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Research review paper

Engineering conditional protein-protein interactions for dynamic cellular control

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ABSTRACT

Conditional protein-protein interactions enable dynamic regulation of cellular activity and are an attractive approach to probe native protein interactions, improve metabolic engineering of microbial factories, and develop smart therapeutics. Conditional protein-protein interactions have been engineered to respond to various chemical, light, and nucleic acid-based stimuli. These interactions have been applied to assemble protein fragments, build protein scaffolds, and spatially organize proteins in many microbial and higher-order hosts. To foster the development of novel conditional protein-protein interactions that respond to new inputs or can be utilized in alternative settings, we provide an overview of the process of designing new engineered protein interactions while showcasing many recently developed computational tools that may accelerate protein engineering in this space.

1. Introduction

Proteins are diverse biomolecular machines with the potential to solve many pressing issues facing society, such as sustainable chemical production (Zhang et al., 2022), plastic degradation (Lu et al., 2022; Yoshida et al., 2016), and disease treatment (Ebrahimi and Samanta, 2023). Proteins readily interface with and regulate all essential cellular processes, including transcription, translation, cell division, viral defense, transport, and metabolism, through their highly specific and conditional interactions (Chubukov et al., 2014; Jinek et al., 2012). This incredible diversity of proteins is enabled by the infinite sequence space of amino acid residues that determine the structure and, thus, the function of a protein. Furthermore, these functionalities can be conditional, as various stimuli can induce conformational shifts in a protein structure to present previously unavailable protein-protein interaction (PPI) interfaces.

Conditional PPIs drive dynamic cellular behavior in nature. For instance, gene expression is often regulated by the conditional dimerization of transcription factors (Mahr and Frunzke, 2016); gene products can also be conditionally targeted for degradation (Nishimura et al., 2009). Recent advances in molecular biology and protein engineering have yielded a host of engineered PPIs that are responsive to physiological inputs such as chemicals (Park et al., 2024), nucleic acids (Mitkas et al., 2022), and light (Tague et al., 2024) as depicted in Fig. 1. These conditional interactions may be direct interactions, such as the reconstitution of a split protein (Zetsche et al., 2015), protein homodimerization (Kaberniuk et al., 2021), protein heterodimerization (Liang et al., 2011), or indirect proximity-based interactions where proteins are colocalized onto a scaffold (Mitkas et al., 2022) or within specific cellular compartments (Haruki et al., 2008). Regulated protein interactions are of particular interest in metabolic engineering as dynamic regulation of cellular activity can improve the allocation of limited cellular resources, leading to enhanced product titers (Tague et al., 2024). Conditional protein activity is also critical for developing advanced therapeutics where only disease-afflicted cells must be treated to minimize off-target toxicity (Lajoie et al., 2020). Environmental synthetic biology also benefits from the rapid and responsive conversion of external stimuli to cellular response for threat detection or pollutant remediation (Brooks and Alper, 2021).

Engineering novel conditional PPIs or adapting existing interactions to new contexts often face many challenges. In some cases, steric clashes or non-optimal spatial orientations between proteins may prevent desired interactions and weaken or abolish protein activity (Chen et al., 2013). Reducing non-specific protein interactions is also critical to lowering background activity when the target stimulus is absent (Ding et al., 2024; Pu et al., 2017). The portability of conditional PPIs to other cellular contexts can be impaired by poor expression of the required protein components (Beygmoradi et al., 2023) or missing cellular

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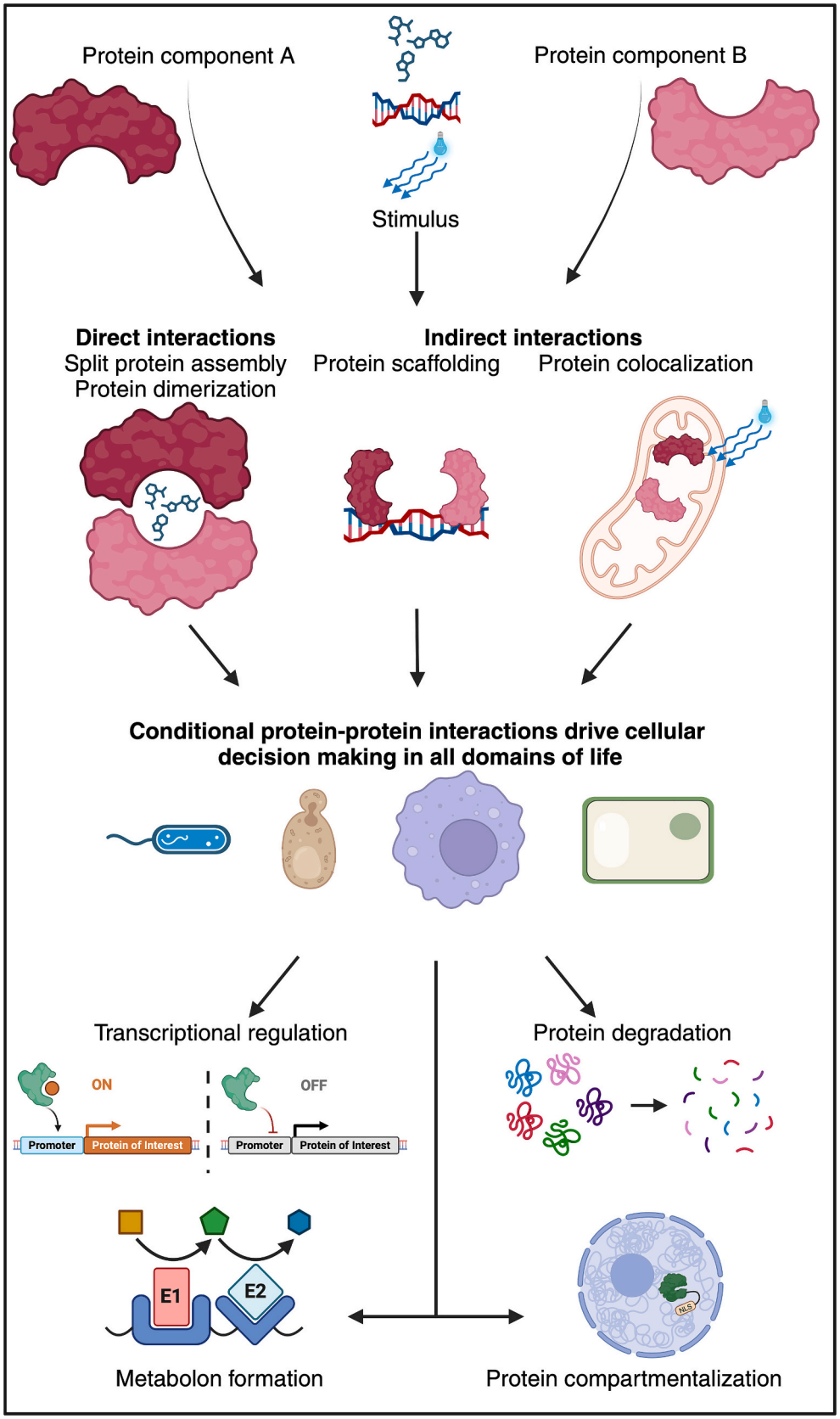

Fig. 1. Conditional protein-protein interactions are driven by a diverse range of stimuli, including chemicals, nucleic acids, and light. These interactions can take a variety of forms to regulate cellular functions.

machinery needed to interface with the PPI (Morreale et al., 2022). Despite these challenges, many successful examples of engineered conditional protein interactions have been developed for diverse applications that are responsive to a wide range of triggers and employed in all kingdoms of life.

