Review Article

Synthetic Biology for Designing Allostery and Its Potential Biomedical Applications ☆

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Abstract

Allosteric regulation of protein function, where a perturbation at one site induces a conformational shift or alters dynamics at a distal functional site, plays a key role in numerous biological processes. The ability to introduce allostery using synthetic biology principles holds significant potential both for biomedical and biotechnological applications, and for advancing our understanding of natural allostery. By customizing target proteins for sensing specific chemical or physical signals, including ligand binding and environmental cues, we aim to allosterically modulate the function of a target protein depending on the selected triggers. This approach, unlike active-site targeting, offers greater specificity and selectivity and can allosterically couple diverse physiological processes. Synthetic biology strategies have been developed recently for designed allosteric protein regulation, including the design of allosteric modulators such as domain insertion, generation of *de novo* allosteric protein switches, and application of engineered allosteric mechanisms to control cellular functions. We examine the application of artificial intelligence (AI)-based generative protein design and other important milestones, challenges and opportunities in this field, highlighting how these approaches could be applied for the development of new therapeutic strategies.

Introduction

Allostery involves the modulation of protein function triggered by binding of an effector molecule or through physical interaction (e.g. light absorption) at a distal site from the functional site of the target protein [1]. This modulation can affect catalysis, binding, and other protein functions, playing a pivotal role in diverse cellular processes such as signal transduction, metabolism, and gene regulation, enabling precise control over protein activity and cellular processes [2–4]. Our understanding of allostery has expanded significantly, revealing its profound role in both normal cellular functions and disease states. Dysregulation of allostery is implicated in diseases including diabetes [5], phenylketonuria [6] or various types of cancers [7], which makes it an attractive target for synthetic biology interventions, particularly for developing novel biomedical tools.

This review highlights recent progress in synthetic biology strategies aimed at the rational design of allosteric mechanisms. Allosteric control has been primarily achieved through point mutations, often relying on random mutations and directed evolution [8,9]. However, domain insertion engineering has more recently become a productive

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rational strategy, enabling the design of allosteric proteins across diverse target proteins and applications [10,11]. This strategy involves genetically inserting a domain into a target protein via molecular cloning, resulting in a chimeric protein whose activity is modulated by a regulator. The allosteric effect is mediated by the inserted domain, whose engagement, whether through regulator binding or a physical effect, triggers a conformational change that alters the host protein's activity. Several insertion domains have been employed for allosteric protein regulation, including coiled coils [12], LOV2, FKBP12 [13], calmodulin (CaM) [14,15], PDZ domain, estradiol binding domain, nanobodies and yellow fluorescent protein [16].

Beyond regulating natural proteins, the engineering of *de novo* synthetic protein switches [17] represents an emerging approach for engineered allosteric regulation. This strategy involves the design of modular and tunable protein systems, with switchable conformation states that are controlled by external inputs [18]. The modularity of these designs allows for specific input signals to be coupled to various output responses, allowing for tailored outputs without the need of redesigning the entire system [19]. This approach involves construction of protein switches based on domain displacement [18], as well as assemblies with switchable functions controlled by binding of effector molecules [17]. These assemblies can be designed to incorporate or eject subunits in response to peptide binding, offering the potential for modular protein delivery.

The review discusses the challenges and opportunities in this field, emphasizing the potential of synthetic biology approaches to drive the development toward new biomedical applications. The increasing integration of computational tools, high-throughput screening technologies and artificial intelligence (AI)/machine learning is crucial for accelerating the discovery and design of allosteric modulators and protein switches. The continued advancement of research in this area promises transformative progress in the application of allosteric regulation.

Light- or Small Molecule-regulated Protein Allosteric Control

Light-sensitive domains have been widely employed in the design of synthetic allosteric modulators, where naturally occurring or engineered light-responsive modules couple light input to the regulation of diverse protein functions, enabling rapid and spatially controlled modulation. Coyote-Maestas et al. [20] used a human inward rectifier K^+ channel to map site-specific permissibility for the domain insertion (Figure 1a). They rendered Kir2.1 activity sensitive to light by inserting a light-responsive Cib81 domain into sites with latent allosteric capacity. Their findings suggest that mapping differential permissibility could serve as a generalizable method for guiding the *de novo* engineering of allosteric regulation in any protein.

Alternatively, light-oxygen-voltage (LOV) domains, one of the most widely used domains for optogenetic regulation, are characterized by a dynamic equilibrium that shifts from the dark-adapted to the light-activated state upon blue-light exposure [21]. Hongdusit et al. [22,23] reported a light-activated method to control protein tyrosine phosphatase 1B (PTP1B), a key regulator of signaling pathways implicated in diabetes, obesity, and cancer. By fusing a LOV2 domain with a regulatory element of PTP1B, they preserved the enzyme's activity, and cellular localization (Figure 1b). In the absence of light, the fused helix allowed normal PTP1B activity without significantly affecting the catalytic domain. However, illumination with blue light induces unwinding of LOV2 terminal helices, destabilizing the PTP1B's α7 helix and disrupting the WPD loop—a region essential for its catalytic activity. This structural distortion facilitated the creation of a photo-inhibited PTP1B. Insertion of a light-sensitive LOV2 domain was also employed by Chen et al. [24], who reported an improved immune cell infiltration into solid tumors by allosteric manipulation of septin-7 functions in mammalian cells. Septin proteins are involved in critical processes such as cell division, membrane dynamics and cytoskeletal organization [25]. The researchers engineered a light-controllable septin-7 hybrid protein (LCS7) by insertion of a blue light-sensitive LOV2 domain. They found that blue-light induced inhibition of LCS7 in live cells causes extended cell protrusions and enhanced cell polarization, ultimately improving cell transmigration efficiency. They demonstrated the potential of their construct for cell-based immunotherapies by showing that LCS7-expressing human natural killer cells (NK92) and mouse primary CD8+ T-cells exhibit increased penetration and cytotoxicity in tumor spheroid models.

Allosteric regulation of kinase activity in living cells has been extensively studied [26,27]. Researchers have developed strategies to allosterically regulate kinase activity within living cells, enabling precise control over their catalytic function. Zhou et al. [28] described the design and application of single-chain cofactor-free kinases with photo-switchable activity. The authors engineered a dimeric protein, pdDronpa, which dissociates in cyan light and re-associates in violet light (Figure 1c). By attaching two pdDronpa domains at selected locations in the kinase domain, they created photoswitchable kinases psRaf1, psMEK1, psMEK2, and psCDK5. Using photoswitchable kinases, the authors established an optical cell-based assay for screening inhibitors, uncovered a direct and rapid inhibitory feedback loop from ERK to MEK1, and mediated developmental changes and synaptic vesicle transport *in vivo* using light. Shaaya et al. [29] developed an optogenetic tool

