dependent, active/inactive, wildtype/mutant protein) while sparing others.

DARPins in clinical trials: Promising therapeutic antibody mimetics

In disease diagnostics and as therapeutics, mAbs as well as engineered Ig-based antibody fragments are still the prevalent format of biologics even though for most of their purposes the complex Ig scaffold became largely dispensable. In the following section, we will provide an overview of therapeutic DARPin formats which are currently in different stages of clinical evaluation. As such, the DARPin molecule is – among others[109,110] - poised to become one of the first non-Ig derived scaffold to not only complement the arsenal of existing mAbs and Ig-derived formats but also to open up new possibilities of drug interventions unavailable to current binders (**Table 2**).

Pharmacokinetics

In contrast to applications in basic research, the small size of DARPins can be disadvantageous for therapeutic applications considering the short plasma half-life through fast renal clearance within minutes. Therefore, several strategies have been devised to prolong the circulation in the blood stream. For instance, the site-specific, covalent attachment of polyethylene glycol (PEG) reduces immunogenicity and can be used to retard kidney filtration.[127,128] A PEGylated DARPin monomer with a nominal molecular weight of 20 kDa has an increased hydrodynamic radius resulting in an apparent molecular weight of 230-350 kDa and, therefore, an increased half-live .[129] Alternatively, avoiding some of the drawbacks of PEGylation such as poor metabolization and the propensity to form intracellular vacuoles, human serum albumin (HSA) binding domains can be fused to the target-specific DARPin entity. Just like DARPin molecules in general, has-binding DARPin modules display high thermal stability and long storage stability which is a crucial property in drug development.[130] By means of fusing HSA-binding DARPins, plasma half-lives of up to 2-3 weeks can be accomplished which is comparable to the pharmacokinetic properties of mAbs.[131] Fusion with defined unstructured polypeptides presents yet another means to extend serum half-life without significant increase in the nominal, yet in the apparent molecular weight. These polypeptides consist of a series of Pro, Ala and Ser residues (PASylation) or Pro, Ala, Ser, Thr, Gly, Glu (termed XTEN) and, like PEG, expand the hydrodynamic volume of the molecule they are conjugated to.[132,133] Despite the larger size, such modified DARPin drug candidates are still comparably small with regards to the classical Ig-based antibody molecule and retain the benefit of greater tissue penetration as demonstrated by a pegylated DARPin targeting HER2-overexpressing tumors.[134] To date, the medical therapy space of DARPins that has been explored ranges from ophthalmology and oncology to infectious disease (e.g., virology). In terms of translation of academic research into clinical benefit, the work of Molecular Partners, the first company to commercialize the DARPin technology, has been fundamental since it granted the scaffold clinical validation. The initial focus centered on the development of a high affinity DARPin against human vascular endothelial growth factor VEGF-A for the treatment of retinal diseases like neovascular (wet) age-related macular degeneration (AMD) which is characterized by a gradual loss of vision.[135]

Monospecific DARPin Abicipar pegol reduces treatment frequency

Existing anti-VEGF-A biologics such as the humanized mAb bevacizumab (“Avastin”), the recombinant Fab ranibizumab (“Lucentis”) or aflibercept (“Zaltrap”), an engineered hybrid between the VEGF receptor and an Fc domain, require frequent dosing through intravitreal injections due to low ocular half-life and, consequently, suffer from shortened efficacy.[136] The frequent, tedious administration results in poor compliance by patients jeopardizing the effectiveness of the treatment. Indeed, clinical data show that at least eight injections per year with ranibizumab or aflibercept are needed to significantly ameliorate disease condition while the actual average number of reported injections are in the range of 5-7 per year.[137] Since the pharmacokinetic profile of VEGF-A inhibitors has a great influence on treatment compliance, Molecular Partners developed a high affinity anti-VEGF-A DARPin drug candidate that is potent at very low drug levels. In addition, due to the high solubility of the DARPin scaffold high doses can be administered delaying ocular clearance. The selected DARPin, named abicipar pegol, contains a PEG-conjugated VEGF-A binding DARPin domain with picomolar affinity to its ligand.[135,138] This fairly simple architecture adds up to 34 kDa, considerably smaller than currently used VEGF-A inhibitors. The pharmaceutically favorable biophysical properties of abicipar pegol (i.e. low picomolar affinity, small size and increased half-life in the eye) translates into the ability to administer increased doses (up to 5.5 times higher than ranibizumab).[139] After successful completion of preclinical trials, abicipar pegol has passed several Phase I and Phase II studies fulfilling

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Table 2. Overview of DARPins in therapeutic development

Name	Target(s)	Therapeutic area	Clinical status	References
Ensovibep	Coronavirus spike protein	COVID-19 (Virology)	Phase III	Rothenberger *et al.*[111], Walser *et al.*[112]
MP0423	Coronavirus spike protein	COVID-19 (Virology)	Preclinical	Rothenberger *et al.*[111], Walser *et al.*[112]
Abicipar pegol	VEGF-A	neovascular age-related macular degeneration (Ophthalmology)	Phase III	Kunimoto *et al.*[113], Khurana *et al.*[114], Hussain *et al.*[115], Moisseiev *et al.*[116]
AMG 506 (MP0310)	FAP, 4-1BB	Immuno-oncology	Phase I	Link *et al.*[117], Tosevski *et al.*[118],
MP0317	FAP, CD40	Immuno-oncology	Phase I	Rigamonti *et al.*[119], Ioannou *et al.*[120]
MP0250	VEGF-A, HGF	Multiple myeloma, lung carcinoma	Phase II	Sennino *et al.*[121], Fiedler U *et al.*[103]
MP0274	HER2 (domains II and IV)	Cancer (Oncology)	Phase I	Fiedler U *et al.*[122], Baird *et al.*[123]
E2_79, bi53_79 (E2_79 + E3_53)	FcεRI, receptor-bound IgE	Acute allergic reaction	Preclinical	Eggel *et al.*[124], Eggel *et al.*[125]
^{99m}Tc-(HE)$_3$-G3	HER2	Cancer diagnostic	Phase I	Bragina O *et al.*[126]

efficacy and safety criteria while demonstrating the benefit of long-lasting VEGF-A inhibition allowing less frequent injection intervals.[135,140–143] In two ensuing Phase III studies (CEDAR and SEQUOIA), abicipar pegol displayed similar potency in comparison to ranibizumab, yet with less frequent treatment: 6-8 versus 12 injections per year.[116,144] However, the frequency of increased, drug-related ocular inflammation (uveitis or vitritis) was 15.3%, which has been attributed to impurities in the manufacturing process.[114] In a first step, using a modified formulation procedure, the incidence of intraocular inflammation could be reduced to 8.9%, though it remained higher compared to existing VEGF-A drugs.[115] In case the rate of inflammation can be further lowered, abicipar pegol shows great potential to decrease treatment burden of patients suffering from AMD.

Abicipar pegol first showcased in a clinical setting several of the unique benefits originating from the excellent biophysical properties of the DARPin scaffold: i) a single DARPin domain was sufficient to achieve picomolar binding affinities for the desired target[135], ii) the absence of cysteine residues in the design of the consensus scaffold enabled the site-specific attachment of PEG through thiol-maleimide reaction[145], iii) the low molecular weight (34 kDa) of abicipar pegol allows higher doses which, as in the case of AMD, directly affects treatment efficacy due to better compliance by patients. It is worth pointing out that since the development of abicipar pegol, the DARPin scaffold has been further optimized – with the latest improvement of the N-Cap sequence significantly increasing its thermostability[80] – which will benefit the design and performance of future DARPin therapeutics. Ultimately, compared to existing drugs the DARPin format affords three advantages resulting in a reduced dosing frequency for treating eye diseases: (i) high potency (single digit pM K_D); (ii) high stability and (iii) long PK in the eye.

Targeting multiple epitopes in *cis* or *trans* with single DARPin molecules

The flexible modular arrangement of multiple, identical DARPin moieties within a single polypeptide chain provides avidity as achieved by two identical antigen binding sites of mAbs; by fusion of two or more DARPins with different specificity, however, novel modes of action can be realized that go beyond the realms of possibility of conventional mAbs. For instance, two epitopes can be connected either in *cis* (on the same target) or in *trans* e.g., binding two cell surface receptors or bringing two cells in close proximity. The former has been employed to prevent the IgE Fc receptor (FcεRI) to bind to its ligand, the Fc domain of IgE antibodies.[124]

To this end, monovalent binders against different epitopes of the alpha chain of FcεRI were selected and fused to each other. In cellular assays monitoring release of pro-inflammatory mediators upon FcεRI activation, only the bispecific combination of two DARPins with paratopes targeting distinct regions on the FcεRI efficiently blocked IgE binding.[124] Conversely, allergic reactions can be mitigated by decreasing the levels of circulating ligands using IgE antibody neutralizing DARPins. The inhibitory capacity IC_{50} of the selected anti-IgE DARPin (IC_{50} = 1.66 nM) proved to

be as high as the therapeutic mAb omalizumab ("Xolair") (IC_{50} = 1.77 nM).[146] Structural analyses revealed that this DARPin molecule bound to two epitopes on the Fc region of IgE attenuating FcεRI activation by two means. On the one hand, antagonizing the interaction with the receptor through sterical hindrance; on the other hand, through the active disruption of pre-formed IgE-FcεRI complexes by a mechanism known as facilitated dissociation.[147] The disruptive potential of the anti-IgE DARPin could be further enhanced by the attachment of an FcεRI-tethering DARPin moiety which was more effective in inhibiting an IgE-dependent allergic response compared to omalizumab due to increased local concentrations.[125] Recently, optimized versions of the DARPin-based disruptive IgE inhibitors have been shown to also terminate IgE-mediated signaling in pre-activated human blood basophils and attenuated pre-initiated allergic reactions *in vivo*.[148] Therefore, biparatopic DARPins which lack an Fc domain are ideally suited to mitigate allergen-induced degranulation of mast cells and basophils.

The combination of DARPins with different specificities - hitting a 'sweet spot' of avidity - has also been applied to the targeting of tumors. In light of the ability of cancer cells to rapidly adapt to the therapeutic intervention of a single target, the ease of introducing multi-specificity in the DARPin format is very attractive. This feature was exploited to create a tetravalent, bi-specific DARPin directed against two distinct epitopes on the epidermal growth factor receptor EGFR.[149] The described binding molecule was able to reduce surface EGFR by inhibiting receptor recycling, leading to a significant decrease in cancer cell viability in EGFR-dependent cell lines. Notably, the multi-specific DARPin construct outperformed the mAb cetuximab that prevents EGFR dimerization and activation through blocking ligand binding.[149]

A bi-specific DARPin targeting the human growth factor receptor HER2 showcases a completely different and novel mode of action to eliminate cancer cells. HER2 overexpression on transformed cells drives tumorigenesis and correlates with poor prognosis. Treatment with HER2-specific mAbs (e.g., trastuzumab ("Herceptin"), pertuzumab ("Perjeta") as well as antibody drug conjugates (trastuzumab emtansine, "Kadcyla") requires additional chemotherapy and is hampered by declining efficacy due to tumor escape via acquiring resistance.[150] In an attempt to overcome above mentioned limitations of HER2-targeting IgGs, monovalent DARPins were selected that recognize different conformational epitopes on the extracellular domain (ECD) of HER2, but do not inhibit the growth of HER2 positive tumor cells as monovalent binders.[89,151] However, the fusion of two of the evolved DARPins binding to subdomain I and IV of the ECD yielded bispecific binders with very strong cytotoxic capacity, that increases with shorter linker length of different constructs.[152] As revealed by structural and biochemical analyses, the bispecific DARPin distorts the HER2 receptor molecules into a conformation that prevents the formation of signaling-proficient receptor dimers (including heterodimers with EGFR and HER3).[152] This DARPin-induced trapping of adjacent HER2 molecules is the basis for the exceptional potency observed experimentally. The cross-linking did not trigger receptor internalization and degradation but induced cell death through apoptosis in all tested HER2-dependent cancer cell lines.[152,153] In contrast to mAbs like trastuzumab, the bispecific DARPin abolishes not only HER3 phosphorylation, which can be bypassed to activate the PI3K/AKT pathway, but also HER2 signaling.[153] The complete lockdown of HER2 complex-dependent signaling renders the bi-specific DARPin a universal (pan) HER inhibitor and precludes resistance due to its cytotoxic effect. It is worth pointing out that inverted constructs and such with longer linker sequences were both considerably less active as were mixtures of homobivalent DARPins.[152] A recent study further assessed the described pro-apoptotic effect on HER2-addicted cancer cells and revealed that the bi-specific DARPin induces a HER2 lockdown into signaling-incompetent, oligomerized receptor molecules.[154] This compelling example illustrates the huge potential of the DARPin format awaiting to be unleashed: the flexibility and immense diversity of the DARPin scaffold not easily attainable by Ig-based proteins open up new modes of action for innovative drugs to more effectively interfere with deregulated physiological processes. Indeed, based on this principle, Molecular Partners has independently developed a similar DARPin drug candidate MP0274, intended to directly kill HER2-addicted tumor cells without the need to engage the immune system or employ cytotoxic drugs, thereby minimizing the risk of side effects.[122] MP0274 consists of a pair of DARPin modules targeting two non-overlapping epitopes on neighboring HER2 molecules (the extracellular domains II and IV, respectively) and two additional moieties binding to albumin. The biparatopic HER2-DARPin domains handcuff the receptors in a signaling-inactive state that leads to apoptotic cell death. MP0274 has been part of a Phase I study and demonstrated favorable antitumor activity in *in vivo* models similar or superior to trastuzumab.[123] Importantly, the different antitumor mechanism of MP0274 which also interferes with HER3-mediated signaling holds promise to overcome acquired resistance to therapeutic HER2 antibodies.

