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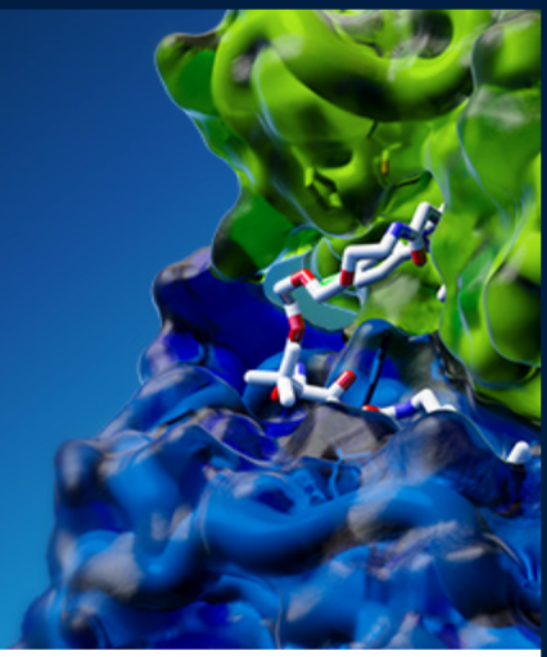

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Received: 18 April 2023 | Revised: 26 August 2023 | Accepted: 29 August 2023

DOI: 10.1002/pro.4774

REVIEW ARTICLE

THE PROTEIN SOCIETY WILEY

Rational design of small-molecule responsive protein switches

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Funding information
Krebsliga Schweiz; National Center of Competence in Research Chemical Biology; Strategic Focus Area "Personalized Health and Related Technologies (PHRT); Swiss Cancer League, Grant/Award Number: KFS-5032-02-2020; Strategic Focus Area "Personalized Health and Related Technologies (PHRT)"; Anniversary Foundation of Swiss Life for Public Health and Medical Research; Swiss National Science Foundation; NCCR in Chemical Biology; NCCR in Molecular Systems Engineering; European Research Council, Grant/Award Number: 716058

Review Editor: Nir Ben-Tal

Abstract

Small-molecule responsive protein switches are powerful tools for controlling cellular processes. These switches are designed to respond rapidly and specifically to their inducer. They have been used in numerous applications, including the regulation of gene expression, post-translational protein modification, and signal transduction. Typically, small-molecule responsive protein switches consist of two proteins that interact with each other in the presence or absence of a small molecule. Recent advances in computational protein design already contributed to the development of protein switches with an expanded range of small-molecule inducers and increasingly sophisticated switch mechanisms. Further progress in the engineering of small-molecule responsive switches is fueled by cutting-edge computational design approaches, which will enable more complex and precise control over cellular processes and advance synthetic biology applications in biotechnology and medicine. Here, we discuss recent milestones and how technological advances are impacting the development of chemical switches.

KEYWORDS

computational protein design, protein switches, protein–ligand interactions, protein–protein interactions, small molecule, synthetic biology

1 | INTRODUCTION

Protein switches are designed to quickly and specifically respond to molecular cues, and are critical tools to control cellular functions in fundamental research and translational studies (Stein and Alexandrov, 2015). Small-molecule responsive protein switches are particularly suitable for remotely controlling engineered cells thanks to their versatile applicability and simple administration. The cell permeability of small molecules plays an essential role in their ability to regulate intracellular functions. Protein switches have been applied to control transcriptional regulation, protein degradation, protein localization, protein transportation, cell receptor engineering, and signaling pathway engineering, among many other purposes.

Sailan Shui, Stephen Buckley, and Leo Scheller contributed equally to this study.

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For example, one of the first known small-molecule-controlled protein switch systems is referred to as a chemically inducible dimerization (CID) complex and is based on the macrolactam natural product FK506 and its derivatives, such as rapamycin. These molecules simultaneously bind to the FK506-binding protein (FKBP12) and the FKBP12-rapamycin-binding (FRB) domain of the FKBP-rapamycin-associated protein (FRAP) (Choi et al., 1996). Fusing FKBP12 and FRB domains to effector domains of interest enables small-molecule-induced dimerization of the complex. However, naturally sourced proteins with such properties are exceedingly rare, with limited chemicals or inducers available. For instance, rapamycin and its analogs used in CID systems are known immunosuppressive drugs and, therefore, suboptimal for several clinical applications (Kolos et al., 2018). Thus, many protein engineering and design approaches are used to facilitate the development of next-generation protein switches to meet application-specific requirements.

Beyond their applications in basic research, protein switches have evolved to a stage where it is important to discuss their potential and limitations for clinical use. We will outline the opportunities, milestones, and current challenges of the described systems regarding their applicability for clinical use. We envision that small-molecule responsive protein switches will increasingly be used to control the activity of protein and cell-based therapies. For protein therapeutics with long half-lives, such as antibodies, or cell therapies, managing activity levels in response to orally bioavailable small molecules could enable more precise dosing and subsequently a superior control of their activity. In addition, we speculate that topical administration of small molecules might help mitigate systemic side effects. For example, CAR-T cells with controllable activity could potentially broaden the therapeutic window of treatments with safety concerns due to on-target, off-tumor activity (Hill et al., 2018). Moreover, incorporating systems for inducible gene expression in cell therapeutics could facilitate on-demand, in-situ production of therapeutic proteins, such as cytokines. Thus, we believe that next-generation protein switches will contribute to more targeted, personalized treatments and represent an exciting frontier in biomedicine.