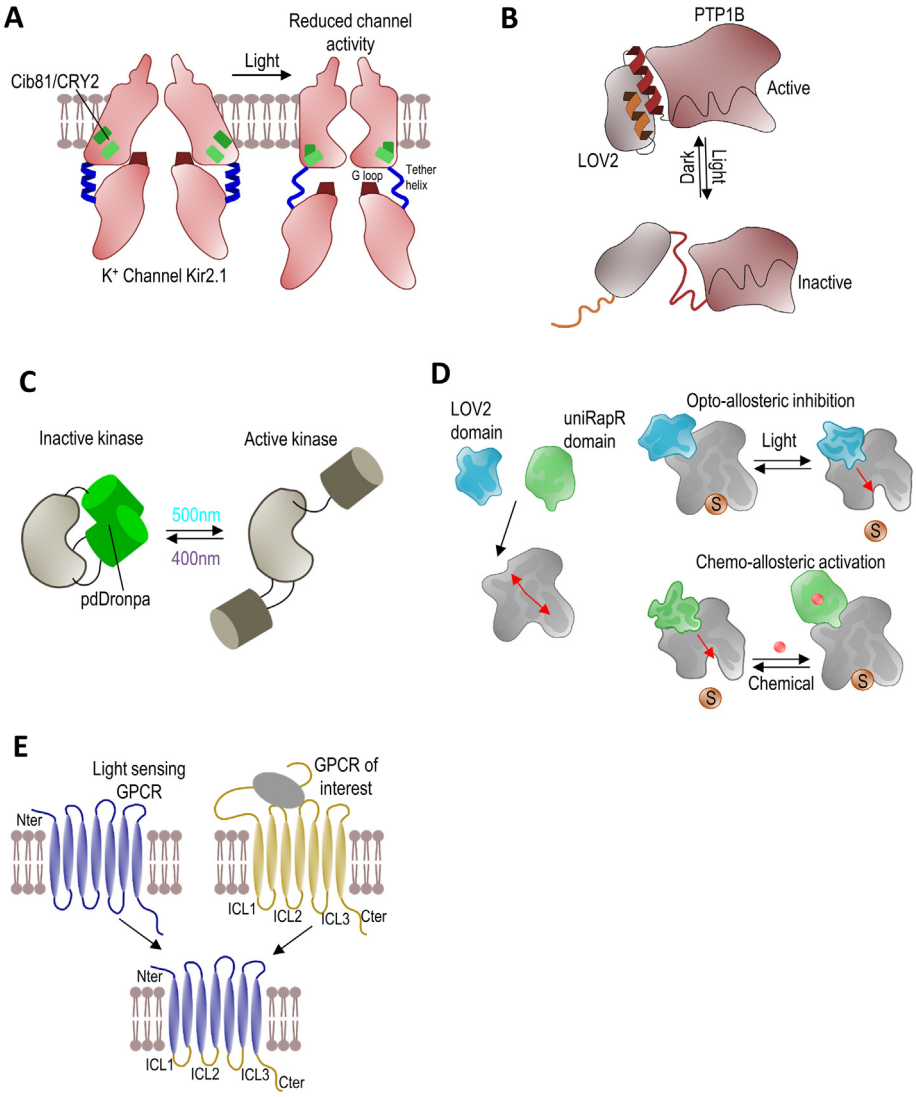


Figure 1. Schematic representation of allosteric regulation of protein function through domain insertion, enabling light or small molecule-induced conformational changes. A. Light-dependent modulation of K+ channel Kir2.1 involves inserting a cryptochrome-interacting basic-helix-loop-helix (Cib81) into target sites. In the channel open state, the tether helix links the C-terminal to the transmembrane domain (TMD), bringing them into close proximity and positioning the G-loop within the TMD. Upon light exposure, Cib81 dimerizes with CRY2, inducing a shift of the channel into a closed state. Adapted from Coyote-Maestas et al. [20]. B. The optogenetic regulation of PTP1B is achieved by fusing a light-oxygen-voltage 2 (LOV2) domain to its C-terminus. Blue light triggers the unwinding of the LOV2 terminal helices, inducing a conformational change that destabilizes PTP1B, suppressing its catalytic activity. Adapted from Hongdusit et al. [23]. C. Single-chain kinases with photoswitchable activity designed by fusion of an engineered photodissociable dimeric Dronpa domain (pdDronpa) to rationally selected sites. Under violet light, pdDronpa dimerizes, inhibiting kinase activity. Exposure to cyan light induces pdDronpa dissociation, restoring protein function. Adapted from Zhou et al. [28]. D. The opto- or chemo-allosteric control of proteins involves incorporating a light-sensitive LOV2 domain or a ligand-binding uniRapR domain into a surface-exposed loop of target protein. The LOV2 domain inhibits catalytic activity by disrupting substrate (S) binding upon light exposure, while the uniRapR domain enables activation through small-molecule binding (pink). Adapted from Dagliyanet al. [31]. E. The design of light-activated chimeric GPCRs involves substituting the C-terminal domain and intracellular loops (ICL1-3) of a light-sensing GPCR with the corresponding regions of a target GPCR to confer light-responsive signaling. Adapted from Tichy et al. [32].

designed to achieve precise spatiotemporal control of enzymatic activity using a light-sensitive allosteric switch module named Light-Regulated (LightR) domain. Composed of two tandemly connected Vivid (VVD) photoreceptor domains, it acts as a clamp that, in the dark, opens to distort the protein