The treatment of acute myeloid leukemia (AML) is hampered by the heterogenous nature of the disease and dose-limiting toxicities of existing therapies resulting in a narrow therapeutic index that excludes robust anti-tumor efficacy and, consequently, results in high relapse rates. The combinatorial assembly of DARPin domains engaging multiple tumor antigens simultaneously provides a means to tackle these problems. Recently, a multi-specific DARPin T cell engager candidate has been developed with highly potent and specific activity on AML cells, a reduced effect on healthy cells and with the potential to counteract target escape mechanisms likely to arise from tumor heterogeneity.[155] This molecule combines three distinct tumor antigen

binding domains – against CD70, CD173 and CD33, respectively – with an anti-CD3 T cell recruiting DARPin domain. An important feature of this avidity-driven multi-specific DARPin lies in its ability to discriminate between healthy tissue (expressing only one antigen) and transformed cancer cells with two or three surface antigens. The increased selectivity directly correlated with a wider therapeutic window: in an *ex vivo* assay using fresh blood from healthy donors, the DARPin-based T cell engager induced considerably less profound side effects (e.g., inflammatory cytokine production, reduction in platelet counts) compared to competitor molecules with similar T cell engager mechanism of action (MOA).[155] Thus, combined multi-specificity promises to effectively target heterogenous cell populations characteristic to AML cells (and the tumor microenvironment in general) with reduced toxicity towards healthy tissue.

Multi-specific DARPins in cancer therapy

Cytokines, growth factors and receptors constitute prominent targets for therapeutic antibodies in cancer therapy. However, improved specificity by the inhibition of a particular pathway often proves insufficient in the long-term treatment of cancer patients, since compensatory pathways and increased mutation rates, enable surviving cancer cells to escape drug-induced selection pressure and acquire resistance. The combined targeting of multiple targets can preemptively counteract the emergence of such escape routes, independently from the actual killing mechanism (e.g., T-cell recruitment or cytotoxic payload delivery). In this respect, multi-specific DARPins constitute an appealing alternative format for the design of more effective and versatile cancer drugs, and several multidomain DARPins have yet proven efficient in pre-clinical and clinical trials.

MP0250, a bi-specific anti-HGF/anti-VEGF DARPin flanked by additional HSA binding domains, has been positively evaluated in Phase II studies.[156] The rationale to synergistically inhibit both ligands came from the observation that resistance to VEGF inhibition can arise through activation of the HGF/cMet signaling pathway.[121] MP0250 has a molecular weight of 62.4 kDa and binds to all of its targets with EC_{50} values in the low two-digit picomolar range. The efficacy, safety and tolerability of MP0250 has been demonstrated in pre-clinical and Phase I clinical trials.[103,157] Two additional Phase II clinical trials of MP0250 have been conducted, one in multiple myeloma patients and one in EGFR-mutated non-squamous non-small lung cancer patients; the former in combination with the proteasome inhibitor bortezomib and the corticosteroid dexamethasone, the latter together with the EGFR kinase inhibitor osimertinib. Overall, the proven antitumor activity, favorable safety and pharmacokinetic profiles of MP0250 indicate that the dual inhibition of HGF/VEGF can benefit patients both as monotherapy or in combination with standard of care treatments, especially in cases of acquired resistance.

Bispecificity can also be exploited to promote proximity between two molecules or distant cells such as the recruitment of immune cells to tumor cells, a concept often used for immune-oncological therapies. The idea is to only activate immune cells locally in the tumor and not anywhere else in the body, avoiding systemic side effects while delivering greater efficacy. The bridging of T cells with a cancer cell triggers the release of cytotoxic molecules (e.g., granzymes, perforin) that penetrate the cell and induce apoptotic cell death. This physiological process is being emulated with the design of bispecific T cell engagers (BiTEs) which comprise two scFvs with distinct specificities. One of the scFv interacts with T cells while the other is directed against tumor cell-specific epitope.[158,159] In an analogous fashion, the bispecific DARPin MP0310 acts as a local agonist by binding to the fibroblast activation protein FAP (abundantly expressed on cancer-associated fibroblasts) and the stimulator 4-1BB on T cells, building on nature's concept of receptor activation by clustering. Only upon simultaneous binding of both target molecules T cells become activated resulting in a locally-confined cytotoxic response.[117] Tumor-restricted activation is thereby ensured as 4-1BB clustering on T cells is only brought about by FAP-overexpressing fibroblasts, likely yielding a larger therapeutic window compared to non-tumor localized activators. After initial preclinical characterization in mice, MP0310 is currently evaluated in patients with advanced solid tumors for its safety and activity (NCT04049903).[118] Except for 12 cases of infusion-related reactions (IRR) no adverse effects of special interest have been reported so far. Often times IRR can be attributed to a reaction of the innate immune system, such as a complement activation-related pseudoallergy.[160] As for Ig-based antibodies, rather than an intrinsic property of the DARPin scaffold IRR appears to be compound specific; for instance, for another bispecific DARPin MP0250 such IRRs were not observed.

In a similar approach, MP0317 is being developed to direct the immune activator CD40 on B cells to FAP-overexpressing tissues.[119] Recent data demonstrated its potency to repolarize macrophages resulting in T cell activation with killing effects comparable to an anti-CD40 antibody.[120] MP0317 is currently evaluated in clinical phase I. While bi-specific T cell engagers are very potent anti-tumor drugs, systemic toxicities due to off-target recruitment of T cells to noncancerous cells hamper their therapeutic window. An established strategy to avoid systemic side effects is the administration of a compound as a prodrug which is only converted into a pharmacologically active drug within the body.[161] This concept has recently been applied to a DARPin based CD3-binding T cell engager in which the CD3 effector function is masked until being activated in the tumor microenvironment.[162] The anti-CD3 prodrug molecule is composed of four distinct DARPin domains: an EGFR- and a CD3-binder as the core T cell redirecting entity, connected by a protease-cleavable linker to a DARPin domain that occupies the CD3-binding interface through intramolecular interactions. A fourth anti-HSA domain ensures prolonged blood circulation. This DARPin prodrug

is unable to bind and recruit T cells in its non-cleaved state in circulation and becomes activated on site upon cleavage of the linker by tumor-associated proteases, ensuring tumor-localized release of stimulated immune cells. Notably, the flexible modular arrangement of the four DARPin domains facilitated a built-in safety mechanism: upon activation (i.e. upon cleavage), the HSA domain is lost and, consequently, the T cell engager entity is quickly eliminated from circulation by renal clearance if not bound to its target, minimizing the exposure of the activated drug outside the tumor.[162] It will be interesting to see how this DARPin-based prodrug compares to 'muted' T cell bispecific antibodies (TCBs) that are similarly activated by cleavage of an anti-idiotypic anti-CD3 mask through tumor-associated proteases.[163]

Ensovibep: a potent tri-specific anti-COVID-19 DARPin

Arguably the most compelling example highlighting the unique benefits of the DARPin platform/technology comes from the recent development of the multi-specific DARPin molecule ensovibep which is designed to inhibit the infectivity of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2).[84,111,112] All current binding proteins inhibiting SARS-CoV-2 are based on neutralizing a single site on the spike protein used by the virus to attach to and enter host cells. While proven effective so far, intrinsic issues like high global abundance and the (treatment-induced) selection pressure promote the emergence of viral escape mutations. Indeed, several mutated strains have been reported that have undergone changes to their spike protein rendering such variants less susceptible to neutralizing antibodies and increasingly contagious.[164,165] While antibody cocktails have the potential to counteract this limitation, such combinations entail time-consuming optimization, production and regulatory challenges. However, time is a critical factor in containing a viral pandemic in today's globalized world. Here, the unique benefit of the rapid development of multifunctional DARPins, capable of binding to multiple epitopes at once, comes into play. MP0420 (ensovibep) is a multidomain DARPin that comprises three DARPin domains recognizing three distinct epitopes on the spike's receptor binding domain (RBD) (**Figure 4**). The cooperative target binding of ensovibep does not only lead to a strong avidity effect with an actual K_D in the fM range, but importantly translates into very potent viral inhibition, impeding viral evasion strategies. Indeed, ensovibep has been shown to retain full potency against all COVID-19 variants of concern, including Omicron.[111] The significance of built-in, preemptive anti-escape measures is further evidenced by the observation that the endogenous antibodies elicited by vaccines (Pfizer or AstraZeneca) or from previous infections against other strains of SARS-CoV-2 show diminished potency against the prevailing Delta variant.[166] It has been suggested that the receptor-binding β-loop-β motif of the Delta strain adopts an altered conformation that weakens the interaction with RBD-targeting antibodies.[167] Similarly, recent data demonstrate that mutations in the Epsilon variants B.1.427/B.1.429 cause reduced or even complete loss of neutralization by Spike protein-binding antibodies.[168] Therefore, ensovibep promises to remain effective against evolving, future variants.

A Phase I study has demonstrated the safety and tolerability of ensovibep and a serum half-life of 2-3 weeks. The latter is achieved through two built-in half-life extending DARPin domains (HSA-binding DARPin domains) and allows for biweekly administration in a preventive setting. Based on the positive results of the Phase II portion of the EMPATHY study – encompassing 407 acute COVID-19 ambulatory patients, ensovibep lowered the viral load monitored over an eight days period and led to an overall 78% reduction in COVID-19-related hospitalization, emergency room visits or death – a request for emergency use authorization for ensovibep has been submitted to the FDA in early 2022. As of today, the emergency use authorization remains under review and the FDA has indicated that Phase III data will be required prior to authorization. Thinking ahead, when looking for the most economical and convenient route of administration, it is very likely that the high thermostability of ensovibep may facilitate the development of aerosol or dry powder inhaler formulations for pulmonary delivery. The fast-paced development of ensovibep that had started in March 2020 and may be granted market approval in the first half of 2022 showcases the flexibility of the DARPin technology to adapt swiftly to unanticipated, quickly arising challenges. Notably, in case of the emergence of viral escape mutations, new DARPin domains can be easily selected against the mutated epitope and replaced or added to existing anti-viral DARPins. MP0423, another anti-COVID-19 DARPin molecule in preclinical trials, binds simultaneously to three different parts of the spike protein. One moiety targets an epitope on the spike's RBD, another one on its S1 N-terminal domain and a third one on its S2 domain (**Figure 4**). In conclusion, the relatively short time during which high-affinity DARPin domains can be obtained (in this particular case in a matter of weeks), the flexible modular nature with arrays of different epitope-targeting domains, the geometric adaptability to fit the target molecule and the ease of manufacturing to rapidly produce batches at sufficient scale for global treatment at low cost are exceptional assets of the DARPin technology. Lastly, the high thermal stability of the DARPin scaffold may render cold-chain requirements during delivery and storage dispensable, making it easily accessible to underdeveloped countries.[80,85]

The untapped potential of the DARPin technology

The motivation that drives the engineering of novel types of binding proteins is fueled by enabling new applications that existing binders cannot fulfil. In light of this, DARPins can be viewed as the next generation 'magic bullets' equipped with a Swiss Army knife, i.e. tailor-made high affinity target binders that are able to integrate multiple functions in addition. The DARPin drug candidates described above are representing only the spearhead of feasible modes of action that can be envisioned. In the following, we will illustrate both

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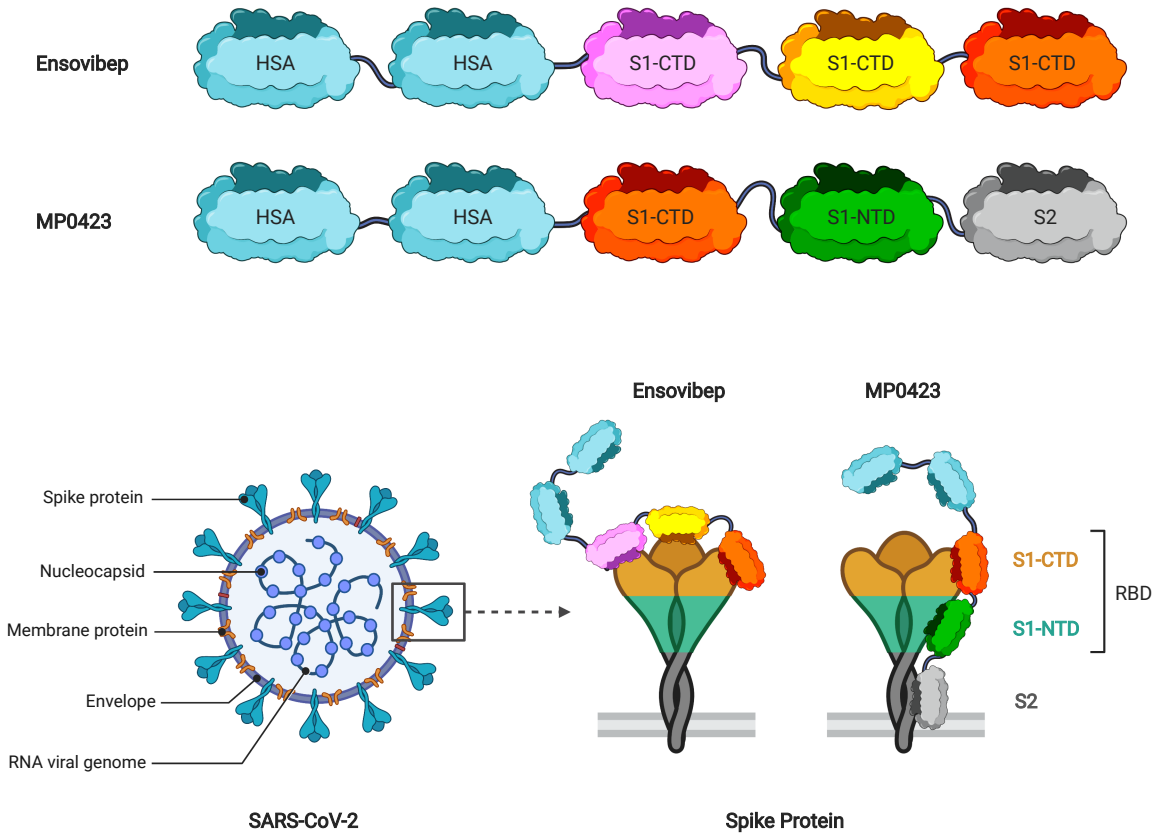

