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Protein binders and their applications in developmental biology

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ABSTRACT

Developmental biology research would benefit greatly from tools that enable protein function to be regulated, both systematically and in a precise spatial and temporal manner, *in vivo*. In recent years, functionalized protein binders have emerged as versatile tools that can be used to target and manipulate proteins. Such protein binders can be based on various scaffolds, such as nanobodies, designed ankyrin repeat proteins (DARPins) and monobodies, and can be used to block or perturb protein function in living cells. In this Primer, we provide an overview of the protein binders that are currently available and highlight recent progress made in applying protein binder-based tools in developmental and synthetic biology.

KEY WORDS: Protein binder, Nanobody, DARPin, Monobody, Protein degradation, GFP

Introduction

Forward and reverse genetic approaches have been key to expanding our knowledge of gene function during development (see Housden et al., 2017 and references therein). However, as more and more proteins and pathways are becoming associated with developmental processes, and as increasingly complex networks of regulatory interactions emerge (see for example Manning and Toker, 2017), the need to study protein function in detail at tissue and single-cell scales in living organisms has become greater than ever. This represents a considerable challenge, in particular when studying proteins that regulate general cellular processes or have broad expression patterns. The availability of tools that allow protein function to be regulated more precisely and acutely in a spatial and temporal manner would be extremely helpful and could provide unprecedented insights into complex developmental processes.

Over the years, several methods have been developed to manipulate proteins directly *in vivo*. These include degradation-inducing applications (Banaszynski et al., 2006; Bonger et al., 2011; Chung et al., 2015; Natsume et al., 2016), protein cleavage using tobacco etch virus (TEV) protease (Harder et al., 2008; Pauli et al., 2008), the 'anchor-away' approach (Haruki et al., 2008), the 'knocksideways' technique (Robinson et al., 2010) and various dimerization tools that allow protein functions to be assembled in an inducible manner (Renicke et al., 2013; van Bergeijk et al., 2015; Wu et al., 2009), to mention just a few. However, an additional, more systematic approach to manipulation of protein function has recently emerged. This approach utilizes protein binders, which are small, protein-based affinity reagents that can selectively recognize and bind to a target protein and that are increasingly being used to study protein function in living cells and organisms (Beghein and Gettemans, 2017; Helma et al., 2015; Plückthun, 2015; Sha et al., 2017). These genetically encodable binders, which are based on various protein scaffolds, can be used to block or mask protein function. Furthermore, such protein binders can be 'functionalized' by fusing them to various effector domains with the ultimate goal of directly visualizing or regulating the function and interaction of target proteins in living cells or organisms.

In the past few years, protein binders have started to be used in developmental biology, and a multitude of novel protein binder-based tools has been reported. In this Primer, we provide an overview of these tools and discuss the possible applications of protein binders in developmental biology research. Furthermore, we highlight the potential and advantages of adding protein binder-based tools to the methodological repertoire of developmental biologists.

Types of protein binders and their generation

For many years, antibodies have been the tools of choice for recognizing a specific protein of interest (POI), and they are still the most widely used reagents for many applications. However, owing to their large size and poor folding and stability properties in the cytoplasm of cells, antibodies are most often used in the extracellular milieu or in fixed tissues, and have proven to be less useful in the context of a live cell. Over the last decade, however, several classes of smaller protein-binding scaffolds have been used to generate protein-specific binders that function when expressed in cells.

The different classes of peptide-based binders that currently exist can be divided into two broad families: (1) those that are based on or derived from immunoglobulins, i.e. antibodies and derivatives thereof, such as single-chain variable fragments (scFvs) and nanobodies; and (2) those that are based on non-immunoglobulin, natural or designed protein scaffolds, such as designed ankyrin repeat proteins (DARPins), monobodies, affibodies, anticalins and others (Boxes 1-3, Fig. 1). In the past decade, tremendous progress has been made in refining these scaffolds for better stability, higher affinity and easier handling (Goldman et al., 2017; Schilling et al., 2014; Schmidt et al., 2016), and libraries of increasing complexity have been generated and characterized (Moutel et al., 2016; Tiede et al., 2017; Yan et al., 2014).

With the exception of immunoglobulin-based binders, which can also be obtained upon immunization (Greenfield, 2014), protein binders are generally selected using *in vitro* display techniques (Boder and Wittrup, 1997; Samuelson et al., 2002; Zhao et al., 2009), with phage display being the most commonly used (Bradbury et al., 2011; Breitling et al., 1991; Romao et al., 2016; Kuhn et al., 2016). Although these techniques use different biological systems, their general principles are shared; they allow large peptide libraries to be screened, the respective protein is coupled to its encoding DNA sequence, and multiple cycles of selection can be used to increase binding specificity (Fig. 2). The isolation of protein binders can also be performed commercially and on large platforms, and many institutes have established core facilities to isolate and characterize specific protein binders, making

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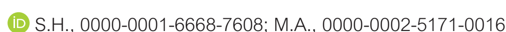
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Box 1. scFv scaffolds

Conventional antibodies (immunoglobulins, IgGs; see Fig. 1A) have been used extensively in basic research and are indispensable tools for protein detection. However, conventional IgGs are unsuitable for intracellular expression for various reasons; the reducing nature of the intracellular environment hampers disulphide bond formation and thus proper antibody folding, and whole IgG antibodies have a complex structure and a high atomic mass (~150 kDa). These drawbacks are partially overcome by connecting the V_H and V_L domain with a peptide linker, forming a so-called single-chain variable fragment (scFv; Bird et al., 1988; Huston et al., 1988; see Fig. 1B) that retains antigen-binding capacity. scFvs are relatively small (~28 kDa) and consist of a single domain that can be expressed in various host systems, such as bacteria, yeast or higher animals. These advantages make scFvs attractive tools in medical applications and in biotechnology (Lyon and Stasevich, 2017; Monnier et al., 2013) but their use as intracellular protein binders (intrabodies) is restricted because scFvs typically contain two highly conserved intra-domain disulphide bonds (Williams and Barclay, 1988). These bonds influence scFv stability and function (Glockshuber et al., 1992; Proba et al., 1997), and so only intrinsically stable scFvs fold correctly within a cell and can be utilized as functional intrabodies (Worn and Pluckthun, 1998). Owing to these limitations, relatively few scFv-based binders have been used to date in developmental biology. Nonetheless, using protein engineering (Tanha et al., 2006) and specific screening strategies, several improved scFv binders have been successfully generated (Lynch et al., 2008; Vielemeyer et al., 2010) and scFvs can now be selected for intracellular use (e.g. SunTag, see main text). These will no doubt start to play an increasingly important role in intracellular protein targeting, especially as scFvs can be derived from the huge pool of existing and validated monoclonal antibodies and used as genetically encoded protein binders in cells and developing organisms.

the task easier and more efficient. For more detailed information on the screening and isolation of different protein binder classes, we refer the reader to several recent publications (Helma et al., 2015; Pardon et al., 2014; Plückthun, 2015; Sha et al., 2017; Tiede et al., 2017; Veugelen et al., 2017).

Protein binders: their applications in developmental biology

The first step towards regulating or modifying a protein is to identify and isolate a binder that can specifically recognize the POI. Generally, protein binders can be generated against two distinct classes of proteins: against a widely used ‘tag’, such as fluorescent proteins or smaller peptide tags, and against endogenous proteins or specific post-translational modifications thereof. The first approach, using a ‘tag-binder’, comes with the advantage that a number of protein binders against protein tags have already been characterized, and their specificity *in vivo* has been carefully evaluated such that they can be used without much further validation (see below). However, this approach requires tagging of the POI (e.g. by genomic engineering using CRISPR/Cas9 or other technologies) and the tag must be inserted at a position at which it does not influence protein function. For certain studies, the tag-binder approach might be the faster and more convenient way to manipulate a target protein. This is the case when either a tagged version of the POI already exists and/or when the tag adds further properties to the protein of interest, such as making it visible in cells by fusing it to a fluorescent protein. For many applications, however, a better and more desirable strategy is to target an endogenous protein specifically with a protein binder. For certain targets, such as those carrying specific post-translational modifications, there is no alternative but to isolate protein binders that recognize these modifications. Although this approach does not require tagging of the POI, it requires the isolation and characterization of a novel binder. The latter then has to be validated with regard to its specificity, efficacy and off-target effects, a task that is not always trivial and has to be carefully designed and executed.

The second step is to functionalize the binder such that it modifies or regulates the target protein in a desired manner. This represents one of the most exciting aspects of protein binders, as there are innumerable possibilities for such functionalization. Over the past few years, the availability of a wide range of high-affinity protein binders has resulted in the development of a versatile repertoire of novel protein binder-based techniques and tools (reviewed by Bieli et al., 2016; Böldicke, 2017; Helma et al., 2015; Kaiser et al., 2014; Marschall et al., 2015; Plückthun, 2015; Sha et al., 2017). Many of these tools have been validated using binders that recognize specific fluorescent proteins. In particular, several binders against green fluorescent protein (GFP) have been isolated and characterized (Fridy et al., 2014; Kirchhofer et al., 2010; Kubala et al., 2010; Rothbauer et al., 2006; Saerens et al., 2005; Brauchle et al., 2014). In addition, several studies have used binders that recognize specific small tags, such as the 19 amino acid (aa) peptide derived from GCN4 used in SunTag (discussed below) (Tanenbaum et al., 2014; Wörn et al., 2000).

However, more and more applications based on binders that recognize endogenous, non-tagged proteins have emerged in the last few years. Below, we focus on the protein-binding tools that have been applied in developmental studies of multicellular systems, and explain how they are starting to be used to answer developmental questions. We also include certain exciting novel approaches in cell biology and systems biology that have potential applications in the field of developmental biology.

Box 2. Nanobodies and DARPins

The discovery of naturally evolved heavy chain antibodies (HcABs) in camelids (Hamers-Casterman et al., 1993) initiated a new era in antibody engineering (Beghein and Gettemans, 2017; Goldman et al., 2017). It was discovered that the antigen-binding domain of HcABs can be reduced to a single variable domain (V_{HH}, also called a nanobody) of only ~12-15 kDa (Fig. 1C,D). Nanobodies have biochemical properties that favour a wide variety of biological applications: they are small in size, they are highly stable, and they do not, in many cases, require disulphide bridges to correctly fold – in such cases, nanobodies can be stably expressed within the cytosol as intrabodies (Dmitriev et al., 2016; Kaiser et al., 2014). Nanobodies are thus ideal candidates for protein engineering and several of the protein binder-based tools already applied in developmental biology utilize binders of the nanobody type (Beghein and Gettemans, 2017). In particular, nanobodies that recognize fluorescent proteins, and other proteins, have been isolated and used in developmental biology (see main text).

Designed ankyrin repeat proteins (DARPins; see Pluckthun, 2015; Fig. 1E) represent a well-known repeat-based protein binder scaffold. In DARPins, the repeat modules are based on the ankyrin fold, which mediates protein-protein interaction *in vivo* and consists of two antiparallel α-helices connected by a β-turn (Pluckthun, 2015). DARPin specificity is determined by defined residues in the α-helices (Fig. 1E), and the selective randomization of these helical residues allows high-affinity binders to be selected (Steiner et al., 2008). The absence of disulphide bonds and the concave binding surface of DARPins make them ideal binders for intracellular applications and for large, conformational epitopes. More recently, a modified DARPin scaffold was introduced that overcomes epitope limitations imposed by the concave shape of the binding surface (Schilling et al., 2014). This modification optimizes screening for high-affinity binders with extended epitope-binding properties. High-affinity DARPins against GFP and mCherry have been isolated and validated (Brauchle et al., 2014) in multicellular organisms.

