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Non-immunoglobulin scaffold proteins: Precision tools for studying protein-protein interactions in cancer

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Abstract

Cancer is frequently characterised by dysregulation of the cellular signalling processes that govern proliferation, survival and attachment. Understanding such dysregulation continues to present a challenge given the importance of protein-protein interactions in intracellular processes. Exploring this protein-protein interactome requires novel tools capable of discriminating between highly homologous proteins, individual domains and post-translational modifications. This review examines the potential of scaffold-based binding proteins to fulfil these requirements. It also explores protein-protein interactions in the context of intracellular signalling pathways and cancer, and demonstrates the uses of scaffold proteins as functional moderators, biosensors and imaging reagents. This review also highlights the timeliness and potential to develop international consortia to develop and validate highly specific "proteome" scaffold-based binding protein reagents with the ultimate aim of developing screening tools for studying the interactome.

Keywords

Affimer, Monobody, DARPins, protein-protein interaction, intracellular signalling.

Abbreviations: PPIs – protein-protein interactions; SBPs – scaffold-based binding proteins; RTK – receptor tyrosine kinase, DARPins – Designed Ankyrin Repeat Proteins.

Introduction

Cancer has six biological hallmarks, namely sustaining proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis [1]. These hallmarks arise from dysregulation of normal cell processes that are governed by complex networks of proteins [2], a detailed understanding of which will provide new therapeutic opportunities. Classically, signalling networks are viewed as a predominantly intracellular cascade of protein modifications [3]. However, proteins do not act in isolation within cells, indeed 80% of proteins act in multimolecular complexes [4]. The importance of protein-protein interactions (PPIs) is thus as great as any other biochemical function of a protein when considering its biology. Consequently cell signalling and the process of transduction of an external stimulus into a cellular response can be viewed as a series of PPIs. When considered from this viewpoint, intracellular signalling becomes an increasingly complex issue as a single protein within the pathway is likely to have multiple domains that can engage in distinct PPIs. For example, the epidermal-growth factor receptor (EGFR) pathway typically consists of 375 proteins, but has been reported to involve more than 1000 PPIs [5]. Understanding these PPIs and their dynamic nature poses one of the major

challenges for cell and cancer cell biology, and provides the potential for developing exciting new therapeutics.

PPIs can be difficult to probe with traditional small molecule approaches as they are frequently more dynamic, and involve larger and often relatively uncontoured surfaces, than conventionally targeted ligand-binding domains and enzyme active sites [6]. However, several approaches have been used to gain understanding of PPIs over the years, notably including small molecule approaches [7, 8], as well as the use of peptides and proteins such as antibody fragments [6, 9-11]. A substantial proportion of this work focuses on modulating PPIs at the initiation of cell signalling, by targeting membrane proteins and their interacting ligands as these are readily accessible [11, 12]. Membrane proteins, however, only account for a small number of the PPIs in a signalling pathway and their inhibition may not have clinical effects as alterations frequently occur downstream of pathway initiation. However, targeting intracellular PPIs presents a more difficult set of challenges, as the cytoplasm of cells is a reducing environment [13] preventing disulphide bridge formation which can sometimes limit the use of antibodies and their derivatives. In addition, families of intracellular proteins frequently consist of highly homologous domains that can be difficult to target specifically with small molecules and thus leading to undesirable off-target effects [14]. Within the last decade great strides have been made to address such issues and here we focus on the newest approach employed to modulate PPIs within cells - the use of artificial binding proteins and specifically non-Ig, scaffold-based binding proteins (SBPs). Nanobodies are not covered in this review as, despite meeting many of the criteria that define SBPs, they derive from Igs. SBPs are small proteins, typically 10-20kDa and with a fixed stable scaffold that constrains variable

regions that may be introduced either by varying existing sequences within the scaffold or by introducing longer loop insertions. The introduction of such variability generates libraries of variants from which specific binders for a defined target protein, protein domain or modification (Figure 1) may be selected usually by phage or ribosomal display.