Figure 4. Architecture and mode of action of ensovibep and MP0423. Schematic depiction of anti-COVID-19 DARPin candidates. Ensovibep comprises three receptor binding domains (RBD) that bind to the same epitope region on the Subunit 1-C-terminal domain (S1-CTD) of the RBD but with different antigen-binding sequences (pink, yellow, orange). By contrast, MP0423 targets three distinct parts of the coronavirus spike protein which include the S1-CTD of the RBD (orange), the Subunit 1-N-terminal domain (S1-NTD) of the RBD (green) and the S2 domain (grey). Both anti-COVID-19 DARPin candidates possess half-life enhancing DARPin domains that bind to human serum albumin (HSA; shown in blue) to support prolonged activity. Mode of action of ensovibep and MP0423 neutralizing SARS-CoV-2. The cooperative target binding of the trispecific DARPin candidates supports potent virus inhibition and preemptively counteracts viral escape mutations.

emerging and unexplored applications that can be conceived based on the huge, still largely unexploited degrees of freedom in the design space of DARPin molecules.

pMHC-targeting DARPins in cancer immunotherapy

A key principle in the selective elimination of tumors sparing healthy cells is the identification of surface-exposed, cancer-specific antigens or neoantigens. Cells constantly present the state of their intracellular proteome to the immune system as small peptides bound to major histocompatibility complex (MHC) class I molecules.[169,170] If these peptides arise from a mutated tumor suppressor or a fusion protein as a result of gene translocation events, patrolling T cells are able to detect such tumor-associated peptides displayed on MHC molecules (pMHC) as nonself (transformed) and trigger the elimination of the diseased cell. Like MP0310 or the BiTE format, such bispecific antibodies redirect T cells to tumors to induce local and selective killing of cancer cells. However, the huge variation of individual peptides (besides very low amounts) presented per cell and the composite nature of the pMHC-epitope impose major challenges to the generation of highly specific binders that recognize exclusively the desired pMHC without exhibiting crossreactivity to other self-antigen-presenting MHC complexes.[171] T cell receptors seem to have an optimal affinity window for their pMHC targets (typically in the micromolar range) whereby higher affinities result in a loss of specificity and activity.[172] In addition, to maintain specificity, it needs to be ensured that the co-recognition of the peptide-MHC target by the selected paratope is not dominated by interactions with the MHC molecule that is expressed on all nucleated cells of vertebrates (diseased and healthy ones). To date, the use of antibody and T-cell receptor-based pMHC binders has been limited due to the low target abundance, weak affinity, promiscuity to other pMHCs or challenging biochemical properties. DARPins, however, possess the characteristics to potentially overcome these obstacles and provide sufficient specificity among a multitude of self-antigens. The slightly concave and rigid binding surface of a DARPin domain is well suited to interact with the small and flat epitope of the peptide embedded within a groove of MHC complexes. The modular nature and geometric flexibility of the DARPin format allows to as-

semble high affinity domains specific to the tumor-associated pMHC and to recruit T cells via another CD3-binding module. In solid tumor indications, pMHC-targeting DARPins would additionally benefit from their small size to penetrate into dense tumor tissues barely accessible to larger molecular formats like bispecific antibodies. Molecular Partners is developing specific pMHC DARPin binders in the T cell engager format to target transformed cancer cells.[173] Architectural (i.e. linker length) and sequence tuning yielded highly potent DARPins built from a DARPin domain specific to the human MHC class I molecule HLA-A2 in association with a peptide derived from NY-ESO-1 cells, and a DARPin domain specific for CD3. Notably, this bispecific DARPin drug candidate potently redirected and activated T cells exclusively at antigen presenting cancer cells.[173] These promising results also suggest that pMHC-targeting DARPins could be useful in a wider range of treatment modalities (e.g., drug conjugates, CAR-T cell therapy, diagnostics).

DARPins as guide molecules in tumor therapy

Antibody drug conjugates are an established class of highly potent biologics with enhanced functional properties that can deliver a payload (e.g., a small molecular weight anticancer drug or another therapeutic agent) in a targeted manner.[150] Once bound to the antibody-specific antigen on the target cell, the antibody, together with the drug, is internalized. This mode of delivery maximizes the efficacy of the treatment while at the same time adverse side effects are kept at a minimum due to target-specific drug exposure.[174,175] In a similar fashion, the favorable properties of the DARPin domain can be exploited as a targeting moiety for a payload discriminating between healthy and diseased tissue. As the consensus DARPin scaffold lacks cysteine residues by design, this feature can be used to site-specifically introduce a cysteine which is amenable to thiol-based conjugation. For instance, an EpCAM-specific DARPin genetically fused to a truncated version of the Pseudomonas exotoxin A exhibited potent antitumor activity at well-tolerated doses in mouse xenograft studies, resulting in complete regression in some animals.[176]

A further iteration of this strategy features an expression protocol that incorporates a single N-terminal azidohomoalanine, a methionine surrogate replacing the initiator Met, and allows for azide-alkyne cycloaddition as additional means of chemical functionalization of the DARPin. Such "clickable" and cysteine-tagged DARPins allow for double-functionalization, a strategy that has been employed to build an EpCAM-targeting DARPin equipped with both a small cytotoxin and serum albumin for half-life extension.[145] Importantly, neither EpCAM-specific binding and internalization nor the cytotoxic potency of the molecule were affected by the site-directed functionalization, which is a consequence of the DARPin topology with its clearly defined paratope and the described sites of functionalization being well-placed outside of the area of target binding.[177]

Radioligand therapy is another prominent therapeutic strategy that combines a tumor-targeting binding moiety and a radioactive isotope, causing DNA damage that inhibits tumor growth and replication. This approach enables targeted delivery of radiation – conjugated to the binding protein via a chelator – to the tumor, while limiting damage to the surrounding normal tissue. Since harsh chemical conditions are needed to load chelators with radioisotopes, DARPins with their very high thermostability are very appealing binders for this approach. Derivatized DARPins can also be used as diagnostic tools. To validate clinical diagnoses and evaluate therapy efficacy, accurate assessment and classification of the aggressiveness of tumor tissues constitute critical factors. For tumor imaging, radionuclides selectively accumulating in the tumor are generally used to generate contrast in imaging techniques (e.g., PET or SPECT). The small size and high affinity make radiolabeled DARPins excellent diagnostics, efficiently infiltrating the dense tumor microenvironment and showing reduced accumulation in the liver.[178]

Another type of payload with a lot of potential are small interfering RNA molecules (siRNA). Tissue-specific delivery and efficient intracellular delivery are major hurdles for the widespread therapeutic use of siRNA mediated gene silencing. To this end, an anti-EpCAM DARPin has been used as a vehicle for siRNA targeting the mRNA of the antiapoptotic factor Bcl-2.[179] Protamine, an unstructured positively charged protein, was thereby fused to DARPin domains to achieve complexation of the siRNA molecules. While the fusion protein effectively decreased Bcl-2 expression and sensitized EpCAM-positive cells towards doxorubicin, no effect was observed for cell lines lacking surface-exposed EpCAM.[179] Thus, DARPin-siRNA bioconjugates are alternative new tumor agents which may fulfil their promise with improved endosomal escape strategies at hand.[180] Of note, in light of the recent success of mRNA-based vaccines, it will be interesting to compare this direct delivery of naked oligonucleotides to specific cells to the delivery with lipid nanoparticles and engineered viruses.

Engineered change in viral tropism and exosome targeting

Gene therapy holds great promise to cure many human diseases and after several initial setbacks the market approval of the first gene transfer vector derived from adeno-associated virus (AAV) in 2012 (Glybera) has reignited the excitement for targeted gene delivery.[181] Due to its supreme safety profile, comparably low immunogenicity and moderate packaging capacity, AAV is a promising vector for virus-mediated gene delivery.[182] However, AAV vectors accumulate predominantly in the liver which results in decreased efficiency due to loss of virus particles to irrelevant cells.[181] To avoid off-targeting, several strategies for cell-specific delivery are employed, including fusions of targeting moieties to viral capsid proteins.[183] With their highly robust and stable fold, that does not depend on disulfide bonds and can thereby be

obtained at high accuracy in the cytoplasm, DARPins are well suited to serve as guiding molecules upon genetic fusion to AAV particles and have been successfully applied in viral retargeting in different experimental settings.[184–186] The alternative approach fusing scFvs as high-affinity targeting ligands to virus coat proteins is very much hampered due to poor display efficiencies and, here, the compact fold of DARPins provides a unique advantage: i.e. the ease and efficient intracellular expression which allows for the straightforward integration into the viral particles when they get assembled. For instance, a HER2-specific DARPin genetically linked to AAV's capsid protein VP2 on virus particles ablated for primary receptor binding transduced target cells with a far higher specificity compared to other AAV systems and could be further improved by obtaining purer AAV preparations devoid of AAV particles lacking the DARPin-VP2 protein fusion.[187,188]

In an entirely different strategy that does not involve the expression of an integral DARPin fusion on the viral surface, bispecific DARPin adapters redirect the virus to any desired cell receptor.[91] In this case, one DARPin domain was designed to bind the receptor-attachment region of adenovirus serotype 5 and the other DARPin domain to bind to the tumor marker HER2. The former bispecific construct was assembled in tandem as bi- or trivalent binders to strengthen the non-covalent interaction with the virus coat protein.[91] This strategy has several advantages over the approach of genetic fusion to viral capsid proteins. Such adapters are rapidly and cost-efficiently produced in bacterial cultures in pharmaceutically-relevant quantities; the viral tropism can be easily rewired to other cell types by replacement of the receptor-targeting DARPin domain and used for the same type of virus carrying different payloads. For example, using this technology the delivery of the adenovirus-based anti-SARS-Cov2 vaccine Vaxzevria (AstraZeneca) could be targeted specifically to the lung and, thus, potentially enhance treatment efficacy.

Lentiviral vectors (LVV) provide an outstanding therapeutic potential by stable long-term transgene expression in nondividing cells. In order to improve selectivity and efficiency of the transduction, so called pseudotyping approaches allow to direct the LVV to the desired target cells and -tissue and to thereby tailor the tropism as needed. Engineered measles virus (MV) glycoproteins (hemagglutinin (H) and fusion protein (F)), expressed on the LVV surface for efficient LVV-transduction, can be extended with a specificity-providing binder to add selectivity. Classically, this has been obtained through display of an scFv as targeting domain by fusion to the MV-H protein. Since LVV quality and yield of production are dependent on the stability of the recombinant pseudotyping moiety, DARPin fusion proteins proved to incorporate very efficiently into LVVs and with high titers.[189] When applied in vivo systemically and featuring anti-HER2 or anti-CD8 binding DARPins, respectively, DARPin-targeted LVVs mediated exclusive transduction in HER2 positive tumor tissue[189] and to CD8-positive T cells[190,191], while vesicular stomatitis virus-glycoprotein (VSV-G) pseudotyped vectors mainly transduced cells in spleen and liver. Thus, DARPins are a very promising alternative to scFvs for retargeting of LVs, especially when it comes to innovative and novel in situ approaches.

The use of DARPins as targeting ligands is not restricted to virus-based gene delivery but has also been shown to efficiently direct siRNA-loaded exosomes to tumor cells.[192] Engineered exosomes equipped with surface-exposed LAMP2b-DARPin fusions (specific to HER2) delivered siRNA complementary to the TPD52 oncogene cell-type specifically to HER2-positive breast cancer cells. Due to their natural, nontoxic, non-immunogenic and biodegradable properties, exosomes constitute excellent vehicles for gene targeting. Similarly, lipid nanoparticles, successfully used as the delivery system of the mRNA COVID-19 vaccines from Pfizer/BioNTech and Moderna, could be modified with guiding DARPins to promote fusion with relevant target cells.

Multi-specific CAR targeting to avoid antigen escape

Chimeric antigen receptor (CAR) engineering is an emerging branch of cellular immunotherapy allowing for adoptive cell therapy in which immune cells (currently mostly T-cells) are endowed with designed receptor chimeras that combine both antigen-binding and T cell activating functions into a single receptor.[193] The typical CAR molecule combines an extracellular antigen-binding domain, classically an scFv, with intracellular signaling domains that activate the T cell in response to antigen recognition and formation of an immunological synapse. In the liquid tumor setting CAR-T cell therapy showed high potency and led to the market authorization of so far six different CAR-T cell products for the treatment of B cell malignancies.[194] In contrast to the successful treatment of circulating tumor cells, treatment of solid tumors with CAR-T cells has thus far been significantly less effective and instances of severe side effects (e.g., neurotoxicity or cytokine release syndrome) demonstrate that this relatively new therapeutic approach still requires improvement for efficient and successful translation into the clinic.[195] While the small size of scFvs and their virtually unlimited binding specificities make them attractive moieties for antigen-recognition, their tendency to oligomerize due to mispairing of the V_H and V_L domains across neighboring molecules, observed for scFvs integrated into CARs, can lead to antigen-independent constitutive (tonic) signaling and T cell exhaustion.[196–198] As there is no biochemical or functional requirement for the use of scFvs, undesired domain crosspairing and clustering could be avoided by using other high affinity binding scaffolds such as DARPins. Two studies with CAR molecules using anti-HER2 DARPins as target-binding domain instead of scFvs illustrate the feasibility of this approach.[199,200] The resulting 'DARPinCARs' targeted HER2-overexpressing cancer cells as effectively as scFv-based CAR-T cells and showed efficient tumor