We will focus on small-molecule responsive protein switches, which comprise of two proteins whose interaction is dependent on the presence or absence of a small molecule. When the small molecule is present, it alters the interactions of proteins (from dissociation to association, or vice versa), thus switching the controlled cellular functions ON or OFF. For this review, we define that protein–protein dissociation facilitated by a small molecule is referred to as an OFF-switch while small-molecule-induced protein–protein association is referred to as an ON-switch. This narrow definition excludes many other systems that can be considered switches but are outside the scope of this review, including GPCRs (Hu et al., 2016), LUMABs (Gräwe and Merkx, 2022), inducible bacterial transcription factors (Resch et al., 2008), hormone receptors (Feil et al., 1996), optogenetic systems (Wehler et al., 2016), and many more. In this review, we discuss various categories of small-molecule controlled protein switches, including naturally-sourced, high-throughput screened, rationally engineered, and computationally designed switches, and we highlight the increasing importance of computational design in the engineering of protein switches.

2 | NATURALLY SOURCED ON-SWITCHES

Homodimerization of FKBP and heterodimerization between FKBP with FRB were some of the first chemically inducible systems (Choi et al., 1996; Spencer et al., 1993) (Figure 1a). This widely known CID based on rapamycin and rapalogs has been explored for various cellular processes at the transcriptional, posttranslational, and signal transduction levels, giving it potential therapeutic applications (Ho et al., 1996; Leung et al., 2019; Spencer et al., 1993; Wu et al., 2015). The protein domains are small, and the dimerization is sensitive, stable, and highly specific to rapamycin (Banaszynski et al., 2005), increasing their versatility. However, this system has several drawbacks including rapamycin's intrinsic immunosuppressive characteristic and ability to bind to native FRB/FKBP in cells, as well as the potentially problematic stability of FRB fusion proteins (Edwards and Wandless, 2007). Rapalogs with reduced cell toxicity or immunosuppressive properties, better pharmacokinetics, and less binding to endogenous proteins are highly desirable. Several promising molecules of this type are now widely used in several research domains (Kolos et al., 2018). Some of them have entered preclinical and clinical studies, raising hopes for human use in the future (Leung et al., 2019).

To generate systems that respond to small molecules that are less toxic and more stable, other popular systems are based on plant proteins. The ABI protein dimerizes with a sextuple mutant of the plant ABA receptor pyrabactin resistance 1 (PYR^{mandi}) in response to the agrochemical mandipropamid (Liang et al., 2011). ABI also dimerizes with the protein PYL in response to the plant hormone abscisic acid (Liang et al., 2011). Similarly, gibberellin, which promotes germination and stem elongation, dimerizes the receptor GID1 with the protein GAI

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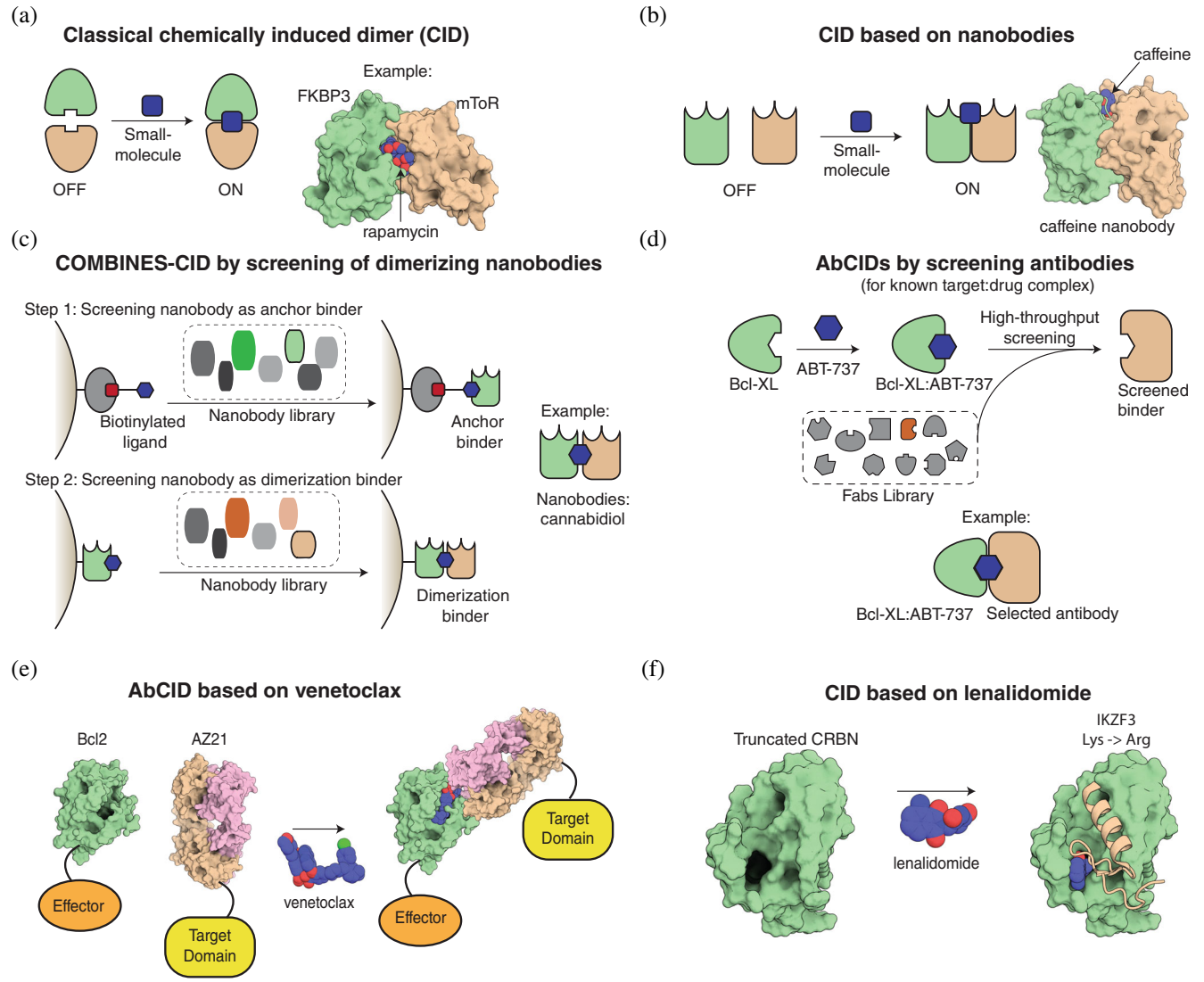