In this review, we examine engineered PPIs responsive to various physiological stimuli and highlight instances where components are generalizable across different hosts or applications. We also explore the necessary considerations to employ and engineer PPIs for dynamic cellular regulation and showcase the recent computational tools developed for this design space.

2. Mechanisms and applications for conditional protein-protein interactions

Induction of conditional PPI by physiological stimuli can lead to dynamic regulation of cellular processes. In theory, a conditional PPI could regulate nearly all cellular activities, given the ubiquitous role of proteins in the cell. In practice, conditional PPIs have been readily applied to regulate transcription, translation, protein compartmentalization, targeted protein degradation, and modulate enzymatic activity (Fig. 1). In some cases, the proteins may directly assemble as in the reconstitution of a split protein or protein-protein binding (Ma et al., 2023; Rihtar et al., 2022). Alternatively, it may be sufficient to colocalize two proteins in an organelle or on a scaffold to engage in a site-specific process or increase the effective molarity of a reaction in the case of catalysis. The division of separate molecular sensing and actuation domains in proteins enables protein engineers to apply regulatory strategies used in one cellular process to many others (Table 1). The means of inducing conditional PPIs and their applications were divided into three primary classes: chemical-responsive (Fig. 2), nucleic-acid-responsive (Fig. 3), and light-responsive (Fig. 4).

2.1. Chemical-responsive protein interactions

The cellular environment contains thousands of unique chemical species including metabolites, cofactors, energy storage, signaling molecules, and anti-microbials. Cells have naturally evolved to sense and respond to changes in this complex environment. To adequately respond to physiological inputs, cells must first detect the specific molecule of interest and then employ the proper response. Proteins are capable of readily binding to their target ligand with high specificity. As a result of protein-ligand binding, an allosteric conformational shift or dimerization event may occur, which can lead to conditional functionality or initiation of a cellular response. These chemical-responsive protein interactions are used to regulate transcription and translation, transduce signals, and control enzyme kinetics.

Perhaps the most well-studied example of chemical-responsive protein interactions is the rapamycin-induced dimerization of FKBP-FRB, an example of chemical-induced dimerization (CID). The inducible heterodimerization of the FKBP and corresponding FKBP-rapamycin-binding domain (FRB) inhibits the kinase activity of TOR1 in yeast and the mammalian homolog, mTOR, leading to growth arrest (Brown et al., 1994; Zheng et al., 1995). The inducible nature of these dimerizing proteins, as well as the high affinity ($K_d = 12$ nM) for FKBP-rapamycin-FRB binding (Banaszynski et al., 2005), has been exploited to study several cellular phenomena such as cell signaling (Graef et al., 1997), protein localization (Haruki et al., 2008), and chromatin memory (Hathaway et al., 2012). Translational fusion of each of the rapamycin-binding proteins to one part of a split-intein enabled rapamycin-responsive protein ligation in mammalian cells; conditional intein splicing could be halted by the addition of ascomycin, a competitive inhibitor of FKBP-FRB binding (Mootz et al., 2003). Using rapamycin-induced dimerization as a test case, conditional intein-splicing has recently been applied to a high-throughput method to detect PPIs and screen small-molecule inhibitors of PPIs (Yao et al., 2020). Zetsche and colleagues split the CRISPR-Cas9 endonuclease and fused each split Cas9 fragment to FKBP or FRB, leading to rapamycin-regulated gene editing with minimized off-target effects in human cells (Zetsche et al., 2015). Similarly, fusion of FKBP and FRB to split catalytically inactive dCas9 enabled rapamycin-inducible gene activation (Zetsche et al., 2015). An alternative approach to CID-regulated transcriptional activation was demonstrated through the rapamycin-induced reconstitution of a split

Table 1
Summary of conditional protein-protein interactions and their regulatory applications.

Class	Input	Protein Interactions	Regulatory Applications
Chemical	Rapamycin	Dimerization of FKBP-FRB; trimerization of split FKBP/FRB fragments	Transcriptional control; cell signaling; protein localization; protein ligation; split protein reconstitution; genetic engineering; PPI and RBP screening
	Abscisic acid (ABA) and other natural products	Dimerization of PYR1-ABI	Transcriptional control; plasticity exploited to identify new inputs
	Gibberellin	Dimerization of GID1-GAI	Transcriptional control
	Small molecule drugs and natural ligands	Reconstitution of split human ligand-binding proteins	Transcriptional control
	Hormones	Dimerization of nuclear hormone receptor domains	Multi-input transcriptional activation
	Auxins	Dimerization of degron tag and TIR1 leading to E2 ligase recruitment	Targeted protein degradation
	PROTACs	Dimerization of target protein and E3 ligase	Targeted protein degradation through ubiquitination; transcriptional control using truncated E3 ligases
	Diverse range of sugars, amino acids, and natural products	Dimerization of allosteric transcription factors	Transcriptional control; scaffold building blocks; plasticity exploited to identify new inputs
Nucleic Acids	Viral RNA hairpins	Viral coat protein colocalization	Enzyme scaffolds; protein reconstitution
	DNA	Zinc finger or TALE protein colocalization	Enzyme scaffolds
	Cas9 RNA hairpins with TMSD	Orthogonal Cas9 protein colocalization	Enzyme scaffolds; enzyme reconstitution
	Cas6 RNA hairpins with TMSD	Orthogonal Cas6 protein colocalization	Enzyme scaffolds; enzyme reconstitution
Light	Infrared and near-infrared	Dimerization of BphPI-PpsR2 or Cph8*	Transcriptional control; protein localization
	Red light	Dimerization of PhyB-PIF3, PSM-FIXJ, or BICYCL-Green	Transcriptional control; protein localization
	Green light	Dimerization of CcaS-CcaR or BICYCL-Red	Transcriptional control; protein localization
	Blue light	Dimerization of EL222, VVD, WC1-VVD, CRY2-C1BI, YF1-FIXJ, PAL, LicV, LOV2, or pMag-nMagH	Transcriptional control; epigenetic remodeling; translational control; RNA editing, splicing, and degradation; protein localization; cell signaling; cell cycle regulation; modulation of enzyme activity; condensate formation; protein degradation
	Ultraviolet	Dimerization of UirR-UirS	Transcriptional control