Box 1. Scaffold-based binding proteins. Scaffold-based binding proteins (SBPs) are small monomeric proteins, typically less than 200 amino acid residues, consisting of a stable scaffold and one or more regions of randomised residues. The scaffolds have a variety of parent proteins (see Table 1 for examples), but have several commonalities as they comprise single or repeated domains that frequently lack cysteine residues, meaning they can easily be expressed in bacterial systems and mammalian cells, and display high thermostability [11, 15-19]. SBPs frequently bind their targets with low nM and pM affinitiesy [17, 20].

The small size of SBPs means rapid tissue distribution and elimination occurs in vivo which is advantageous for diagnostic imaging, but not ideal for therapeutic use [21]. However SBPs can be site-specifically conjugated to a number of molecules including PEG which increases their half-life [22]. Immunogenecity is another concern for SBPs derived from non-human scaffolds [17].

To date 20 different scaffolds have been used in a variety of applications from crystallization chaperones and antibody replacements in basic research to diagnostics and biopharmaceuticals [11]. Here we focus on a subset of SBPs that have been used to target intracellular signalling proteins (**Table 1 and Figure 1**) (For a comprehensive review of all 20 SBPs and their uses the reader is directed to [11]). SBPs present advantages over other protein-based approaches in the reducing intracellular environment as they frequently have no cysteine residues [11] and are small proteins that can be easily delivered by standard cell biology techniques including transient transfection, viral transduction, or even modified to be cell-penetrating proteins [20]. These properties make SBPs exciting tools for exploring

PPIs in the context of intracellular signalling networks and to identify new therapeutic targets for cancer as illustrated below.

SBPs and Receptor-Tyrosine Kinase initiated pathways

Receptor-tyrosine kinase (RTK) signalling has important roles in the control of fundamental cellular activity including proliferation, differentiation, migration, metabolism and survival [49] (**Figure 2**) and frequently underlies changes in cell behaviour associated with cancer [2]. To this end SBPs have been isolated against a number of receptor tyrosine kinases that are readily accessible due to their extracellular domains, and some of these have entered Phase III clinical trials [11, 12]. Here we focus on the use of SBPs to modulate intracellular signalling cascades downstream of activated RTKs as these are particularly challenging due to their intracellular nature. SBPs can also be used as imaging reagents against intracellular proteins as recently reviewed by Bedford et al. [50] although this topic is not covered here.

Mitogen-Activated Protein Kinase Pathway

The mitogen-activated protein kinase (MAPK) pathway (Figure 2) can be considered the "classical" signal transduction pathway and remains the most studied [51]. Dysregulation of this pathway occurs in about one third of all human cancers [51] and the Ras family members (H-Ras, K-Ras and N-Ras) are key in this transduction cascade. These proteins are small GTPases which cycle between inactive GDP-bound and active GTP-bound forms (**Figure 3**). Ras isoforms are mutated in approximately 30% of all human cancers, commonly causing decreased hydrolysis of GTP leading to constitutive activation [52], in theory making Ras an excellent candidate for anti-

cancer drugs. Unfortunately, Ras has proved to be a particularly difficult protein to modulate by traditional drug development approaches and to date no drugs have successfully progressed through clinical trials [52], resulting in Ras being labelled an 'undruggable' protein. This label has arisen for a number of reasons including the pM affinity for GTP and that Ras has more than one mechanism to localise to the plasma membrane [19]. This failure to date has made it an attractive but challenging target for a number of research groups who work with SBPs [52].

The premise that SBPs may be used to target Ras and other intracellular proteins has developed from the use of a single chain variable fragment of an antibody (ScFv), termed an "intrabody" [53]. This was then followed by inhibition of Ras intracellularly with the small protein, iDab6 (a single V_H protein similar to a nanobody)[54]. X-ray crystallography revealed that iDab6 binds in the switch 1 region of H-Ras and prevents the binding of the Ras effector protein Raf1 (cRaf). Functional inhibition was shown in vitro and in vivo using retroviral expression of iDab6 which rescued transformed NIH3T3-EJ cells and tumour formation in nude mice.