FIGURE 1 Small-molecule responsive switches derived from natural components or high-throughput screening through in vitro evolution. (a) Chemically inducible dimerization (CID) based on FKBP and FRB proteins, which are controlled by rapamycin and its derivatives. The example shows the interaction of FKBP3 (green) and the FRB domain of mToR (beige) in the presence of rapamycin (magenta) (PDB ID: 5GPG). (b) CIDs derived or engineered from nanobody homodimers. The example shows nanobody homodimerization induced by caffeine (PDB ID: 6QTL). (c) Rationally engineered CIDs through screening dimerizing nanobodies by phage display; nanobody libraries were designed with rationally randomized amino acids in their CDR regions: Step 1, select ligand-binding nanobodies for a ligand of interest. A nanobody library is displayed on phages and screened against the biotinylated ligand. Selected nanobodies are captured by streptavidin-coated beads. Step 2, the nanobody selected from the first step is coated on magnetic beads to select dimerization binders in the presence of non-biotinylated ligands. The example shows homodimerizing nanobodies induced by cannabidiol. (d) Engineering of Fab-based CIDs by selecting binders for known target:drug complexes. The Bcl-XL:ABT-737 complex was used to screen human Fabs that can bind to Bcl-XL only in the presence of ABT-737. The example shows a nanobody selected to bind Bcl-XL:ABT-737. (e) AbCIDs controlled by venetoclax. The Fab (AZ21) was selected to bind to the Bcl-2:venetoclax complex. This venetoclax-induced interaction can dimerize fused protein domains, such as a targeting domain (e.g., anti-HER2 Fab) and an effector domain (e.g., a cell engager like anti-CD3 or a half-life extender such as an Fc domain). The schematic structural representations were based on the PDB structures for Bcl-2 (60OK) and an unrelated fab (1F8T). (f) CID based on the FDA-approved drug lenalidomide, where CRBN and the C2H2 zinc finger degron motif are used to construct the lenalidomide-dependent CID degradation system (PDB ID: 6H0F).

(Miyamoto et al., 2012). These protein switches have been used in synthetic biology applications, including targeted protein translocation (Miyamoto et al., 2012), split CAR receptors (Wu et al., 2015), and two-hybrid systems for activating gene expression (Gao et al., 2016). In contrast to rapamycin-based systems, the orthogonality of the proteins and small molecules of plant-sourced switches make off-target effects unlikely. However,

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potential immunogenicity of plant-derived domains might reduce translational potential.

Naturally dimerizing systems have also inspired the design of bivalent ligands to introduce either homo- or hetero-dimerization. Other systems include bis-methotrexate (bisMTX), which homodimerizes human dihydrofolate reductase (DHFR) (Kopytek et al., 2000), coumermycin, which binds the bacterial DNA gyrase subunit B (GyrB) in a stoichiometry of 1:2 (Farrar et al., 1996), and the heterologous bivalent reactive cross-linking molecule (HaXS) which induces the covalent heterodimerization between the SNAP-tag and Halo-tag (Erhart et al., 2013). More recently, the development of proteolysis targeting chimeras (PROTACs), in which a small molecule with two functional domains links a target of interest with E3 ubiquitin ligase (Cermakova and Hodges, 2018), can also be seen as an inducible protein switch with a singular function in degrading targeted proteins.

3 | DEVELOPMENT OF CHEMICALLY INDUCED DIMERS USING IN VITRO EVOLUTION

Naturally sourced proteins are limited by the availability of their inducers. As outlined in the previous section, all naturally sourced protein switches have drawbacks for the development of therapeutic applications. To fill this gap, several high-throughput screening and structure-based engineering approaches were used to expand the array of available ON-switches.

Nanobodies that bind to small molecules can be selected from animal immunization via carrier-protein coupled haptens and, in some cases, can be used to create small-molecule responsive nanobody-based switches (Figure 1b). For example, nanobodies were selected against RR120, a red dye consisting of two RR1 molecules. Each nanobody can bind to a single RR1 molecule (Frenken et al., 2000; Spinelli et al., 2001). This feature was later applied to switchable cytokine receptors that are based on the RR120-induced dimerization of two such nanobodies (Scheller et al., 2018). RR120 is a very cheap, highly soluble, and stable compound. However, for clinical purposes, this very large and negatively charged molecule that has never been tested in humans is not a likely candidate. Caffeine, on the other hand, is one of the best-studied small molecules for human use. It is cheap, highly soluble, stable, has low toxicity, and very rapidly diffuses through the whole body, including the brain, upon consumption. A nanobody selected for caffeine (Ladenson et al., 2006) was found to bind as a homodimer (Sonneson and Horn, 2009), which was an unexpected finding and unusual in response to an asymmetric molecule. The binding mode was studied in a crystal structure, revealing a tyrosine-rich binding pocket and highlighting H-bonds of Tyr104 of both chains that stabilize the interaction (Lesne et al., 2019). A cell-based high-throughput screening method was used to further improve the sensitivity of the system, from an EC_{50} of 560–100 nM (Wang et al., 2021). By now, the caffeine-controlled nanobody dimerization system has been used in an increasing number of applications spanning from controlling cell receptors (Bojar et al., 2018; Chang et al., 2018) and dimerization-dependent enzymes (Scheller et al., 2020) to protein re-localization (Wang et al., 2021). One concern of this system is that homodimerization systems may be less effective in controlling heterodimerization-dependent applications, such as two-hybrid systems for transcription factor-based inducible gene expression or the assembly of split CARs.