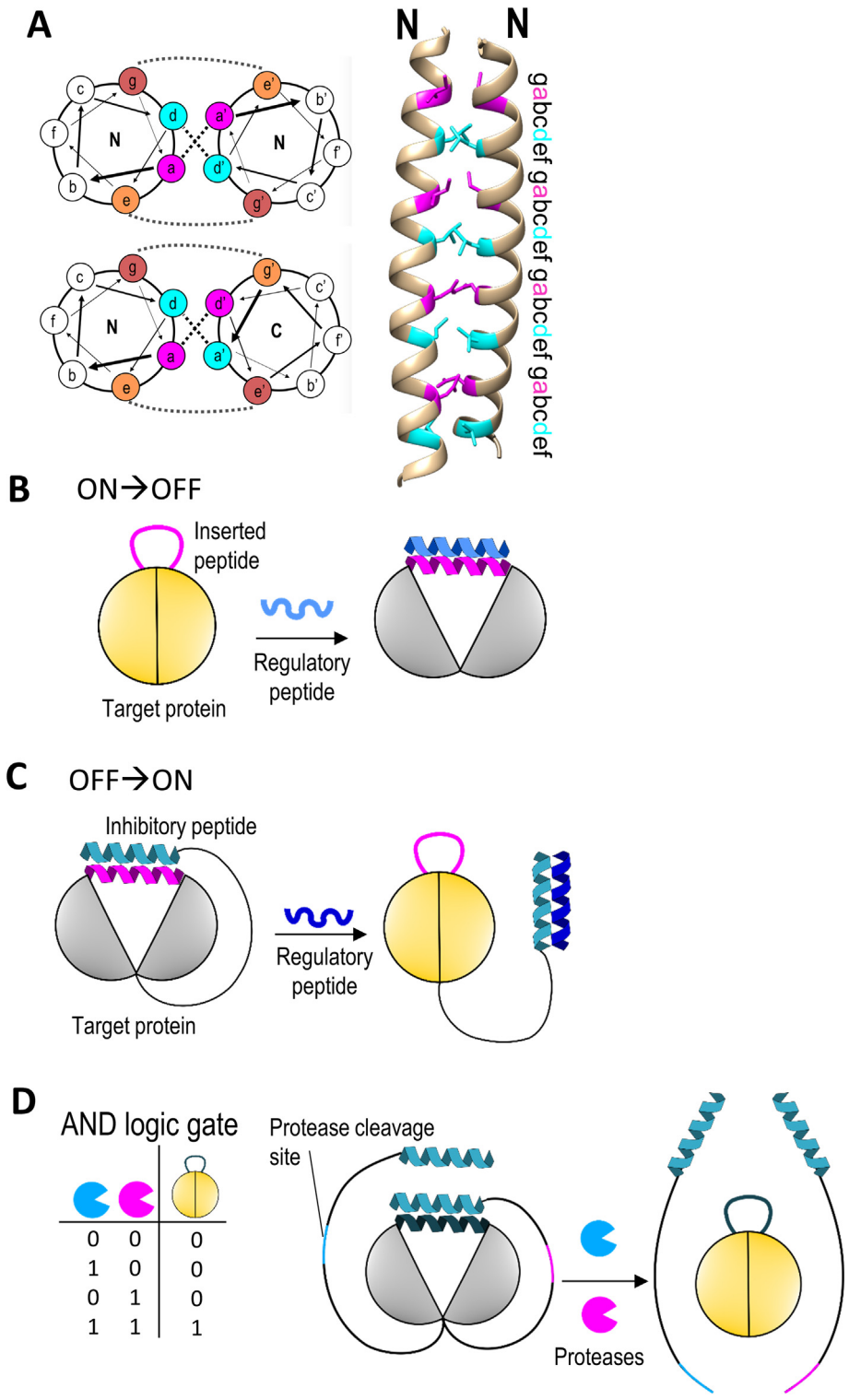
and render it inactive, while in response to blue light, it closes to restore the protein's native structure and activity. The utility of LightR was demonstrated by engineering light-regulated variants of Src, Abl, and bRaf kinases, as well as Cre recombinase. Karginov et al. [30] engineered kinases by inserting a modified FKBP12 protein fragment (iFKBP) into the kinase domain, which inactivates the kinase. Its activity is then restored through rapamycin, providing a means for precise temporal and spatial control. This approach was demonstrated using FAK, Src, and p38 kinases, with evidence suggesting that iFKBP insertion increases the flexibility of the catalytic domain, while rapamycin binding promotes rigidity. In a related approach, Dagliyan et al. [31] inserted light- or drug-responsive domains into surface-exposed loops of proteins that were computationally predicted to be allosterically coupled to active sites. This strategy allows for the modulation of structural disorder to create robust allosteric switches. For example, Src kinase activity was inhibited using a light-responsive LOV2 domain and guanine exchange factors (GEFs) were activated with a rapamycin-responsive domain (uniRapR) (Figure 1d). Collectively, these studies illustrate the utility of allosteric regulation in manipulating kinase activity, employing distinct methods such as drug-inducible domain insertion [30] and light- or drug-responsive domains in surface loops [31].

Chimeric G-protein coupled receptors (GPCRs) are another example of engineered allosteric modulation of protein function. GPCRs are involved in a wide range of physiological processes and serve as important therapeutic targets. Their function relies on structural rearrangements of receptor domains upon stimulation, which allosterically modulate downstream signalling. Different GPCRs respond to various stimuli, including light, and their structural similarity has been leveraged to design custom-tailored inputs and outputs in GPCR signalling [1]. In this manner, light-activated chimeric G protein-coupled receptors (GPCRs) called OptoXRs were created by replacing intracellular domains of a GPCR of interest with corresponding domains from a light-sensitive GPCR, like rhodopsin, which allows control of GPCR with light (Figure 1e). OptoXRs have been used to study various cellular processes, including neural circuits, cell migration, and vision restoration. Additionally, they have proven valuable in studying orphan GPCRs, for which ligands and downstream signalling pathways are unknown [32,33].

Designed Coiled Coil Mediated Allosteric Control and Introduction of Protein Logic

A strategy for regulating protein function through a method called INSRTR (Insertion of a peptide to regulate protein function) was published by Plaper et al. [12]. This method involves inserting a coiled coil (CC) forming peptide into a loop of a target protein in a way that the protein retains its function and is regulated by the formation of CC dimers. CC peptides are a common structural motif in proteins [34], present in approximately 8 % of proteins involved in various biological functions. They possess an aminoacid heptad repeat pattern that forms an amphipathic structure (Figure 2a). This arrangement of alternating hydrophobic and polar residues drives the assembly of stable CCs, where the hydrophobic residues form a protected core, and polar residues contribute to stability and selective interactions [35,36]. By understanding these rules, CC peptides can be designed with specific properties, such as tunable stability and orthogonal pairing, making them attractive as allosteric modulators. Compared to similar strategies of domain insertion, such as photosensitive domains, rapamycin binding domain [13] or calmodulin [14,37], they are much smaller, allowing for weaker structure perturbation and a larger number of permissible insertion sites. Their well-defined design rules enable orthogonality, allowing the peptides to exclusively interact with their designed binding partners. This minimizes unintended interactions with other cell components and enables the simultaneous use of multiple CC peptides [38,39]. Direct protein regulation has been challenging due to the unique characteristics of different protein folds. Strategies such as degrons [40] and split proteases [41] often require extensive optimization and have limited applicability. INSRTR addressed these limitations by providing a broadly applicable and robust platform for protein regulation. By inserting a peptide into a solvent-exposed loop of the target protein, remote from the active site, the target protein retains its function. Upon binding with a complementary regulatory peptide, the inserted peptide forms a rigid helical conformation, disrupting the local structure of the protein and affecting its function through allostery.

The INSRTR technology demonstrated versatility as evidenced by successful application across more than ten unrelated proteins with functions ranging from enzymatic activity, signal transduction, transcriptional regulation, fluorescence, and antibody binding. For each protein tested, multiple viable insertion sites for regulatory peptides were identified, enabling precise control over their activity. This broad applicability underscores the potential of INSRTR as a powerful tool for allosteric protein regulation across various biological contexts. A particular advantage of the INSRTR platform was that it can generate both ON and OFF switches triggered by the addition of a regulatory molecule (Figure 2b, c) and can be used to implement two-input logic functions in mammalian cells (Figure 2d). This was achieved by the fusion of an autoinhibitory peptide to the host protein, which generated a


A
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D
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0 0 0
1 0 0
0 1 0
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Protease cleavage site
Proteases

protein that is initially inactive and can be activated either by the addition of a CC peptide with high affinity to the inhibitory peptide or by a proteolytic cleavage of the linker between the protein and an inhibitory peptide. This modular strategy enabled the generation of a full set of a binary Boolean logic functions that can process different combinations of proteases, which either originate from natural processes or can be chemically regulated. The response of logic gates was demonstrated in living mammalian cells, with the response generated within minutes after the addition of a small molecule regulator of the protease.

Introduction of Allosteric Control of Antibody Binding Domains

Antibodies are widely applied for therapy, either as proteins or integrated into the cellular therapy [42]. CAR T cell therapy utilizes engineered T cells equipped with synthetic receptors known as chimeric antigen receptors (CARs). CARs enable T cells to recognize and destroy cancer cells by targeting specific surface antigens via the scFv. The binding affinity of the scFv significantly influences the overall effectiveness of CAR T cell therapy and treatment outcomes. CAR T cells designed to target CD19 antigen have shown a remarkable success in treating patients with relapsed B cell malignancies [43]. Binding capacity of antibody variable domains was also addressed by the coiled-coil mediated INSRTR platform [12]. To address the risk of cytokine storms caused by overactive CAR T cells, researchers have applied the INSRTR platform for controlling CAR T cell activity. This allowed for rapid and temporary inactivation of CAR T cells. By inserting a peptide into the variable loop of anti-CD19 scFv that is adjacent to the CDR loops, they successfully modulated binding to the target antigen and also CAR T cell function. The dynamic response range of the system was further enhanced by the addition of the second heterodimeric CC that recruited the regulatory CC to the scFv (Figure 3a). Upon addition of the regulatory peptide, IL-2 production was significantly reduced. The researchers observed similar results when applying the INSRTR platform to α-Her2 CAR, demonstrating the platform's potential to regulate the activity of various therapeutically relevant CARs and enhance their safety profile.