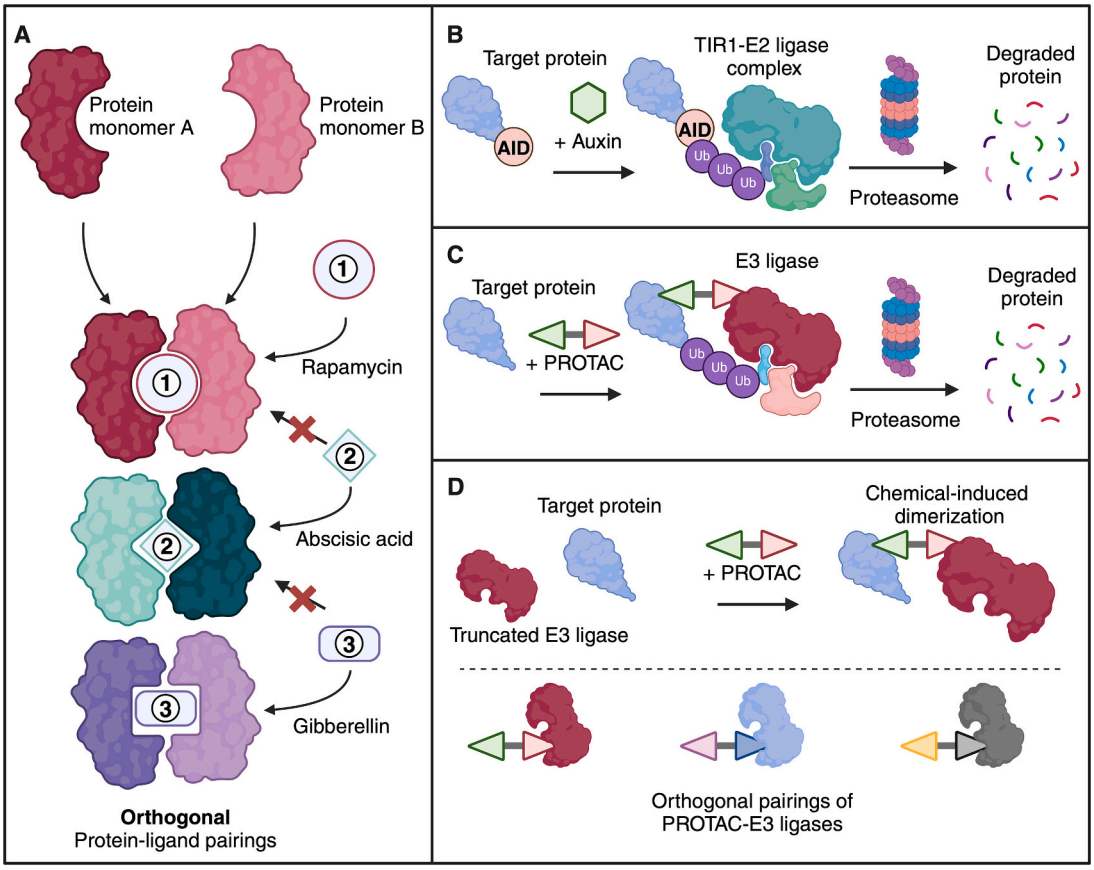

Fig. 2. Many protein-protein interactions are regulated by chemical species in the cellular environment. (a) In chemical-induced dimerization (CID), a pair of protein monomers conditionally dimerize in the presence of the target ligand. The specificity of the ligand preference can be utilized to develop logic-gated cellular regulation strategies. (b) In auxin-inducible degradation, the target protein is tagged with an auxin-inducible degron that only recruits the TIR1-E2 ligase complex when auxin is present; targeted recruitment of the E2 ligase leads to polyubiquitination and subsequent degradation. (c) Proteolysis targeting chimeras (PROTAC) are a similar strategy that utilizes a PROTAC warhead that can specifically bind to a target protein and an E3 ligase to initiate polyubiquitination. (d) Truncation of the ubiquitination domain from an E3 ligase facilitates PROTAC-CID in which CID occurs between the target and truncated E3 ligase but does not induce proteolysis. The PROTAC-CID system can use many orthogonal PROTAC-E3 ligase pairings for multi-layer and logic-gated control.

T7 RNA polymerase and corresponding transcription of both fluorescent protein and small interfering RNA (Pu et al., 2017). Another application of rapamycin CID is identifying RNA targets for RNA-binding proteins. Fusion of FRB to the RNA-binding protein of interest and FKBP to an adenosine deaminase acting on RNA (ADAR) led to an enrichment of base edited RNA substrates indicating likely targets for the tested RNA-binding protein as well as identification of small-molecule that inhibited RNA-protein binding (Seo and Kleiner, 2023). Classical rapamycin-induced protein dimerization was further expanded to develop chemically-induced trimerization by rationally splitting either of the rapamycin-binding proteins and employed to control the formation of tri-organelle junctions in primate cells (Wu et al., 2020). Applications of rapamycin-inducible PPIs are limited due to the inherent growth inhibitory effect of rapamycin on mammalian and yeast cells, which can be somewhat mitigated through the use of non-toxic rapamycin analogs or mutations in the yeast *TOR1* gene (Putyrski and Schultz, 2012), and poor diffusion through bacterial membranes (Kemp et al., 2023).

Plant-derived phytohormones, such as abscisic acid and gibberellin, are alternative chemical inducers of dimerization. Abscisic acid (ABA) induces the heterodimerization of the pyrabactin resistance domain (PYR1) of the ABA receptor and the ABA-insensitive phosphate (ABI). The ABA-responsive heterodimerization is completely orthogonal with the rapamycin CID system and can be used to regulate protein localization (Liang et al., 2011). Compared to rapamycin, ABA-induced dimerization has a more linear dynamic range, is non-toxic (and cheaper than rapamycin analogs) at high concentrations, and is readily available in mammalian diets (Liang et al., 2011). Similarly, GID1 and GAI heterodimerization is induced by gibberellin and is orthogonal with the previously described CID systems (Miyamoto et al., 2012). It is important to note that gibberellin must be chemically modified with an acetoxymethyl group to permeate the cell membrane (Miyamoto et al., 2012). ABA- and gibberellin-inducible CRISPR activation was achieved in mammalian (Gao et al., 2016), yeast (Cunningham-Bryant et al., 2019), and bacterial cells (Alba Burbano et al., 2023). The development of multiple orthogonal CID systems (Fig. 2a) enabled logic-gated control of gene expression through the fusion of heterodimerization protein pairings to orthogonal dCas9 with either a transcriptional activator, VPR, or repressor, KRAB; with this approach, Gao and colleagues demonstrated AND, OR, NOR (not OR), and NAND (not AND) logic gates (Gao et al., 2016).

There is broad utility in developing additional CID platforms regulated by alternative molecules beyond rapamycin, ABA, and gibberellin. In the ABA heterodimerization system, PYR1 is solely responsible for ligand recognition and has been engineered to bind to a set of small molecules structurally distinct from the original ligand. Site-saturated mutagenesis of the ligand recognition pocket paired with a computational protein stability screen led to the identification of PYR1 variants for 21 out of 38 chemicals screened, several of which were banned pesticides or synthetic cannabinoids relevant for environmental biosensing (Beltrán et al., 2022). Additional engineering of PYR1 and its dimerization partner led to an additional orthogonal set of dimerizers that did not respond to their original ligand or engage with the natural