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Box 3. Other protein binder scaffolds

Many other protein scaffolds have been used to generate specific protein binders; we discuss here just a few that have been used for functional studies in multicellular systems and direct the reader to recent reviews for further information (Huet et al., 2015; Kalichuk et al., 2016; Lee et al., 2012; Škrlec et al., 2015; Valerio-Lepiniec et al., 2015).

One popular type of small binder scaffold is the monobody (Koide et al., 2012), which is engineered from the 10-kDa fibronectin protein fold (Fig. 1F). Similar to the IgG domain, monobodies possess three complementarity-determining region (CDR)-like loops that extend from a barrel-like scaffold consisting of seven β-sheets. In contrast to IgG domains, the fibronectin fold does not depend on disulphide bonds and thus allows monobodies to be used as affinity binders in living cells without further manipulation (Sha et al., 2017). Monobody scaffolds have been used to isolate a large number of protein binders that can act as allosteric inhibitors blocking specific protein-protein interaction or protein function (Ji et al., 2017; Kükenshöner et al., 2017; Spencer-Smith et al., 2017; Turman and Stockbridge, 2017), making monobodies interesting candidates for interfering with developmentally relevant pathways.

Anticalins are another class of synthetic binders that are based on the 20-kDa lipocalin-fold (Gebauer and Skerra, 2012; Richter et al., 2014). The anticalin structure consists of an eight-stranded β-barrel that contains four naturally occurring loops (Fig. 1G) that can be randomized to recognize specific antigens with high affinity. Owing to their structural properties, anticalins bind well to small proteins (Eggenstein et al., 2014). Anticalins have recently been used as binding reagents that interfere with molecular function and interaction, for example by blocking vascular endothelial growth factor receptor function (Richter and Skerra, 2017).

One of the smallest classes of protein binders are the Affibodies (Hansson et al., 1999; Stahl et al., 2017), with a molecular weight of around 7 kDa (Fig. 1H). Affibodies are based on *Staphylococcus aureus* protein A, consist of three α-helixes and contain no disulphide bonds. Affibodies have been used extensively, but mostly as protein drugs for biomedical applications (Frejd and Kim, 2017).

Protein visualization

Protein visualization *in vivo* is a powerful approach for investigating protein dynamics, localization and interactions. In general, visualization *in vivo* relies on the fusion of a fluorescent reporter protein to the POI. Despite recent developments in CRISPR/Cas9 technology, however, the modification and tagging of a POI by modifying its endogenous locus remains complex and labour intensive. Also, the use of heterologous promoters/enhancers to drive the expression of tagged proteins rarely recapitulates endogenous expression levels. Fusions between protein binders and fluorescent proteins, which are known as chromobodies, thus provide novel alternatives as *in vivo* biosensors (Fig. 3A). A major advantage of this approach is that the endogenous locus does not need to be modified. Furthermore, protein labelling can be restricted to the tissue of interest by expressing the chromobody in a tissue-specific manner in a developing organism. However, careful evaluations are needed to ensure that the binding of the chromobody to the target protein does not interfere with the function of the target protein. For instance, it is known that protein binder interactions can induce a change in target protein size and shape, and this could potentially alter protein distribution and/or dynamics (Schornack et al., 2009). Furthermore, a binder could potentially mask an interaction surface or an active site (De Genst et al., 2006), thereby impairing protein interactions and/or function. The appropriate controls, which vary for individual protein types, thus need to be carefully designed.

Pioneering studies have shown that protein-binding chromobodies can trace antigens in several cell compartments and throughout the cell cycle in living cells (Nizak et al., 2003a,b; Rothbauer et al., 2006). In another landmark paper, a 17-aa peptide from a yeast actin-binding protein was fused at the C terminal to GFP; the resulting actin binder, called LifeAct, represents a useful and much used *in vivo* marker to visualize F-actin dynamics in a wide range of animal systems (Riedl et al., 2008). In this case, the binder is derived from an endogenous protein, and not from a scaffold via screening. In more recent studies, chromobodies specific to endogenous protein targets have been used in multicellular organisms. For example, Rothbauer and colleagues used chromobodies to follow endogenous actin and proliferating cell nuclear antigen (PCNA) proteins in living zebrafish embryos (Panza et al., 2015).

Chromobodies are particularly valuable if they are targeted to ‘marker’ proteins of particular relevance to specific research areas (such as actin in the case of LifeAct, for example). Arnold and colleagues have recently demonstrated the power of chromobodies by using them to visualize endogenous synaptic proteins *in vivo* (Gross et al., 2013). Based on the monobody scaffold, they isolated recombinant proteins, termed Fibronectin intrabodies generated with mRNA display (FingRs), that bind the endogenous neuronal proteins PSD-95 (also known as DLG4) and Gephyrin. When fused to GFP, these FingRs allow excitatory and inhibitory synapses, respectively, to be visualized in living cortical neurons in culture. These tools can thus report the localization and amount of endogenous synaptic proteins and may be used to study changes in synaptic strength *in vivo*. FingRs were subsequently used in living zebrafish embryos to map synaptic dynamics in genetically defined neurons (Son et al., 2016), confirming that the methods do indeed also work in the complex environment of a living organism. Furthermore, by redesigning the FingRs and replacing the fluorescent protein with a degradation-inducing domain (see below), a powerful method was developed to ablate inhibitory synapses *in vivo* and study the resulting developmental or behavioural alterations (Gross et al., 2016).

In another recent variation of this approach, a chromatin-binding nanobody, called chromatibody, was fused to a fluorescent protein (Jullien et al., 2016). Chromatibody binds specifically to the endogenous H2A-H2B histone heterodimer in a non-intercalating manner, allowing real-time imaging of chromatin in live *Drosophila* embryos, larvae and adult individuals. By fusing chromatibody to an E3 ubiquitin ligase, this particular ligase activity was targeted to native chromatin, allowing the latter to be modified *in vivo*. These studies show that chromatibodies can be used as a universal, non-invasive tool for chromatin imaging or to manipulate the chromatin landscape *in vivo*.

In order for chromobodies to report the *in vivo* localization and trafficking of their endogenous targets accurately, it is important that their expression levels are such that excess, unbound chromobodies are minimized. In most cases, this has been achieved by low level expression of the chromobodies used. However, this problem was directly addressed in the FingR design by incorporating a transcriptional regulation system that ties chromobody (FingR) expression to the level of the target proteins (PSD-95 and Gephyrin, respectively) in order to reduce background fluorescence (see Gross et al., 2013 for more information). Using a different approach to solve this potential problem, Cepko and colleagues used a GFP nanobody derivative that was isolated using a conditional screening system; this nanobody is only stably expressed in cells in the presence of GFP and is degraded in its absence (Tang et al., 2016). The identified nanobody framework mutations leading to instability can be used to rapidly create destabilized versions of nanobodies recognizing other proteins and will be very useful for future studies in the protein binder field.

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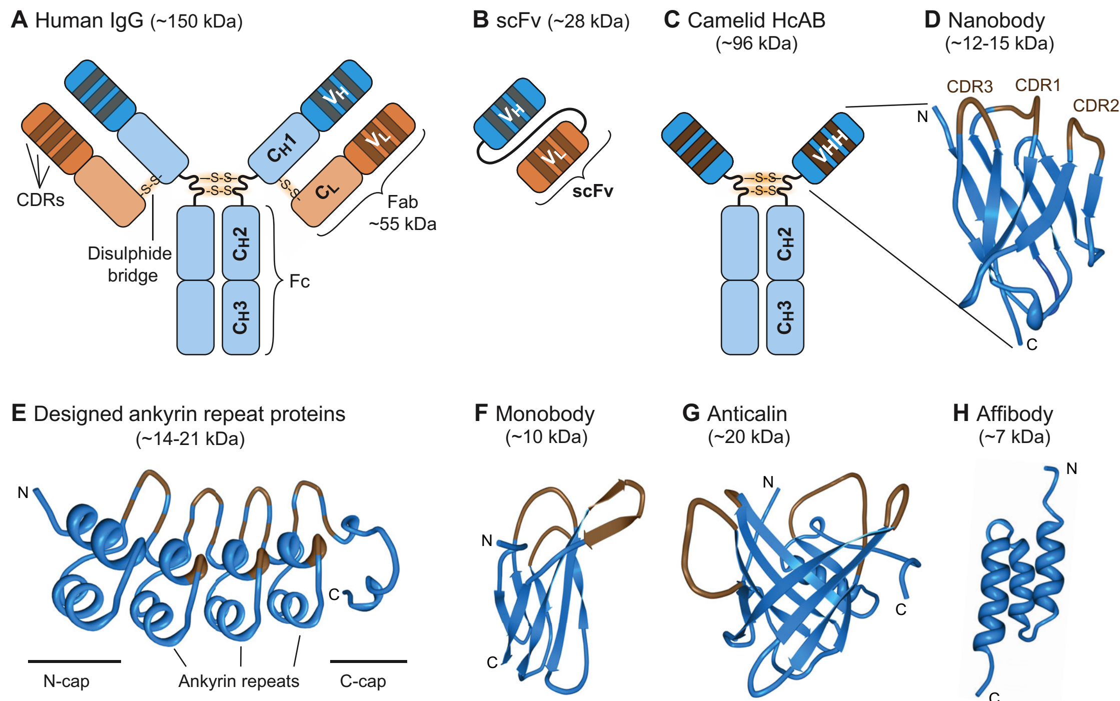

Fig. 1. The structure and size of selected protein binder scaffolds. The structures of different protein binder scaffolds are shown; variable regions are marked in brown. (A) Conventional human immunoglobulin (IgG) antibody, consisting of two heavy (blue) and two light (orange) chains connected by disulphide bridges (S-S). Both chains consist of several constant domains (C_L and C_H1-C_H3) and the variable domains (V_L and V_H). The variable domains contain the complementarity-determining regions (CDRs), which are the three hypervariable domains that determine antibody-binding specificity. (B) The single-chain variable fragment (scFv) is a synthetic fusion of the V_H and V_L domains via a linker. (C) Camelid heavy-chain antibodies (HcABs) are made up of only two heavy chains, each of which consists of two constant (C_H2 and C_H3) domains and a single variable (VHH) domain. (D) Structure of an anti-GFP nanobody (vhhGFP4, PDB ID:3OGO; Kubala et al., 2010). The positions of the three CDRs are highlighted. (E) Structure of the AR_3a DARPin (PDB ID:2BKK; Kohl et al., 2005). DARPins typically consist of constant N- and C-capping repeats motives (N-/C-cap), encapsulating two or three variable ankyrin repeats (three are shown here). (F) Structure of the monobody ySMB-1 (PDB ID: 3QHT; Gilbreth et al., 2011). (G) Anticalin US7 structure (PDB ID: 4MVI; Rauth et al., 2016). (H) Structure of the ZHER2 affibody (PDB ID: 2KZJ; Eigenbrot et al., 2010).

Protein binders that recognize specific post-transcriptional modifications have also been generated. The isolation of a DARPin-based binder that specifically recognizes the phosphorylated form of ERK (pERK) allowed Plückthun and colleagues to create an activity biosensor for ERK (also known as MAPK1), which provided a sensitive readout of ERK activation and localization in cultured mouse embryo fibroblasts (Kummer et al., 2013). It will be interesting to use this sensor in complex animal systems to determine the spatial and temporal activation pattern of ERK. Such studies, including similar sensors for other signalling pathways, will allow signal integration in developing tissues to be examined at a much more detailed level.