As another strategy to regulate antibody binding, Kellmann et al. [14] explored regulation of antibody binding by developing switchable antibody fragments using a calmodulin linker (Figure 3b). This approach involves replacing the linker that typically connects the variable heavy (VH) and light (VL) chains of the single-chain variable fragment (scFv) with permuted calmodulin (CaM) variants. Calmodulin's conformation changes upon binding calcium and calmodulin-binding peptides (CBPs), and this conformational shift is exploited to allosterically modulate the scFv's affinity for its antigen. The authors tested this strategy on scFvs with different specificities and demonstrated that the addition of different CBPs could modulate affinity in five out of the six tested scFvs. The study also found that effective modulation required identifying specific combinations of calmodulin permutations and peptides for different scFvs, highlighting the need for optimization.

Khowsathit et al. [44] reported a computationally designed allosteric antibody switch, enabling modulation of antigen-binding activity through the addition of an exogenous small molecule ligand (Figure 3c). They developed a computational strategy to design cavities within the antibody structure by identifying sets of multiple adjacent large-to-small amino acid mutations. Previously, the authors explored the introduction of a deactivating tryptophan-to-glycine mutation at a selected position, which could be reactivated by the addition of indole [45]. However, a high concentration of indole was required to restore activity, which limited the potential applications of these switch proteins. To overcome this limitation, the authors developed a

Figure 2. Design of coiled coil mediated allosteric control and protein logic functions for engineered protein systems. A. (Left) Helical wheel diagram for parallel (top) and antiparallel (bottom) coiled-coil. Characterized by a pattern of amino acids, creating an amphipathic structure with alternating hydrophobic and polar residues. Hydrophobic residues (magenta and cyan) are typically at the 'a' and 'd'. Polar or charged residues at the 'e' and 'g' positions (orange and pink) contribute to stability and selective interactions through electrostatic forces. The remaining positions ('b', 'c', and 'f') are solvent-exposed and influence stability. (Right) Parallel coiled coil pair, highlighted hydrophobic core. Adapted from Drobnak et al. [36]. B. The INSRTR OFF strategy involves inserting an unstructured peptide into a solvent-exposed loop of the target protein. Upon the addition of a regulatory peptide, a coiled coil (CC) dimer forms, inducing a structural perturbation that inactivates the target protein. Adapted from Plaper et al. [12]. C. The INSRTR ON strategy involves fusing an autoinhibitory loop with an inhibitory peptide to the C-terminus of a target protein. The two peptides form a dimer, inhibiting the target protein. The dimer dissociates upon the addition of a regulatory peptide with higher affinity for the inhibitory peptide, thereby restoring target protein activity. Adapted from Plaper et al. [12]. D. An example of AND logic gate generated by fusion of autoinhibitory CC peptides to both termini of a target protein, coupled with site-specific loop cleavage by orthogonal proteases. Adapted from Plaper et al. [12].

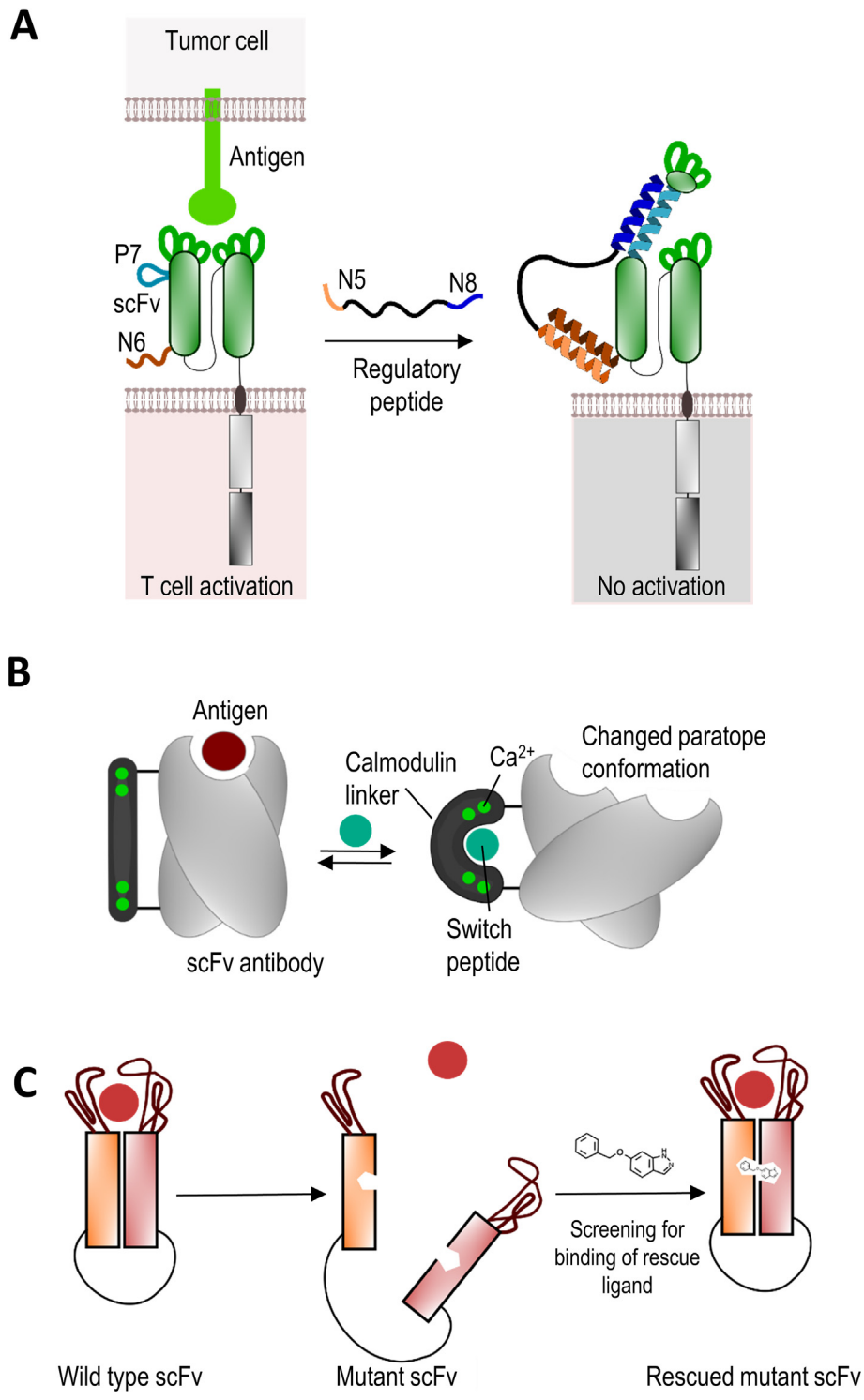

Figure 3. Examples of engineering allosteric regulation of antibody binding domains, allowing selective activation and tunable affinity. A. The INSRTR-regulated CAR T receptor is designed by inserting P7 and N6 peptides into the single-chain variable fragment (scFv) heavy chain loop and N-terminus, respectively, allowing CAR T cell activity to be inhibited by the addition of a regulatory N5-N8 fusion peptide. Adapted from Plaper et al. [12]. B. Engineering switchable scFv-CaM with allosterically modulated antigen affinity involves replacing scFv linker with cyclically permuted calmodulin variants, enabling calcium binding and peptide interactions to induce paratope conformational changes by altering scFv domain arrangement. Adapted from Kellmann et al. [14]. C. Ligand-dependent antigen recognition in antibodies is engineered by computationally introducing large cavities at the heavy-light chain interface to disrupt antigen binding. Mutations are then screened for their ability to bind a complementary rescuing ligand that restores the interface. Adapted from Khowsathit et al. [44].