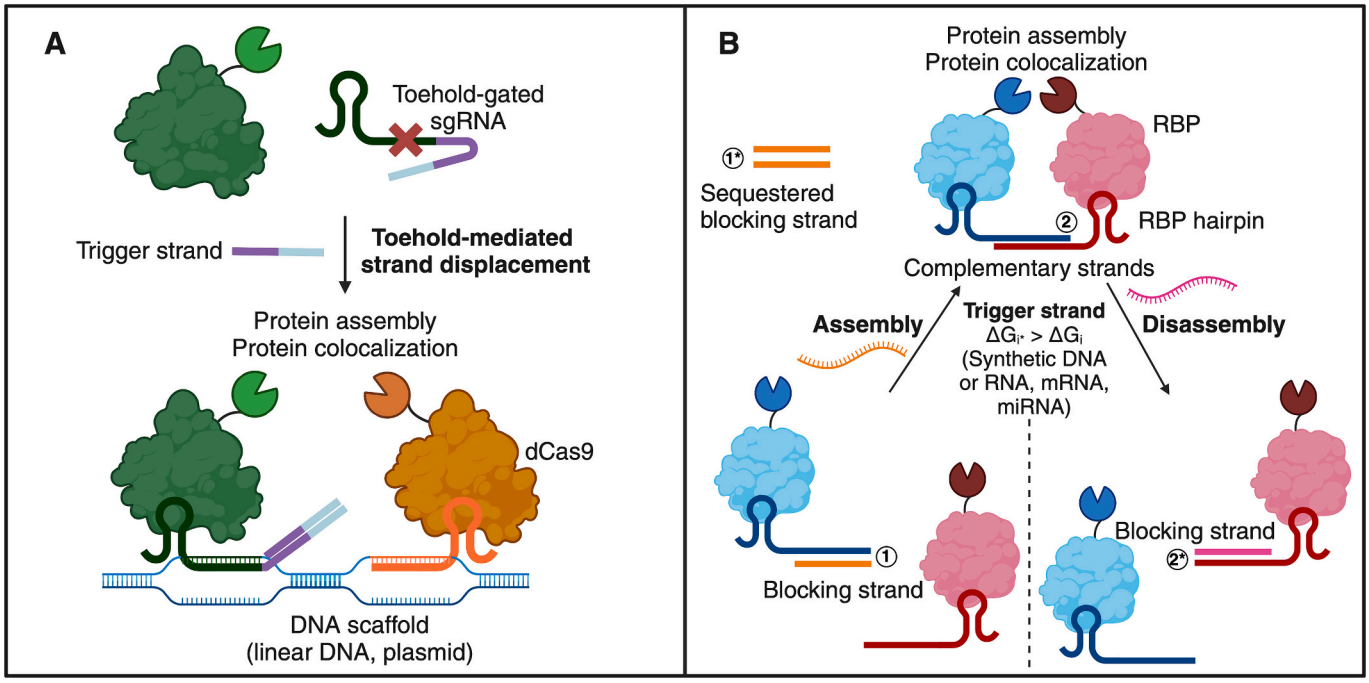

Fig. 3. Nucleic-acid responsive protein-protein interactions can exploit the programmability of DNA and RNA strand interactions to conditionally assemble proteins onto nucleic-acid scaffolds. Toehold-mediated strand displacement is used to "unlock" motifs when a trigger strand is present in the cellular environment as demonstrated by (a) conditional recruitment of dCas9 protein fusions to a DNA scaffold and (b) assembly and disassembly of synthetic RNA scaffolds.

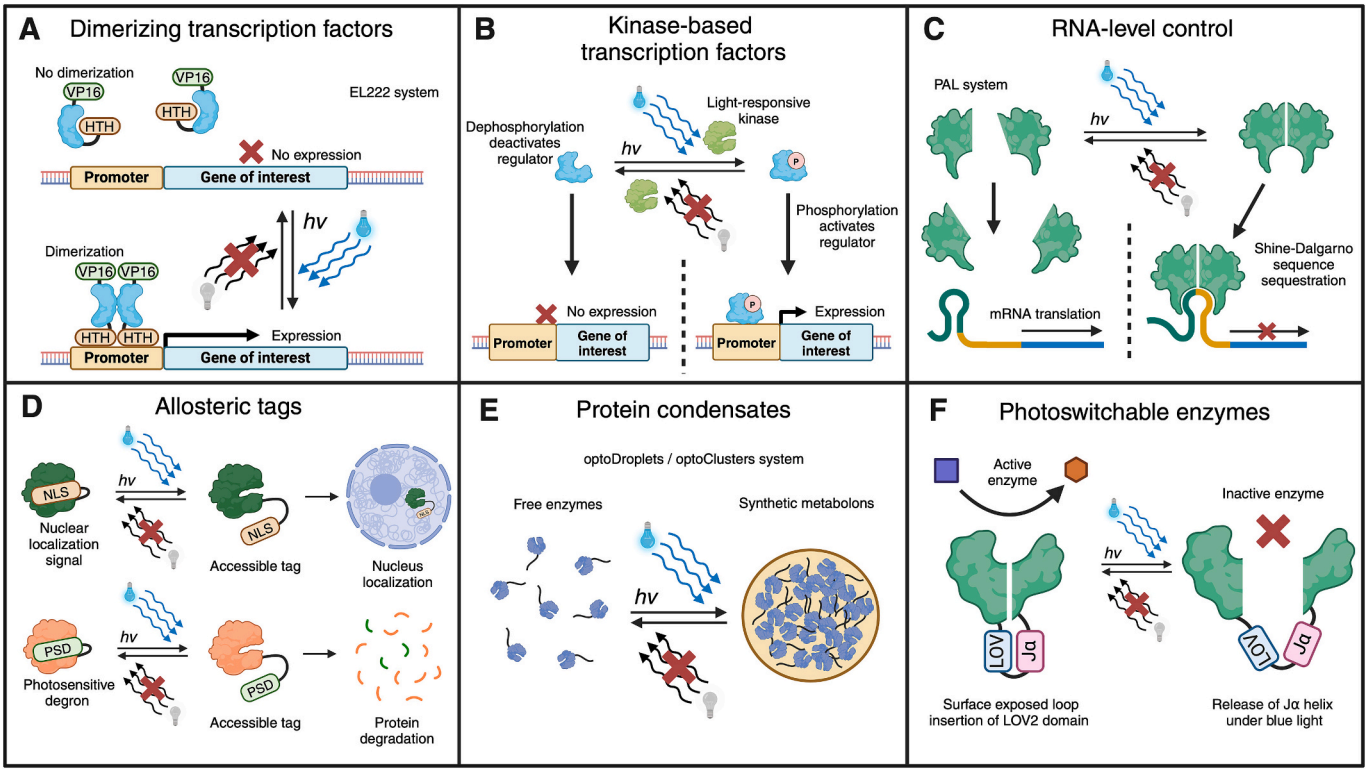

Fig. 4. Light-responsive protein-protein interactions enable reversible non-invasive regulation of various cellular processes. (a) Light-induced dimerization of the EL222 transcription factor can conditionally activate gene expression when stimulated by blue light. (b) Using light-responsive kinases to activate kinase-based transcription factors adds an additional layer of control while amplifying signals. (c) Light-inducible RNA-binding proteins can be used for RNA-level control strategies including Shine-Dalgarno sequence sequestration caused by binding of the PAL protein to inhibit translation. (d) Photocaging of localization signals or degron tags enables light-responsive protein localization or targeted protein degradation, respectively. (e) Conditional protein condensate assembly can be applied to develop inducible synthetic metabolons for dynamic metabolic engineering. (f) Loop insertion of the LOV2 domain into enzymes can modulate enzymatic activity by introducing disorder of the enzyme in response to blue light. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

plant hormone signaling pathway and were applied to build multi-input circuits in both a model yeast, *Saccharomyces cerevisiae*, as well as a plant, *Arabidopsis thaliana* (Park et al., 2024). An alternative approach to developing new CID modules is rationally splitting human ligand-binding proteins into two split fragments that conditionally reassemble in the presence of the target ligand. This approach yielded six novel orthogonal CID platforms that could regulate transcriptional activity in both human cells and a mouse model (Rihtar et al., 2022). Ligand-binding domains from separate human nuclear hormone receptors were readily swapped to permit AND-gated dimerization and corresponding transcriptional activation in response to two separate molecules (Kretschmer et al., 2023). Bertschi and colleagues demonstrated that the ligand-binding domains from bacterial transcription factors can be repurposed as modular building blocks for inducible protein dimerization. Tethering ligand-binding domains from multiple transcription factors led to multi-input AND logic-gated computation with up to five input chemicals when coupled with existing rapamycin, ABA, and gibberellin CID systems (Bertschi et al., 2023). The specificity of a chemically responsive PPI to its target ligand means that most of these conditional PPIs are orthogonal and can be used to design complex multi-input regulatory programs with minimal crosstalk. Furthermore, protein engineering strategies such as mutagenesis or directed evolution can be applied to exploit the plasticity of ligand-binding pockets to generate chemically responsive PPI for new target molecules as demonstrated in the PYR1 CID system.