Kang et al. (2019) described a phage display approach that selects dimerizing nanobodies for any given ligand. It consists of (i) an "anchor binder" that first binds to the given ligand and (ii) a "dimerization binder" that only binds to the anchor binder:ligand complex but not the unbound anchor binder. It was used with a randomly mutated nanobody library to select binders for cannabidiol that form a ligand-dependent dimerization system (Figure 1c). The authors used these binders to develop diagnostic tests with a limit of detection of 0.8 nM. Cannabidiol is also a highly promising candidate for an inducer with a very favorable safety profile. A possible future direction could be to start the selection process with a humanized nanobody library to facilitate translational use. In a related method, phage display of human fragment antigen-binding regions (Fabs) was used to find binders for the human Bcl-XL protein in the presence of its inhibitor, ABT-737. This type of protein switch is referred to as antibody-based chemically induced dimerizers (AbCIDs) (Hill et al., 2018) (Figure 1d) and has been applied to the chemical induction of CARs in Jurkat T cells and to generate transcriptional switches, both with an EC_{50} in the range of 10 nM. While ABT-737, unfortunately, failed in clinical trials, this study serves as an important proof of concept. Their workflow starts with finding proteins bound to small molecules of which large parts are solvent exposed. This first step ensures that the surface of the protein is sufficiently different in the drug-bound state compared to the drug-free state, enabling the selection of antibodies that only bind to the drug-bound state.

Following a similar approach, it should be possible to generate switches that bind to small molecules that are already FDA-approved. The follow-up from the same group was to apply their approach to the very similar

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Bcl-2 protein bound to the approved drug venetoclax (Martinko et al., 2022) (Figure 1e). While this cancer drug can still have unwanted side effects, this is one of the first candidates suitable for clinical use. The clinically approved drug lenalidomide was also used to control both OFF- and ON-switches. Lenalidomide can dimerize the $CRL4^{CRBN}$ E3 ubiquitin ligase and a C2H2 zinc finger, thus degrading target proteins and turning the respective function OFF (Figure 1f). They also constructed a lenalidomide-dependent dimerization system as an ON-switch in which they truncated the CRBN to diminish the interaction site of DDB1. Moreover, all lysine residues of IKZF3 were mutated to arginine to avoid ubiquitination by the endogenous $CRL4^{CRBN}$ E3 ubiquitin ligase (Jan et al., 2021). Lenalidomide is considered one of the few drugs, like rapamycin (Dagliyan et al., 2013) and venetoclax (Shui et al., 2021), capable of controlling both ON- and OFF-switches.

4 | RATIONALLY ENGINEERED SMALL-MOLECULE CONTROLLED SWITCHES

High-throughput screening-based approaches for directed evolution can select small-molecule binders. However, they are limited by low hit rates due to limited libraries and potential false positives, which need to be eliminated from the selection rounds. Rational engineering of protein switches aims to introduce specific domain arrangements or targeted point mutations to achieve or modulate the desired switching behavior. In this section, we will discuss (i) OFF switches that react to small-molecule binding with protein–protein dissociation and (ii) ON/OFF switches that involve conformational alterations upon binding to small molecules.

The retention using selective hooks (RUSH), a biotin-controlled protein trafficking system, can be considered as an OFF-switch according to our definition, as it relies on induced protein dissociation. It is based on the interaction between a streptavidin-binding peptide (SBP) and the streptavidin protein, which can be disrupted by the potent competitor biotin (Pacheco-Fernandez et al., 2021) (Figure 2a). The very high affinity between streptavidin and biotin makes this system attractive for research settings, but the use of the bacterial protein streptavidin and varying levels of endogenous biotin that is normally circulating in the blood would be problematic for potential therapeutic use. Therefore, OFF switches based on human proteins and approved or experimental drugs are an active field of research. An FKBP mutant was found serendipitously by introducing a single point mutation (Phe36 → Met) in the ligand-binding site that converted the monomeric proteins into a preassembled and ligand-disruptable dimer (Rollins et al., 2000) (Figure 2b). The homodimer has a micromolar affinity and can be dissociated in the presence of rapamycin. This switch faces the same advantages and disadvantages of rapamycin-based systems that were already discussed, and the dimerization affinity of the mutant was very weak, requiring the use of multiple domains for avidity.

Park and colleagues engineered a conditionally active scFv that interacts with tumor-associated antigens (TAAs) in the absence of a clinically approved drug, methotrexate. They integrated the binding site of a methotrexate binding nanobody into the highly homologous heavy chain of an anti-CD33 scFv to generate scFvs that bind to both methotrexate and CD33. Methotrexate binds to the scFv and modulates the CDR's configuration, resulting in an OFF switch (Figure 2c). The chemically controlled antibody was used in a CAR to modulate CAR-T cell activity in response to methotrexate (Park et al., 2021). The toxicity of methotrexate may be limiting for some applications, but the approach has the potential to find or select nanobodies and scFvs for similar switching behaviors for more favorable molecules.

Farrants and colleagues developed an OFF-switch named ligand-modulated antibody fragments (LAMAs) in which a circularly permuted bacterial dihydrofolate reductase (cpDHFR) was integrated into nanobodies to control the binding affinity between the nanobody and its target (Farrants et al., 2020). The cpDHFR is partially unfolded, allowing nanobody:target binding. This interaction can be disrupted by adding the cofactors nicotinamide adenine dinucleotide phosphate (NADPH) and DHFR inhibitors, such as trimethoprim (TMP) (Figure 2d). LAMAs rely on the conformational change of cpDHFR upon binding to TMP and NADPH, which allosterically influences the interaction between the nanobody and its target. This approach was demonstrated using GFP and its binding nanobody and has the potential to be applied to controlling the activities of the many GFP fusion proteins that are routinely used. Another engineered allosteric switch is UniRapR, which fuses insertable FKBP12 (iFKBP12) and FRB domains into a single polypeptide chain (Figure 2e). When inserted into a kinase, UniRapR can allosterically regulate activity dependent on the presence of rapamycin, whose binding decreases the distance between the N and C termini, stabilizing the active conformation of the kinase (Dagliyan et al., 2013, 2019). This paper presents a proof of concept for generating single-chain switches using a domain-insertion strategy. It is particularly effective for structures that involve tight, short loops connecting interacting structural units.