computational strategy for identifying larger and more complex cavities in proteins that can be created by multiple simultaneous large-to-small amino acid mutations. They applied this strategy to a model antibody system, a fluorescein-binding single-chain variable fragment (scFv). A triple mutant (Rip-3) having reduced antigen affinity successfully restored binding using a complementary ligand (Stitch-3). This Rip-3/Stitch-3 pairing was found to be transferrable to scFv derived from a completely unrelated framework, due to the design targeting conserved residues in the antibody framework.

Allosteric Regulation of Designed Protein Switches

Beyond the principles of allostery observed in natural proteins, recent advances in computational strategies have enabled the design of *de novo* synthetic proteins with allosterically regulated functions [46]. While accurately modeling the subtle coordinated conformational shifts that mediate allosteric transitions in natural proteins remains a significant challenge, *de novo* designs successfully generated switchable protein systems that transition between distinct conformational states in response to an external input [18]. To date, this has involved engineering of helical bundles as the basis for switchable designs [18,47], with more recent developments extending to modular allosteric assemblies composed of monomeric units that oligomerize upon ligand binding [17].

Pillai et al. [17] demonstrated the design of protein assemblies with switchable functions controlled by effector molecules which have the potential for feedback control of signaling in adoptive cell therapies. They focused on creating cyclic rings and dihedral cages that can incorporate or eject subunits in response to peptide binding (Figure 4a). Inspired by the Monod-Wyman-Changeux (MWC) model, the researchers focused on building systems where molecules can switch between the two states – 'X' and 'Y' – with the Y state stabilized by a peptide effector. They first designed switchable ring proteins that adopt different oligomeric states based on peptide binding. The designed rings incorporated hinge modules that toggle between X and Y conformations and interaction modules (LHDs) [48] that dictate the assembly into cyclic oligomers. These rings were computationally designed to be strained or imperfectly closed in the X state, ensuring that peptide binding drives a transition to the Y state and a change in oligomerization. The study investigated whether these switchable rings exhibit ligand-binding cooperativity. The researchers hypothesized that the energetic penalty associated with partially ligated states would lead to cooperative behavior. A series of experiments demonstrated that a designed ring that transitions from a trimer (X3) to a tetramer (Y4) upon peptide binding, exhibits strong positive cooperativity. The authors further explored allosteric control of protein dimerization by designing an inducible homodimer (IHA10) that assembles only in the presence of the peptide effector. They extended this to design protein cages that disassemble upon effector binding. These assemblies were composed of cyclic oligomers linked by weak dihedral interfaces. The addition of a peptide or a high-affinity effector protein disrupted these interfaces, leading to the disassembly of cages into their constituent components. This design principle offers the potential for developing drug delivery systems that release molecular cargo upon encountering specific signals.

Cross et al. [49] designed a conformational switch in the kinesin-1 motor protein, enabling its allosteric activation. They targeted the flexible "elbow" region within the coiled-coil architecture of kinesin-1, which enables the transition between its open (active) and closed (autoinhibited) state. They engineered a system where a *de novo* designed peptide could bind to the elbow region and induce a conformational change from the closed to the open state, thereby regulating the motor activity (Figure 4b). The team utilized computational modeling with AlphaFold2 to predict the structural changes in kinesin-1 variants, followed by experimental validation using biophysical techniques. They successfully designed several kinesin-1 variants, ultimately creating a variant, Kinesin-1EL-PA-Ala, that could be switched from a closed conformation to an open one upon binding of a *de novo* designed peptide, pB. They also demonstrated the functionality of this switch in living cells. The addition of cell-penetrating pB peptide to cells expressing Kinesin-1EL-PA-Ala led to a significant redistribution of kinesin-1 complexes, indicating activation of the motor. This approach provides valuable insights into cellular biology and may facilitate the development of novel biomedical strategies for modulating motor protein activity, with potential applications in conditions characterized by dysregulated motor function [50].

LOCKR Allosteric Platform

Langan et al. [18] showcased the *de novo* design of protein switches with functions activated by an external trigger. The article describes the development of a new, tunable protein switch platform called LOCKR (Latching Orthogonal Cage-Key pRoteins) (Figure 4c). The system is based on the principle of competition between intramolecular and intermolecular interactions. It consists of a "cage" (a protein structure with five or six alpha helices that forms a binding site for the latch), a "latch" (contains a functional motif that can be a peptide, a protein, or another molecular fragment), and a "key" (a peptide that can bind to the cage with a higher affinity than the latch). When the key is added, it binds to the cage and displaces the latch, which releases the functional motif and allows it to