Chemically induced PPIs can also be applied to targeted protein degradation. The auxin-inducible degron system utilizes the well-conserved Skp, Cullin, F-box (SCF) protein degradation pathway coupled with heterologous expression of the plant-derived F-box transport inhibitor response 1 (TIR1) protein to enable conditional protein degradation in eukaryotes (Fig. 2b). TIR1 only binds to target proteins tagged with an auxin-inducible degron when an auxin phytohormone, such as indole-3-acetic acid, is present; once bound, TIR1 recruits a native E2 ligase leading to polyubiquitination and degradation of the target protein (Nishimura et al., 2009). Auxin-induced protein degradation enabled significant or complete target ablation within 30 min in both yeast and mammalian cells (Nishimura et al., 2009). Li and colleagues reduced basal degradation and improved inducible degradation in human cells by identifying an optimal degron tag and F-box protein pairing from *A. thaliana*, miniIAA7, and AFB2, respectively (Li et al., 2019). Auxin-induced protein degradation can be stimulated by the metabolic activity within a microbial consortium; in a synthetic co-culture, one strain of *S. cerevisiae* synthesized indole-3-acetic acid from its precursor, indole-3-acetamide, which induced auxin-mediated degradation within a second receiver strain (Khakhar et al., 2016). The conditional nature of the auxin-inducible degron system enabled the degradation of an essential enzyme, ERG20p, that catalyzes a competing reaction to redirect flux towards terpene synthesis. Further expansion of the degradation targets to Hxk2p and Acc1p reduced carbon catabolite repression and induced growth arrest, improving nerolidol bioproduction (Lu et al., 2021).

Similar to the auxin-inducible degron system, proteolysis targeting chimeras (PROTACs) form a ternary complex with a target protein and an E3 ligase to initiate polyubiquitination and subsequent protein degradation by the proteasome (Fig. 2c). An advantage of the PROTAC system is the diversity of chemical ligands that can be used to induce protein degradation. Since the original discovery of PROTACs (Sakamoto et al., 2001), a large number of orthogonal E3 ligase—PROTAC—target pairings have been characterized and applied to targeted protein degradation of clinically relevant proteins (Schapira et al., 2019; Sun et al., 2019a). The expanded ligand portfolio developed for PROTAC systems offers an array of options for conditional PPI. Truncation of E3 ligases to remove the native ubiquitination functionality enabled Ma and colleagues to generate a PROTAC mimic of the CID systems described earlier (Fig. 2d). Translational fusions of the truncated E3 ligase to VPR and the target protein to a DNA-binding protein led to transcriptional activation induced by the presence of the PROTAC molecule. The diversity of orthogonal E3 ligase-PROTAC-target pairings was exploited to develop logic-gated transcriptional regulation programs in mammalian cells and mouse models (Ma et al., 2023). Decoupling the PROTAC platform from polyubiquitination expands the range of applications from solely chemical-responsive degradation to the many functions developed previously for CID systems. Additionally, the development of PROTAC-CID should enable the use of PROTAC-inducible systems in hosts that lack the ubiquitin proteasome pathway, such as bacteria.

2.2. Nucleic acid-responsive protein interactions

Proteins interact with nucleic acids, both DNA and RNA, to engage in many essential cellular processes, including transcription, translation, and viral defense. These interactions are driven by the recognition of a specific nucleic acid sequence or structural motif by a protein binding domain. As demonstrated by the targeted regulation of gene expression by transcription factors or cleavage by restriction enzymes and CRISPR-Cas9, proteins are capable of highly specific binding to the cognate nucleic acid motif even within the vast sequence space of large genomes (Chen et al., 2017). Thus, proteins can be engineered to bind precisely to a particular genomic locus, episomal or plasmid site, or mRNA transcript.

Multiple proteins can be docked onto a nucleic acid scaffold, allowing for precise spatial organization of enzymes or protein reconstitution. Viral RNA-binding proteins (RBP), such as the MS2 and PP7 coat proteins, bind to distinct RNA hairpins, permitting orthogonal binding to an RNA scaffold. Fusing each viral RBP to a split fragment of GFP led to protein complementation and restoration of fluorescence only when an RNA scaffold containing the corresponding hairpins was present (Delebecque et al., 2011). The colocalization of hydrogenase and ferredoxin onto an RNA scaffold enhanced hydrogen bioproduction; increasing the dimensionality through polymerization of the RNA scaffolds further elevated hydrogen bioproduction (Delebecque et al., 2011). Due to the helical nature of nucleic acids, the number of nucleotides between the RNA hairpins changes the orientation and distance between the scaffolded proteins, which significantly affects protein complementation or metabolic channeling between pathway enzymes (Mitkas et al., 2022; Sachdeva et al., 2014). The use of DNA-binding proteins (DBP) enables DNA to be used as a scaffold for improved biochemical production of propylene glycol, mevalonate, and resveratrol using zinc fingers as the DBP anchor for these pathway enzymes (Conrado et al., 2012) or increased biosynthesis of indole-3-acetic acid using transcription activator-like effectors (TALE) scaffolded onto TALE-binding sequences (Zhu et al., 2016). As demonstrated in these efforts, the user-defined nature of plasmid DNA scaffold sequences permits a facile approach towards identifying the proper density of colocalized enzymes for efficient biosynthesis.

The assembly of protein-nucleic acid scaffolds can be regulated by DNA or RNA triggers through toehold-mediated strand displacement (TMSD). In TMSD, a short “toehold” of accessible DNA or RNA complementary to a trigger strand enables the trigger strand to hybridize and displace the original strand. Any complementary sequence can be used for TMSD if it is thermodynamically favorable. This limitation can be partially mitigated for DNA scaffolds by adding a TMSD enabled conditional cellulosome assembly on a DNA scaffold. Cellulolytic enzymes were fused to orthogonal dCas9 and targeted to the scaffold by a sgRNA, leading to a 2.6-fold increase in the production of reducing sugars (Berckman and Chen, 2019). A turn-on scaffold architecture was achieved by replacing the sgRNA with a toehold-gated sgRNA, a modified sgRNA where the spacer region that binds to the dCas9 protein is sequestered in a hairpin (Siu and Chen, 2019), providing a complementary trigger strand that induces branch migration along the toehold-gated sgRNA. This frees the spacer region which leads to formation of the dCas9-sgRNA complex and binding to the target sequence on the

DNA scaffold (Fig. 3a). Similarly, cancer-associated micro RNA controlled the conditional reconstitution of the enzyme prodrug split yeast cytosine deaminase on a DNA scaffold (Chen et al., 2018). With a turn-off architecture, the protein-binding motif is available initially and then displaced when the more thermodynamically favorable trigger nucleic acid is present. As demonstrated by FRET, a turn-on and turn-off architecture may be combined to generate a reversible circuit with four or more cycles (Chen et al., 2018). The predictable programmability of nucleic acid strand interactions permits existing trigger stands to be swapped for theoretically any desired input sequence, thermodynamics permitting. This constraint limits the reversibility and number of possible successive triggers that can be implemented. However, recent work by Thompson and colleagues developed an engineered helicase, Rep-X, that enabled thermodynamically unfavorable strand displacement at the expense of ATP (Hall-Thomsen et al., 2023).