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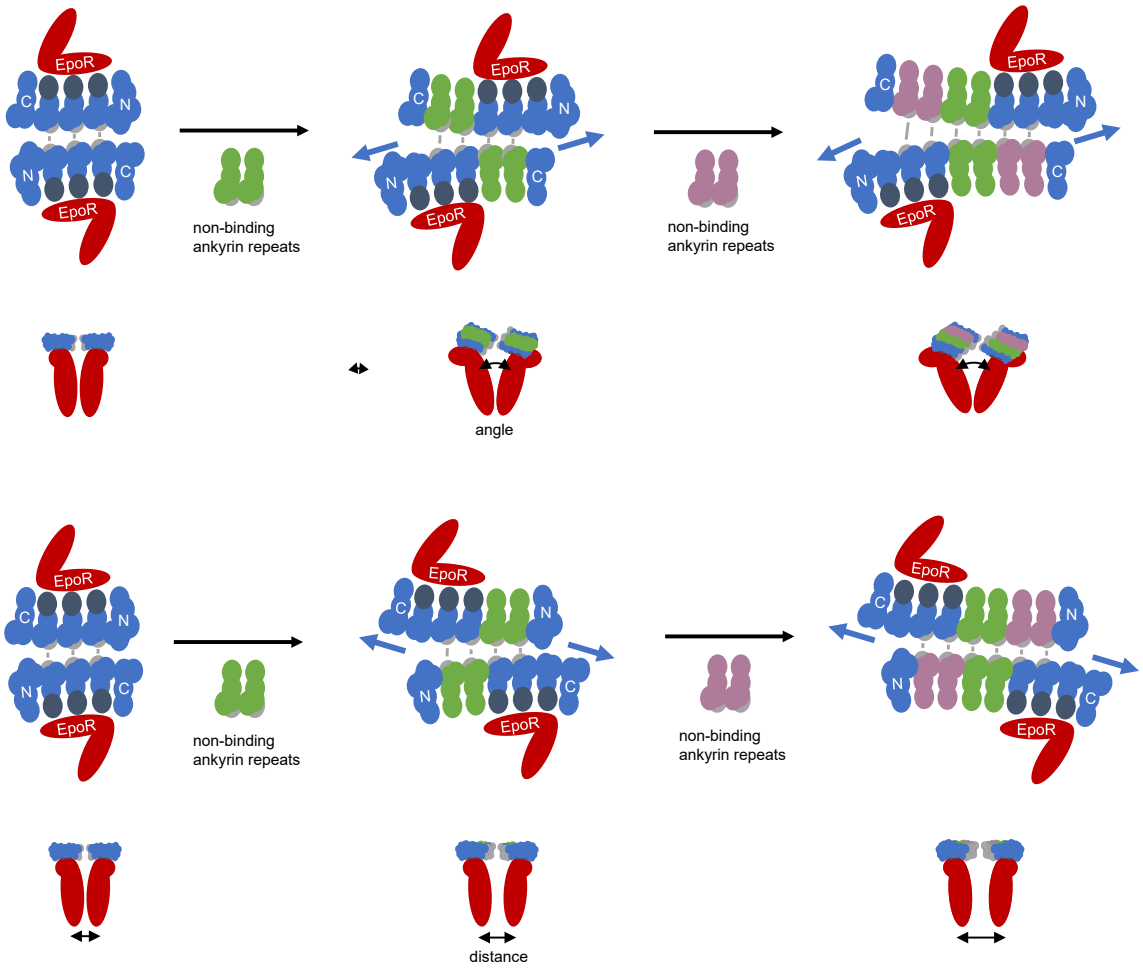

Figure 5. Interrogation of signaling as a function of rotation (angle) and proximity (distance) between erythropoietin receptor (EpoR) monomers. A series of DARPin homodimers can be designed to either pivot the EpoR dimer in a scissor-like manner (top panel) or separate the EpoR monomers without affecting the dimer angle (bottom panel). This is achieved by the insertion of non-binding ankyrin-repeats, altering the relative positions of the EpoR binding repeats across the designed dimerization interface. Successive addition of non-binding ankyrin-repeats to the C-terminal end results in a set of dimers with variation in the angle between the two EpoR subunits. Conversely, to engineer the distance series, the non-binding ankyrin-repeats are integrated stepwise at the N-terminus, moving the binding repeats further apart. Here, the modular nature of the ankyrin repeat protein scaffold enables not only size variation but also allows to build geometrically-defined ligand dimers, an increasingly important feature to systematically elucidate how ligand-induced topological changes of dimeric receptors influence signaling outputs.

clearance *in vivo*.

As outlined above, loss of expression of a cell surface tumor antigen due to drug-induced selective pressure desensitizes the tumor to the treatment, and asks for targeting two or more antigens simultaneously. To this end, so-called tandem CARs (TanCARs), in which two scFvs are fused to each other, have been devised to obtain bispecific CAR molecules.[201] The architecture of TanCARs, however, suffers even more from the potential risk of mispairing between different scFvs and, hence, the design of such molecules is a challenging task.[202] The ease with which multiple DARPin domains with different ligand specificities can be assembled in a single polypeptide chain makes this scaffold an attractive alternative for the construction of multi-specific CARs. Taking advantage of this feature, a tri-specific CAR molecule composed of DARPin moieties targeting EGFR, EpCAM and HER2 has been generated and evaluated for the treatment of tumors with heterogeneous antigen expression.[203] The resulting tri-specific DARPinCAR-T cells targeted effectively a mixture of heterogeneous tumor cells, each expressing a single antigen and displayed synergistic activity when tumor cells expressed more than one target antigen.[203] Therefore, CARs built with DARPins that impart multiple antigen specificity to CAR-T cells promise to synergistically reduce tumor escape.

DARPins open up novel modes of action for next-generation pharmaceuticals

Functional selectivity is an emerging concept that illustrates this need for more refined therapeutics to interfere with biological processes in a desired manner.[204] In receptor-

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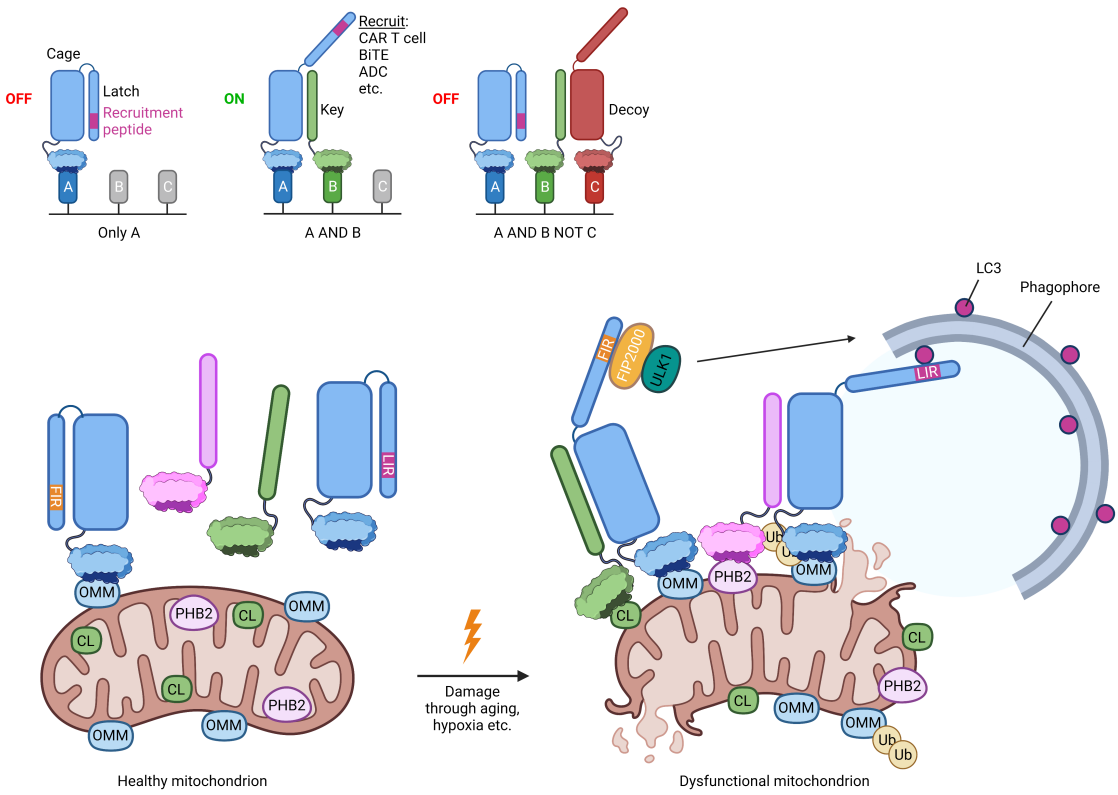

Figure 6. Colocalization-dependent protein switches are tuned so that cage and key do not interact in solution but strongly interact when colocalized on a surface by DARPin-based targeting domains. Top left: In the absence of a colocalizing key due to a missing biomarker (only A) the cage remains locked, i.e. unable to recruit effector molecules. Center: An effector protein is recruited only when cage and key are colocalized on the surface of the same cell (AND logic). Right: A decoy acts as a sponge to sequester the key, thereby preventing cage activation (AND and NOT logic). Therefore, Co-LOCKR is able to perform two- and three-input logics in heterogenous cell populations (OR logic by the addition of a second key to another DARPin-targeting domain is also possible (not depicted)). Lower panel: Potential application of an intracellular DARPin-based Co-LOCKR to sense and eliminate aged/damaged mitochondria. A cage that recognizes an outer mitochondrial membrane (OMM) protein is delivered together with a damage-surveying key (e.g. binding to the inner mitochondrial membrane-restricted lipid cardiolipin (CL) or prohibitin 2 (PHB2) protein). In the absence of damage, intact mitochondria are invisible to the key(s) which therefore localize diffusely within the cell. In the event of mitochondrial injury, CL becomes externalized to the OMM while PHB2 can be exposed to the cytosol due to membrane rupture. Consequently, the keys are recruited specifically to damaged mitochondria and can unlock the cage due to organelle-restricted colocalization. This in turn promotes the formation of autophagosomes either through the recruitment of the ULK1-FIP200 complex or via binding to LC3-coated phagophores. Analogous systems can be envisioned for the detection and lysosomal removal of cytosol-dwelling bacteria and virus particles. FIR: FIP200 interacting region; LIR: LC3-interacting region.

mediated signaling, a ligand has been viewed traditionally either as an agonist (stimulatory) or as an antagonist (inhibitory). More recent data, however, suggest that this classification does not apply to every ligand and is oftentimes an oversimplification.[205]

The notion of functional selectivity holds that different conformations adopted by receptors after associating with specific ligands can determine which intracellular signaling pathways get activated and which do not. This phenomenon, also known as biased signaling, has been best studied for G protein-coupled receptors (GPCR). Here, a number of ligands have been identified that selectively activate some downstream pathways while blocking others.[205,206] The appeal of such biased ligands is immense considering that many disease states might require the subtle manipulation of some but not all downstream events arising from specific receptor activation. Additionally, a better understanding of biased signaling would likely improve the R&D process, as it would allow screening for compounds that selectively activate or deactivate subsets of downstream pathways.

While the elucidation of structure-activity relationships has made considerable progress for GPCR agonists, the same approach cannot be used to investigate the tunable signaling upon ligand-induced receptor dimerization.[207] Exploiting the modular nature of DARPins, a recent study devised an elegant strategy to systematically explore the relationship between ligand-receptor dimer geometry and signaling output.[208] To this end, a series of geometrically controlled cytokine mimetics was used to modulate erythropoietin receptor (EpoR) dimerization orientation and distance between receptor monomers in a controlled manner. After selection of high-affinity anti-EpoR DARPins, the monomeric domains were converted into homodimeric agonists by the incorporation of designed dimerization interfaces

to the DARPin backside (**Figure 5**).[209] This non-covalently associated DARPin homodimer served as a building block to design two different DARPin dimer extension series to interrogate signaling as a function of receptor orientation (angle) and proximity (distance). Systematic variation in each of these parameters was achieved by inserting nonbinding repeats derived from the consensus ankyrin repeat sequence, which changes the relative positions of the EpoR binding repeats across the designed dimerization interface. Because of the nonplanar, helical nature of the DARPin molecule this results in dimers in which the two EpoR binding interfaces assume different separations and orientations. To engineer the "angle" series, one or more nonbinding repeats were inserted at the C-terminal end while the "distance" series was obtained by the successive addition of nonbinding repeats at the N-terminus (**Figure 5**).[208] he systematic variation of angular and distance parameters generated a range of full, biased, and partial agonism of EpoR signaling as assessed by detecting the phosphorylation status of various downstream effectors. Overall, the authors observed a strong correlation between increasing angles or distances and progressive partial agonism. The differentiation and proliferation of hematopoietic stem cells into red blood cells served as another readout to gain insight into the topological control of cytokine receptor signaling. Interestingly, the partial DARPin agonists showed stage-selective effects on erythrocyte maturation, whereas the biased agonists more selectively promoted signaling at either the early or late stages of differentiation.[208] In general terms, the same agonists can result in different downstream signaling depending on cell state. This creative approach could be used to study cytokine receptor dimer geometries for the identification of signaling events of clinical interest in other cytokine receptor systems. The unique features of the DARPin scaffold capitalized on elegantly by the authors could be used as a preclinical pharmacological tool to determine whether different degrees of agonism are optimal for a given cytokine in a particular therapeutic indication. The study also showcases the simple spatial re-arrangement of binding sites that DARPins facilitate; this can hardly be accomplished to the same extent using Ig-based scaffolds.