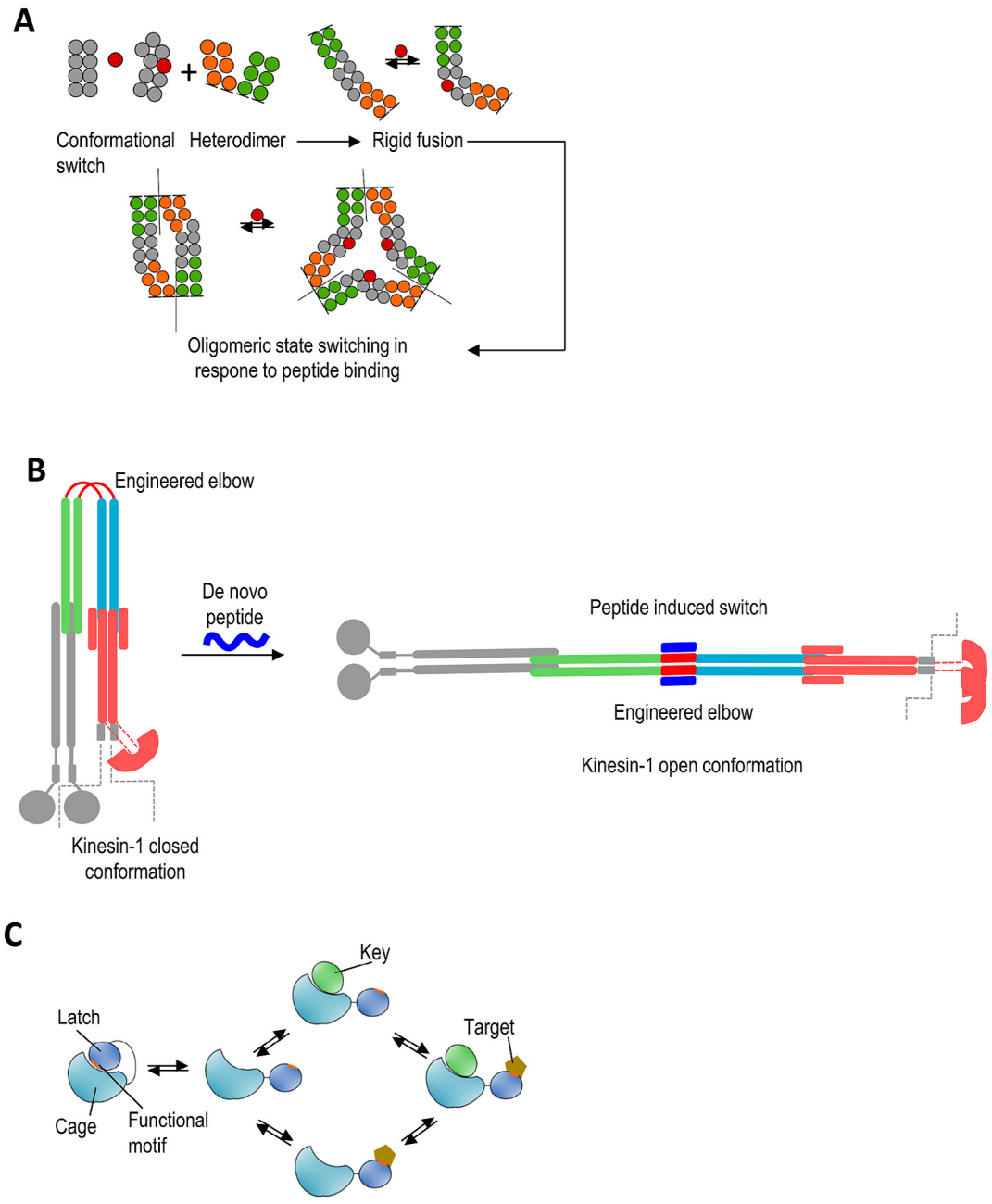

Figure 4. Design and engineering of allosteric protein switches for dynamic control of protein function. A. The design of allosterically coupled oligomeric assemblies uses a 'hinge' building block (gray) that switches between two conformations, with one state stabilized by effector peptide binding (red). Heterodimers, composed of two monomers (orange and green), are fused rigidly with the hinge to create a chimeric protein that adopts different oligomeric states in response to effector peptide binding. Adapted from Pillai et al. [17]. B. The design of a peptide-inducible conformational switch in kinesin-1 involves engineering the elbow region to adopt a closed conformation, which can be switched to an open state upon the addition of a de novo-designed peptide. Adapted from Cross et al. [49]. C. The de novo design of a switchable protein system features a "cage" with a single interface that interacts either with a "latch" or a separate "key" peptide. The latch contains a functional motif that can bind a target, which is inaccessible when the cage-latch interaction occurs. By modulating the cage-latch and cage-key affinities, the system enables target binding upon addition of the key peptide. Adapted from Langan et al. [18].

interact with other partners and renders them active. The authors demonstrated the functionality of the LOCKR system by caging the pro-apoptotic peptide Bim (a peptide that binds to the protein Bcl2 and triggers apoptosis), the cODC degron (signaling sequence that triggers protein degradation

by the proteasome which enabled control over protein degradation in living cells) and the nuclear export signal (NES; a signaling sequence that triggers the export of proteins from the cell nucleus. The LOCKR system was demonstrated in yeast and mammalian cells.

Building further on LOCKR technology, Woodall et al. [51] designed two types of protein switches that link phosphorylation to protein–protein association. The first type of switch relies on a caging mechanism, in which an ITAM phosphorylation target site is caged by a four helical bundle. The ITAM contains a dual-tyrosine motif that, when phosphorylated, initiates T-cell activation. A four-helix bundle was designed in which the C-terminal tyrosine in the ITAM sequence motif forms part of the central core and hence is not accessible to the kinase. The addition of a synthetic key peptide displaces the helical bundle and exposes the ITAM site, allowing phosphorylation of this domain. The second type of switch creates a phosphorylation-dependent latch mechanism, in which phosphorylation of the *de novo* helical bundle results in a conformational change and exposes a binding region for a reporter or POI (protein of interest). Designed phosphorylation switches, capable of interacting with native cellular proteins, offer promising prospects for engineered T cells. The article proposes using binding-dependent phosphorylation switches to modulate CAR-T signaling during excessive activation. One potential approach involves expressing a key peptide under the control of repressors associated with T-cell exhaustion, whereby reduced key peptide levels could inhibit caged ITAMs and attenuate CAR signaling. Alternatively, a phosphorylation-dependent binding switch could be engineered to respond to the Lck kinase, which initiates T-cell activation, to cage an inhibitor of T-cell activation and mitigate excess CAR signaling. Authors conclude that improving the dynamic range of these switches will enhance their applications in engineered T cells and broader synthetic biology contexts.

Quijano-Rubio et al. [47] further used the LOCKR for developing modular biosensors that can be adapted for a range of targets, including clinically relevant anti-apoptosis protein BCL-2, HER2 receptor, and botulinum neurotoxin B. These biosensors are constructed from a “lucCage” and a “lucKey”, whose binding reconstitutes the luciferase activity in the presence of the target. Based on this principle, the authors developed a diagnostic tool for detecting severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) using a *de novo*-designed spike receptor binding domain (RBD) binder. With advancements in computational design, such engineered sensors are expected to detect an increasingly broader range of targets with greater sensitivity.

Machine Learning for the Allosteric Insertion Site Prediction

Identifying allosteric sites and understanding their influence on protein function, particularly at the active site, has driven the development of both experimental and computational methods. While experimental approaches can be time-consuming and expensive, computational methods offer a more efficient alternative. Several computational methods have been developed [52,53] (Table 1), including AlloSigMA [54], a structure-based statistical mechanical model developed by Guarnera et al. to explore allosteric pathways. McClendon et al.’s “MutInf” [55] identifies allosteric sites by analyzing correlated motions from molecular dynamics simulations. Goncearenco et al.’s SPACER [56] server uses geometric features and protein dynamics for prediction and the Dokholyan group’s Ohm platform [57] offers a comprehensive suite of tools for identifying allosteric sites, analyzing communication pathways, predicting critical residues, and calculating allosteric correlations. Tan et al. [58], reported AlloMAPS 2, which is based on the Structure-Based Statistical Mechanical Model of Allostery (SBSMMA). It provides the quantification of allosteric signaling and tune ligand-site interactions to obtain desired allosteric effects.

INSRTR-based CC inserts are relatively tolerant to their specific location within the host protein due to the flexibility provided by linkers. The resulting CC dimer formation generates tension that propagates to the enzyme’s active site, influencing

Table 1 Machine learning allosteric modeling tools.