Subsequent to these proof of concept studies, Affibodies (SBPs derived from staphylococcal Protein A [16]) have been isolated that also inhibit the Ras:Raf1 interaction in biochemical assays [24] and that displayed low to mid-nanomolar binding affinities for Ras and Raf1. However, only those Affibodies that were isolated against the Ras-binding domain of Raf1 showed dose-dependent inhibition of the Ras:Raf1 PPI in real-time biospecific interaction analysis [24]. Unexpectedly, the Affibodies that showed binding to Ras had no effect in this assay. Interestingly, both

the Ras and Raf-binding Affibodies demonstrated effectiveness at reducing tumour-necrosis factor-α (TNFα)-induced interleukin-6 (IL-6) and prostaglandin E2 production in synovial cells when introduced by either plasmid or protein transfection [23]. In addition, one Affibody, Z_{Ras220}, was shown to inhibit the signal transduction associated with TNFα stimulation by reducing phosphorylation of downstream extracellular signal-regulated kinase (ERK). The Raf1 Affibody also reduced MH7A cell proliferation when delivered as a purified protein, but failed to mediate this effect when introduced in an expression plasmid, despite detectable protein expression [23]. It is interesting that although the Raf1-binding Affibody inhibits the Ras:Raf interaction, it has little effect on ERK activation and its effects on cell proliferation are dependent on delivery method and thus may depend on total levels of the Affibody which were not compared in this study, indicating that further work is needed to fully elucidate the mechanism and specificity of these inhibitors.

The development of novel Ras protein inhibitors has been further investigated by the work of Spencer-Smith et al.[44] who developed a monobody, NS1 (with a scaffold derived from the 10^{th} domain of fibronectin (Table 1) [18]), that binds to H-Ras and K-Ras irrespective of their nucleotide status. In a separate study DARPins (based on an ankyrin repeat scaffold (Table 1) [17]) were isolated, with K27 and K55 respectively inhibiting nucleotide exchange and the Ras:Raf interaction [32]. These studies further highlight the importance of the Ras:Raf PPI, as NS1 binds at a novel allosteric site, the α**4**-β**6**-α**5** region, leading to disruption of Ras dimerization and nanoclustering at the plasma membrane, preventing the binding of cRaf/bRaf [44]. DARPin K55 interacts with both switch regions and its binding to the switch 1 region resembles that of Raf, and directly competes for Raf binding [32]. In a similar manner

to iDab6 and the Ras:Raf Affibodies these binders have been characterised in cell-based assays and show inhibition of downstream phosphorylation of ERK and AKT [32] and also block Ras-driven transformation when expressed in cells [32, 44]. Even more exciting is the ability of DARPin K27 to inhibit nucleotide exchange. With a binding affinity of 4nM for GDP-loaded Ras, this inhibitory ability appears to be due to binding to a region very similar to that of the nucleotide exchange factor Sos, but without causing the conformational change to the switch 1 region that is mediated by Sos binding. Consequently K27 binding "traps" Ras in an inactive conformation [44]. It will be interesting to see whether other SBPs can inhibit nucleotide exchange in similar ways. This work on Ras/Raf has provided important insights into Ras biology, which has recently been supplemented by the development of a monobody inhibitor of isoprenylcysteine carboxyl methyltransferase, the final protein involved in the maturation of Ras [48], demonstrating that SBPs represent useful tools for targeting intracellular proteins both in vitro and in vivo, and showing that intracellular modulation of Ras is not as intransigent a problem as previously thought.