Tumors are complex cell populations characterized by the expression of heterogenous surface antigens. To target such mixed populations of closely related cells while sparing healthy cells therefore usually requires the specific recognition of several (more than one) marker proteins. Building on the concept of avidity, bispecific binders are able to engage two targets with weak to moderate binding affinities such that only cells expressing both antigens are recognized. A functionally more diverse system is to use AND, OR and NOT logic gates built into modular protein systems that are activated only upon respective target engagement on the cell surface. In an iteration of such bioactive protein modules, a colocalization-dependent protein switch (Co-LOCKR) has been recently designed using DARPins as antigen-targeting moieties (**Figure 6**).[210] Co-LOCKR is composed of two entities: a structural "cage" protein that contains a latch domain to sequester a functional peptide in an inactive conformation until binding of a separate "key" protein induces a conformational change that enables binding to an effector protein (e.g., antibody-drug conjugates, CAR-T cells). Cage, key, and effector interact in a three-way equilibrium such that the sensitivity of the switch can be tuned by adjusting the relative cage–latch and cage–key affinities. Fusion of the cage and key modules to DARPin domains specific to either HER2 or EGFR yielded a proximity-sensor able to discriminate between HER2-, EGFR- and HER2/EGFR-expressing cells in a mixed cell population. Importantly, only when bound to their respective targets, cage and key proteins are favored to interact. Through the addition of another key module targeting yet another antigen (e.g., EpCAM) OR operations can be introduced while NOT logics are achieved by a decoy protein fused to a targeting domain against a surface marker that should be avoided. The decoy protein functions to sequester the key module, preventing the activation of the cage.[210] As such, Co-LOCKR proteins can perform logic autonomously responding to coherent as well as competing inputs which enables targeting of specific subpopulations of cells. As a proof of principle, the authors successfully applied Co-LOCKR to direct CAR-T cells to heterogeneous cancer cells (Raji and K562 cells) using two- and three-input logic operations *in vitro*. In addition, as Co-LOCKR actuation is thermodynamically controlled, the therapeutic index will depend on the affinity of the targeting domains used to direct the Co-LOCKR proteins to antigens on the target cells: subnanomolar affinities are a prerequisite to facilitate dosing below the 40 nM level where activation starts to occur in solution. The use of DARPins is beneficial to mitigate both issues as high-affinity binders in the picomolar range can be obtained.[81]

Intracellular applications

Binding the intracellular target space is presently restricted to small molecules with much less selectivity and, therefore, higher chance of adverse effects than Ig-based antibodies. The fact that DARPins can fold and remain active in the cytoplasm is arguably a key advantage over most Ig-based antibody formats which due to the presence of disulfide bonds have only limited intracellular use. Many pharmaceutically relevant targets are inside the cell and, therefore, precluded from Ig-based antibody-based intervention. With the emergence of improved delivery systems (viral vehicles, lipid nanoparticles, engineered exosomes) and the increasing efficacy of endosomal escape mechanisms, exciting possibilities arise for the use of DARPins in targeting disease-relevant intracellular processes.

Because of their modular flexibility, DARPins are ideally suited to induce proximity of two or more ligands. For instance, analogous to the concept of proteolysis targeting chimeras (PROTACs), bispecific DARPins could promote the selective intracellular proteolysis of mutated oncogenes, abnormal fusion proteins derived from gene translocation events or polyQ expansions (e.g., huntingtin, ataxins),

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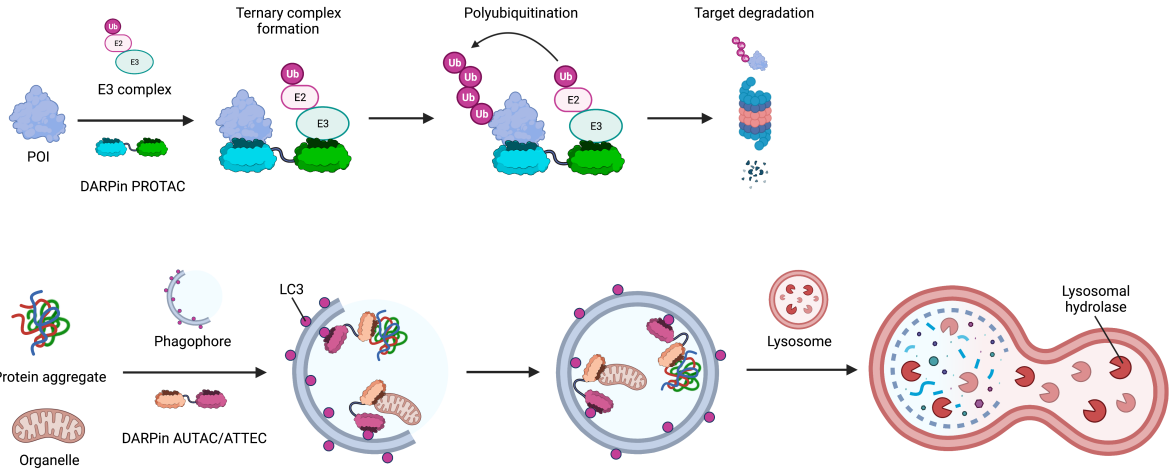

Figure 7. PROTACs are bifunctional molecules that simultaneously bind to a protein of interest (POI) and an E3 ubiquitin ligase complex, leading to ubiquitination and degradation of the POI via the proteasome. The fusion of two distinct DARPin domains enables the formation of DARPin-based PROTACs that can target any desired endogenous protein for degradation but also promote the specific turnover of protein isoforms, protein complexes and mutated or modified (active/inactive) targets (top panel). Similarly, DARPin-based ATTECs (autophagosome-tethering compounds) could direct large cellular targets - that cannot be processed by the proteasome - for degradation to the lysosome by binding simultaneously to LC3-coated nascent autophagosomes (phagophore). For instance, an LC3-targeting DARPin can be fused to another substrate-specific domain that recognizes cytotoxic protein aggregates or dysfunctional organelles such as dysfunctional mitochondria or cytosol-colonizing bacteria (bottom panel).

preventing the formation of toxic protein aggregates (**Figure 7**).[211] PROTACs are commonly based on heterobifunctional small molecules that simultaneously engage an E3 ubiquitin ligase and a protein destined for degradation. Similarly, a DARPin domain specific to a target of interest, combined with another DARPin moiety recruiting the ubiquitin ligase would result in the proteasomal degradation of the bound substrate. To date, PROTACs have been developed to degrade a variety of cancer targets with unprecedented efficacy against a multitude of tumor types.[212] Notably, the target protein does not have to be a natural substrate of the ubiquitin ligase; merely, induced proximity suffices to trigger its ubiquitination. An appealing feature of this proteolysis strategy is that only a specific binder is required which does not need to inhibit the function of the protein.

This concept has been applied to create a DARPin-based degrader specific to the KRAS oncogene.[213] The GTPase KRAS is the most frequently mutated oncogene found in pancreatic, colorectal and lung cancers accounting for 20% of human cancers. Effective cancer therapy against KRAS-driven tumors is hindered by several challenges: KRAS is a member of the RAS family comprised of three isoforms (> 80% sequence identity), each of which can be mutated in numerous tumors.[214] A variety of KRAS mutants arises through single amino acid changes spread throughout the protein concentrated at mutational hotspots.[215] Furthermore, RAS proteins lack well-defined pockets available for high-affinity small-molecule binding and, therefore, RAS is often referred to as "undruggable". Hence, no isoform specific RAS degraders have been identified.[216,217] Recently, the fusion of a DARPin domain selected to specifically bind to a mutant form of KRAS with a ubiquitin E3 ligase invoked efficient proteasomal degradation of KRAS.[213] In multiple cell lines tested, the DARPin-E3 ligase fusion led to KRAS-specific depletion while the protein levels of the other RAS isoforms (HRAS and NRAS) remained unaffected. Although the KRAS-specific DARPin degrader induced turnover of both mutant and wildtype KRAS, it only prohibited the proliferation of cancer cells expressing mutant KRAS in vivo, resulting in rapid regression of KRAS-driven tumors. Due to the functional redundancy within the RAS protein family, the loss of KRAS is likely compensated for in RAS wildtype cells. Importantly, a pan-RAS degrader, based on a single domain antibody and tested in parallel, showed no specificity towards any RAS isoform mutant protein and, consequently, induced programmed cell death in cells irrespective of RAS mutation.[213] Thus, DARPin-mediated KRAS proteolysis provides an attractive therapeutic strategy for KRAS-driven cancers that is not limited to any specific residue change. Though the direct therapeutic use of protein-based PROTACs to target intracellular proteins such as KRAS is currently limited, the efforts being made for the delivery of macromolecules by mRNA or other delivery strategies mentioned above promises tremendous potential to be leveraged in the near future. While PROTACs show great pharmaceutical potential and the first PROTAC-based degraders have entered clinical trials, their use is limited to soluble proteins that can fit through the narrow proteasomal channel. To overcome these size constraints, so-called autophagy-targeting chimeras (AUTACs) and autophagosome-tethering compounds (ATTECs) have been designed which can induce the selective removal of not only single proteins but also protein aggregates and entire organelles such as mitochondria (**Figure 7**).[218,219] Instead of proteasomal turnover, this system relies on the lysosomal

degradation of the bound target mediated by the autophagy machinery. Cytotoxic protein aggregates and dysfunctional organelles are attractive drug targets and the demand for such therapeutics is consequently high. For instance, defects in the selective elimination of damaged or aged mitochondria by autophagy (mitophagy) is associated with a number of neurodegenerative diseases such as Alzheimer's and Parkinson's disease.[220] Here, bifunctional DARPins can be envisioned that act as quality-control surveillance proteins: upon mitochondrial damage, mitochondria-specific proteins and lipids (e.g., PHB2, PINK1, cardiolipin) become exposed at the outer mitochondrial membrane. Such danger signals could be sensed, i.e., recognized, by a DARPin molecule that simultaneously engages the autophagy machinery (e.g., ULK1 complex, LC3 proteins). In such a scenario, only damaged mitochondria would activate the bifunctional DARPin protein, sparing the healthy ones. This is reminiscent of the Co-LOCKR system which could be employed in this setting in a similar fashion. Other modes of action can be envisioned such as the design of a bifunctional DARPin that is connected through a protease-specific cleavable linker sequence. Here, the release of one DARPin domain harboring a nuclear localization signal could lead to the inhibition of a nuclear enzyme upon damage- or cell cycle stage-specific activation of a protease. In conclusion, the increasingly efficient delivery of DNA- and mRNA-containing vehicles for intracellular expression holds great promise that the excellent properties of the DARPin scaffold can also be harnessed to manipulate disease-relevant biological processes in the cytoplasm.

Concluding remarks

The development of the DARPin format over the last two decades may culminate with the market approval of ensovibep in the following months. Ensovibep has been clinically validated undergoing Phase I/II/III studies with more than 400 patients. Thus, ensovibep showcases the unique benefits the DARPin scaffold affords: the robust biophysical properties that enables the generation of multi-specific formats and the ease of production of DARPin molecules not only expand the application range beyond what is possible with traditional antibodies but also enable the design and development of a drug candidate at unprecedented speed. Imperative to the success of the DARPin format has been the thorough design of a robust consensus scaffold that underwent several iterations throughout the past years. It is thus crucial to continuously improve its stability and robustness, likely to benefit current and as yet unanticipated, future applications.[80]

ABBREVIATIONS

AAV	adeno-associated virus
AMD	age-related macular degeneration
AML	acute myeloid leukemia
ANK	ankyrin
ARM	armadillo
AUTAC	autophagy-targeting chimera
ATTEC	autophagosome-tethering compounds
BiTE	bispecific T cell engager
CAR	chimeric antigen receptor
CDR	complementarity-determining region
COVID-19	coronavirus disease 19
DARPin	designed ankyrin repeat protein
ECD	extracellular domain
Fab	antigen binding fragment
FcεRI	IgE Fc receptor
GPCR	G protein-coupled receptor
HC	heavy chain
HEAT	huntingtin, elongation factor 3, protein phosphatase 2A, PI3-kinase TOR1
HSA	human serum albumin
Ig	immunoglobulin
IRR	infusion-related reactions
LC	light chain
LRR	leucine-rich repeat
LVV	lentiviral vector
mAb	monoclonal antibody
MHC	major histocompatibility complex
MV	measles virus
PEG	polyethylene glycol
PROTAC	proteolysis targeting chimera
PTM	posttranslational modification
SARS-CoV-2	severe acute respiratory syndrome coronavirus type 2
scFv	single chain variable fragment
TCB	T cell bispecific antibody
TPR	tetratricopeptide repeat
VLR	variable lymphocyte receptor

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DISCLOSURE OF INTEREST

PF and CJ are co-founders and shareholders of Athebio AG; PF is a co-founder and shareholder of Molecular Partners AG.

ORCID IDENTIFIERS

0000-0001-7269-3633: Rohan S. Eapen
0000-0002-9717-0991: Patrik Forrer
0000-0002-4169-5879: Christian Jost

References

1. Pancer, Z., et al. Somatic diversification of variable lymphocyte receptors in the agnathan sea lamprey. *Nature*, 430(6996):174–80, 2004.
2. Schwartz, R. S. Paul Ehrlich's magic bullets. *N Engl J Med*, 350(11):1079–80, 2004.
3. Mullard, A. FDA approves 100th monoclonal antibody product. *Nat Rev Drug Discov*, 20 (7):491–495, 2021.
4. Lu, R. M., et al. Development of therapeutic antibodies for the treatment of diseases. *J Biomed Sci*, 27(1):1, 2020.
5. Bradbury, A. and Plückthun, A. Reproducibility: Standardize antibodies used in research. *Nature*, 518(7537):27–9, 2015.
6. Pardridge, W. M. Biopharmaceutical drug targeting to the brain. *J Drug Target*, 18(3): 157–67, 2010.
7. Ridgway, J. B., Presta, L. G., and Carter, P. 'Knobs-into-holes' engineering of antibody CH3 domains for heavy chain heterodimerization. *Protein Eng*, 9(7):617–21, 1996.
8. Schaefer, W., et al. Immunoglobulin domain crossover as a generic approach for the production of bispecific IgG antibodies. *Proc Natl Acad Sci U S A*, 108(27):11187–92, 2011.
9. Woof, J. M. and Burton, D. R. Human antibody-Fc receptor interactions illuminated by crystal structures. *Nat Rev Immunol*, 4(2):89–99, 2004.
10. Ward, E. S., et al. From sorting endosomes to exocytosis: association of Rab4 and Rab11 GTPases with the Fc receptor, FcRn, during recycling. *Mol Biol Cell*, 16(4):2028–38, 2005.
11. Diebolder, C. A., et al. Complement is activated by IgG hexamers assembled at the cell surface. *Science*, 343(6176):1260–3, 2014.
12. Ames, R. S., et al. Conversion of murine Fabs isolated from a combinatorial phage display library to full length immunoglobulins. *J Immunol Methods*, 184(2):177–86, 1995.
13. Huls, G. A., et al. A recombinant, fully human monoclonal antibody with antitumor activity constructed from phage-displayed antibody fragments. *Nat Biotechnol*, 17(3):276–81, 1999.
14. Thie, H., et al. Rise and fall of an anti-MUC1 specific antibody. *PLoS One*, 6(1):e15921, 2011.
15. Jostock, T., et al. Rapid generation of functional human IgG antibodies derived from Fab-on-phage display libraries. *J Immunol Methods*, 289(1-2):65–80, 2004.
16. Renaut, L., et al. Affinity maturation of antibodies: optimized methods to generate high-quality ScFv libraries and isolate IgG candidates by high-throughput screening. *Methods Mol Biol*, 907:451–61, 2012.
17. Steinwand, M., et al. The influence of antibody fragment format on phage display based affinity maturation of IgG. *MAbs*, 6(1):204–18, 2014.
18. Leung, K. M., et al. A HER2-specific Modified Fc Fragment (Fcab) Induces Antitumor Effects Through Degradation of HER2 and Apoptosis. *Mol Ther*, 23(11):1722–1733, 2015.