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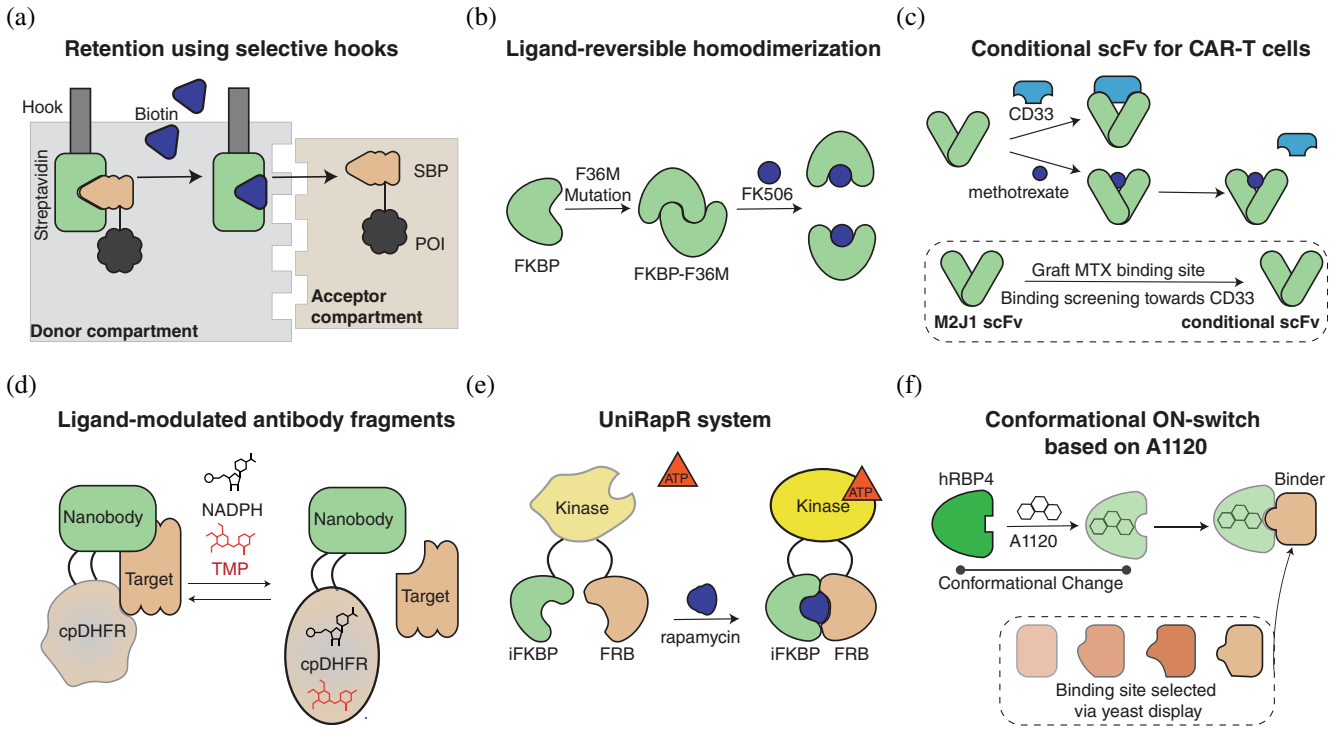

FIGURE 2 Rationally engineered small-molecule-controlled switches. (a) Retention using selective hooks (RUSH) is based on the interaction of streptavidin and streptavidin-binding peptide (SBP), which can be disrupted by biotin. RUSH functions as a controllable protein trafficking system in which the protein of interest can be released from the donor compartment and diffuse to the acceptor compartment upon the addition of biotin. (b) The F36M mutation on the FKBP protein induces ligand reversible protein dimerization. (c) An engineered conditional scFv binds to CD33. The methotrexate binding site is grafted from a nanobody to M2J1-scFv and followed with several rounds of yeast display against CD33 to select the conditional scFv that binds to CD33 and can be disrupted by methotrexate. (d) Ligand-modulated antibody fragments (LAMAs) are based on the conformational change of cpDHFR after binding to TMP and NADPH. When integrated into a nanobody, the conformational change can allosterically disrupt the interaction between the nanobody and its target protein. (e) UniRapR was engineered by rationally fusing the insertable FKBP (iFKBP), FRB, and a kinase into a single chain. The conformational change induced by rapamycin binding controls kinase stability and allosterically modulates the kinase's ATP binding site. (f) Conformational ON-switch based on A1120. The hRBP4 changes its conformation upon binding to A1120; this feature is used to design a specific binder that only interacts with the hRBP4:A1120 complex.

Recently, Zajc et al. presented another allosteric CID system responding to the investigational drug A1120 and used it to control CAR-T cell functions (Zajc et al., 2020). The human retinol-binding protein 4 (hRBP4) undergoes conformational changes when bound to A1120 (Figure 2f). Binders from a randomly mutated scaffold library were selected for interacting with the hRBP4:A1120 complex. These binders showed specific interactions with hRBP4 in the presence of A1120, as demonstrated by a 500-fold increase (from ~5 to ~10 nM) in binding affinity. This switch was used on the extracellular site of a split CAR to recruit the antigen recognition domain in response to A1120. Despite only a two to three-fold increase in target cell killing in response to A1120 and even though A1120 is not yet approved for clinical use, this study illustrates the development of protein switches based on conformational changes in hRBP4.