In addition to Ras, SBPs have been isolated against upstream adaptor proteins that link RTK-activation to Ras and against other effector proteins downstream of Ras. For example, Affimer proteins (SBPs based on plant protein phytocystatin and human Stefin A (see Table 1) [19, 28]) have been generated against a number of Grb binding proteins [25] showing high selectivity of binding between isoforms that have approximately 70% sequence identity [55]. These Affimer reagents are specifically targeted to the Grb SH2 domains and are able to isolate their respective full length Grb proteins from U-2 OS cell lysates and are currently being tested for intracellular activity [25].

Downstream of MEK, the MAPK pathway diverges to three families of closely related effectors, ERK, JNK or p38 (Figure 2), all of which have been used to isolate SBPs, although p38 has yet to be followed up by intracellular studies [29, 37, 43]. Both monobody and DARPin binders have been selected against ERK2, and show low nM binding affinities as well as specificity for ERK2 over JNK and p38 proteins [28-30]. The monobody binders demonstrated inhibition of phosphorylation of the downstream protein Elk1 when co-transfected into HEK293 cells that were subsequently stimulated with epidermal growth factor (EGF), but did not inhibit phosphorylation of ERK2 itself. Interestingly they were functional at the whole organism level, leading to formation of multiple vulva in C. elegans transfected with constructs that express monobodies. This developmental process involves loss of function of the ERK homologue Ce-MPK-1 [43]. DARPin binders have taken ERK2 recognition further with versions identified that can distinguish between ERK2 in its unmodified conformation and in its phosphorylated conformation and displaying low nanomolar K_D values for their respective conformers [37]. The ability of these binders to function intracellularly was tested by bioluminescence resonance energy transfer (BRET) in COS7 cells with an increase in BRET signal observed for the pERK2 specific binder when the cells were stimulated with serum [37]. Subsequently, this binder has been developed for use as a biosensor in live cell assays with the addition of a merocyanine dye that demonstrated localisation of pERK2 in the nucleus of serum-stimulated NIH3T3 cells [36]. This work shows that SBPs can detect the smallest but arguably one of the most important protein modifications in live cells and can provide functional read-outs to allow monitoring of real-time pathway activation.

DARPin binders for the c-Jun N-terminal kinase (JNK) family of proteins have also been identified [33]. This family of proteins has three members JNK1, JNK2 and JNK3 each with multiple isoforms that share 80% homology at the protein level [56]. Whilst small molecule inhibitors that display low nM binding affinities are available for the JNKs, they cannot distinguish between the three family members [57]. By contrast the DARPin binders show isoform specificity between the JNK1 and JNK2 isoforms and also possess the desirable characteristics of a 1:1 binding ratio and low nM affinities. In addition, they can inhibit phosphorylation of JNK and its downstream effector c-Jun [33]. The ability of these DARPins to bind the specific JNK isoform endogenously was again demonstrated by BRET in HEK293 cells. Intracellularly they retained their isoform specificity and ability to inhibit JNK phosphorylation [33]. To date several key members of the MAPK pathway have been targeted, demonstrating the utility of SBPs to probe and modulate intracellular signalling pathways with high specificity but without altering the levels of the endogenous target protein.

Phosphoinositide 3-kinase pathway

The phosphoinositide 3-kinase (PI-3K) pathway is a parallel pathway to MAPK that can be activated by RTKs, GPCRs or by Ras (Figure 2). Given that this pathway also regulates cell proliferation and survival, it is not surprising that it is one of the most commonly activated pathways in cancer [58]. The ability of SBPs to modulate components of this pathway have not yet been assessed in detail, with SBPs only isolated against PI-3K. This kinase is a heterodimer consisting of a p110 catalytic subunit and a p85 regulatory subunit, both of which have multiple isoforms [58].