19. Beckmann, R., et al. DutaFabs are engineered therapeutic Fab fragments that can bind two targets simultaneously. *Nat Commun*, 12(1):708, 2021.
20. Koenig, P. A., et al. Structure-guided multivalent nanobodies block SARS-CoV-2 infection and suppress mutational escape. *Science*, 371(6530):eabe6230, 2021.
21. Manstein, V. v., et al. Resistance of Cancer Cells to Targeted Therapies Through the Activation of Compensating Signaling Loops. *Curr Signal Transduct Ther*, 8(3):193–202, 2013.
22. Boumahdi, S. and Sauvage, F. J. d. The great escape: tumour cell plasticity in resistance to targeted therapy. *Nat Rev Drug Discov*, 19(1):39–56, 2020.
23. Harvey, W. T., et al. SARS-CoV-2 variants, spike mutations and immune escape. *Nat Rev Microbiol*, 19(7):409–424, 2021.
24. Alder, M. N., et al. Diversity and function of adaptive immune receptors in a jawless vertebrate. *Science*, 310(5756):1970–3, 2005.
25. Nagawa, F., et al. Antigen-receptor genes of the agnathan lamprey are assembled by a process involving copy choice. *Nat Immunol*, 8(2):206–13, 2007.
26. Rogozin, I. B., et al. Evolution and diversification of lamprey antigen receptors: evidence for involvement of an AID-APOBEC family cytosine deaminase. *Nat Immunol*, 8(6):647–56, 2007.
27. Tasumi, S., et al. High-affinity lamprey VLRA and VLRB monoclonal antibodies. *Proc Natl Acad Sci U S A*, 106(31):12891–6, 2009.
28. Velikovsky, C. A., et al. Structure of a lamprey variable lymphocyte receptor in complex with a protein antigen. *Nat Struct Mol Biol*, 16(7):725–30, 2009.
29. Mariuzza, R. A., Velikovsky, C. A., Deng, L., Xu, G., and Pancer, Z. Structural insights into the evolution of the adaptive immune system: the variable lymphocyte receptors of jawless vertebrates. *Biol Chem*, 391(7):753–60, 2010.
30. Wezner-Ptasinska, M. and Otlewski, J. Selection of specific interactors from phage display library based on sea lamprey variable lymphocyte receptor sequences. *Biochim Biophys Acta*, 1854(12):1833–1841, 2015.
31. Hassan, K. M. A., Hansen, J. D., Herrin, B. R., and Amemiya, C. T. Generation of Lamprey Monoclonal Antibodies (Lampribodies) Using the Phage Display System. *Biomolecules*, 9 (12):868, 2019.
32. Wit, J. d., Hong, W., Luo, L., and Ghosh, A. Role of leucine-rich repeat proteins in the development and function of neural circuits. *Annu Rev Cell Dev Biol*, 27(1):697–729, 2011.
33. Marcotte, E. M., Pellegrini, M., Yeates, T. O., and Eisenberg, D. A census of protein repeats. *J Mol Biol*, 293(1):151–60, 1999.
34. Schaper, E., Kajava, A. V., Hauser, A., and Anisimova, M. Repeat or not repeat? – Statistical validation of tandem repeat prediction in genomic sequences. *Nucleic Acids Res*, 40(20):10005–17, 2012.
35. Björklund, A. K., Ekman, D., and Elofsson, A. Expansion of protein domain repeats. *PLoS Comput Biol*, 2(8):e114, 2006.
36. Grove, T. Z., Cortajarena, A. L., and Regan, L. Ligand binding by repeat proteins: natural and designed. *Curr Opin Struct Biol*, 18(4):507–15, 2008.
37. Brunette, T. J., et al. Exploring the repeat protein universe through computational protein design. *Nature*, 528(7583):580–4, 2015.
38. Kobe, B. and Kajava, A. V. The leucine-rich repeat as a protein recognition motif. *Curr Opin Struct Biol*, 11(6):725–32, 2001.
39. Bella, J., Hindle, K. L., McEwan, P. A., and Lovell, S. C. The leucine-rich repeat structure. *Cell Mol Life Sci*, 65(15):2307–33, 2008.
40. Johnson, R. J., McCoy, J. G., Bingman, C. A., Phillips, G. N., and Raines, R. T. Inhibition of human pancreatic ribonuclease by the human ribonuclease inhibitor protein. *J Mol Biol*, 368(2):434–49, 2007.
41. Lee, F. S. and Vallee, B. L. Binding of placental ribonuclease inhibitor to the active site of angiogenin. *Biochemistry*, 28(8):3556–61, 1989.
42. Liker, E., Fernandez, E., Izaurralde, E., and Conti, E. The structure of the mRNA export factor TAP reveals a cis arrangement of a non-canonical RNP domain and an LRR domain. *EMBO J*, 19(21):5587–98, 2000.
43. DeYoung, B. J. and Innes, R. W. Plant NBS-LRR proteins in pathogen sensing and host defense. *Nat Immunol*, 7(12):1243–9, 2006.
44. Hohenester, E., Hussain, S., and Howitt, J. A. Interaction of the guidance molecule Slit with cellular receptors. *Biochem Soc Trans*, 34(Pt 3):418–21, 2006.
45. West, A. P., Koblansky, A. A., and Ghosh, S. Recognition and signaling by toll-like receptors. *Annu Rev Cell Dev Biol*, 22(1):409–37, 2006.
46. Ko, J. and Kim, E. Leucine-rich repeat proteins of synapses. *J Neurosci Res*, 85(13): 2824–32, 2007.
47. Perez-Riba, A. and Itzhaki, L. S. The tetratricopeptide-repeat motif is a versatile platform that enables diverse modes of molecular recognition. *Curr Opin Struct Biol*, 54:43–49, 2019.
48. Main, E. R., Jackson, S. E., and Regan, L. The folding and design of repeat proteins: reaching a consensus. *Curr Opin Struct Biol*, 13(4):482–9, 2003.
49. Lapouge, K., et al. Structure of the TPR domain of p67phox in complex with Rac.GTP. *Mol Cell*, 6(4):899–907, 2000.
50. Yang, J., et al. Molecular basis for TPR domain-mediated regulation of protein phosphatase 5. *EMBO J*, 24(1):1–10, 2005.
51. Allan, R. K. and Ratajczak, T. Versatile TPR domains accommodate different modes of target protein recognition and function. *Cell Stress Chaperones*, 16(4):353–67, 2011.
52. Cortajarena, A. L., Wang, J., and Regan, L. Crystal structure of a designed tetratricopeptide repeat module in complex with its peptide ligand. *FEBS J*, 277(4):1058–66, 2010.
53. Mapelli, M. and Gonzalez, C. On the inscrutable role of Inscuteable: structural basis and functional implications for the competitive binding of NuMA and Inscuteable to LGN. *Open Biol*, 2(8):120102, 2012.
54. Baskin, J. M., et al. The leukodystrophy protein FAM126A (hyccin) regulates PtdIns(4)P synthesis at the plasma membrane. *Nat Cell Biol*, 18(1):132–8, 2016.
55. Tewari, R., Bailes, E., Bunting, K. A., and Coates, J. C. Armadillo-repeat protein functions: questions for little creatures. *Trends Cell Biol*, 20(8):470–81, 2010.
56. Kidd, A. R., Miskowski, J. A., Siegfried, K. R., Sawa, H., and Kimble, J. A beta-catenin identified by functional rather than sequence criteria and its role in Wnt/MAPK signaling. *Cell*, 121(5):761–72, 2005.
57. Parmeggiani, F., et al. Designed armadillo repeat proteins as general peptide-binding scaffolds: consensus design and computational optimization of the hydrophobic core. *J Mol Biol*, 376(5):1282–304, 2008.
58. Ernst, P., et al. Structure-Guided Design of a Peptide Lock for Modular Peptide Binders. *ACS Chem Biol*, 15(2):457–468, 2020.
59. Andrade, M. A., Petosa, C., O'Donoghue, S. I., Müller, C. W., and Bork, P. Comparison of ARM and HEAT protein repeats. *J Mol Biol*, 309(1):1–18, 2001.
60. Andrade, M. A., Perez-Iratxeta, C., and Ponting, C. P. Protein repeats: structures, functions, and evolution. *J Struct Biol*, 134(2-3):117–31, 2001.
61. Malik, H. S., Eickbush, T. H., and Goldfarb, D. S. Evolutionary specialization of the nuclear targeting apparatus. *Proc Natl Acad Sci U S A*, 94(25):13738–42, 1997.
62. Cingolani, G., Petosa, C., Weis, K., and Müller, C. W. Structure of importin-beta bound to the IBB domain of importin-alpha. *Nature*, 399(6733):221–9, 1999.
63. Bennett, V. The molecular basis for membrane - cytoskeleton association in human erythrocytes. *J Cell Biochem*, 18(1):49–65, 1982.
64. Li, J., Mahajan, A., and Tsai, M. D. Ankyrin repeat: a unique motif mediating protein-protein interactions. *Biochemistry*, 45(51):15168–78, 2006.
65. Bennett, V. Ankyrins. Adaptors between diverse plasma membrane proteins and the cytoplasm. *J Biol Chem*, 267(13):8703–6, 1992.
66. Michaely, P., Tomchick, D. R., Machius, M., and Anderson, R. G. Crystal structure of a 12 ANK repeat stack from human ankyrinR. *EMBO J*, 21(23):6387–96, 2002.
67. Lux, S. E., John, K. M., and Bennett, V. Analysis of cDNA for human erythrocyte ankyrin indicates a repeated structure with homology to tissue-differentiation and cell-cycle control proteins. *Nature*, 344(6261):36–42, 1990.
68. Kobe, B. and Kajava, A. V. When protein folding is simplified to protein coiling: the continuum of solenoid protein structures. *Trends Biochem Sci*, 25(10):509–15, 2000.
69. Sonnberg, S., Seet, B. T., Pawson, T., Fleming, S. B., and Mercer, A. A. Poxvirus ankyrin repeat proteins are a unique class of F-box proteins that associate with cellular SCF1 ubiquitin ligase complexes. *Proc Natl Acad Sci U S A*, 105(31):10955–60, 2008.
70. Huxford, T. and Ghosh, G. A structural guide to proteins of the NF-kappaB signaling module. *Cold Spring Harb Perspect Biol*, 1(3):a000075, 2009.
71. Cánepa, E. T., et al. INK4 proteins, a family of mammalian CDK inhibitors with novel biological functions. *IUBMB Life*, 59(7):419–26, 2007.
72. Plaxco, K. W., Simons, K. T., and Baker, D. Contact order, transition state placement and the refolding rates of single domain proteins. *J Mol Biol*, 277(4):985–94, 1998.
73. Binz, H. K., Stumpp, M. T., Forrer, P., Amstutz, P., and Plückthun, A. Designing repeat proteins: well-expressed, soluble and stable proteins from combinatorial libraries of consensus ankyrin repeat proteins. *J Mol Biol*, 332(2):489–503, 2003.
74. Forrer, P., Stumpp, M. T., Binz, H. K., and Plückthun, A. A novel strategy to design binding molecules harnessing the modular nature of repeat proteins. *FEBS Lett*, 539(1-3):2–6, 2003.
75. Stumpp, M. T., Forrer, P., Binz, H. K., and Plückthun, A. Designing repeat proteins: modular leucine-rich repeat protein libraries based on the mammalian ribonuclease inhibitor family. *J Mol Biol*, 332(2):471–87, 2003.
76. Kohl, A., et al. Designed to be stable: crystal structure of a consensus ankyrin repeat protein. *Proc Natl Acad Sci U S A*, 100(4):1700–5, 2003.
77. Forrer, P., Binz, H. K., Stumpp, M. T., and Plückthun, A. Consensus design of repeat proteins. *Chembiochem*, 5(2):183–9, 2004.
78. Binz, H. K., et al. High-affinity binders selected from designed ankyrin repeat protein libraries. *Nat Biotechnol*, 22(5):575–82, 2004.
79. Schilling, J., Schöppe, J., and Plückthun, A. From DARPins to LoopDARPins: novel LoopDARPin design allows the selection of low picomolar binders in a single round of ribosome display. *J Mol Biol*, 426(3):691–721, 2014.
80. Schilling, J., et al. Thermostable designed ankyrin repeat proteins (DARPins) as building blocks for innovative drugs. *J Biol Chem*, 298(1):101403, 2022.
81. Zahnd, C., et al. A designed ankyrin repeat protein evolved to picomolar affinity to Her2. *J Mol Biol*, 369(4):1015–28, 2007.
82. Interlandi, G., Wetzel, S. K., Settanni, G., Plückthun, A., and Caflisch, A. Characterization and further stabilization of designed ankyrin repeat proteins by combining molecular dynamics simulations and experiments. *J Mol Biol*, 375(3):837–54, 2008.
83. Kramer, M. A., Wetzel, S. K., Plückthun, A., Mittl, P. R., and Grütter, M. G. Structural determinants for improved stability of designed ankyrin repeat proteins with a redesigned C-capping module. *J Mol Biol*, 404(3):381–91, 2010.
84. ensovibepum: WHO Drug Information. volume 34, pages 968–970, 2020.
85. Wetzel, S. K., Settanni, G., Kenig, M., Binz, H. K., and Plückthun, A. Folding and unfolding mechanism of highly stable full-consensus ankyrin repeat proteins. *J Mol Biol*, 376(1): 241–57, 2008.
86. Steiner, D., Forrer, P., Stumpp, M. T., and Plückthun, A. Signal sequences directing cotranslational translocation expand the range of proteins amenable to phage display. *Nat Biotechnol*, 24(7):823–31, 2006.
87. Dreier, B. and Plückthun, A. Ribosome display: a technology for selecting and evolving proteins from large libraries. *Methods Mol Biol*, 687:283–306, 2011.
88. Schütz, M., et al. Generation of Fluorogen-Activating Designed Ankyrin Repeat Proteins (FADAs) as Versatile Sensor Tools. *J Mol Biol*, 428(6):1272–1289, 2016.
89. Zahnd, C., Amstutz, P., and Plückthun, A. Ribosome display: selecting and evolving proteins in vitro that specifically bind to a target. *Nat Methods*, 4(3):269–79, 2007.
90. Hanes, J., Schaffitzel, C., Knappik, A., and Plückthun, A. Picomolar affinity antibodies from a fully synthetic naive library selected and evolved by ribosome display. *Nat Biotechnol*, 18(12):1287–92, 2000.
91. Dreier, B., et al. Her2-specific multivalent adapters confer designed tropism to adenovirus for gene targeting. *J Mol Biol*, 405(2):410–26, 2011.
92. Amstutz, P., et al. Intracellular kinase inhibitors selected from combinatorial libraries of designed ankyrin repeat proteins. *J Biol Chem*, 280(26):24715–22, 2005.
93. Amstutz, P., Koch, H., Binz, H. K., Deuber, S. A., and Plückthun, A. Rapid selection of specific MAP kinase-binders from designed ankyrin repeat protein libraries. *Protein Eng*