Computational tool	Application
AlloSigMA [54]	Structure-based statistical mechanical model for allosteric pathways exploration.
MutInf [55]	Identification of allosteric sites by analyzing correlated motions from molecular dynamics simulations.
SPACER [56]	Prediction server based on geometric features and protein dynamics.
Ohm platform [57]	Tools for identifying allosteric sites, analyzing communication pathways, predicting critical residues, and calculating allosteric correlations.
AlloMAPS 2 [58]	Based on SBSMMA, provides the quantification of allosteric signaling and tune ligand-site interactions.
INSRTR prediction model [12]	Model implementing gradient boosting trees (GBT) algorithm for predicting CC insertion sites.
ProDomino [59]	Allosteric protein switches design based on large language model to predict suitable domain insertion sites within proteins.

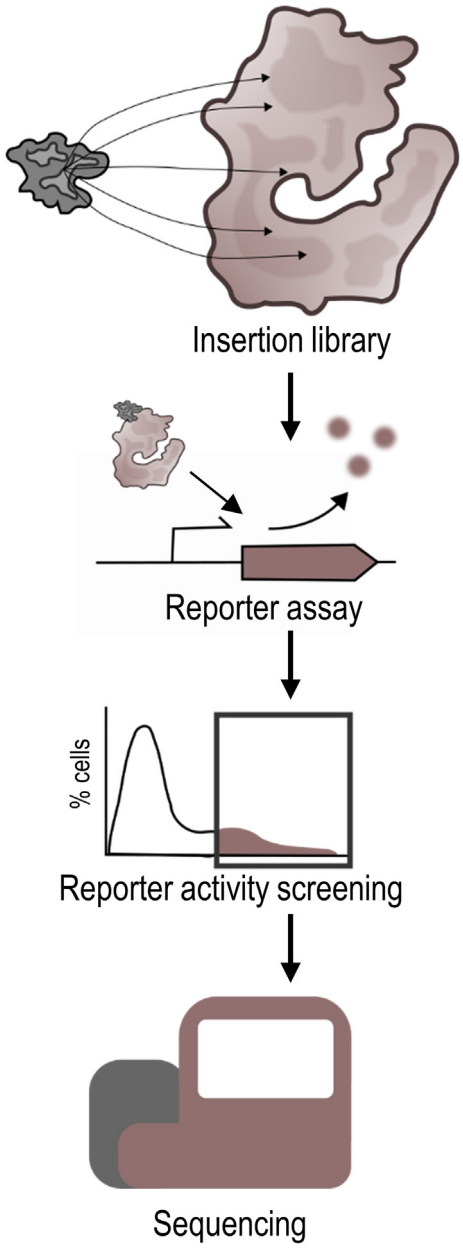

Figure 5. High-throughput screening pipeline for assessing domain insertion tolerance and functional compatibility. Profiling domain insertion permissibility involves generating a library composed of a selected effector protein carrying insertions at all possible sequence positions. Active protein hybrids are identified through a reporter assay that links protein activity to fluorescent marker expression. This is followed by fluorescence-activated cell sorting and next-generation sequencing to map the insertion sites. Figure adapted from Mathony et al. [16].

its activity. To predict the effects of these insertions, the authors employed machine learning based on experimental data from tens of designs and 4D structures and identified key descriptors that determined the successful insertion and regulation. This analysis yielded a predictive model, implemented using a gradient boosting trees (GBT) algorithm, and made available through an online server (Table 1).

Mathony et al. [16] developed a high-throughput screening pipeline that combines fluorescence-activated cell sorting (FACS) and next-generation sequencing (NGS) to screen vast libraries of domain insertion engineering of hybrid proteins (Figure 5). To predict domain insertion tolerance, machine learning models were employed, incorporating features such as biophysical properties of amino acids and linker propensity indices. The models achieved good accuracy in predicting insertion site suitability, with sequence conservation statistics emerging as the most crucial predictor. The team selected four structurally and functionally diverse proteins as scaffolds for domain insertion: AraC transcription factor, Flp recombinase,TVMV protease and σ-factor F (SigF). As insert domains, five structurally and functionally distinct domains were used: the PDZ domain from murine α1-syntrophin, the AsLOV2 domain, the estradiol binding domain from the human estrogen receptor (ERD), an enhanced yellow fluorescent protein (eYFP), and the synthetic rapamycin receptor uniRapR. Insertion sites were not necessarily located on surface-exposed, unstructured loops, contrary to common assumptions underlying domain insertion engineering strategies. Additionally, domain insertion tolerance was significantly affected by the size of the insert domain, with smaller domains like PDZ and LOV2 exhibiting greater tolerance than larger domains.

Wolf et al. [59] developed a computational tool ProDomino (Table 1), for designing allosteric protein switches leveraging a large language model to predict suitable domain insertion sites within proteins, enabling the creation of customized protein switches with controllable activity. Due to the scarcity of experimental data on designed inserted domains, natural folds with inserted domains were used to generate the learnings of permissible insertion sites. Surprisingly, that set did not exhibit a bias towards the disordered loops as insertions were equally found also within the secondary structure elements. ProDomino's accuracy was validated through experimental testing on four proteins from diverse structural and evolutionary families. The model successfully predicted insertion sites for puromycin acetyltransferase and chloramphenicol acetyltransferase, enzymes mediating antibiotic resistance, CRISPR-Cas9 and CRISPR-Cas12a. ProDomino identified insertion sites that resulted in functional hybrid proteins with switchable activity. The researchers demonstrated the practical application of ProDomino by engineering optogenetic and chemogenetic protein switches. They inserted receptor domains responsive to blue light (AsLOV2) or a chemical inducer (GR2) into the effector proteins at the predicted sites.

Translational Barriers and Potential Therapeutic Applicability of Allosterically Engineered Proteins

Engineered allosteric proteins achieved through domain insertion and traditional small molecule allosteric modulators represent powerful approaches to modulating protein function. Domain insertion methods face hurdles related to immunogenicity, protein stability and potential off-target effects of the inserted domain. These challenges are often more complex and unique compared to the issues faced by small molecule allosteric modulators.

Domain insertion has certain advantages over drug-based allosteric modulators, however, none of the methods of protein domain insertion have reached clinical trials. Most trials targeting allosteric modulation are focused on allosteric drug modulators [60,61] or mutagenesis-based modulation [62]. Domain insertions can be engineered to target specific regions within a protein, leading to highly selective modulation of its function. The effects can be reversible [12,22], depending on the design and the specific interaction between the inserted domain and the target protein. This allows for dynamic control over protein function. Additionally, the strength of the allosteric effect can be tuned by modifying the inserted domain or its interaction interface. Once a domain insertion is engineered into a protein, the allosteric modulation becomes an inherent property of the protein itself. In order to engineer allosteric proteins, identifying suitable insertion sites and ensuring allosteric communication is necessary. This is often a laborious process of screening and directed evolution, while small molecule discovery involves screening chemical libraries for binders to sometimes elusive allosteric sites [63,64]. Engineered proteins offer the potential for high specificity due to their larger interaction surfaces, whereas small molecules can struggle with specificity, leading to unwanted side effects.