Conditional protein assembly on RNA scaffolds was made possible by using CRISPR-Cas6 proteins, a class of orthogonal RBP with endonuclease capabilities, and TMSD. Again, turn-on and turn-off architectures (Fig. 3b) were established and validated through colocalization and assembly of a split nanoluciferase using synthetic RNA triggers as well as a full-length mRNA transcript (Mitkas et al., 2022). These conditional RNA-protein scaffolds proved useful as synthetic metabolons in *Escherichia coli* for improved indole-3-acetic acid production; the large multimeric enzymes involved in malate synthesis were also able to be conditionally colocalized to achieve a 2.9-fold increase in malate titers (Mitkas et al., 2022). Conditional nucleic acid-based protein assembly and colocalization appear generalizable across kingdoms of life and adaptable for many different metabolic pathways as the Cas6-based scaffolds were readily adapted for use in *S. cerevisiae* as well as a non-model yeast, *Kluyveromyces marxianus*, for violacein production (Pham et al., 2023). Regulating the transcription of RNA triggers with chemically induced PPI is a potential strategy to provide logic-gated cellular control in response to both chemicals and nucleic acids.

2.3. Light-responsive protein interactions

External light signals can stimulate light-responsive PPIs for fine-tuned cellular control. Light-responsive PPIs use photoreceptor proteins that allow organisms to sense light signals and convert these signals into physiological responses. These proteins require specific chromophores to absorb light energy (Möglich et al., 2010). Photoreceptor proteins typically contain light-responsive and effector domains that interact with other macromolecules. The rearrangement of peptide residues upon light detection is often coupled with effector outputs such as phosphorylation or transcriptional regulation. Light-responsive domains can be modified through engineering efforts to detect narrow wavelengths and form precise interactions with other proteins and nucleic acids for optogenetic control (Hartzell et al., 2021).

Light-responsive transcriptional regulation leverages two distinct mechanisms to couple light sensing to gene expression: dimerization of transcription factor-like devices and kinase-based transcription regulation systems. The first mechanism is exemplified by the OptoEXP circuit in which the fusion of the activation domain VP16 to the EL222 light-sensitive transcription factor enabled blue light-dependent gene expression (Fig. 4a) and production of isobutanol and 2-methyl-1-butanol in *S. cerevisiae* (Zhao et al., 2018). Further studies of the EL222 system incorporated inverted circuit designs, engineered circuit amplifications, improved scalability, and demonstrated portability to non-conventional hosts, such as the yeast *Yarrowia lipolytica* (Pouzet et al., 2023; Wang et al., 2022; Zhao et al., 2020, 2021). One challenge of the EL222 system is limited reversibility, which can be mediated by adding a protein degradation control layer, which is discussed in a later section (Tague et al., 2024). Light-sensitive cryptochrome 2 protein (Cry2) and its partner CIB1 have also been exploited for two-hybrid transcriptional regulation under blue light. Fusion to TALE and VP64 allows light-dependent gene activation in neurons. Epigenetic-level regulation was also achieved by replacing VP64 with histone effectors (Konermann et al., 2013). The Cry2 light-responsive PPI is also used for fermentation as evidenced by the light-controlled production the sweet protein monellin in *S. cerevisiae* (Gramazio et al., 2022). Phytochrome-based optical dimers are another attractive optogenetic device due to their capability to sense red light with the heterodimer PhyB and PIF3 pair used to modulate CRISPR activation in *S. cerevisiae* when fused to dCas9 and VPR respectively (Machens et al., 2023). Light penetration is severely limited in high-density cell cultures, which has encouraged the exploration of near-infrared (NIR) optogenetic systems. Bacteriophytochrome photoreceptor 1 (BphP1) can sense NIR light and activate transcription by inhibiting the transcriptional repressor PpsR2. Adaptation of this system in *E. coli* created the most red-shifted optogenetic tool available in bacterial systems (Ong et al., 2018). The portability of these NIR PPI was demonstrated by the evolution of a truncated BphP variant leading to NIR-induced gene regulation in mammalian systems with maximal activation of 65-fold in cell culture and 6-fold in deep mice tissues (Kaberniuk et al., 2021). NIR-responsive phytochrome PPIs hold great promise in tackling the light penetration issue for in vivo therapeutic applications as well as microbial fermentation. One challenge with these phytochrome-based systems is the requirement of a bilin-type chromophore, which is not present in many hosts and requires exogeneous supplementation or heterologous expression of the bilin synthesis pathways.

Kinase-based transcriptional regulation provides an alternative method for light-induced gene expression. Generally, a kinase system includes a light-responsive kinase, the corresponding kinase-regulated transcription factor, and the regulated promoter (Fig. 4b). This two-layer architecture increases output strength through signal amplification which is often compromised by the requirement for more biological components, higher background, and slower kinetics. The CcaS-CcaR two-component system, which responds to green and red light, is native to *Synechocystis* and its endogenous regulatory strategy was repurposed to enable light-controlled production of isobutanol and 3-methyl-1-butanol in this host (Kobayashi et al., 2022). The CcaS-CcaR PPI was used as a light-inducible metabolic switch to regulate metabolic flux between the oxidative pentose phosphate and Embden-Meyerhof-Parnas pathways in *E. coli* (Tandar et al., 2019). An orthogonal ultraviolet-violet-green light-responsive PPI pair, UirS-UirR, was also sourced from *Synechocystis* and then repurposed as a transcriptional regulator in *E. coli* (Ramakrishnan and Tabor, 2016). The UirS-UirR PPI is currently the most blue-shifted photoreversible optogenetic system available. The orthogonality of kinase-based light-inducible transcriptional regulators controlled with different wavelengths was proven when an RGB color vision circuit was engineered in *E. coli*. The system utilized Cph8* (705 nm infrared light on, 650 nm red light off), CcaS-CcaR (535 nm green light on, 672 nm far-red off), and YF1-FixJ (darkness on, 470 nm blue light off) that were integrated into the genome with their required chromophores, respectively. Expression of red, green, and blue color pigments under these three light-sensitive promoters generated “color photographs” in a spatially distant manner (Fernandez-Rodriguez et al., 2017). The development of orthogonal light-responsive PPI systems provides the ability to execute complex cellular programs with finely controlled intensity and duration specified by the user. While light-inducible PPI has been demonstrated to regulate transcription across many hosts and in response to a wide range of wavelengths, challenges such as poor reversibility, limited light penetration, and excessive generation of heat caused by light stimulation remain.

RNA-level control provides another knob to tune physiological responses. One of the few RNA-level systems available makes use of PAL, a novel protein from *Nakamurella multipartite* that contains a light-oxygen-voltage (LOV) photosensor and an RNA-binding domain that targets an antitermination hairpin (Fig. 4c). The specificity of the RNA-binding activity in response to blue light was confirmed by screening against multiple RNA libraries. Blue light-induced inhibition of translation was

achieved in both bacterial and mammalian cells by inserting the PAL cognate sequence upstream of the ribosome binding site; the ribosome binding site is blocked by PAL upon blue light stimulation (Ranzani et al., 2022; Weber et al., 2019). Another system using a similar mechanism makes use of the photoswitchable RNA-binding protein LicV, which contains a Vivid domain that dimerizes under blue light fused to a monomer of the RNA-binding domain from dimeric LicT. These dimerization events enabled light-inducible RNA translocation, splicing, and degradation in human cells. Additionally, appending the cognate RNA sequence for LicV to the 3′ end of a sgRNA enabled light-responsive CRISPR activation when LicV was fused to VPR and genomic locus labeling when LicV was fused to GFP (Liu et al., 2022). An alternative strategy to use blue light for RNA-level control instead used the light-responsive pMag and nMagH domains to conditionally recruit split Cas13. The use of catalytically active Cas13 enabled light-inducible degradation of RNA targets while instead using catalytically dead Cas13 fused to the ADAR2 base editor enabled blue light-induced RNA editing (Yu et al., 2024). Further work is needed to develop optogenetic PPIs usable for RNA-level control beyond those that respond to the shorter waves of blue light.