Des Sel, 19(5):219–29, 2006.
94. Schweizer, A., et al. Inhibition of caspase-2 by a designed ankyrin repeat protein: specificity, structure, and inhibition mechanism. *Structure*, 15(5):625–36, 2007.
95. Parizek, P., et al. Designed ankyrin repeat proteins (DARPins) as novel isoform-specific intracellular inhibitors of c-Jun N-terminal kinases. *ACS Chem Biol*, 7(8):1356–66, 2012.
96. Kummer, L., et al. Structural and functional analysis of phosphorylation-specific binders of the kinase ERK from designed ankyrin repeat protein libraries. *Proc Natl Acad Sci U S A*, 109(34):E2248–57, 2012.
97. Seeger, M. A., et al. Design, construction, and characterization of a second-generation DARPin library with reduced hydrophobicity. *Protein Sci*, 22(9):1239–57, 2013.
98. Batyuk, A., Wu, Y., Honegger, A., Heberling, M. M., and Plückthun, A. DARPin-Based Crystallization Chaperones Exploit Molecular Geometry as a Screening Dimension in Protein Crystallography. *J Mol Biol*, 428(8):1574–88, 2016.
99. Plückthun, A. Designed ankyrin repeat proteins (DARPins): binding proteins for research, diagnostics, and therapy. *Annu Rev Pharmacol Toxicol*, 55(1):489–511, 2015.
100. Boersma, Y. L. Advances in the Application of Designed Ankyrin Repeat Proteins (DARPins) as Research Tools and Protein Therapeutics. *Methods Mol Biol*, 1798:307–327, 2018.
101. Harmansa, S. and Affolter, M. Protein binders and their applications in developmental biology. *Development*, 145(2):dev148874, 2018.
102. Mittl, P. R., Ernst, P., and Plückthun, A. Chaperone-assisted structure elucidation with DARPins. *Curr Opin Struct Biol*, 60:93–100, 2020.
103. Fiedler, U., et al. MP0250, a VEGF and HGF neutralizing DARPin. *Oncotarget*, 8(58): 98371–98383, 2017.
104. Baird, R. D., et al. First-in-Human Phase I Study of MP0250, a First-in-Class DARPin Drug Candidate Targeting VEGF and HGF, in Patients With Advanced Solid Tumors. *J Clin Oncol*, 39(2):145–154, 2021.
105. Atiqi, S., Hooijberg, F., Loeff, F. C., Rispens, T., and Wolbink, G. J. Immunogenicity of TNF-Inhibitors. *Front Immunol*, 11:312, 2020.
106. Kummer, L., et al. Knowledge-based design of a biosensor to quantify localized ERK activation in living cells. *Chem Biol*, 20(6):847–56, 2013.
107. Kohl, A., et al. Allosteric inhibition of aminoglycoside phosphotransferase by a designed ankyrin repeat protein. *Structure*, 13(8):1131–41, 2005.
108. Stephens, E. A., et al. Engineering Single Pan-Specific Ubiquibodies for Targeted Degradation of All Forms of Endogenous ERK Protein Kinase. *ACS Synthetic Biology*, 10(9): 2396–2408, 2021.
109. Frejd, F. Y. and Kim, K. T. Affibody molecules as engineered protein drugs. *Exp Mol Med*, 49(3):e306, 2017.
110. Deuschle, F. C., Ilyukhina, E., and Skerra, A. Anticalin® proteins: from bench to bedside. *Expert Opin Biol Ther*, 21(4):509–518, 2021.
111. Rothenberger, S., et al. Ensovibep, a novel trispecific DARPin candidate that protects against SARS-CoV-2 variants. *bioRxiv*, page 2021.02.03.429164, Feb 2021.
112. Walser, M., et al. Highly potent anti-SARS-CoV-2 multivalent DARPin therapeutic candidates. *bioRxiv*, page 2020.08.25.256339, 2020.
113. Kunimoto, D., et al. Efficacy and Safety of Abicipar in Neovascular Age-Related Macular Degeneration: 52-Week Results of Phase 3 Randomized Controlled Study. *Ophthalmology*, 127(10):1331–1344, 2020.
114. Khurana, R. N., et al. Two-Year Results of the Phase 3 Randomized Controlled Study of Abicipar in Neovascular Age-Related Macular Degeneration. *Ophthalmology*, 128(7): 1027–1038, 2020.
115. Hussain, R. M., Weng, C. Y., Wykoff, C. C., Gandhi, R. A., and Hariprasad, S. M. Abicipar pegol for neovascular age-related macular degeneration. *Expert Opin Biol Ther*, 20(9): 999–1008, 2020.
116. Moisseiev, E. and Loewenstein, A. Abicipar pegol-a novel anti-VEGF therapy with a long duration of action. *Eye (Lond)*, 34(4):605–606, 2020.
117. Link, A., et al. Preclinical pharmacology of MP0310: a 4-1BB/FAP bispecific DARPin drug candidate promoting tumor-restricted T cell co-stimulation. 2018. doi: 10.1158/1538-7445.am2018-3752.
118. Tosevski, I., et al. Preclinical identification of the pharmacologically active dose range of the tumor targeted 4-1BB agonist MP0310. 2018.
119. Rigamonti, N., et al. Abstract 3251: Fibroblast activation protein (FAP)-selective delivery of CD40 agonistic DARPin® molecule for tumor-localized immune activation. Immunology, pages 3251–3251, 2019. doi: 10.1158/1538-7445.am2019-3251.
120. Ioannou, K., et al. MP0317, a FAPxCD40 targeting multi-specific DARPin® therapeutic, drives immune activation and leads to macrophage repolarization in vitro and ex vivo. 2021.
121. Sennino, B., et al. Suppression of tumor invasion and metastasis by concurrent inhibition of c-Met and VEGF signaling in pancreatic neuroendocrine tumors. *Cancer Discov*, 2(3): 270–87, 2012.
122. Fiedler, U., et al. Pre-clinical antitumor activity, tumor localization, and pharmacokinetics of MP0274, an apoptosis inducing, biparatopic HER2-targeting DARPin. 2021. doi: 10.1158/1538-7445.sabcs16-p4-21-18.
123. Baird, R., et al. MP0274-CP101: a phase 1, first-in-human, single-arm, multi-center, open-label, dose escalation study to assess safety, tolerability, and pharmacokinetics of MP0274 in patients with advanced HER2-positive solid tumors. 2021. doi: 10.1158/1538-7445.sabcs17-ot1-03-02.
124. Eggel, A., Baumann, M. J., Amstutz, P., Stadler, B. M., and Vogel, M. DARPins as bispecific receptor antagonists analyzed for immunoglobulin E receptor blockage. *J Mol Biol*, 393(3): 598–607, 2009.
125. Eggel, A., et al. Accelerated dissociation of IgE-FcεRI complexes by disruptive inhibitors actively desensitizes allergic effector cells. *J Allergy Clin Immunol*, 133(6):1709–19.e8, 2014.
126. Bragina, O., et al. Phase I Trial of. *J Nucl Med*, 63(4):528–535, 2022.
127. Chapman, A. P. PEGylated antibodies and antibody fragments for improved therapy: a review. *Adv Drug Deliv Rev*, 54(4):531–45, 2002.
128. Simon, M., Stefan, N., Borsig, L., Plückthun, A., and Zangemeister-Wittke, U. Increasing the antitumor effect of an EpCAM-targeting fusion toxin by facile click PEGylation. *Mol Cancer Ther*, 13(2):375–85, 2014.
129. Kubetzko, S., Sarkar, C. A., and Plückthun, A. Protein PEGylation decreases observed target association rates via a dual blocking mechanism. *Mol Pharmacol*, 68(5):1439–54, 2005.
130. Steiner, D., et al. Half-life extension using serum albumin-binding DARPin® domains. *Protein Eng Des Sel*, 30(9):583–591, 2017.
131. Ryman, J. T. and Meibohm, B. Pharmacokinetics of Monoclonal Antibodies. *CPT Pharmacometrics Syst Pharmacol*, 6(9):576–588, 2017.
132. Schlapschy, M., et al. PASylation: a biological alternative to PEGylation for extending the plasma half-life of pharmaceutically active proteins. *Protein Eng Des Sel*, 26(8):489–501, 2013.
133. Brandl, F., et al. Influence of size and charge of unstructured polypeptides on pharmacokinetics and biodistribution of targeted fusion proteins. *J Control Release*, 307:379–392, 2019.
134. Zahnd, C., et al. Efficient tumor targeting with high-affinity designed ankyrin repeat proteins: effects of affinity and molecular size. *Cancer Res*, 70(4):1595–605, 2010.
135. Stahl, A., et al. Highly potent VEGF-A-antagonistic DARPins as anti-angiogenic agents for topical and intravitreal applications. *Angiogenesis*, 16(1):101–11, 2013.
136. Caputi, A. P. and Navarra, P. Beyond antibodies: ankyrins and DARPins. From basic research to drug approval. *Curr Opin Pharmacol*, 51:93–101, 2020.
137. Carrasco, J., et al. Real-World Effectiveness and Real-World Cost-Effectiveness of Intravitreal Aflibercept and Intravitreal Ranibizumab in Neovascular Age-Related Macular Degeneration: Systematic Review and Meta-Analysis of Real-World Studies. *Adv Ther*, 37(1):300–315, 2020.
138. Rodrigues, G. A., et al. Functional Characterization of Abicipar-Pegol, an Anti-VEGF DARPin Therapeutic That Potently Inhibits Angiogenesis and Vascular Permeability. *Invest Ophthalmol Vis Sci*, 59(15):5836–5846, 2018.
139. Callanan, D., et al. Double-Masked, Randomized, Phase 2 Evaluation of Abicipar Pegol (an Anti-VEGF DARPin Therapeutic) in Neovascular Age-Related Macular Degeneration. *J Ocul Pharmacol Ther*, 34(10):700–709, 2018.
140. Campochiaro, P. A., et al. Treatment of diabetic macular edema with a designed ankyrin repeat protein that binds vascular endothelial growth factor: a phase I/II study. *Am J Ophthalmol*, 155(4):697–704, 704.e1–2, 2013.
141. Souied, E. H., et al. Treatment of exudative age-related macular degeneration with a designed ankyrin repeat protein that binds vascular endothelial growth factor: a phase I/II study. *Am J Ophthalmol*, 158(4):724–732.e2, 2014.
142. Pecen, P. E. and Kaiser, P. K. Current phase 1/2 research for neovascular age-related macular degeneration. *Curr Opin Ophthalmol*, 26(3):188–93, 2015.
143. Amadio, M., Govoni, S., and Pascale, A. Targeting VEGF in eye neovascularization: What's new?: A comprehensive review on current therapies and oligonucleotide-based interventions under development. *Pharmacol Res*, 103:253–69, 2016.
144. Sharma, A., Kumar, N., Kuppermann, B. D., and Bandello, F. Abicipar pegol: the non-monoclonal antibody anti-VEGF. *Eye (Lond)*, 34(5):797–801, 2020.
145. Simon, M., Zangemeister-Wittke, U., and Plückthun, A. Facile double-functionalization of designed ankyrin repeat proteins using click and thiol chemistries. *Bioconjug Chem*, 23 (2):279–86, 2012.
146. Baumann, M. J., Eggel, A., Amstutz, P., Stadler, B. M., and Vogel, M. DARPins against a functional IgE epitope. *Immunol Lett*, 133(2):78–84, 2010.
147. Kim, B., et al. Accelerated disassembly of IgE-receptor complexes by a disruptive macromolecular inhibitor. *Nature*, 491(7425):613–7, 2012.
148. Pennington, L. F., et al. Structure-guided design of ultrapotent disruptive IgE inhibitors to rapidly terminate acute allergic reactions. *J Allergy Clin Immunol*, 148(4):1049–1060, 2021.
149. Boersma, Y. L., Chao, G., Steiner, D., Wittrup, K. D., and Plückthun, A. Bispecific designed ankyrin repeat proteins (DARPins) targeting epidermal growth factor receptor inhibit A431 cell proliferation and receptor recycling. *J Biol Chem*, 286(48):41273–41285, 2011.
150. Kümler, I., Tuxen, M. K., and Nielsen, D. L. A systematic review of dual targeting in HER2-positive breast cancer. *Cancer Treat Rev*, 40(2):259–70, 2014.
151. Zahnd, C., Pecorari, F., Straumann, N., Wyler, E., and Plückthun, A. Selection and characterization of Her2 binding-designed ankyrin repeat proteins. *J Biol Chem*, 281(46):35167–75, 2006.
152. Jost, C., et al. Structural basis for eliciting a cytotoxic effect in HER2-overexpressing cancer cells via binding to the extracellular domain of HER2. *Structure*, 21(11):1979–91, 2013.
153. Tamaskovic, R., et al. Intermolecular biparatopic trapping of ErbB2 prevents compensatory activation of PI3K/AKT via RAS-p110 crosstalk. *Nat Commun*, 7(1):11672, 2016.
154. Stüber, J. C., et al. Apoptosis-inducing anti-HER2 agents operate through oligomerization-induced receptor immobilization. *Commun Biol*, 4(1):762, 2021.
155. Reschke, N., et al. Abstract 525: Novel DARPin multi-specific T-cell engager with an improved therapeutic window to overcome dose limiting toxicities in AML therapies. volume 81 of *Cancer Research*, pages 525–525, 2021. doi: 10.1158/1538-7445.am2021-525.
156. Binz, H. K., et al. Design and characterization of MP0250, a tri-specific anti-HGF/anti-VEGF DARPin® drug candidate. *MAbs*, 9(8):1262–1269, 2017.
157. Rao, L., et al. Targeting angiogenesis in multiple myeloma by the VEGF and HGF blocking DARPin. *Oncotarget*, 9(17):13366–13381, 2018.
158. Heiss, M. M., et al. The trifunctional antibody catumaxomab for the treatment of malignant ascites due to epithelial cancer: Results of a prospective randomized phase II/III trial. *Int J Cancer*, 127(9):2209–21, 2010.
159. Nagorsen, D., Kufer, P., Baeuerle, P. A., and Bargou, R. Blinatumomab: a historical perspective. *Pharmacol Ther*, 136(3):334–42, 2012.
160. Szebeni, J. Complement activation-related pseudoallergy: A stress reaction in blood triggered by nanomedicines and biologicals. *Molecular Immunology*, 61(2):163–173, 2014.
161. Rautio, J., Meanwell, N. A., Di, L., and Hageman, M. J. The expanding role of prodrugs in contemporary drug design and development. *Nat Rev Drug Discov*, 17(8):559–587, 2018.
162. Bosshart, A., et al. A solution to T-cell engager toxicity: An anti-CD3 Prodrug DARPin® (CD3-PDD) shows no toxicity, but potent anti-tumor activity in a humanized mouse model.