Affimer reagents have been developed to bind to the p85 subunit of PI-3K including those specific for the N-terminal SH2 domain of the p85 α-isoform. These binding proteins show not only isoform specificity, but also domain specificity as they do not cross-react with the C-terminal SH2 domain within the same protein [25]. These Affimers can block p85 function intracellularly as demonstrated by increased Akt phosphorylation in transfected NIH3T3 cells but without disrupting the formation of the p85:p110 complex [25]. This further demonstrates the ability of SBPs to inhibit function without disrupting protein complex formation and/or scaffolding function, a property that is likely to be important in dissecting some regulatory pathways.

Src Signalling

Domain-specific binding illustrates why SBPs represent a new way to complement existing approaches to the study of protein function. Their ability to target individual domains within endogenous proteins should allow determination of the role of those domains in protein function and signalling cascades. An example of this is shown by SBPs that have been selected against SH3 and SH2 domains for several members of the Src family of proteins [38-42]. This family of non-receptor tyrosine kinases has nine members, Src, Yes, Fyn, Fgr, Yrk, Lyn, Blk, Hck, and Lck, that contain both SH2 and SH3 binding domains in addition to their kinase domain and an SH4 domain [59]. Activation of Src is a feature of multiple cancers including breast, pancreatic, liver, lung and colorectal cancer [60]. Indeed 80% of colorectal cancers have elevated Src levels [61], while Yes and Lck have also been implicated in this disease [62]. The relevance of this protein family as cancer targets can be seen from the number of small molecule inhibitors currently undergoing clinical trials, despite the fact that such inhibitors show only modest effects when used as a monotherapy [63]. This

highlights the need for development of further more potent and selective means to studying PPIs. The ability of SBPs to specifically block a particular domain without affecting protein level will ultimately aid in identifying functionally important domains and potentially provide novel therapeutics in their own right, or define new target sites for small molecule design.

Monobody reagents have been identified that bind with high specificity and nM affinities to either the SH3 or the SH2 domains within several family members, notably Src, Fyn, Lyn and Lck, [38-42]. The majority of the SH3-binding reagents contained the classical proline-rich SH3 binding motif xxPxxP whereas the SH2 binding reagents were strongly antagonistic to the substrate pYEEI, but did not show a consensus sequence [38-42]. Interestingly, specificity was greater for the SH3 monobody that lacked the consensus sequence, whilst the binder displaying the highest affinity showed a degree of cross-reactivity to a number of family members, namely Yes, Fyn, Lyn and Lck [41]. The most potent SH3 binders for Src, Fyn and Lyn could bind their respective targets in cell lysates [39-41], but not all binders altered the function of their target as assessed by kinase assay [38, 39]. The utility of these 'non-active' binders was subsequently explored and the most potent of Src SH3 binders, 1F11, which bound with similar affinity to Src intracellular binding partners, was found to preferentially bind activated Src, as the SH3 domain is more exposed in this conformation [38]. These characteristics mean that it is ideally suited for biosensor development and its use as an intracellular fluorescence biosensor was demonstrated by attaching an mCerulean tag and modifying the scaffold backbone for use in live cell imaging [35]. This novel biosensor was used to monitor Src localisation at the cell membrane in PDGF-stimulated mouse NIH3T3 cells, a difficult

location to monitor with good spatiotemporal resolution, leading to the finding that activated Src has an important role in ruffle formation and movement [38]. In contrast to the SH3 binders that have been tested intracellularly and shown to be inactive, the monobody binders for the SH2 domains in the Src family do show intracellular activity. Most recently, an Lck binder has been reported to cause a decrease in phosphorylation of downstream protein Zap70 in stimulated Jurkat cells [42]. These various studies demonstrate that SBPs can bind specifically to individual, highly homologous domains in proteins, and mediate functional effects intracellularly.