5 | COMPUTATIONALLY DESIGNED PROTEIN SWITCHES

Foight et al. (2019) computationally designed CIDs based on the viral NS3a protease and its inhibitors. The procedure started with selecting suitable scaffolds to bind to the interface of the NS3a:drug-bound complex using PatchDock/RIFDock. Then, Rosetta interface design and yeast-display screening were combined to optimize the binding affinity between the selected scaffold and the NS3a:drug complex (Figure 3a). They designed binders to specifically interact with NS3a in the presence of the drugs danoprevir and grazoprevir, therefore functioning as CIDs for their respective drug, while the other drug functioned as a competitive inhibitor. These switches all use the NS3a protease, but can be used with orthogonal inducers and designed binders. While the overall models for these designed proteins only

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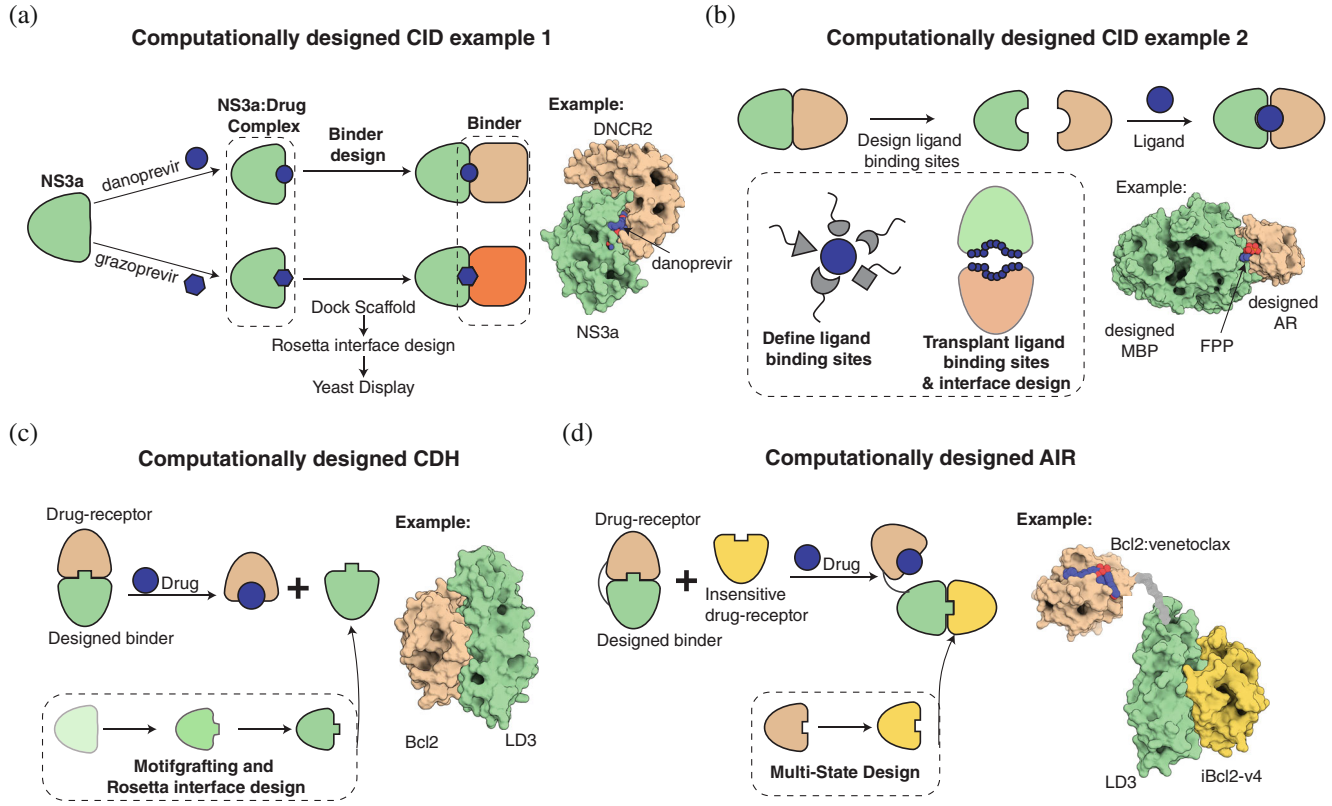

FIGURE 3 Computationally designed protein switches. (a) Computationally designed CID based on NS3a protease and its inhibitors. The binders were designed with steps of docking and scaffold design to bind to the NS3a:drug complex, and screened by yeast display to optimize the binding affinity and specificity. The example shows DNCR2 (beige) in complex with NS3a (green) in the presence of danoprevir (blue and red) (PDB ID: 6N4N). (b) Computationally designed CID through ligand binding site transplantation. The binding site of the ligand of interest is defined and grafted to existing dimers. The example shows the designed AR (ankyrin repeat; beige) and designed MBP (green), in the presence of the ligand FPP (blue and red) (PDB ID: 6OB5). (c) Computationally designed CDH based on known receptor:drug complexes through motif-grafting and Rosetta interface sequence design. The example shows Bcl-2 (beige) and designed LD3 (green) which can be disrupted by a drug (PDB ID: 6IWB). (d) Activation by inhibition release switches (AIRs) which converts CDHs into ON-switches. They consist of a single-chain CDH and an insensitive drug-receptor. Upon drug addition, the preassembled CDH will open and release the designed binder to interact with the insensitive drug-receptor. The example shows that the LD3 protein (green) binds to the iBcl2-v4 design (gold) while venetoclax (blue and red) inhibits binding to the wild type Bcl-2 protein (beige). CDHs, chemically disruptable heterodimers; CID, chemically inducible dimerization; FPP, farnesyl pyrophosphate.

moderately aligned to the determined structures, the interface residue predictions were more accurate. Another example of rational CID design was performed by Glasgow et al. (2019) by transplanting the binding site of a ligand to an existing protein dimer (Figure 3b). The ligand-binding site for farnesyl pyrophosphate (FPP) was transplanted to the interface of a protein–protein heterodimer complex. A few designs reached a >100-fold binding increase in response to FPP. Although computational and experimental optimization of this approach was complex and time-consuming, it represented a first step towards computational CID design that will benefit from the rapidly improving computational methodologies.