Protein localization is an effective technique to spatially control PPI, especially within eukaryotic hosts where regulatory proteins often exist on organelle membranes. Light-responsive protein localization can be engineered by fusing localization signals to chimeric light-sensing protein effectors. LINuS is a LOV2 domain that photocages a nuclear localization signal (NLS) at the end of the Jα helix (Fig. 4d). The helix unfolds upon blue light stimulation, which unveils the NLS; N-terminal fusion of LINuS to a protein of interest enables conditional nuclear localization. To demonstrate this, a fusion of cyclin B1-CDK1 to LINuS granted light-dependent commitment to mitosis in HeLa cells (Niopek et al., 2014). Another similar mechanism was demonstrated with LANS and applied in *S. cerevisiae* and the model nematode *Caenorhabditis elegans* (Yumerefendi et al., 2015). Conversely, the NLS can be replaced with a nuclear export signal (NES) to rapidly remove proteins in response to light (Yumerefendi et al., 2016). Localization can also be achieved using the same light-inducible dimerizing partners used for transcriptional activation. The red-light responsive PhyB-PIF protein pair was fused to a mitochondrial membrane protein and Bem1 to regulate bud emergence in yeast (Jost and Weiner, 2015).

Photosensitive PPIs are an emerging technique to conditionally generate protein condensates that can sequester cellular effectors into synthetic compartments through phase separation. Conditional condensate formation can be used to generate synthetic organelles in organisms that lack them such as most bacteria or provide orthogonal compartments in eukaryotes to minimize interactions with native processes (Fig. 4e). Towards that end, fusion of the Cry2 with an intrinsically disordered region from the fused in sarcoma protein produced light-responsive liquid-like condensates called optoDroplets (Shin et al., 2017); mutating a single residue in Cry2 enables more enhanced oligomerization leading to optoClusters, a more rigid condensates (Taslimi et al., 2014). Photosensitive PPI pair PixD and PixE with an inverted response to light irradiation were used to make the PixELL system in which droplets dissociate in response to light (Dine et al., 2018). There is particular interest in developing optogenetic enzyme clustering systems for dynamic control of metabolism to take advantage of metabolic channeling and increased effective molarity of pathway intermediates. To achieve this, the optoDroplets, optoClusters, and PixELL systems were adapted for use in yeast and used to cluster enzymes in the violacein pathway. These light-inducible condensates improved product titers by 6.1-fold as well as minimizing by-product formation (Zhao et al., 2019a). Light-responsive protein condensates were also demonstrated in *E. coli* to improve the biosynthesis of luciferin and catechol (Huang et al., 2022). Condensate dynamics are rapid, with assembly and disassembly occurring in the order of seconds for PixELLs and in the order of minutes for optoDroplets (Zhao et al., 2019b). In order to develop effective condensate systems, careful optimization of the expression levels of the oligomerizing proteins is required to minimize background condensate formation.

Targeted protein degradation controlled by light is achieved by engineering light-responsive degron tags. Similar to the localization strategy, fusing the ornithine decarboxylase (ODC) degron tag to the Jα helix of the LOV2 domain creates a photosensitive degron (psd) module (Fig. 4d). Tagging the cell cycle regulators Clb2 and Sic1 with the psd module permitted light-responsive growth arrest and cell death in *S. cerevisiae* (Renicke et al., 2013). Fusing the psd module to the ubiquitin-proteasome system hub Cdc48 instead arrested yeast cells in the G2/M phase, which increased the abundance of metabolic enzymes and ultimately increased the yield of beta-carotene by 11 to 18-fold (Bezold et al., 2023). In applying light-activated protein degradation in *E. coli*, Tague and colleagues modified the C-terminus of the LOV2 protein for tighter dark state caging of an unstructured degron and used this LOVdeg platform to conditionally deplete LacI, AcrB, and an activator for CRISPRa (Tague et al., 2024). A combined approach using light-induced protein degradation with LOVdeg and light-responsive transcriptional repression with EL222 led to both a stronger and faster response towards regulating the target protein (Tague et al., 2024).

In contrast to the previous methods of post-translational regulation, photoswitchable allosteric regulation directly modulates the activity of a protein by conditionally inducing conformation shifts. Comparatively, this regulatory strategy is more challenging to achieve as it requires a thorough understanding of the protein structure, function, and mechanism of action. Insertion of the LOV2 domain into surface-exposed loops can disrupt protein activity by inducing disorder in the target protein as C-terminus of the LOV2 protein becomes more flexible in the presence of blue light. This was demonstrated in several distinct classes of mammalian signaling proteins including a Src kinase, Rho family GTPase, and guanine exchange factor (Dagliyan et al., 2016). Another allosteric approach also used LOV2 to instead induce an off-to-on confirmational shift by photocaging the binding and activating motif of a G-family-protein-receptor (GPCR) unless stimulated by light (Garcia-Marcos et al., 2020). Enzymatic activity can also be regulated by insertion of the LOV2 domain demonstrating potential use in developing dynamic metabolic engineering strategies (Fig. 4f). Reversible optical repression of isocitrate dehydrogenase (Li et al., 2022) and alcohol dehydrogenase (Sun et al., 2019b) in *E. coli* was achieved using an approach similar to Dagliyan and colleagues. Photoswitchable allosteric regulation of the isocitrate dehydrogenase led to dynamic flux diversion between the competing tricarboxylic acid (TCA) pathway and itaconic acid biosynthesis increasing product yield by 79 %. A primary challenge of photoswitchable allosteric regulation is recovering the full activity of the regulated protein in the on-state, which is often impaired by steric issues imposed by insertion of the photoreceptor domain. The relatively small size of LOV2 (16.5 kDa) alleviates this challenge, but many photoreceptors, including red light responsive phytochromes, are significantly larger in size. The recent development of bidirectional, cyanobacteriochrome-based light-inducible dimers (BICYCLs) produced the smallest red/green optogenetic tool to date with a size of only 161 residues. BICYCLs have been utilized for protein localization and condensate formation induced by red light (Jang et al., 2023). Further work will be needed to assess whether these light-responsive phytochromes can also be implemented in photoswitchable allosteric regulation, but their smaller size may resolve common fusion protein issues such as unwanted steric interactions and poor solubility.

Light-responsive PPIs have several distinct advantages in that cellular control can be carefully controlled by exogenous light stimulation that has minimal impact on native cellular processes. Additionally, the use of multiple PPIs responding to different wavelengths provides users with a set of orthogonal regulatory mechanisms. However, poor depth penetration is an inherent challenge in optogenetics that limits applications in industrial fermentation and therapeutic settings necessitating the demand for near-infrared PPI.