Wnt signalling

The Wnt signalling pathway has fundamental roles in embryonic development and regulates processes as diverse as cell fate determination, axis formation and organogenesis [64]. Over the last 20 years it has become clear that Wnt signalling is dysregulated in a number of cancers, notably colorectal but also breast and hepatocellular cancer [65]. This pathway has been targeted with monobody binders against β-catenin and dishevelled (Figure 2) [45]. A β-catenin binder, B8, showed inhibition in a TOPflash reporter assay in HEK293 and DLD1 colorectal cancer cells and this translated to in vivo inhibition of Drosophila wing disc development [45]. This work provides a further demonstration of the range of pathways that can be modulated by SBPs.

Apoptosis

Evasion of apoptosis and cell death is a key hallmark of cancer and is usually the result of a change in the balance of anti-apoptotic and pro-apoptotic signals [1]. SBPs have been developed to target both of these forms of signal with Alphabodies selected

against the anti-apoptotic Mcl-1 [20] and DARPins against the pro-apoptotic caspases [31]. Alphabodies, artificial SBPs of three anti-parallel helices [15], targeting Mcl-1, an anti-apoptotic member of the Bcl-2 family that sequesters the pro-apoptotic Bax [66], show pM binding affinities [20]. Mcl-1 is implicated in a number of cancers including myeloid leukaemia and breast cancer [67] and when tested in NCI-H929 bone marrow cells, the Alphabodies reduced cell viability with IC_{50} values of 0.5-2 µM [20]. This translated to in vivo mouse models with 33% tumour inhibition compared to vehicle controls [20]. In comparison with other SBPs, Alphabodies are cell-penetrative without the need for a plasmid-based delivery mechanism, demonstrating how some SBPs have the potential to be more readily translated to the clinic.

Caspases are a homologous family of proteases that function as the intracellular effectors of apoptosis. They have been targeted by DARPin binders against the various caspases with the exception of caspase 4 [48]. However, not all of the binders were able to modulate caspase function, demonstrating that inhibition/activation is not a prerequisite of binding and emphasising the importance of performing functional assays before undertaking studies to examine SBPs' intracellular properties. Indeed, functional assays can provide insights into the mode of action of SBPs and which may consequently affect the likelihood that a particular SBP will display intracellular effectiveness. For example, the caspase 2 inhibitory DARPin has an allosteric effect that stabilises the enzyme in an inactive conformation [35]. By contrast, the caspase 3 DARPins show competitive inhibition [34] and very similar Ki values to small molecule inhibitors of caspase 3, but greater Ki than the natural substrate XIAP [68]. The caspase 7 binding DARPins show a third mode of action by inhibiting the

cleavage of procaspase 7 to its active caspase 7 form [30]. The caspase 3 and caspase 7 inhibitory DARPins have been tested in combination for in vitro functionality in HeLa cells and showed the ability to reduce by 70-80% both caspase 3 and 7 activity against the synthetic substrate DEVD [30]. This work shows that pro-apoptotic proteins can be targeted by SBPs which will be useful for addressing diseases characterised by overactive apoptotic pathways, such as autoimmune diseases [69]. However, in their current format these binders do not have direct applications for informing cancer therapeutics as they are targeted to inhibit apoptosis, which is a hallmark of cancer where SBPs that activate apoptosis would be more beneficial.

Other cancer protein targets

SBPs have also been isolated against other intracellular proteins and post-translocation modifications (PTMs) implicated in cancer, including monobodies against the SH2 domain of Abl kinase [70, 71], DARPins against tubulin caps [72] and Affimers and monobodies against sumoylated and ubiquitnated proteins [26, 27, 46, 47]. Abl kinase is a non-receptor tyrosine kinase that links external stimuli to cellular responses, notably proliferation, migration and survival [73]. Abl is frequently found as a component of fusion proteins in leukaemias and is responsible for transformation in these cells, but it also occurs in solid tumours with links to resistance in these tumours [73]. Small molecule inhibitors do exist as clinical therapies for Abl-fusion-associated leukaemias, however, they are not specific for Abl and cross-react with other tyrosine kinases [73]. By contrast, the Abl binding monobody, HA4, shows highly specific binding for the SH2 domain of Abl [71]. HA4 has inhibitory effects on phosphorylation of a downstream kinase, paxillin, and

reduced STAT activation when expressed in HEK293 and K562 cells respectively [71]. The effectiveness of this binder was then enhanced by combination with a second binder targeting a distinct epitope to form a tandem binder fusion that has the ability to induce apoptosis in both the K562 cell-line and peripheral blood cells from patients with chronic myeloid leukaemia [70].