An exciting trend in the use of protein binders relies on the development of novel methods, as recently demonstrated by visualizing single protein molecules and genomic loci in living cells. Notably, Tanenbaum et al. designed a protein scaffold, called SunTag, which can recruit up to 24 copies of an scFv fusion protein (Tanenbaum et al., 2014). SunTag is based on the specific recognition by an scFv intrabody of a 19-aa peptide region of the yeast transcriptional regulator GCN4 (see Wörn et al., 2000); the recruitment of several scFv-GFP fusion proteins to oligomerized versions of the short peptide antigen results in strong signal amplification and allows the detection of individual proteins by live-cell imaging (Fig. 3B, left). SunTag can also function as a synthetic transcription factor by recruiting several units of a transcriptional activator fused to the scFv domain (Tanenbaum et al., 2014). The SunTag principle was recently utilized by several groups to visualize the dynamics of translation of single RNA molecules in cultured cells and cultured neurons (Morisaki et al., 2016; Wang et al., 2016; Wu et al., 2016; Yan et al., 2016). Such experiments were not possible before and represent the beginning of a new area in single-molecule analysis in cell and developmental biology. SunTag can also be utilized to visualize single genomic loci (Fig. 3B, right). This was achieved using a custom guide RNA (gRNA) to target a fusion of the dCas9 protein and the SunTag to a defined genomic locus. The subsequent recruitment of several copies of scFv-GFP chromobodies by SunTag to the location at which the gRNA associates with Cas9 allowed detection of the targeted locus. Using this approach, a single genomic locus was visualized *in vivo* and tracked throughout the cell cycle in HeLa cells; furthermore, looking at different loci, it was possible to follow the differential positioning of active and inactive regions in the nucleus (Qin et al., 2017). We expect to see many more such novel and ingenious applications emerge with the availability of more and more protein binders.

Protein relocalization

The subcellular localization and relocalization of many proteins has been studied in detail by immunohistochemistry and/or by using fluorescently tagged versions of the POI (Lye et al., 2014; Stadler et al., 2013). Although these studies have revealed that many proteins show restricted localization patterns, for example within the cell (e.g. components of the Hippo pathway: Richardson and Portela, 2017; Su et al., 2017), along the cell membrane (e.g.

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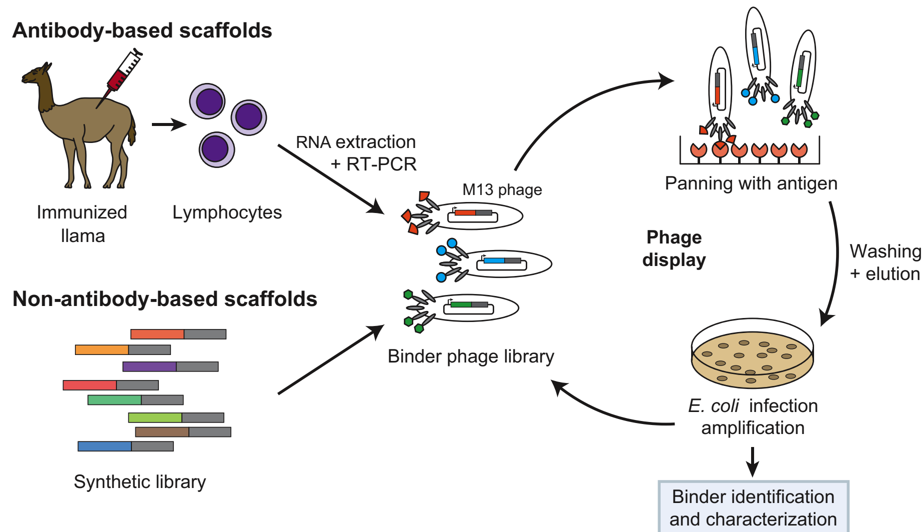

Fig. 2. Selection of high-affinity protein binders using phage display. Protein binder selection by phage display. In the case of antibody-based scaffolds (top), an antibody library (i.e. a nanobody library) is generated using lymphocytes (B cells) obtained from camelids (a llama is shown here). DNA fragments encoding protein binders are extracted from the B cells by RT-PCR. A binder phage library is then created by ligation of the protein binder-encoding sequences into the M13 phage pIII or pVIII genes, which encode phage coat proteins, allowing protein binders to be presented on the phage surface. DNA encoding the respective binder is encapsulated in the phage genome (protein and DNA are thus physically linked). Binder-presenting phages are immobilized and isolated by antigens in a process called panning, eluted and then amplified by infection in *Escherichia coli*. The selected binders are directly used for further characterization or undergo another round of panning, which might include a mutagenesis step (e.g. error prone PCR, not shown here) for affinity maturation. Several cycles of panning are commonly employed for the selection of high-affinity binders. For non-antibody-based scaffolds (bottom), synthetic peptide libraries are used (Hoogenboom, 2005). These are constructed *in vitro* by introducing tailored degeneracy in the sites responsible for antigen binding. Once cloned into a phage display vector, the panning process is the same as for scFvs and nanobodies.

receptors: Gui et al., 2016) or in the extracellular space (e.g. diffusible growth factors: Matsuo and Kimura-Yoshida, 2014), the functional implications or importance of protein localization are more difficult to assess. Recently, however, protein binders fused to localization domains have been successfully used to relocalize POIs in a controlled manner, and have thus allowed the systematic study of protein localization/mislocalization during animal development (Berry et al., 2016; Chen et al., 2017; Harmansa et al., 2017; Rothbauer et al., 2008). In these studies, protein relocalization is achieved by fusing a GFP-binding nanobody to a protein or a protein domain that localizes to a defined position within the cell or the tissue. Such ‘localized’ protein binders can act as traps for GFP-tagged POIs and can superimpose a novel localization on the POI, determined by the properties and the position of the localized binder (Fig. 4A).

This approach was first demonstrated in HeLa cells in which a GFP-tagged version of the promyelocytic leukemia protein was relocalized to the nuclear membrane by a localized protein binder

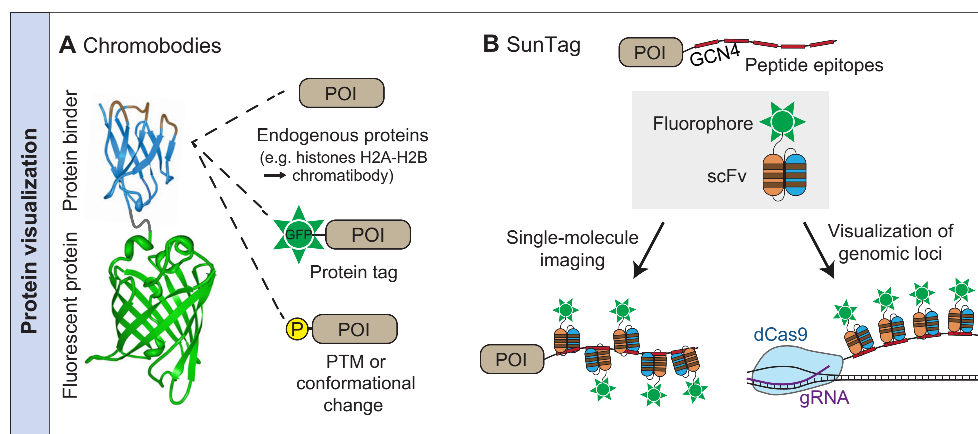

Fig. 3. Examples of protein visualization using protein binder-based tools. (A) Fusion proteins between a protein binder and a fluorescent protein are referred to as chromobodies. Chromobodies can be expressed *in vivo* and, depending on the protein binder used, target endogenous proteins, protein tags (e.g. GFP) or a post-translational modification (PTM). (B) The SunTag system consists of two components: a POI that contains up to 24 copies of a peptide epitope (GCN4), and a single-chain fragment (scFv) that specifically recognizes the GCN4 peptide epitope. As multiple copies of the scFv will be recruited by the oligomerized peptide epitope, using a fusion of a fluorescent protein (e.g. GFP) and the scFv allows the visualization of single molecules (left). Single genomic loci can also be visualized using the SunTag system (right). For this purpose, the SunTag is fused to the dCas9 protein, which can form a complex with a so-called guide RNA (gRNA) to recognize specific DNA sequences. The recruitment of several chromobodies by the SunTag results in signal amplification and improved signal-to-noise ratio. Image based on Tanenbaum et al. (2014).

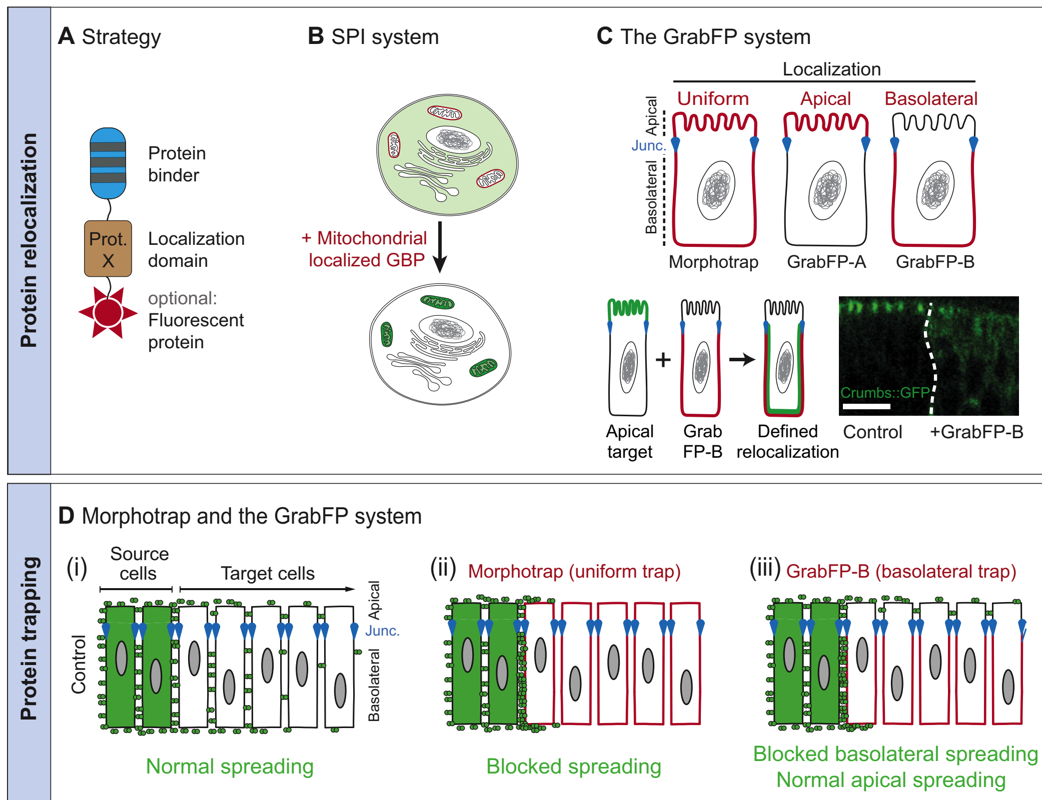

Fig. 4. Examples of protein relocalization and trapping using protein binder-based tools. (A) Protein binders (blue/brown) can be localized to specific subcellular or membrane compartments by fusion to proteins of known localization (Prot. X) or to specific localization domains. Such a localized protein binder can act as a protein trap for the POI and impose a novel localization on the POI, resulting in its controlled relocalization. A fluorescent protein can be optionally included in the protein trap to visualize fusion protein localization. (B) The synthetic physical interaction (SPI) system provides several GFP-binding proteins (GBPs) that localize to defined cellular compartments in yeast cells. In the example shown, a mitochondrial localized GBP (red) can relocalize a cytosolic GFP-tagged protein (green) to the mitochondria. (C) The GrabFP system consists of several membrane-bound GFP traps (red, based on a GFP-nanobody) that localize either uniformly around epithelial cells (morphotrap) or show a localization bias to either the apical (GrabFP-A) or basolateral (GrabFP-B) compartment in the *Drosophila* wing disc epithelium. Expression of GrabFP-B with a GFP-tagged version of the apical protein Crumbs (Crumbs::GFP) results in the relocalization of Crumbs::GFP to the basolateral compartment (bottom right). Scale bar: 5 µm. Image courtesy of Ilaria Alborelli. Epithelial cell junctions (Junc.) are shown in blue. (D) In the *Drosophila* wing imaginal disc, a GFP-tagged morphogen (green circles) is secreted from source cells (shaded green) and disperses into the target tissue (i). In experiments using either morphotrap (ii), or components of the GrabFP system (iii), the morphogen can be immobilized at defined structures within the tissue. Image based on Harmansa et al. (2017).

consisting of a GFP-binding protein (GBP) and the nuclear lamina protein lamin B1 (Rothbauer et al., 2008). The same approach was used by Berry et al. to systematically relocalize each of the ∼6000 yeast proteins to all the major cellular compartments (Berry et al., 2016). Relocalization was achieved by a set of 23 GFP traps that localize to defined cellular compartments (e.g. the mitochondria, see Fig. 4B). Interestingly, forced protein relocalization resulted in drastic effects in only a few cases; in most cases, cells seemed to be remarkably tolerant to protein relocalization. An analogous approach was recently used to study the role of protein localization along the apical-basal axis of epithelial cells in the wing imaginal disc of *Drosophila melanogaster*. This study used the Grab green fluorescent protein (GrabFP) system (Harmansa et al., 2017), which consists of three nanobody-based GFP traps that localize to defined positions along the apical-basal axis of epithelial cells (Fig. 4C). Using this approach, GFP-tagged POIs could be effectively relocalized along the apical-basal axis of epithelial cells (see Fig. 4C, bottom right). It would be particularly interesting to use such a protein-trap approach to relocalize POIs in cases in which knowledge of the molecular control of protein localization is missing or when endogenous modification of the POI is not feasible or is time consuming (e.g. in systematic screens). However, one has to bear in mind that binding (as well as the subsequent relocalization) of the protein trap could potentially interfere with the function of the POI and this needs to be assessed in each individual case.