The *chemically disruptable heterodimers* (CDHs) from our own work were designed based on known drug-receptor:motif interactions and their pre-clinically or clinically approved small-molecule inhibitors, including Bcl-XL:BH3, Bcl-2:BH3, and mdm2:p53 interactions. The BH3 or p53 motif was computationally grafted onto an unrelated protein to enable the interaction with the respective drug-receptors (Figure 3c). This approach stabilizes the peptide motif and improves the interaction strength with drug-receptors by presenting a more restrained conformation of the peptide motif and incorporating additional interactions from interface residues. It resulted in low nanomolar to picomolar binding affinity between binders and drug-receptors. The chemical inhibitors compete with the motif-presenting designs for binding the target interface, leading to the disruption of the complex. These CDHs were used to control cell surface receptor activity, gene transcription, and split CARs (Giordano-Attianese et al., 2020; Shui et al., 2021). One

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CDH is based on Bcl-2 and the FDA-approved drug venetoclax, making this the OFF-switch counterpart to the AbCIDs described above. To generate ON-switches, the CDHs were converted into a multi-domain architecture named activation by inhibitor release switches (AIRs) (Shui et al., 2021). Each AIR switch consists of fused CDH domains and a rationally designed insensitive drug-receptor. Upon the drug stimulation, the fused CDH will open up and free the designed binder to associate with the insensitive drug-receptor (Figure 3d). This presents a generalizable OFF- and ON-switch architecture based on known drug-receptor:motif interactions.

6 | COMPUTATIONAL DESIGN FOR NEXT-GENERATION SMALL-MOLECULE PROTEIN SWITCHES

With all the described small-molecule responsive protein switches, engineering problems often fall into two categories: (i) predict and incorporate conformational changes for allostery-based switches, and (ii) design specific chemically controlled protein–protein interactions for proximity-based switches.

Many current approaches for protein design build on AlphaFold2 (AF2), a deep learning-based protein sequence to structure prediction program (Jumper et al., 2021). AF2 was trained on structures in the PDB and uses multiple sequence alignments (MSAs) to determine the coevolutionary relationship between amino acid pairs. These MSAs have been adjusted to predict multiple possible conformations from a single input amino acid sequence (del Alamo et al. 2022; Stein and Mchaourab, 2022; Wayment-Steele et al., 2022). The ability to accurately model multiple conformations of a protein will allow for more realistic depictions of the energetic landscape of proteins and grant protein designers the opportunity to design switches based on a protein's possible conformations. This ability to simulate the different conformational states of a protein would facilitate the design of proteins that regulate activity through structural changes. It also creates the opportunity to target the protein through sites that may not be accessible in current structure prediction algorithms that output a single conformation. The emergence of structure-based drug design (SBDD) and computer-aided drug design (CADD) would complement multi-state protein structure prediction where a de novo designed site-specific small molecule could drive a population shift towards a specific conformation. In addition, molecular dynamics (MDs) simulations will likely be increasingly used, with programs like CHARMM (Brooks et al., 2009), AMBER (Wang et al., 2004), and GROMACS (Van Der Spoel et al., 2005) capable of modeling both protein:small-molecule docking and protein conformational changes (Leman et al., 2020). However, MD simulations are limited in that they are computationally expensive, and running simulations at potentially relevant timescales of protein conformational changes (sometimes milliseconds to seconds) is currently unachievable (Jin et al., 2021). Recently, some labs have started to address these MD limitations by applying deep learning approaches coupled with physics-based weights to more efficiently sample protein conformations within an energetic landscape (Jin et al., 2021; Ramaswamy et al., 2021).

For proximity-based switches, it is key to design proteins that bind to specific sites of target proteins or target protein:small-molecule complexes and to yield PPIs that are either dependent on or disrupted by a small molecule. For instance, CDHs can be developed by designing a protein binder that competitively binds to a small-molecule binding site. Therefore, the presence of a small molecule will inhibit the interaction between the target and binder proteins (Figure 3d). In contrast, CIDs need the extra step of incorporating a small molecule into the design pipeline, as the designed protein binder should bind specifically to the target protein at the drug-bound interface (Figure 3b). Recent breakthroughs in the development of computational methods have increased the success rates of designed binders to specific sites of target proteins. For example, Cao et al. (2022) have designed site-specific de novo protein binders by docking billions of disembodied amino acid rotamers onto the target's site of interest to find suitable binding motifs. This is one of the first publications that incorporated de novo motif generation with a mini-protein scaffold library to develop protein-based therapeutics. This approach will help to design binders to target proteins independent of existing binding partners. Different from rotamer docking, the MaSIF approach from our own work generates fingerprints of protein surface patches that describe geometric and chemical features (Gainza et al., 2020). This approach uses a geometric deep learning algorithm to identify surface patches that have a high propensity to be targeted by a protein binder. Once a target patch is identified, MaSIF-seed can identify motifs from a protein fragment library that are complementary to the target patch of interest, using multiple metrics such as shape complementarity, hydrophobicity, and electrostatic satisfaction (Gainza et al., 2023). Such site-specific approaches will be crucial for future development of tools capable of designing PPIs dependent on the presence or absence of a small molecule.