2021. doi: 10.1158/1538-7445.am2021-1890.
163. Geiger, M., et al. Protease-activation using anti-idiotypic masks enables tumor specificity of a folate receptor 1-T cell bispecific antibody. *Nat Commun*, 11(1):3196, 2020.
164. Andreano, E., et al. SARS-CoV-2 escape from a highly neutralizing COVID-19 convalescent plasma. *Proceedings of the National Academy of Sciences*, 118(36):e2103154118, 2021.
165. Kemp, S. A., et al. SARS-CoV-2 evolution during treatment of chronic infection. *Nature*, 592(7853):277–282, 2021.
166. Planas, D., et al. Reduced sensitivity of SARS-CoV-2 variant Delta to antibody neutralization. *Nature*, 596(7871):276–280, 2021.
167. Baral, P., et al. Mutation-induced Changes in the Receptor-binding Interface of the SARS-CoV-2 Delta Variant B.1.617.2 and Implications for Immune Evasion. *bioRxiv*, page 2021.07.17.452576, 2021.
168. McCallum, M., et al. SARS-CoV-2 immune evasion by the B.1.427/B.1.429 variant of concern. *Science*, 373(6555):648–654, 2021.
169. Heemskerk, B., Kvistborg, P., and Schumacher, T. N. The cancer antigenome. *EMBO J*, 32(2):194–203, 2013.
170. Comber, J. D. and Philip, R. MHC class I antigen presentation and implications for developing a new generation of therapeutic vaccines. *Ther Adv Vaccines*, 2(3):77–89, 2014.
171. Dhanik, A., et al. In-silico discovery of cancer-specific peptide-HLA complexes for targeted therapy. *BMC Bioinformatics*, 17(1):286, 2016.
172. Oren, R., et al. Functional comparison of engineered T cells carrying a native TCR versus TCR-like antibody-based chimeric antigen receptors indicates affinity/avidity thresholds. *J Immunol*, 193(11):5733–43, 2014.
173. Venetz, N., et al. Application of the DARPin® technology for specific targeting of tumor-associated MHC class I: peptide complexes. doi: 10.1158/1538-7445.am2021-1349.
174. Alley, S. C., Okeley, N. M., and Senter, P. D. Antibody-drug conjugates: targeted drug delivery for cancer. *Curr Opin Chem Biol*, 14(4):529–37, 2010.
175. Hamilton, G. S. Antibody-drug conjugates for cancer therapy: The technological and regulatory challenges of developing drug-biologic hybrids. *Biologicals*, 43(5):318–32, 2015.
176. Martin-Killias, P., et al. A novel fusion toxin derived from an EpCAM-specific designed ankyrin repeat protein has potent antitumor activity. *Clin Cancer Res*, 17(1):100–10, 2011.
177. Simon, M., Frey, R., Zangemeister-Wittke, U., and Plückthun, A. Orthogonal assembly of a designed ankyrin repeat protein-cytotoxin conjugate with a clickable serum albumin module for half-life extension. *Bioconjug Chem*, 24(11):1955–66, 2013.
178. Goldstein, R., et al. Development of the designed ankyrin repeat protein (DARPin) G3 for HER2 molecular imaging. *Eur J Nucl Med Mol Imaging*, 42(2):288–301, 2015.
179. Winkler, J., Martin-Killias, P., Plückthun, A., and Zangemeister-Wittke, U. EpCAM-targeted delivery of nanocomplexed siRNA to tumor cells with designed ankyrin repeat proteins. *Mol Cancer Ther*, 8(9):2674–83, 2009.
180. Lorenzer, C., et al. Targeted delivery and endosomal cellular uptake of DARPin-siRNA bioconjugates: Influence of linker stability on gene silencing. *Eur J Pharm Biopharm*, 141: 37–50, 2019.
181. Kaufmann, K. B., Büning, H., Galy, A., Schambach, A., and Grez, M. Gene therapy on the move. *EMBO Mol Med*, 5(11):1642–61, 2013.
182. Mingozzi, F. and High, K. A. Therapeutic in vivo gene transfer for genetic disease using AAV: progress and challenges. *Nat Rev Genet*, 12(5):341–55, 2011.
183. Liu, Y., Siriwon, N., Rohrs, J. A., and Wang, P. Generation of Targeted Adeno-Associated Virus (AAV) Vectors for Human Gene Therapy. *Curr Pharm Des*, 21(22):3248–56, 2015.
184. Muik, A., et al. Covalent coupling of high-affinity ligands to the surface of viral vector particles by protein trans-splicing mediates cell type-specific gene transfer. *Biomaterials*, 144:84–94, 2017.
185. Hartmann, J., et al. GluA4-Targeted AAV Vectors Deliver Genes Selectively to Interneurons while Relying on the AAV Receptor for Entry. *Mol Ther Methods Clin Dev*, 14:252–260, 2019.
186. Stone, D., et al. Gene Transfer in Adeno-Associated Virus Seropositive Rhesus Macaques Following Rapamycin Treatment and Subcutaneous Delivery of AAV6, but Not Retargeted AAV6 Vectors. *Hum Gene Ther*, 32(1-2):96–112, 2021.
187. Münch, R. C., et al. Displaying high-affinity ligands on adeno-associated viral vectors enables tumor cell-specific and safe gene transfer. *Mol Ther*, 21(1):109–18, 2013.
188. Münch, R. C., et al. Off-target-free gene delivery by affinity-purified receptor-targeted viral vectors. *Nat Commun*, 6(1):6246, 2015.
189. Münch, R. C., et al. DARPins: an efficient targeting domain for lentiviral vectors. *Mol Ther*, 19(4):686–93, 2011.
190. Agarwal, S., et al. In Vivo Generation of CAR T Cells Selectively in Human CD4. *Mol Ther*, 28(8):1783–1794, 2020.
191. Frank, A. M., et al. CD8-Specific Designed Ankyrin Repeat Proteins Improve Selective Gene Delivery into Human and Primate T Lymphocytes. *Hum Gene Ther*, 31(11-12):679–691, 2020.
192. Limoni, S. K., Moghadam, M. F., Moazzeni, S. M., Gomari, H., and Salimi, F. Engineered Exosomes for Targeted Transfer of siRNA to HER2 Positive Breast Cancer Cells. *Appl Biochem Biotechnol*, 187(1):352–364, 2019.
193. June, C. H. and Sadelain, M. Chimeric Antigen Receptor Therapy. *N Engl J Med*, 379(1): 64–73, 2018.
194. CAR T-cells: an exciting frontier in cancer therapy. *Lancet*, 390(10099):1006, 2017.
195. Neelapu, S. S., et al. Chimeric antigen receptor T-cell therapy - assessment and management of toxicities. *Nat Rev Clin Oncol*, 15(1):47–62, 2018.
196. Long, A. H., et al. 4-1BB costimulation ameliorates T cell exhaustion induced by tonic signaling of chimeric antigen receptors. *Nat Med*, 21(6):581–90, 2015.
197. Ajina, A. and Maher, J. Strategies to Address Chimeric Antigen Receptor Tonic Signaling. *Mol Cancer Ther*, 17(9):1795–1815, 2018.
198. Salzer, B., et al. Engineering AvidCARs for combinatorial antigen recognition and reversible control of CAR function. *Nat Commun*, 11(1):4166, 2020.
199. Hammill, J. A., et al. Designed ankyrin repeat proteins are effective targeting elements for chimeric antigen receptors. *J Immunother Cancer*, 3(1):55, 2015.
200. Siegler, E., Li, S., Kim, Y. J., and Wang, P. Designed Ankyrin Repeat Proteins as Her2 Targeting Domains in Chimeric Antigen Receptor-Engineered T Cells. *Hum Gene Ther*, 28(9):726–736, 2017.
201. Grada, Z., et al. TanCAR: A Novel Bispecific Chimeric Antigen Receptor for Cancer Immunotherapy. *Mol Ther Nucleic Acids*, 2(7):e105, 2013.
202. Qin, H., et al. Preclinical Development of Bivalent Chimeric Antigen Receptors Targeting Both CD19 and CD22. *Mol Ther Oncolytics*, 11:127–137, 2018.
203. Balakrishnan, A., et al. Multispecific Targeting with Synthetic Ankyrin Repeat Motif Chimeric Antigen Receptors. *Clin Cancer Res*, 25(24):7506–7516, 2019.
204. Simmons, M. A. Functional selectivity, ligand-directed trafficking, conformation-specific agonism: what's in a name? *Mol Interv*, 5(3):154–7, 2005.
205. Smith, J. S., Lefkowitz, R. J., and Rajagopal, S. Biased signalling: from simple switches to allosteric microprocessors. *Nat Rev Drug Discov*, 17(4):243–260, 2018.
206. Luttrell, L. M. Minireview: More than just a hammer: ligand "bias" and pharmaceutical discovery. *Mol Endocrinol*, 28(3):281–94, 2014.
207. Wisler, J. W., Xiao, K., Thomsen, A. R., and Lefkowitz, R. J. Recent developments in biased agonism. *Curr Opin Cell Biol*, 27:18–24, 2014.
208. Mohan, K., et al. Topological control of cytokine receptor signaling induces differential effects in hematopoiesis. *Science*, 364(6442):eaav7532, 2019.
209. Fallas, J. A., et al. Computational design of self-assembling cyclic protein homo-oligomers. *Nat Chem*, 9(4):353–360, 2017.
210. Lajoie, M. J., et al. Designed protein logic to target cells with precise combinations of surface antigens. *Science*, 369(6511):1637–1643, 2020.
211. Sakamoto, K. M., et al. Protacs: chimeric molecules that target proteins to the Skp1-Cullin-F box complex for ubiquitination and degradation. *Proc Natl Acad Sci U S A*, 98(15): 8554–9, 2001.
212. Khan, S., et al. PROteolysis TArgeting Chimeras (PROTACs) as emerging anticancer therapeutics. *Oncogene*, 39(26):4909–4924, 2020.
213. Bery, N., Miller, A., and Rabbitts, T. A potent KRAS macromolecule degrader specifically targeting tumours with mutant KRAS. *Nat Commun*, 11(1):3233, 2020.
214. Hobbs, G. A., Der, C. J., and Rossman, K. L. RAS isoforms and mutations in cancer at a glance. *J Cell Sci*, 129(7):1287–92, 2016.
215. Cox, A. D., Fesik, S. W., Kimmelman, A. C., Luo, J., and Der, C. J. Drugging the undruggable RAS: Mission possible? *Nat Rev Drug Discov*, 13(11):828–51, 2014.
216. Röth, S., et al. Targeting Endogenous K-RAS for Degradation through the Affinity-Directed Protein Missile System. *Cell Chem Biol*, 27(9):1151–1163.e6, 2020.
217. Zeng, M., et al. Exploring Targeted Degradation Strategy for Oncogenic KRAS. *Cell Chem Biol*, 27(1):19–31.e6, 2020.
218. Li, Z., et al. Allele-selective lowering of mutant HTT protein by HTT-LC3 linker compounds. *Nature*, 575(7781):203–209, 2019.
219. Takahashi, D., et al. AUTACs: Cargo-Specific Degraders Using Selective Autophagy. *Mol Cell*, 76(5):797–810.e10, 2019.
220. Fritsch, L. E., Moore, M. E., Sarraf, S. A., and Pickrell, A. M. Ubiquitin and Receptor-Dependent Mitophagy Pathways and Their Implication in Neurodegeneration. *J Mol Biol*, 432(8):2510–2524, 2020.