Extracellular protein trapping

Secreted signalling molecules, such as morphogens and hormones, play crucial roles during animal development and homeostasis. Being able to interfere with their extracellular distribution directly would help us to understand better how secreted molecules function, to dissect how and when morphogen gradient formation is important, and to form gradients of altered shapes and investigate the consequences of such manipulations. In the last few years, protein binder-based approaches have proved to be useful in achieving these aims.

For example, a nanobody-based method called morphotrap has been used to retain secreted GFP-tagged proteins on the surface of producing cells, or to immobilize them in the surrounding target tissue, in *Drosophila* (Fig. 4D; Harmansa et al., 2015). Conceptually, morphotrap consists of an anti-GFP-nanobody (Rothbauer et al., 2006) that is presented along the extracellular surface by coupling it to the extracellular portion of the mouse CD8

transmembrane protein; this ‘GFP trap’ can bind and immobilize secreted GFP-tagged proteins of interest in the extracellular space. Using morphotrap, it was possible to retain the secreted morphogen Decapentaplegic (Dpp), a homologue of the vertebrate Bmp2/4 signalling molecule, in those cells that express and secrete it during *Drosophila* wing imaginal disc development. These studies showed that although Dpp dispersal is required for patterning of the wing imaginal disc and for growth of medial cells, dispersal is not required for growth of the lateral structures of the disc (Harmansa et al., 2015). Following on from this, the Grab green fluorescent protein (GrabFP) system (discussed above), which is based on the morphotrap concept, was used to directly modify different sub-pools of the Dpp morphogen gradient in the developing *Drosophila* wing disc (Harmansa et al., 2017). This approach revealed that Dpp in the basolateral plane of the wing imaginal disc is crucial for wing development (Harmansa et al., 2017). The morphotrap and the GrabFP approaches will be valuable for future investigations concerning the role of dispersal and the subcellular functions of secreted signalling factors in diverse developmental contexts.

Protein interference and degradation

Approaches that use genetic or RNA interference (RNAi) tools to intervene with protein function often depend heavily on the turnover of the protein of interest. However, many proteins are maternally provided to the egg and/or are rather stable in non-proliferating cells, making it difficult to deplete them or inactivate them using standard genetic techniques. In order to achieve fast, tissue-specific and temporally controlled protein degradation, previous studies have used destabilizing or inducible degradation signal (degron) domains (Banaszynski et al., 2006; Bonger et al., 2011; Chung et al., 2015; Natsume et al., 2016), but these approaches require the POI to be modified and therefore are time and work intensive. Several protein binder-based methods have been developed to overcome some of these limitations. These methods generally rely on two approaches, either using inhibitory protein binders or using protein binders to hitchhike the endogenous proteasomal degradation pathway.

The first approach relies on the ability to screen for and select protein binders that recognize protein-protein interaction surfaces, enzymatic domains or protein phosphorylation sites, and that can thus interfere with a specific protein function by masking important functional domains upon binding. Importantly, this approach does not require modification of the endogenous locus encoding the POI, but does require screening for an appropriate binder with the desired properties. Many protein binders that mask disease-associated protein functions have been isolated over the past years (Marschall et al., 2015). In many cases, however, these reagents were used to show that the function of a given protein is masked *in vitro* or in cultured cells, and they have been used less often in the context of developmental biology. One such approach that has been applied to this research field used peptide aptamer interference (PAPTi), which makes use of monobodies that shield specific protein-protein contact surfaces, to interfere with protein-protein interactions in living *Drosophila* larvae without depleting the protein of interest (Yeh et al., 2013). In this study, peptide binders against several molecules involved in cell-cell signalling, including Notch, Dishevelled and β-catenin (Armadillo), were identified and tested *in vivo*. This approach demonstrated that one of the binders against the ankyrin region of Notch increases the inhibitory activity of Notch towards Wingless signalling, highlighting that this approach can reveal important protein interactions within signalling networks. A more recent example of the use of an inhibitory or disruptive binder is the DeActs method. DeActs consists of genetically encoded actin-modifying peptides fused to GFP (Harterink et al., 2017), which effectively induce actin disassembly upon binding. DeAct has been used successfully in cultured cells, as well as in *Caenorhabditis elegans* and mouse neuronal cells, to investigate the role of actin dynamics. Further advances in the isolation and use of inhibitory protein binders have also been made recently (reviewed by Böldicke, 2017).

The general principle of the second approach, which aims to inactivate proteins, is to direct the POI to the ubiquitylation machinery, and hence to proteasome-mediated degradation, by fusing a protein binder to a subunit of the E3 ubiquitin ligase complex (Fig. 5A). This recruits the POI to the complex, resulting in polyubiquitylation and subsequent degradation of the POI. In one application of this technique, called deGradFP, an anti-GFP nanobody was used to target GFP-tagged proteins for degradation in cultured cells and in living *Drosophila* embryos and larvae (Caussinus et al., 2011). Similar approaches using fusion of a protein binder to a degradation-inducing partner have been shown to be effective in human and mouse cell lines (Fulcher et al., 2017, 2016; Portnoff et al., 2014), in zebrafish (Shin et al., 2015) and against GFP-fusion proteins in *C. elegans* (Wang et al., 2017, 2015). Tissue-specific protein degradation based on the aforementioned method has also been used in *Drosophila* to look at the role of force requirements during dorsal closure (Pasakarnis et al., 2016). Because classical genetic approaches are not feasible in this particular case, deGradFP was used to interfere with Myosin II (MyoII) function. In particular, the deGradFP-mediated, tissue-specific depletion of MyoII activity, via the inactivation of the Myosin regulatory light chain Spaghetti Squash (Sqh), was used to show that the forces required for dorsal closure are provided in a tissue-autonomous manner by amnioserosa cells (Pasakarnis et al., 2016). The same deGradFP-mediated MyoII inactivation approach has also been used to show that Myosin-mediated forces are not required during branch elongation and cell intercalation in the developing tracheal system in *Drosophila* embryos (Ochoa-Espinosa et al., 2017). In another recent investigation, an E3 ubiquitin ligase was fused to a FingR binding protein that targets the neuronal protein Gephyrin (called GFE3), which then targets the latter for ubiquitylation and degradation (Gross et al., 2016). By temporarily expressing GFE3 in cultured cells or in living zebrafish embryos, it was shown that inhibitory synapses retract upon Gephyrin depletion; however, these synapses regrow when Gephyrin is re-stabilized.

In summary, binder-mediated protein degradation allows the functions and levels of a POI to be altered in a region-specific and temporally controlled manner. Furthermore, the reversibility of this approach allows protein function to be depleted for a defined period of time, allowing the phenotypic consequences upon protein recovery to be studied. This has been shown in a very elegant way using the Dunce (Dnc) protein (Nagarkar-Jaiswal et al., 2015), which is expressed in mushroom bodies (MBs) of the adult *Drosophila* brain and is involved in learning and memory. Specifically, it was shown that the expression of deGradFP for 3 days (using a temperature-dependent driver) in GFP-Dnc-carrying flies causes a 70% decrease in learning score. Strikingly, these defects could be rescued by shifting the flies back to the restrictive temperature for 2 days, which restores GFP-Dnc levels to physiological conditions owing to the absence of deGradFP (Nagarkar-Jaiswal et al., 2015).

Protein/DNA modification

Many protein kinases have several substrates, and it can thus be challenging to control uniquely the phosphorylation state of a specific substrate of a particular kinase. One way to address this is to

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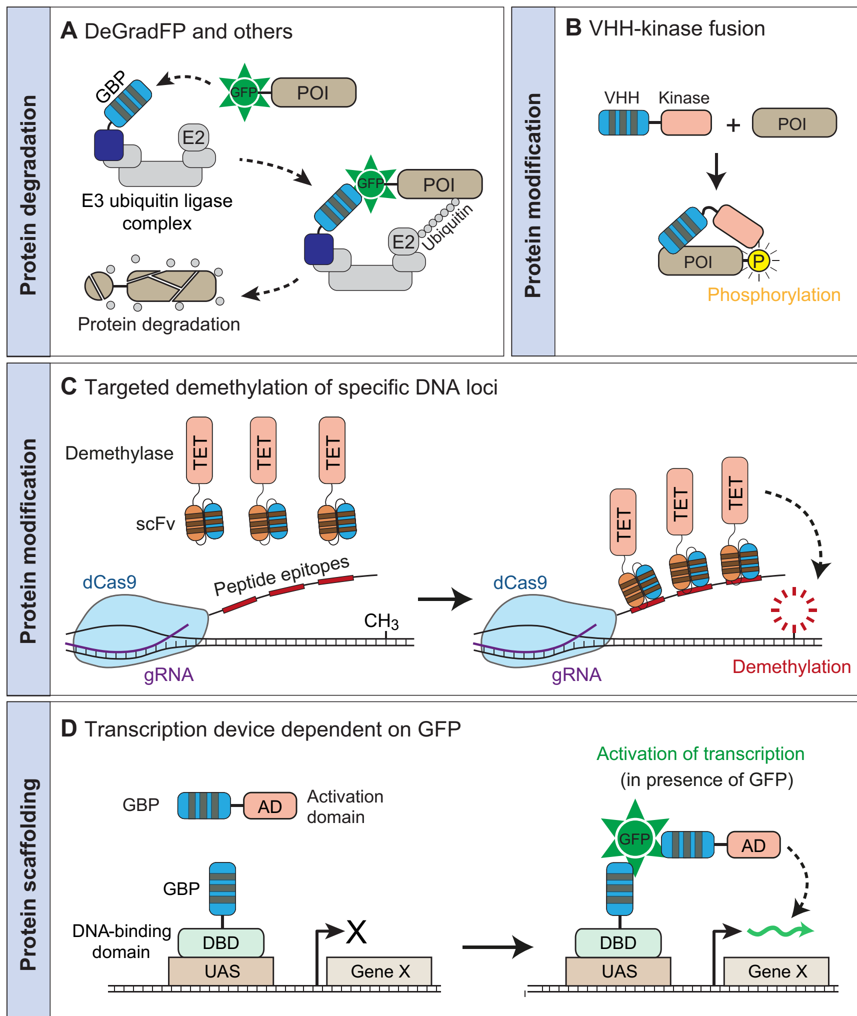