Both site-specific methods ultimately rely on using Rosetta's motif grafting method to incorporate the complementary motif onto a supporting scaffold, making the pool of available scaffolds a limiting factor for these

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approaches. It would therefore be highly advantageous to be able to design tailor-made de novo scaffolds. Early examples to investigate this approach include FunFolDes and TopoBuilder, which have stabilized motifs of interest by building a series of secondary structures around them to create stable scaffolds (Bonet et al., 2018; Yang et al., 2021). Recently, methods based on machine learning algorithms, such as protein hallucination and inpainting, have been shown to be capable of generating de novo proteins around a defined motif of interest. Protein hallucination leverages the structure prediction network trRosetta to optimize inter-residue distances by starting with a random amino acid sequence, iteratively mutating random residues, and selecting changes that increase the resolution of an inter-residue distance map to create a de novo protein (Anishchenko et al., 2021). Alternatively, inpainting was trained to fill in both sequence and structure of missing parts of a protein through its network that was trained on masked protein segments (Wang et al., 2022). However, these methods have limitations, such as protein hallucination being computationally expensive and inpainting, while much faster, struggles with small functional sites and is deterministic, limiting the number of potential designs.

These limitations have been recently addressed by incorporating denoising diffusion probabilistic models (DPPMs). RFDiffusion is a Rosetta-based deep learning network that uses DPPMs to de-noise randomness until a pattern is recognized to generate entirely de novo proteins, scaffold structures around motifs of interest that look like real proteins, and design de novo binders with very high success rates (Torres et al., 2022; Watson et al., 2022). An amino acid sequence is then generated by ProteinMPNN (protein message passing neural network), a deep learning-based sequence predictor for specified protein folds (Ingraham et al., 2019; Dauparas et al., 2022). Diffusion overcomes many challenges from the previously mentioned approaches by its ability to generate a binding motif to a specific target of interest or to generate a suitable de novo scaffold for an existing motif while also being computationally efficient. To date, this approach has generated the highest affinity protein binders, designed completely computationally without further experimentation needed. We anticipate that a pipeline consisting of diffusion scaffold generation, small-molecule incorporation, and site specificity will ultimately be suitable for the design of small-molecule-induced protein switches.

7 | DISCUSSION AND OUTLOOK

The time scale of cellular responses of protein-based switches has the potential to be orders of magnitude faster than genetically dependent switches, which are reliant on both transcription and translation. This improved speed can be vital for biochemical readouts such as imaging, environmental monitoring, or in some clinical settings (Adamson and Jeuken, 2020; Adeniran et al., 2018; Marchand et al., 2023). Protein switches of natural origin are likely to continue to be relevant, as such systems have already evolved to perform well with many relevant molecules. These proteins are of particular interest for potential clinical applications if they are humanized or derived from human proteins, where the hurdles due to potential immunogenicity are likely to be lower.

We are already observing an increasing blending of the rough categorizations of protein switches that we used in this review, such as naturally sourced, found by high throughput screening, engineered, or de novo designed. We expect this trend to continue, especially through the integration of user-friendly computational tools, such as ProteinMPNN and ColabFold (Mirdita et al., 2022), into the more traditional switch-building pipelines. Machine learning will play a critical role in advancing small-molecule-based de novo protein switches, but the models they create are still mostly static in nature, which is particularly limiting for allosteric switch design. While a protein has a specific sequence, it may have multiple energetically stable conformations, which may or may not be dependent upon a stimulus. While some progress has been made (Norn et al., 2021; Sala et al., 2022), future protein design will model conformational changes in response to small molecules through approaches such as newly developed Rosetta methods, incorporation of MD simulations, and fine-tuning the weights of protein structure prediction neural networks.

Over the next few years, we expect deep learning-based technologies to develop to the point where a de novo protein can be designed to target a specific protein:small-molecule interface only present once a conformational change takes place or a protein interface that only becomes accessible after a small-molecule-induced conformational change; addressing the current limitation of modeling multi-state energy levels controlled by a chemical input. Such technologies will also facilitate the development of tailor-made small-molecule-based protein switches for prespecified purposes. For example, a small-molecule-induced protein switch-based therapeutic could be designed for a clinically approved small molecule of choice.

Prediction of affinities of PPIs and protein:small-molecule interactions must be improved in the next generations of algorithms to be able to rank designs in order of affinity. Such a feature would be specifically useful for CIDs where high affinity to the small molecule is necessary, and often low-affinity interactions between the

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protein partners stabilize the complex (Rube et al., 2022; Volkov et al., 2022; Yasuda et al., 2022). These approaches will greatly profit from the tools developed in the emerging field of CADD. Ideal computational tools for designing protein components of small-molecule-induced protein switches should be fast, accurate, target specific sites, incorporate and dock small molecules, and portray the conformational changes based upon stimuli. Such tools will enable researchers with the capability to design de novo protein switches for purposes such as next-generation switchable therapeutics and rapid biosensors. Moreover, these tools should also be capable of modeling other inducible elements such as temperature, light, pH, and posttranslational modifications. Over the coming years, an integrative approach will be facilitated by the deep-learning methods mentioned and ones yet to be developed to revolutionize complex tasks as de novo protein switch design as well as many other challenges.

ACKNOWLEDGMENTS

Sailan Shui was supported by Swiss Cancer League grant no. KFS-5032-02-2020. Leo Scheller was supported by the grant #2021-446 of the Strategic Focus Area "Personalized Health and Related Technologies (PHRT)" of the ETH Domain and by the Anniversary Foundation of Swiss Life for Public Health and Medical Research. Bruno E. Correia was supported by the Swiss National Science Foundation, the NCCR in Chemical Biology, the NCCR in Molecular Systems Engineering, the Swiss Cancer League grant no. KFS-5032-02-2020, and the ERC Starting grant no. 716058. Open access funding provided by Ecole Polytechnique Federale de Lausanne.

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How to cite this article: Shui S, Buckley S, Scheller L, Correia BE. Rational design of small-molecule responsive protein switches. Protein Science. 2023;32(10):e4774. https://doi.org/10.1002/pro.4774

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