Tubulin is a critical cytoskeletal protein that has important roles in mitosis, governing spindle formation and the correct separation of chromosomes. Inhibition of tubulin can induce apoptosis and cell death and tubulin-binding agents have been used as cancer therapeutics for almost 50 years [74]. However, resistance to these agents occurs and the mechanisms by which they induce apoptosis are not fully understood [74]. SBPs have the potential to address these issues. For example, the DARPin binder D1 binds the longitudinal interface β-subunit of tubulin and inhibits tubulin assembly in a biochemical assay [72]. A tandem fusion of two molecules of this binder was shown to inhibit tubulin growth at the + end of microtubules and to induce tubulin depolymerisation in Xenopus egg extract [72]. This depolymerisation due to + end binding is similar to that mediated by the clinically used tubulin-binding agent vinblastine [75] that has a number of side-effects. SBPs could be used to further understand the mechanism of action of tubulin-binding agents.

In addition to targeting specific signalling proteins SBPs have been developed to probe PTMs that can mediate signal transduction, notably sumoylation and ubiquitnation (Figure 4). Both are functionally important in a variety of cancers modifying well-known tumor suppressors and oncogenes such as p53 and mTOR [76-78]. The ability to modulate these processes is providing valuable insights into cancer

biology. To this end Small Ubiquitin-like Modifier (SUMO) proteins have been targeted by both monobodies and Affimers to disrupt the SUMO/SIM (SUMO-interacting motif) interactions with reagents identified that bind in vitro with isoform specificity and nM affinities [26, 47]. These reagents show co-localisation with the appropriate SUMO isoforms in the nuclei of transfected HEK293 cells and the ability to pull-down SUMO isoforms from cell lysates [26, 46]. Despite these similarities, there is one important difference between these reagents - the monobodies inhibit SUMO conjugation whilst the Affimers do not [26, 47], making the latter potential more useful for assessing the impacts of the SUMO/SIM PPI.

Another important PTM is ubiquitination which not only serves to target proteins for degradation, but can also play important roles in cell signalling that are only just being delineated [77, 78]. Ubiquitination can occur as both monoubiquitination and polyubiquitination, the latter having eight linkage variants (Figure 4B). To enable the study of these variations of polyubiquitination, it is necessary to have linkage-specific reagents and to date specific antibodies only exist for five variants; subsequently SBPs could be identified for the remaining linkages [27]. Indeed Affimers have been identified to specifically target two of these untargeted linkages, K6 and K33, with pM affinities. These reagents identified specifically assembled ubiquitin chains in vitro and from cell lysates, especially when used in dimeric forms. The K6-specific Affimers also co-localised with TOM20, a protein known to be polyubiquitinated at this linkage, in HEK293 cells [27]. Pulldown of K6-linked proteins from HEK293 lysate with this Affimer followed by shotgun proteomics identified a number of proteins, including HUWE1 which is overexpressed in several cancers [27, 79],

highlighting the utility of these reagents for gathering further insights into this signalling pathway.