Fig. 5. Functionalized protein binders that target protein and DNA modifications. (A) A fusion protein between a GFP-binding protein (GBP) and a subunit (dark blue) of the E3 ubiquitin ligase complex can recruit a GFP-tagged POI to the ubiquitin ligase complex, resulting in polyubiquitylation and subsequent degradation of the POI. (B) Fusion proteins between protein binders (a VHH domain is shown here) and kinase domains can result in controlled phosphorylation of a specific substrate or POI. (C) Using a combination of a guide RNA (gRNA, purple) and a fusion protein between dCas9 (light blue) and the SunTag (peptide epitopes, red) allows the recruitment of several copies of an scFv-demethylase (TET) fusion protein to defined genomic loci; this results in demethylation of specified chromatin regions. Image based on Morita et al. (2016). (D) In the T-DDOG system, two protein binders recognizing different epitopes on the GFP surface (GBPs) are used to assemble a split Gal4 protein in the presence of GFP. One binder is fused to the Gal4 DNA-binding domain (DBD), the other is fused to the Gal4 activation domain (AD). In the presence of GFP, the split Gal4 is reconstituted and the two activities (DNA binding and transcriptional activation) are combined, resulting in the activation of transcription. Image based on Tang et al. (2013).

bring a minimal kinase domain into close proximity of a chosen substrate to direct phosphorylation exclusively towards this particular target, and to assess the effect of this induced phosphorylation. In a recent study (Roubinet et al., 2017), it was shown that the Myosin regulatory light chain Sqh, when fused to GFP, can be activated (phosphorylated) by a fusion protein consisting of the minimal domain of Rho kinase and an anti-GFP nanobody (see Fig. 5B). In order to restrict Sqh activation to the apical domain of *Drosophila* neuroblasts *in vivo*, the nanobody fusion protein was further linked to an apical membrane localization domain. This approach resulted in the phosphorylation and activation of the Sqh-GFP target protein exclusively on the apical side of a neuroblast, confirming the important role of temporal myosin activation and inactivation on the apical cortex for asymmetric cell division. These experiments also highlight that the same binder can be multi-functionalized via fusion with several effector domains (i.e. localization and post-translation modification), thereby further increasing the versatility of the protein binder approach. In a similar vein, a recent study utilized the previously described combination of a gRNA with dCas9-SunTag to recruit a fusion protein consisting of a DNA demethylase fused to an scFv that recognizes the SunTag (Fig. 5C; Morita et al., 2016). This approach allowed the controlled demethylation of defined DNA loci in cell culture and *in vivo* in mouse embryos. Many more enzymatic activities are involved in the modification of proteins, DNA or RNA; with further advances in protein binder-based technologies, such activities could be directed to specific places in time and space.

Protein scaffolding

Scaffold proteins are common to many cellular processes and provide the framework to bring together two or more proteins in a stable configuration (Garbett and Bretscher, 2014). Recently, protein binders and their targets have been used to create synthetic protein scaffolds that can bring together two or more proteins of defined functions. The basic requirement for this approach is the availability of two or more binders that recognize the same 'scaffold' in a non-overlapping manner. This concept has been used successfully in 'transcription device dependent on GFP' (T-DDOG; Tang et al., 2013), a tool that allows gene expression to be controlled (activated or repressed) specifically in those cells in which GFP is present. T-DDOG uses two anti-GFP nanobodies that bind to GFP in a non-overlapping and non-competitive manner (Kirchhofer et al., 2010). One of the nanobodies is coupled to a DNA-binding domain (from Gal4 or LexA, for example), and the other is coupled to the activation domain of the viral protein VP16. These two components come together only in the presence of the GFP 'scaffold' and function as an artificial transcription factor that drives gene expression in GFP-expressing cells (Fig. 5D). The T-DDOG system has been used to control transcription in mice and zebrafish (Tang et al., 2013). Given the existence of a multitude of transgenic lines that express GFP in a tissue-specific manner in

zebrafish and mouse (Abe and Fujimori, 2013), T-DDOG provides a ready-to-implement tool that can be utilized to control transcription. The same approach has also been used to create a split Cre recombinase that can be reconstituted and activated by the assembly on a GFP scaffold in living mice (Tang et al., 2015). Such synthetic modules, which act as 'sensors' (the presence of GFP, in this particular case), will likely be used more often in the field of developmental biology to detect the presence of specific proteins or protein subspecies in complex tissues.

Synergies between developmental biology and synthetic biology

Synthetic biology is an engineering discipline that aims to find solutions for biological problems by creating new biological circuits, based on both biomolecules and synthetic materials, to perform customized tasks (Ausländer et al., 2017; Davies, 2017). Synthetic biology exploits the vast diversity of functional components found in the genetic material of all life forms, including protein domains of different functions, and uses them to generate molecules with novel biological properties. Protein binders, in most cases scFvs, have been used in synthetic biology for many years, often as part of novel synthetic receptors, triggering signalling upon recognition of a ligand of interest (Barrett et al., 2014; Eyquem et al., 2017; Maus et al., 2014; Srivastava and Riddell, 2015). Few intracellular applications have been reported until now, mostly owing to the limited availability of such functional binders. However, as protein binders have a high degree of target specificity, and can now be isolated against virtually every protein, protein isoform and post-transcriptional modification, it is likely that many more protein binders will find a use in synthetic biology circuits.

In a recent effort to engineer a customized cell sensing and response behaviour, Lim and colleagues showed that chimeric forms of Notch (synthetic Notch receptors; or SynNotch), in which the extracellular sensor module and the intracellular transcriptional module are replaced with heterologous protein domains, can serve as a general platform for novel cell-cell contact signalling pathways (see Fig. 6). In particular, the replacement of the extracellular domain of Notch by binders that recognize different ligands (such as scFvs that recognize CD19 or mesothelin, or a nanobody that recognizes GFP) enabled the generation of cells that have a customized sensing behaviour (Morsut et al., 2016). This leaves room for the generation of cells that respond to user-specified extracellular cues (see also Roybal et al., 2016), and it is clear that the isolation and use of novel specific protein binders will increase the potential use of such chimeric receptors. SynNotch receptors have also been used in the field of developmental biology to ask more basic questions. Two studies (He et al., 2017; Huang et al., 2016) reported that optimized SynNotch receptors can indeed be activated by direct cell-cell contact *in vivo* in *Drosophila*. Both studies used the SynNotch system to reveal such direct cell interactions using binary expression systems to express the ligand (GFP) in one subset of cells, while expressing the SynNotch receptor (harbouring a GFP nanobody and an intracellular transcription activation domain) in another subset of cells (He et al., 2017; Huang et al., 2016). Depending on the downstream response of the SynNotch receptor, this setup can reveal cell-cell contacts by inducing a detectable response in the interacting cells (such as the expression of a fluorescent protein; He et al., 2017; Huang et al., 2016) or can act as a synthetic signalling system that induces the expression of a POI (Huang et al., 2016). Without doubt, the power of this system will be further explored, as it is possible to encode novel transcriptional circuits in the responding cell, as reported in the experiments described above, and reveal direct cell-cell interactions *in vivo* that would be difficult to detect otherwise.

Conclusions

In the past two decades, tremendous progress has been made in generating diverse protein scaffolds that can successfully be used to isolate binding proteins for virtually any target protein (Plückthun, 2015; Sha et al., 2017). The recent generation of various platforms that allow the isolation of such high-affinity binders, both in academia as well as in biotech companies, should thus permit the rapid and efficient identification of high-affinity protein binders that can target any protein of interest. Recent progress in the application of protein binders in cell and developmental biology has demonstrated that such binders can be functionalized in many different ways to regulate the function of the bound target protein. This functionalization allows the controlled degradation of proteins of interest and their controlled localization, and can also allow the tracking of endogenous proteins *in vivo* as well as their controlled post-transcriptional modification. Without doubt, many more such engineered functionalizations will surface in the near future allowing for a broad range of protein manipulations *in vivo*.

Of note, many of the binders that have been used thus far in the field of developmental biology are directed against fluorescent proteins. This is due to the fact that such binders have been available for a number of years and have thus been well characterized, representing ready-to-use reagents for the community. In addition, many lines expressing GFP, either alone or fused to a POI, have been generated in model organisms. In the future, it will be exciting to see more applications of protein binders that recognize endogenous targets in animal models. Obviously, many of the

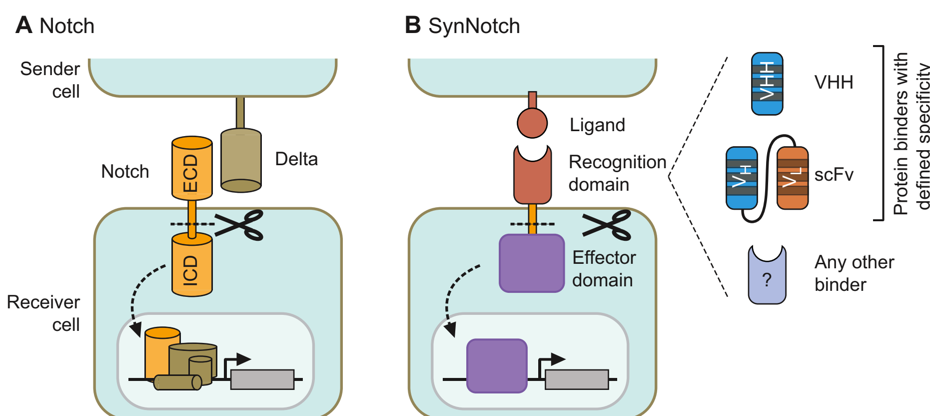

Fig. 6. Application of protein binders in synthetic biology. (A) Upon Notch interaction with its ligand Delta, the intracellular domain (ICD) of Notch is cleaved off and translocates to the nucleus, where it acts as a transcriptional regulator. (B) In the synthetic Notch (SynNotch) pathway, the extracellular recognition domain (ECD) is replaced by a protein binder with defined specificity (such as VHH or scFv) and the ICD is replaced by an effector domain (e.g. a transcription factor from bacteria or yeast). Importantly, both the recognition domain, as well as the effector domain, are exchangeable, enabling the generation of individualized, custom-made responses; any protein binder (VHH, scFv, etc.) for a desired ligand can be utilized. Image based on Morsut et al. (2016).

applications designed to manipulate fluorescently tagged proteins can be applied to endogenous proteins by exchanging the binder against the fluorescent protein with a binder against a target POI. As binder isolation nowadays is relatively straightforward, it should not represent a huge barrier to start working with these novel reagents.

Excitingly, several novel methods based on protein binders have been developed. For example, the availability of two binders against the same proteins (e.g. GFP) provides the option of using the latter to serve as a scaffold to combine split moieties of a molecular activity (Cre recombinase, transcriptional activator and DNA binder, for example) only in the presence of the scaffolding protein. Owing to the versatility of this approach, many novel applications making use of this principle are expected to surface in the near future. Binders against small peptide tags are also being developed and have contributed to the establishment of novel methods. The best example of such a binder is the SunTag. Owing to its small size, multiple copies of this tag can be inserted into a target protein, thereby allowing single molecule visualization in live cells. It is likely that this method will soon be used in developing organisms to follow single molecules in defined tissues and under different experimental paradigms, thereby providing valuable new insight into developmental processes.

With recent advances in the isolation of functional scFv-based intrabodies from monoclonal antibodies (Vielemeyer et al., 2010; Wörn et al., 2000), it should also be possible to isolate numerous scFv-based binders starting from the huge collections of existing monoclonal antibodies. This could result in the availability of countless novel binding proteins that can be functionalized. Other avenues for future development include equipping protein binders with domains for which activity can be regulated by acute interventions, such as illumination with light of a particular wavelength, or with domains that can be functionally modulated by small chemicals. It might also be interesting to generate so-called ‘split’ binders that would only be functional when the two moieties come together. The assembly of the functional binder could then potentially be controlled by light- or chemical-induced dimerization, eventually allowing for more acute regulation of target protein function (Renicke et al., 2013; van Bergeijk et al., 2015; Wu et al., 2009).