A significant issue of engineered proteins is the risk of immunogenicity, including antibody production and cell-mediated immune response. These could neutralize the therapeutic protein, reduce its efficacy, and even cause adverse events. Strategies like humanization of the inserted domain or the use of self-derived domains could mitigate this risk. Additionally, small allosteric domains, such as coiled-coil peptides likely have low immunogenicity as the noninteracting residues are hydrophilic and do not contain aromatic residues which can contribute to B cell epitopes [65]. Small molecule drugs, generally have a lower risk of eliciting a strong adaptive immune response, although hypersensitivity reactions can occur [66]. The pharmaceutical industry has a well-established infrastructure and expertise in drug discovery and development. There is a greater familiarity with drug-based approaches among clinicians and regulatory agencies. Drug-based therapies, while also subject to off-target effects [67], often have a more established framework for assessing and managing these risks. Engineered proteins can be less stable than native proteins and may be prone to degradation or aggregation, which affects their efficacy. For both approaches, the occurrence of mutations presents a risk for losing therapeutic effect. Delivery and pharmacokinetics often present challenges for large engineered proteins, such as *de novo* designed protein switches [68], whereas small molecules can be optimized for oral administration and designed to improve bioavailability.

Cell-based assays serve as the primary platform for demonstrating that an engineered protein can exert its intended modulatory effect within a biological context. While protein domain insertions have not yet reached the clinical trials, genome editing tools such as CRISPR/Cas9 [69,70] could be used in the future to insert protein domains into target endogenous proteins, enabling the proteins to be regulated by various stimuli within the body. If the domain insertion involves permanent genetic modification of somatic cells, there are long-term safety considerations regarding potential genotoxicity [71]. While not always the case for transient protein delivery, this is a concern for gene-editing-based domain insertion. In contrast, small molecule drugs generally do not alter the patient's genome. The successful application of domain-inserted proteins *in vivo* is therefore often intertwined with the capabilities of gene therapy delivery technologies [72,73].

Leveraging domain insertion strategies, an activatable Protein C (PC) for treating congenital PC deficiency was developed by insertion of a self-cleaving peptide sequence [74]. The self-cleaving peptide was inserted between the light and heavy chains of PC which ensures that upon expression (in the liver following AAV-mediated gene delivery or CRISPR/Cas9-mediated genome editing), the protein is efficiently processed into its constitutively active form. This approach represents a clever adaptation of insertion logic and broadens the conceptual application of domain insertion for therapeutic benefit.

Allosteric regulation also offers promising solutions for enhancing the safety of engineered cell therapies, such as CAR T cells. While highly effective for certain hematological malignancies, these therapies require control mechanisms to prevent potentially life-threatening off-target cytotoxicity [75]. Incorporating allosteric regulation could enable transient inactivation of therapeutic functions, minizing off-target toxicity while preserving efficacy. Optogenetic engineering approaches—such as light-regulated CAR T cell activation—have already demonstrated promising results in *in vivo* models [76]. Unlike small molecules, which act systemically, optogenetic tools

offer real-time and spatially precise control of cellular functions. Altogether, engineered systems for allosteric regulation represent a fundamentally distinct therapeutic paradigm. They are centered on dynamic and programmable control, and their successful therapeutic translation will depend on overcoming the challenges outlined above.

Conclusions and Future Outlook

The fundamental regulatory mechanism of allostery has become a powerful tool in synthetic biology, as evidenced by the successful design of several novel regulated proteins. Nevertheless, several challenges persist. Allosteric engineering through domain insertion seeks to introduce specific modifications to a target protein, enabling precise regulation of its function in response to selected inputs while preserving its structural integrity and activity at levels comparable to its native state. However, successful implementation requires careful selection of insertion sites and a comprehensive understanding of the structural and functional consequences of such perturbations.

Predicting the effects of allosteric modulation remains difficult due to the unpredictable nature of target protein activation or deactivation. Conformational changes induced by protein modulation alter inter-domain interactions, impacting the protein's overall activity, localization, and stability [77]. Furthermore, the modulator can influence the target protein's interactions with other proteins, such as signaling partners, regulatory proteins, or even other copies of itself. Because different cell types express different protein sets, the same allosteric modulator can elicit varying downstream effects. The complex cellular environment, with its multitude of other molecules, can further influence the activity of the modulated protein. These molecules may compete for binding sites or interact with the target protein in ways that can influence the allosteric effect. Finally, post-translational modifications, which can alter protein conformation [78,79] also play a crucial role in determining how a protein responds to allosteric modulation.

Although computational modeling is advancing, it still grapples with complexity [80]. A key area of rapid development is the integration of artificial intelligence (AI) into prediction models, particularly for predicting domain insertion sites and the potential outcomes of protein modifications. Machine learning models, crucial for building predictive models, require substantial amounts of data [16,59]. High-throughput and large-scale screening of designed protein libraries are needed. They can also identify modified proteins exhibiting desired allosteric properties, even without a mechanistic understanding of the precise mechanism. Structure-guided design, leveraging knowledge of the protein's structure and allosteric site location, facilitates the development of more selective and effective modulators. However, designing allosteric protein regulation faces challenges in structural prediction. While high resolution techniques like X-ray crystallography and cryo-EM offer an insight into protein structures, predicting the dynamic conformational changes upon allosteric modulator binding remains difficult.

In comparison to traditional small molecule allosteric modualtors, engineered proteins with allosteric domain insertions are generally further from *in vivo* models and human trials. While the field has seen significant progress in engineering functional allosteric switches *in vitro* and in cellular contexts, the challenges of immunogenicity, delivery, and off-target effects need to be carefully addressed for *in vivo* applications.

Allosterically modified proteins hold the potential for linking diverse biological processes and signaling pathways. Harnessing the power of signal transduction engineering holds immense promise due to the inherent modularity of signaling pathways, their rapid response capabilities, signal amplification, and strategic utilization of spatial localization [81]. By strategically selecting key proteins within specific signaling pathways and modulating them, we can modulate downstream signaling events and create novel connections between different biological processes. However, predicting the consequences of allosteric modification remains a challenge. For example, Wu et al. [82] introduced a computational method that utilizes structural data to predict allosteric communication pathways and identify key functional residues in proteins. This method employs bond-to-bond propensity analysis to quantify the coupling between different sites on a protein, offering a strategy to address this challenge.

The *de novo* approach to protein design is emerging as an important advancement in engineering allosteric regulation. Recent progress in computational design, coupled with a deeper understanding of allosteric mechanisms, has attracted considerable interest in applying allosteric modulation to drug discovery [83]. While discovered serendipitously, Liu et al. [84] reported the use of a synthetic antibody as an effective antagonist of proinflammatory cytokine interleukin-18 (IL-18), operating via an allosteric mechanism. This discovery opens a new possibility for using *de novo* designed miniproteins to directly bind and modulate proteins of interest through allostery, offering a promising strategy for targeting natural proteins. However, this approach requires custom design for each target, limiting its modularity compared to domain insertions for introducing allosterically regulated functions. Additionally, it necessitates a comprehensive understanding of both *de novo* protein design and the principles of

allosteric coupling to accurately predict how protein activity will be modulated.

Taken together, the field of engineered allosteric mechanisms has advanced toward developing practical solutions for biomedical applications, leveraging these mechanisms for drug discovery, therapeutic interventions, and diagnostic tools. This transition reflects increasing efforts to translate fundamental insights into applicable strategies, where synthetic biology and computational design play key roles in creating precise and programmable allosteric regulators tailored to specific biomedical challenges.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the authors used ChatGPT and Gemini to improve readability. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

CRediT authorship contribution statement

Tjaša Plaper: Writing – review & editing, Writing – original draft, Conceptualization. **Urška Knez Štibler:** Writing – review & editing, Conceptualization. **Roman Jerala:** Writing – review & editing, Writing – original draft, Funding acquisition, Conceptualization.

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DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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