Conclusion

SBPs represent a new avenue to assist in dissecting signalling pathways and exploring cancer biology. As described here they have the ability to bind in a highly specific manner to proteins that engage in PPIs and to PTMs in the intracellular environment. They have the functional capacity to act as modulators, intracellular biosensors and as imaging reagents. They present advantages over other methods for exploring PPIs as they can be rapidly generated in vitro, display high specificity and can bind to sites other than binding pockets that are commonly the target for small molecule effectors. As the protein-protein "interactome" has been reported to involve some 650,000 interactions [6], there is a clear imperative to generate selective probes to facilitate study of PPIs, particularly in relation to diseases, including cancer. We argue that SBPs represent such selective tools. There is now a need to develop catalogues of validated SBPs that target proteins involved in PPIs of importance in diseases such as cancer. There are examples of such development and validation for antibodies by international consortia, including the Structural Genomics Consortium and Human Protein Atlas [80, 81]. It is now timely to develop international consortia to develop and validate highly specific "proteome" reagents based on SBPs. The novelty of SBPs is that, in addition to providing highly specific recognition reagents, they can be used as intracellular probes and modulators. Ideally SBPs can be configured to allow simple direct intracellular delivery to facilitate rapid high throughput screening for

functional clones. Such biotechnological advances will ultimately improve target validation and provide important tools for the drug discovery pipeline.

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Figure Legends

Figure 1. Scaffold-based binding proteins. A) Structure of the SBPs discussed in this review, showing a variety of different scaffolds with different binding domains shown in orange. These examples are an Affibody against HER2 (PBD: 3MZW), the Affimer scaffold (PBD: 4D6T), an Alphabody against IL23 (PBD: 5MJ4), a DARPin against IL4 (PBD: 4YDY) and a monobody against SH2 (4JRG). **B)** Structures of the SBPs shown in complexes with a relevant target protein, with the exceptions of the

Affibody and Alphabody as these have not been crystallised with signalling proteins to date. These examples are an affibody with HER2 (PBD: 3MZW), an Affimer with SUMO (PBD: 5ELJ), an Affimer with diubiquitin (PBD: 5OHV), an Alphabody with IL23 (PBD: 5MJ4), a DARPin with K-Ras (PBD: 502T) a DARPin with ERK2 (PBD: 32U7) and a monobody with H-Ras (5E95). (DARPin - Designed Ankyrin Repeat Protein). PyMOL (Schrödinger, LLC, New York, NY) was used to create structural diagrams.

Figure 2. Common intracellular signalling pathways. Illustration of the five major pathways discussed in this review. The RTK activated pathways of MAPK in purple, PI3K pathway in green, and SRC in pink, which can also be activated by integrins and GPCRs (not shown). The canonical Wnt pathway is shown in orange, while the apoptotic pathways are shown in yellow. Scaffold-based binding proteins have been selected against the proteins shown in bold. (Dsh – dishevelled, β-cat –β-catenin).

Figure 3. Ras - A molecular switch. K-Ras (green) acts as a molecular switch, cycling between inactive GDP-bound, shown associated with catalytic domain of the guanine exchange factor Sos1 (light pink), and active GTP-bound form shown in complex with RBD region of Raf1 (orange). The switch regions of K-Ras are shown in yellow (switch I) and red (switch II) respectively with the NS1 monobody interface (α4, β6, α5) highlighted in blue. GTP is shown in cyan and Mg^{2+} ion as a hot pink sphere. Ras-Sos structure PDB: 1XD2, Ras-Raf structure PDB: 3KUD.

Figure 4. Sumoylation and ubiquitination. A) The processes of SUMO and ubiquitin conjugation to a target protein are very similar, as shown with

SUMO/Ubiquitin binding to an E1 activating enzyme followed by transfer of the SUMO/Ubiquitin protein to an E2 conjugating protein and then an E3 ligase completing the transfer to the target protein. Target proteins are deubiqutinated or the SUMO protein deconjugated by DUB and SENP enzymes respectively [76, 77]. B) A ribbon diagram (PBD: 1UBQ) showing the 8 locations that ubiquitin linkages can form and a selection of their delineated functions [77]. (SUMO – Small Ubiquitin-like Modifier; Ub – Ubiquitin; DUB – deubiquitinating enzyme; SENP - SUMO specific protease).