Considering the incredible versatility that protein binder-based tools offer, combined with the available opportunities to obtain a binder for a POI, we expect more and more developmental biologists to test, validate and include these novel tools in their experimental repertoire. Certainly, protein binder-based tools are a valuable addition to currently available tools for modifying protein function and will allow us to improve our understanding of developmental phenomena that otherwise would be difficult to dissect and investigate.

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Competing interests
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References
Abe, T. and Fujimori, T. (2013). Reporter mouse lines for fluorescence imaging. *Dev. Growth Differ.* **55**, 390-405.

Ausländer, S., Ausländer, D. and Fussenegger, M. (2017). Synthetic biology-the synthesis of biology. *Angew. Chem.* **56**, 6396-6419.

Banaszynski, L. A., Chen, L.-C., Maynard-Smith, L. A., Ooi, A. G. L. and Wandless, T. J. (2006). A rapid, reversible, and tunable method to regulate protein function in living cells using synthetic small molecules. *Cell* **126**, 995-1004.

Barrett, D. M., Singh, N., Porter, D. L., Grupp, S. A. and June, C. H. (2014). Chimeric antigen receptor therapy for cancer. *Annu. Rev. Med.* **65**, 333-347.

Beghein, E. and Gettemans, J. (2017). Nanobody technology: a versatile toolkit for microscopic imaging, protein-protein interaction analysis, and protein function exploration. *Front. Immunol.* **8**, 771.

Berry, L. K., Ólafsson, G., Ledesma-Fernández, E. and Thorpe, P. H. (2016). Synthetic protein interactions reveal a functional map of the cell. *eLife* **5**, e13053.

Bieli, D., Alborelli, I., Harmansa, S., Matsuda, S., Caussinus, E. and Affolter, M. (2016). Development and application of functionalized protein binders in multicellular organisms. *Intl. Rev. Cell Mol. Biol.* **325**, 181-213.

Bird, R. E., Hardman, K. D., Jacobson, J. W., Johnson, S., Kaufman, B. M., Lee, S. M., Lee, T., Pope, S. H., Riordan, G. S. and Whitlow, M. (1988). Single-chain antigen-binding proteins. *Science* **242**, 423-426.

Boder, E. T. and Wittrup, K. D. (1997). Yeast surface display for screening combinatorial polypeptide libraries. *Nat. Biotechnol.* **15**, 553-557.

Böldicke, T. (2017). Single domain antibodies for the knockdown of cytosolic and nuclear proteins. *Protein Sci.* **26**, 925-945.

Bonger, K. M., Chen, L.-C., Liu, C. W. and Wandless, T. J. (2011). Small-molecule displacement of a cryptic degron causes conditional protein degradation. *Nat. Chem. Biol.* **7**, 531-537.

Bradbury, A. R. M., Sidhu, S., Dübel, S. and McCafferty, J. (2011). Beyond natural antibodies: the power of in vitro display technologies. *Nat. Biotechnol.* **29**, 245-254.

Brauchle, M., Hansen, S., Caussinus, E., Lenard, A., Ochoa-Espinosa, A., Scholz, O., Sprecher, S. G., Plückthun, A. and Affolter, M. (2014). Protein interference applications in cellular and developmental biology using DARPins that recognize GFP and mCherry. *Biol. Open* **3**, 1252-1261.

Breitling, F., Dübel, S., Seehaus, T., Klewinghaus, I. and Little, M. (1991). A surface expression vector for antibody screening. *Gene* **104**, 147-153.

Caussinus, E., Kanca, O. and Affolter, M. (2011). Fluorescent fusion protein knockout mediated by anti-GFP nanobody. *Nat. Struct. Mol. Biol.* **19**, 117-121.

Chen, Y.-H., Wang, G.-Y., Hao, H.-C., Chao, C.-J., Wang, Y. and Jin, Q.-W. (2017). Facile manipulation of protein localization in fission yeast through binding of GFP-binding protein to GFP. *J. Cell Sci.* **130**, 1003-1015.

Chung, H. K., Jacobs, C. L., Huo, Y., Yang, J., Krumm, S. A., Plemper, R. K., Tsien, R. Y. and Lin, M. Z. (2015). Tunable and reversible drug control of protein production via a self-excising degron. *Nat. Chem. Biol.* **11**, 713-720.

Davies, J. (2017). Using synthetic biology to explore principles of development. *Development* **144**, 1146-1158.

De Genst, E., Silence, K., Decanniere, K., Conrath, K., Loris, R., Kinne, J., Muyldermans, S. and Wyns, L. (2006). Molecular basis for the preferential cleft recognition by dromedary heavy-chain antibodies. *Proc. Natl. Acad. Sci. USA* **103**, 4586-4591.

Dmitriev, O. Y., Lutsenko, S. and Muyldermans, S. (2016). Nanobodies as probes for protein dynamics in vitro and in cells. *J. Biol. Chem.* **291**, 3767-3775.

Eggenstein, E., Eichinger, A., Kim, H.-J. and Skerra, A. (2014). Structure-guided engineering of Anticalins with improved binding behavior and biochemical characteristics for application in radio-immuno imaging and/or therapy. *J. Struct. Biol.* **185**, 203-214.

Eigenbrot, C., Ultsch, M., Dubnovitsky, A., Abrahmsen, L. and Hard, T. (2010). Structural basis for high-affinity HER2 receptor binding by an engineered protein. *Proc. Natl. Acad. Sci. USA* **107**, 15039-15044.

Eyquem, J., Mansilla-Soto, J., Giavridis, T., van der Stegen, S. J. C., Hamieh, M., Cunanan, K. M., Odak, A., Gönen, M. and Sadelain, M. (2017). Targeting a CAR to the TRAC locus with CRISPR/Cas9 enhances tumour rejection. *Nature* **543**, 113-117.

Frejd, F. Y. and Kim, K.-T. (2017). Affibody molecules as engineered protein drugs. *Exp. Mol. Med.* **49**, e306.

Fridy, P. C., Li, Y., Keegan, S., Thompson, M. K., Nudelman, I., Scheid, J. F., Oeffinger, M., Nussenzweig, M. C., Fenyö, D., Chait, B. T. et al. (2014). A robust pipeline for rapid production of versatile nanobody repertoires. *Nat. Methods* **11**, 1253-1260.

Fulcher, L. J., Macartney, T., Bozatzi, P., Hornberger, A., Rojas-Fernandez, A. and Sapkota, G. P. (2016). An affinity-directed protein missile system for targeted proteolysis. *Open Biol.* **6**, 160255.

Fulcher, L. J., Hutchinson, L. D., Macartney, T. J., Turnbull, C. and Sapkota, G. P. (2017). Targeting endogenous proteins for degradation through the affinity-directed protein missile system. *Open Biol.* **7**, 170066.

Garbett, D. and Bretscher, A. (2014). The surprising dynamics of scaffolding proteins. *Mol. Biol. Cell* **25**, 2315-2319.

Gebauer, M. and Skerra, A. (2012). Anticalins small engineered binding proteins based on the lipocalin scaffold. *Methods Enzymol.* **503**, 157-188.

Gilbreth, R. N., Truong, K., Madu, I., Koide, A., Wojcik, J. B., Li, N.-S., Piccirilli, J. A., Chen, Y. and Koide, S. (2011). Isoform-specific monobody inhibitors of small ubiquitin-related modifiers engineered using structure-guided library design. *Proc. Natl. Acad. Sci. USA* **108**, 7751-7756.
Glockshuber, R., Schmidt, T. and Plückthun, A. (1992). The disulfide bonds in antibody variable domains: effects on stability, folding in vitro, and functional expression in Escherichia coli. *Biochemistry* **31**, 1270-1279.
Goldman, E. R., Liu, J. L., Zabetakis, D. and Anderson, G. P. (2017). Enhancing stability of camelid and shark single domain antibodies: an overview. *Front. Immunol.* **8**, 865.
Greenfield, E. A. (2014). *Antibodies: A Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
Gross, G. G., Junge, J. A., Mora, R. J., Kwon, H.-B., Olson, C. A., Takahashi, T. T., Liman, E. R., Ellis-Davies, G. C., McGee, A. W., Sabatini, B. L. et al. (2013). Recombinant probes for visualizing endogenous synaptic proteins in living neurons. *Neuron* **78**, 971-985.
Gross, G. G., Straub, C., Perez-Sanchez, J., Dempsey, W. P., Junge, J. A., Roberts, R. W., Trinh le, A., Fraser, S. E., De Koninck, Y., De Koninck, P. et al. (2016). An E3-ligase-based method for ablating inhibitory synapses. *Nat. Methods* **13**, 673-678.
Gui, J., Huang, Y. and Shimmi, O. (2016). Scribbled optimizes BMP signaling through its receptor internalization to the Rab5 endosome and promote robust epithelial morphogenesis. *PLoS Genet.* **12**, e1006424.
Hamers-Casterman, C., Atarhouch, T., Muyldermans, S., Robinson, G., Hamers, C., Songa, E. B., Bendahman, N. and Hamers, R. (1993). Naturally occurring antibodies devoid of light chains. *Nature* **363**, 446-448.
Hansson, M., Ringdahl, J., Robert, A., Power, U., Goetsch, L., Nguyen, T. N., Uhlén, M., Ståhl, S. and Nygren, P. A. (1999). An in vitro selected binding protein (affibody) shows conformation-dependent recognition of the respiratory syncytial virus (RSV) G protein. *Immunotechnology* **4**, 237-252.
Harder, B., Schomburg, A., Pflanz, R., Küstner, K., Gerlach, N. and Schuh, R. (2008). TEV protease-mediated cleavage in Drosophila as a tool to analyze protein functions in living organisms. *BioTechniques* **44**, 765-772.
Harmansa, S., Hamaratoglu, F., Affolter, M. and Caussinus, E. (2015). Dpp spreading is required for medial but not for lateral wing disc growth. *Nature* **527**, 317-322.
Harmansa, S., Alborelli, I., Bieli, D., Caussinus, E. and Affolter, M. (2017). A nanobody-based toolset to investigate the role of protein localization and dispersal in Drosophila. *eLife* **6**, e22549.
Harterink, M., da Silva, M. E., Will, L., Turan, J., Ibrahim, A., Lang, A. E., van Battum, E. Y., Pasterkamp, R. J., Kapitein, L. C., Kudryashov, D. et al. (2017). DeActs: genetically encoded tools for perturbing the actin cytoskeleton in single cells. *Nat. Methods* **14**, 479-482.
Haruki, H., Nishikawa, J. and Laemmli, U. K. (2008). The anchor-away technique: rapid, conditional establishment of yeast mutant phenotypes. *Mol. Cell* **31**, 925-932.
He, L., Huang, J. and Perrimon, N. (2017). Development of an optimized synthetic Notch receptor as an in vivo cell-cell contact sensor. *Proc. Natl. Acad. Sci. USA* **114**, 5467-5472.
Helma, J., Cardoso, M. C., Muyldermans, S. and Leonhardt, H. (2015). Nanobodies and recombinant binders in cell biology. *J. Cell Biol.* **209**, 633-644.
Hoogenboom, H. R. (2005). Selecting and screening recombinant antibody libraries. *Nat. Biotechnol.* **23**, 1105-1116.
Housden, B. E., Muhar, M., Gemberling, M., Gersbach, C. A., Stainier, D. Y. R., Seydoux, G., Mohr, S. E., Zuber, J. and Perrimon, N. (2017). Loss-of-function genetic tools for animal models: cross-species and cross-platform differences. *Nat. Rev. Genet.* **18**, 24-40.
Huang, T.-H., Velho, T. and Lois, C. (2016). Monitoring cell-cell contacts in vivo in transgenic animals. *Development* **143**, 4073-4084.
Huet, S., Gorre, H., Perrocheau, A., Picot, J. and Cinier, M. (2015). Use of the nanofitin alternative scaffold as a GFP-ready fusion tag. *PLoS ONE* **10**, e0142304.
Huston, J. S., Levinson, D., Mudgett-Hunter, M., Tai, M. S., Novotny, J., Margolies, M. N., Ridge, R. J., Bruccoleri, R. E., Haber, E., Crea, R. et al. (1988). Protein engineering of antibody binding sites: recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in Escherichia coli. *Proc. Natl. Acad. Sci. USA* **85**, 5879-5883.
Ji, M., Zheng, G., Li, X., Zhang, Z., Jv, G., Wang, X. and Wang, J. (2017). Computational dissection of allosteric inhibition of the SH2 domain of Bcr-Abl kinase by the monobody inhibitor AS25. *J. Mol. Model.* **23**, 183.
Jullien, D., Vignard, J., Fedor, Y., Béry, N., Olichon, A., Crozatier, M., Erard, M., Cassard, H., Ducommun, B., Salles, B. et al. (2016). Chromatibody, a novel non-invasive molecular tool to explore and manipulate chromatin in living cells. *J. Cell Sci.* **129**, 2673-2683.
Kaiser, P. D., Maier, J., Traenkle, B., Emele, F. and Rothbauer, U. (2014). Recent progress in generating intracellular functional antibody fragments to target and trace cellular components in living cells. *Biochim. Biophys. Acta* **1844**, 1933-1942.
Kalichuk, V., Béhar, G., Renodon-Cornière, A., Danovski, G., Obal, G., Barbet, J., Mouratou, B. and Pecorari, F. (2016). The archaeal "7 kDa DNA-binding" proteins: extended characterization of an old gifted family. *Sci. Rep.* **6**, 37274.
Kirchhofer, A., Helma, J., Schmidthals, K., Frauer, C., Cui, S., Karcher, A., Pellis, M., Muyldermans, S., Casas-Delucchi, C. S., Cardoso, M. C. et al. (2010). Modulation of protein properties in living cells using nanobodies. *Nat. Struct. Mol. Biol.* **17**, 133-138.
Kohl, A., Amstutz, P., Parizek, P., Binz, H. K., Briand, C., Capitani, G., Forrer, P., Plückthun, A. and Grutter, M. G. (2005). Allosteric inhibition of aminoglycoside phosphotransferase by a designed ankyrin repeat protein. *Structure* **13**, 1131-1141.
Koide, A., Wojcik, J., Gilbreth, R. N., Hoey, R. J. and Koide, S. (2012). Teaching an old scaffold new tricks: monobodies constructed using alternative surfaces of the FN3 scaffold. *J. Mol. Biol.* **415**, 393-405.
Kubala, M. H., Kovtun, O., Alexandrov, K. and Collins, B. M. (2010). Structural and thermodynamic analysis of the GFP:GFP-nanobody complex. *Protein Sci.* **19**, 2389-2401.
Kuhn, P., Fuhner, V., Unkauf, T., Moreira, G. M., Frenzel, A., Miethe, S. and Hust, M. (2016). Recombinant antibodies for diagnostics and therapy against pathogens and toxins generated by phage display. *Proteomics. Clin. Appl.* **10**, 922-948.
Kükenshöner, T., Schmit, N. E., Bouda, E., Sha, F., Pojer, F., Koide, A., Seeliger, M., Koide, S. and Hantschel, O. (2017). Selective targeting of SH2 domain-phosphotyrosine interactions of src family tyrosine kinases with monobodies. *J. Mol. Biol.* **429**, 1364-1380.
Kummer, L., Hsu, C.-W., Dagliyan, O., MacNevin, C., Kaufholz, M., Zimmermann, B., Dokholyan, N. V., Hahn, K. M. and Plückthun, A. (2013). Knowledge-based design of a biosensor to quantify localized ERK activation in living cells. *Chem. Biol.* **20**, 847-856.
Lee, S.-C., Park, K., Han, J., Lee, J.-J., Kim, H. J., Hong, S., Heu, W., Kim, Y. J., Ha, J.-S., Lee, S.-G. et al. (2012). Design of a binding scaffold based on variable lymphocyte receptors of jawless vertebrates by module engineering. *Proc. Natl. Acad. Sci. USA* **109**, 3299-3304.
Lye, C. M., Naylor, H. W. and Sanson, B. (2014). Subcellular localisations of the CPTI collection of YFP-tagged proteins in Drosophila embryos. *Development* **141**, 4006-4017.
Lynch, S. M., Zhou, C. and Messer, A. (2008). An scFv intrabody against the nonamyloid component of alpha-synuclein reduces intracellular aggregation and toxicity. *J. Mol. Biol.* **377**, 136-147.
Lyon, K. and Stasevich, T. J. (2017). Imaging translational and post-translational gene regulatory dynamics in living cells with antibody-based probes. *Trends Genet.* **33**, 322-335.
Manning, B. D. and Toker, A. (2017). AKT/PKB signaling: navigating the network. *Cell* **169**, 381-405.
Marschall, A. L., Dubel, S. and Böldicke, T. (2015). Specific in vivo knockdown of protein function by intrabodies. *mAbs* **7**, 1010-1035.
Matsuo, I. and Kimura-Yoshida, C. (2014). Extracellular distribution of diffusible growth factors controlled by heparan sulfate proteoglycans during mammalian embryogenesis. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **369**, 20130545.
Maus, M. V., Fraietta, J. A., Levine, B. L., Kalos, M., Zhao, Y. and June, C. H. (2014). Adoptive immunotherapy for cancer or viruses. *Annu. Rev. Immunol.* **32**, 189-225.
Monnier, P. P., Vigouroux, R. J. and Tassew, N. G. (2013). In vivo applications of single chain Fv (Variable Domain) (scFv) fragments. *Antibodies* **2**, 193-208.
Morisaki, T., Lyon, K., DeLuca, K. F., DeLuca, J. G., English, B. P., Zhang, Z., Lavis, L. D., Grimm, J. B., Viswanathan, S., Looger, L. L. et al. (2016). Real-time quantification of single RNA translation dynamics in living cells. *Science* **352**, 1425-1429.
Morita, S., Noguchi, H., Horii, T., Nakabayashi, K., Kimura, M., Okamura, K., Sakai, A., Nakashima, H., Hata, K., Nakashima, K. et al. (2016). Targeted DNA demethylation in vivo using dCas9-peptide repeat and scFv-TET1 catalytic domain fusions. *Nat. Biotechnol.* **34**, 1060-1065.
Morsut, L., Roybal, K. T., Xiong, X., Gordley, R. M., Coyle, S. M., Thomson, M. and Lim, W. A. (2016). Engineering customized cell sensing and response behaviors using synthetic notch receptors. *Cell* **164**, 780-791.
Moutel, S., Bery, N., Bernard, V., Keller, L., Lemesre, E., de Marco, A., Ligat, L., Rain, J. C., Favre, G., Olichon, A. et al. (2016). NaLi-H1: A universal synthetic library of humanized nanobodies providing highly functional antibodies and intrabodies. *eLife* **5**, e16228.
Nagarkar-Jaiswal, S., Lee, P. T., Campbell, M. E., Chen, K., Anguiano-Zarate, S., Gutierrez, M. C., Busby, T., Lin, W. W., He, Y., Schulze, K. L. et al. (2015). A library of MiMICs allows tagging of genes and reversible, spatial and temporal knockdown of proteins in Drosophila. *eLife* **4**, e05338.
Natsume, T., Kiyomitsu, T., Saga, Y. and Kanemaki, M. T. (2016). Rapid protein depletion in human cells by auxin-inducible degron tagging with short homology donors. *Cell Rep.* **15**, 210-218.
Nizak, C., Martin-Lluesma, S., Moutel, S., Roux, A., Kreis, T. E., Goud, B. and Perez, F. (2003a). Recombinant antibodies against subcellular fractions used to track endogenous Golgi protein dynamics in vivo. *Traffic* **4**, 739-753.
Nizak, C., Monier, S., del Nery, E., Moutel, S., Goud, B. and Perez, F., (2003b). Recombinant antibodies to the small GTPase Rab6 as conformation sensors. *Science* **300**, 984-987.

Ochoa-Espinosa, A., Harmansa, S., Caussinus, E., Affolter, M. (2017). Myosin II activity is not required for Drosophila tracheal branch elongation and cell intercalation. *Development* **144**, 2961-2968.
Panza, P., Maier, J., Schmees, C., Rothbauer, U. and Sollner, C. (2015). Live imaging of endogenous protein dynamics in zebrafish using chromobodies. *Development* **142**, 1879-1884.
Pardon, E., Laeremans, T., Triest, S., Rasmussen, S. G. F., Wohlkönig, A., Ruf, A., Muyldermans, S., Hol, W. G. J., Kobilka, B. K. and Steyaert, J. (2014). A general protocol for the generation of Nanobodies for structural biology. *Nat. Protoc.* **9**, 674-693.
Pasakarnis, L., Frei, E., Caussinus, E., Affolter, M. and Brunner, D. (2016). Amnioserosa cell constriction but not epidermal actin cable tension autonomously drives dorsal closure. *Nat. Cell Biol.* **18**, 1161-1172.
Pauli, A., Althoff, F., Oliveira, R. A., Heidmann, S., Schuldiner, O., Lehner, C. F., Dickson, B. J. and Nasmyth, K. (2008). Cell-type-specific TEV protease cleavage reveals cohesin functions in Drosophila neurons. *Dev. Cell* **14**, 239-251.
Plückthun, A. (2015). Designed ankyrin repeat proteins (DARPins): binding proteins for research, diagnostics, and therapy. *Annu. Rev. Pharmacol. Toxicol.* **55**, 489-511.
Portnoff, A. D., Stephens, E. A., Varner, J. D. and DeLisa, M. P. (2014). Ubiquibodies, synthetic E3 ubiquitin ligases endowed with unnatural substrate specificity for targeted protein silencing. *J. Biol. Chem.* **289**, 7844-7855.
Proba, K., Honegger, A. and Plückthun, A. (1997). A natural antibody missing a cysteine in VH: consequences for thermodynamic stability and folding. *J. Mol. Biol.* **265**, 161-172.
Qin, P., Parlak, M., Kuscu, C., Bandaria, J., Mir, M., Szlachta, K., Singh, R., Darzacq, X., Yildiz, A. and Adli, M. (2017). Live cell imaging of low- and non-repetitive chromosome loci using CRISPR-Cas9. *Nat. Commun.* **8**, 14725.
Rauth, S., Hinz, D., Borger, M., Uhrig, M., Mayhaus, M., Riemenschneider, M. and Skerra, A. (2016). High-affinity Anticalins with aggregation-blocking activity directed against the Alzheimer beta-amyloid peptide. *Biochem. J.* **473**, 1563-1578.
Renicke, C., Schuster, D., Usherenko, S., Essen, L.-O. and Taxis, C. (2013). A LOV2 domain-based optogenetic tool to control protein degradation and cellular function. *Chem. Biol.* **20**, 619-626.
Richardson, H. E. and Portela, M. (2017). Tissue growth and tumorigenesis in Drosophila: cell polarity and the Hippo pathway. *Curr. Opin. Cell Biol.* **48**, 1-9.
Richter, A. and Skerra, A. (2017). Anticalins directed against vascular endothelial growth factor receptor 3 (VEGFR-3) with picomolar affinities show potential for medical therapy and in vivo imaging. *Biol. Chem.* **398**, 39-55.
Richter, A., Eggenstein, E. and Skerra, A. (2014). Anticalins: exploiting a non-Ig scaffold with hypervariable loops for the engineering of binding proteins. *FEBS Lett.* **588**, 213-218.
Riedl, J., Crevenna, A. H., Kessenbrock, K., Yu, J. H., Neukirchen, D., Bista, M., Bradke, F., Jenne, D., Holak, T. A., Werb, Z. et al. (2008). Lifeact: a versatile marker to visualize F-actin. *Nat. Methods* **5**, 605-607.
Robinson, M. S., Sahlender, D. A. and Foster, S. D. (2010). Rapid inactivation of proteins by rapamycin-induced rerouting to mitochondria. *Dev. Cell* **18**, 324-331.
Romao, E., Morales-Yanez, F., Hu, Y., Crauwels, M., De Pauw, P., Hassanzadeh, G. G., Devoogdt, N., Ackaert, C., Vincke, C. and Muyldermans, S., (2016). Identification of useful nanobodies by phage display of immune single domain libraries derived from camelid heavy chain antibodies. *Curr. Pharm. Des.* **22**, 6500-6518.
Rothbauer, U., Zolghadr, K., Tillib, S., Nowak, D., Schermelleh, L., Gahl, A., Backmann, N., Conrath, K., Muyldermans, S., Cardoso, M. C. et al. (2006). Targeting and tracing antigens in live cells with fluorescent nanobodies. *Nat. Methods* **3**, 887-889.
Rothbauer, U., Zolghadr, K., Muyldermans, S., Schepers, A., Cardoso, M. C. and Leonhardt, H. (2008). A versatile nanotrap for biochemical and functional studies with fluorescent fusion proteins. *Mol. Cell. Proteomics* **7**, 282-289.
Roubinet, C., Tsankova, A., Than Pham, T., Monnard, A., Caussinus, E., Affolter, M. and Cabernard, C. (2017). Spatio-temporally separated cortical flows and spindle asymmetry/positioning establish physical asymmetry in fly neural stem cells. *Nat. Commun.* (in press) **8**, 1383.
Roybal, K. T., Williams, J. Z., Morsut, L., Rupp, L. J., Kolinko, I., Choe, J. H., Walker, W. J., McNally, K. A. and Lim, W. A. (2016). Engineering T cells with customized therapeutic response programs using synthetic notch receptors. *Cell* **167**, 419-432 e416.
Saerens, D., Pellis, M., Loris, R., Pardon, E., Dumoulin, M., Matagne, A., Wyns, L., Muyldermans, S. and Conrath, K. (2005). Identification of a universal VHH framework to graft non-canonical antigen-binding loops of camel single-domain antibodies. *J. Mol. Biol.* **352**, 597-607.
Samuelson, P., Gunneriusson, E., Nygren, P.-A. and Ståhl, S. (2002). Display of proteins on bacteria. *J. Biotechnol.* **96**, 129-154.
Schilling, J., Schöppe, J. and Plückthun, A. (2014). 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**, 691-721.
Schmidt, F. I., Hanke, L., Morin, B., Brewer, R., Brusic, V., Whelan, S. P. J. and Ploegh, H. L. (2016). Phenotypic lentivirus screens to identify functional single domain antibodies. *Nat. Microbiol.* **1**, 16080.
Schornack, S., Fuchs, R., Huitema, E., Rothbauer, U., Lipka, V. and Kamoun, S. (2009). Protein mislocalization in plant cells using a GFP-binding chromobody. *Plant J.* **60**, 744-754.
Sha, F., Salzman, G., Gupta, A. and Koide, S. (2017). Monobodies and other synthetic binding proteins for expanding protein science. *Protein Sci.* **26**, 910-924.
Shin, Y. J., Park, S. K., Jung, Y. J., Kim, Y. N., Kim, K. S., Park, O. K., Kwon, S.-H., Jeon, S. H., Trinh le, A., Fraser, S. E. et al. (2015). Nanobody-targeted E3-ubiquitin ligase complex degrades nuclear proteins. *Sci. Rep.* **5**, 14269.
Škrlec, K., Štrukelj, B. and Berlec, A. (2015). Non-immunoglobulin scaffolds: a focus on their targets. *Trends Biotechnol.* **33**, 408-418.
Son, J.-H., Keefe, M. D., Stevenson, T. J., Barrios, J. P., Anjewierden, S., Newton, J. B., Douglass, A. D. and Bonkowsky, J. L. (2016). Transgenic FingRs for live mapping of synaptic dynamics in genetically-defined neurons. *Sci. Rep.* **6**, 18734.
Spencer-Smith, R., Koide, A., Zhou, Y., Eguchi, R. R., Sha, F., Gajwani, P., Santana, D., Gupta, A., Jacobs, M., Herrero-Garcia, E. et al. (2017). Inhibition of RAS function through targeting an allosteric regulatory site. *Nat. Chem. Biol.* **13**, 62-68.
Srivastava, S. and Riddell, S. R. (2015). Engineering CAR-T cells: design concepts. *Trends Immunol.* **36**, 494-502.
Stadler, C., Rexhepaj, E., Singan, V. R., Murphy, R. F., Pepperkok, R., Uhlen, M., Simpson, J. C. and Lundberg, E. (2013). Immunofluorescence and fluorescent-protein tagging show high correlation for protein localization in mammalian cells. *Nat. Methods* **10**, 315-323.
Ståhl, S., Graslund, T., Eriksson Karlstrom, A., Frejd, F. Y., Nygren, P. A. and Lofblom, J. (2017). Affibody molecules in biotechnological and medical applications. *Trends Biotechnol.* **35**, 691-712.
Steiner, D., Forrer, P. and Plückthun, A. (2008). Efficient selection of DARPins with sub-nanomolar affinities using SRP phage display. *J. Mol. Biol.* **382**, 1211-1227.
Su, T., Ludwig, M. Z., Xu, J. and Fehon, R. G. (2017). Kibra and merlin activate the hippo pathway spatially distinct from and independent of expanded. *Dev. Cell* **40**, 478-490 e473.
Tanenbaum, M. E., Gilbert, L. A., Qi, L. S., Weissman, J. S. and Vale, R. D. (2014). A protein-tagging system for signal amplification in gene expression and fluorescence imaging. *Cell* **159**, 635-646.
Tang, J. C. Y., Szikra, T., Kozorovitskiy, Y., Teixiera, M., Sabatini, B. L., Roska, B. and Cepko, C. L. (2013). A nanobody-based system using fluorescent proteins as scaffolds for cell-specific gene manipulation. *Cell* **154**, 928-939.
Tang, J. C. Y., Rudolph, S., Dhande, O. S., Abraira, V. E., Choi, S., Lapan, S. W., Drew, I. R., Drokhlyansky, E., Huberman, A. D., Regehr, W. G. et al. (2015). Cell type-specific manipulation with GFP-dependent Cre recombinase. *Nat. Neurosci.* **18**, 1334-1341.
Tang, J. C., Drokhlyansky, E., Etemad, B., Rudolph, S., Guo, B., Wang, S., Ellis, E. G., Li, J. Z. and Cepko, C. L. (2016). Detection and manipulation of live antigen-expressing cells using conditionally stable nanobodies. *eLife* **5**, e15312.
Tanha, J., Nguyen, T.-D., Ng, A., Ryan, S., Ni, F. and Mackenzie, R. (2006). Improving solubility and refolding efficiency of human V(H)s by a novel mutational approach. *Protein Eng. Des. Sel.* **19**, 503-509.
Tiede, C., Bedford, R., Heseltine, S. J., Smith, G., Wijetunga, I., Ross, R., AlQallaf, D., Roberts, A. P., Balls, A., Curd, A. et al. (2017). Affimer proteins are versatile and renewable affinity reagents. *eLife* **6**, e24903.
Turman, D. L. and Stockbridge, R. B. (2017). Mechanism of single- and double-sided inhibition of dual topology fluoride channels by synthetic monobodies. *J. Gen. Physiol.* **149**, 511-522.
Valerio-Lepiniec, M., Urvoas, A., Chevrel, A., Guellouz, A., Ferrandez, Y., Mesneau, A., de la Sierra-Gallay, I. L., Aumont-Nicaise, M., Desmadril, M., van Tilbeurgh, H. et al. (2015). The alphaRep artificial repeat protein scaffold: a new tool for crystallization and live cell applications. *Biochem. Soc. Trans.* **43**, 819-824.
van Bergeijk, P., Adrian, M., Hoogenraad, C. C. and Kapitein, L. C. (2015). Optogenetic control of organelle transport and positioning. *Nature* **518**, 111-114.
Veugelen, S., Dewilde, M., De Strooper, B. and Chávez-Gutiérrez, L., (2017). Screening and characterization strategies for nanobodies targeting membrane proteins. *Methods Enzymol.* **584**, 59-97.
Vielemeyer, O., Nizak, C., Jimenez, A. J., Echard, A., Goud, B., Camonis, J., Rain, J. C. and Perez, F. (2010). Characterization of single chain antibody targets through yeast two hybrid. *BMC Biotechnol.* **10**, 59.
Wang, S., Wu, D., Quintin, S., Green, R. A., Cheerambathur, D. K., Ochoa, S. D., Desai, A. and Oegema, K. (2015). NOCA-1 functions with gamma-tubulin and in parallel to Patronin to assemble non-centrosomal microtubule arrays in C. elegans. *eLife* **4**, e08649.
Wang, C., Han, B., Zhou, R. and Zhuang, X. (2016). Real-time imaging of translation on single mRNA transcripts in live cells. *Cell* **165**, 990-1001.
Wang, S., Tang, N. H., Lara-Gonzalez, P., Prevo, B., Cheerambathur, D. K., Chisholm, A. D., Desai, A. and Oegema, K. (2017). A toolkit for tissue-specific protein degradation in C. elegans. *Development* **144**, 2694-2701.
Williams, A. F. and Barclay, A. N. (1988). The immunoglobulin superfamily–domains for cell surface recognition. *Annu. Rev. Immunol.* **6**, 381-405.
Wörn, A. and Plückthun, A. (1998). An intrinsically stable antibody scFv fragment can tolerate the loss of both disulfide bonds and fold correctly. *FEBS Lett.* **427**, 357-361.
Wörn, A., Auf der Maur, A., Escher, D., Honegger, A., Barberis, A. and Plückthun, A., (2000). Correlation between in vitro stability and in vivo

performance of anti-GCN4 intrabodies as cytoplasmic inhibitors. *J. Biol. Chem.* **275**, 2795-2803.

Wu, Y. I., Frey, D., Lungu, O. I., Jaehrig, A., Schlichting, I., Kuhlman, B. and Hahn, K. M. (2009). A genetically encoded photoactivatable Rac controls the motility of living cells. *Nature* **461**, 104-108.

Wu, B., Eliscovich, C., Yoon, Y. J. and Singer, R. H. (2016). Translation dynamics of single mRNAs in live cells and neurons. *Science* **352**, 1430-1435.

Yan, J., Li, G., Hu, Y., Ou, W. and Wan, Y. (2014). Construction of a synthetic phage-displayed Nanobody library with CDR3 regions randomized by trinucleotide cassettes for diagnostic applications. *J. Transl. Med.* **12**, 343.

Yan, X., Hoek, T. A., Vale, R. D. and Tanenbaum, M. E. (2016). Dynamics of translation of single mRNA molecules in vivo. *Cell* **165**, 976-989.

Yeh, J. T.-H., Binari, R., Gocha, T., Dasgupta, R. and Perrimon, N. (2013). PAPTi: a peptide aptamer interference toolkit for perturbation of protein-protein interaction networks. *Sci. Rep.* **3**, 1156.

Zhao, X.-L., Chen, W.-Q., Yang, Z.-H., Li, J.-M., Zhang, S.-J. and Tian, L.-F. (2009). Selection and affinity maturation of human antibodies against rabies virus from a scFv gene library using ribosome display. *J. Biotechnol.* **144**, 253-258.