3. Design and testing of novel conditional protein-protein interactions

As demonstrated by the examples discussed in this review, there has been substantial progress in employing conditional PPI to regulate different cellular activities across many species. Converting an existing conditional PPI to regulate an alternative cellular process or function in another organism requires a thorough analysis of the PPI mechanism and the regulatory strategy to confirm that it is appropriate for the desired application. To select a suitable stimulus, consideration must be given to whether the user will exogenously supply the stimulus or whether the cells will autoregulate their behavior in response to an endogenous stimulus. The former approach is often useful for characterizing PPIs of interest or designing conditional PPIs while the latter offers potential for dynamic metabolic engineering or targeted therapeutics. Additionally, all system components must be able to function in the cellular environment; protein components must be able to be solubly expressed or delivered at sufficient concentrations in the host cell. Once a feasible system is designed, the conditional PPI should be tested to determine sensitivity to the stimulus, background activity in the absence of the stimulus, response kinetics, and reversibility of the system. Protein complementation assays are a powerful tool to confirm proper conditional PPI in vitro or in vivo; fusion of the conditional PPI pair to a split luminescent or fluorescent reporter such as split nanoluciferase (Dixon et al., 2016) or split fluorescence-activating and absorption-shifting tag (FAST) (Rakotoarison et al., 2024; Tebo and Gautier, 2019), respectively, provides researchers with a quick and high throughput manner to assess the key system parameters. Conditional PPIs used to regulate transcription or translation can be tested by analyzing the expression of a reporter gene while induction of targeted protein degradation can be assessed by measuring the depletion of a reporter protein. Conditional gene editing, including gene knockouts, base editing, or RNA editing, can also be determined by monitoring fluorescent reporter levels that are elevated or repressed as the result of the genetic edit. Most of the systems described in this review have been ported from one application or host to another and are readily adaptable for implementation in alternative contexts using these common testing strategies. However, generating potential conditional PPI de novo and identifying likely candidates that respond to new stimuli is significantly more challenging. Here, we present many recently developed computational tools that facilitate the design of novel conditional PPIs (Table 2).

Due to the difficulty of constructing a conditional PPI de novo, building from existing platforms is preferable, if available. There is a wealth of resources found in online databases for transcription factors (d'Oelsnitz et al., 2022), PROTACs (Weng et al., 2021), and optogenetics (Kolar et al., 2018). Literature searches can be accelerated by applying open-source artificial intelligence chatbots, such as ChatGPT-4, to mine for specific features of interest (Xiao et al., 2023). With advances in genomics and metagenomics, there is a potentially significant source of unknown natural transcription factors responsive to new chemical ligands that can be exploited for conditional PPIs. New metabolite-responsive transcription factors can be identified with TFBMiner, a program that analyzes gene clusters annotated to produce the metabolite of interest; the application of this tool led to the identification of a previously unknown biosensor for S-mandelic acid (Hanko et al., 2023). In tandem with this approach, the DNA operator sites for uncharacterized transcription factors can be predicted from inverted repeats present in the nearby inter-operon region that are conserved across homologs of the transcription factor using Snowprint (d'Oelsnitz et al., 2024). The generation of single-protein biosensors responsive to new inputs can be used to generate novel conditional PPIs by splitting them into fragments. The ideal split protein for conditional PPI would have minimal interactions between fragments in the absence of the inducer molecule. Split sites that preserve protein function and minimize basal assembly can be determined by extensive trial and error-based approaches. Alternatively, computational identification of split sites can be performed using the SPELL algorithm that explores the split energy of a potential set of split fragments, which is the difference between the energies of the intact protein and its split fragments. This algorithm also accounted for sequence conservation, surface exposure, and loop tightness and was used to successfully split TEV protease, tyrosine kinase, GDI1, and Vav2 (Dagliyan et al., 2018). SPORT is another split protein computational design tool that introduces mutations to interfacial residues to identify mutations that have significant differences in the solvent-accessible surface area and stability of the interface as well as the entire protein (Dolberg et al., 2021).

The advent of highly accurate protein structure prediction tools such as AlphaFold (Jumper et al., 2021) and RoseTTAFold (Baek et al., 2021) allows for rationale design and analysis at every step of the conditional PPI engineering pathway. The ability to generate protein structures from sequence alone permits thorough probing of the impact of mutations on the shape of binding pockets or examination of potential split sites. The latter can be further explored through the predicted PPIs identified by

Table 2
Computational tools and databases useful for the design of novel conditional protein-protein interactions.

Tool	Functionality	Reference
GroovDB	Database for transcription factors	d'Oelsnitz et al., 2022
PROTAC-DB	Database for PROTACs, targets, and ligases	Weng et al., 2021
OptoBase	Database for optogenetics curated by subject matter experts	Kolar et al., 2018
AlphaFold Protein Structure Database	Database with over 200 million protein structure predictions generated by AlphaFold	Varadi et al., 2022
ChatGPT4	AI tool for literature mining	Xiao et al., 2023
TFBMiner	Identifies potential regulatory proteins by scanning annotated biosynthetic gene clusters	Hanko et al., 2023
SnowPrint	Predicts DNA operators for transcription factors	d'Oelsnitz et al., 2024
SPELL	Predicts potential protein split sites by determination of total energies, solvent accessibility, sequence conservation, and loop tightness	Dagliyan et al., 2018
SPORT	Predicts potential protein split sites by identification of junctions with large changes in solvent-accessible surface area coupled with mutation recommendations	Dolberg et al., 2021
AlphaFold	Highly accurate protein structure predictions; Current version predicts structures of protein complexes containing chemical ligands, post-translational modifications, and nucleic acids	Jumper et al., 2021; Abramson et al., 2024
AlphaFold Multimer	Extension of AlphaFold to predict protein-protein interactions	Evans et al., 2022
RoseTTAFold	Protein structure predictions	Baek et al., 2021
Foldseek	Protein structure similarity search	van Kempen et al., 2024
AlphaFind	Protein structure similarity search	Procházka et al., 2024
AlphaCluster	Extension of AlphaFold to identify alternative protein conformations	Wayment-Steele et al., 2023
AlphaFill	Prediction of ligand-binding sites in protein structures	Hekkelman et al., 2022
Autodock	Protein and ligand docking	Forli et al., 2016
DynamicBind	Prediction of ligand-binding conformational shifts	Lu et al., 2024
RoseTTAFold-NA	Predicts structures of protein and nucleic acid complexes	Baek et al., 2024
RFdiffusion	Generative model for de novo protein design	Watson et al., 2023
Chroma	Generative model for de novo protein design	Ingraham et al., 2023

the AlphaFold multimer model (Evans et al., 2022). AlphaFold has generated structures of over two hundred million proteins; each structure contains per-residue confidence metrics, pLDDT, as well as the predicted aligned error, which is useful in determining the relative position of domains within a protein (Jumper et al., 2021; Varadi et al., 2022). These large databases can be quickly searched to identify structurally similar proteins using recently developed tools such as Foldseek (van Kempen et al., 2024) and AlphaFind (Procházka et al., 2024). It is important to note that while these structural predictions may be powerful tools to guide rational engineering, there can be significant deviations between the predicted and actual structures. These deviations can arise from unstructured regions, which are often poorly predicted, or from different protein conformations due to temperature variations, ligand-binding, or as an artifact of crystallography conditions (Terwilliger et al., 2024). By clustering the distribution of structures predicted for a single protein by AlphaFold, Wayment-Steele and colleagues were able to identify multiple conformations for the fold-switching protein KaiB, and then used this same approach to screen protein families to identify previously unknown fold-switching proteins (Wayment-Steele et al., 2023). This tool can be applied to identify alternative protein conformations when target ligands are bound to provide more accurate structures and, thus, a more explicit depiction for rational design. Of particular interest to the design of chemical-inducible PPIs, potential ligand-binding sites for several thousand unique molecules can be identified with AlphaFill (Hekkelman et al., 2022). Alternatively, molecular docking programs, such as AutoDock, can be applied to determine ligand binding (Forli et al., 2016). DynamicBind can also be used to predict ligand-binding sites on target proteins and recover the protein conformation structure in the bound and unbound states (Lu et al., 2024). A recent extension of RoseTTAFold has been developed that enables predictions of protein-nucleic acid binding (Baek et al., 2024), which can guide the design of nucleic acid-induced PPI. De novo protein design can be conducted using generative models that produce a protein structure that meets the design objective, such as grafting functional sites onto a protein scaffold or potent binding to a target protein. Recent generative models rely on an iterative diffusive process that denoises a random initial starting point and gradually refines the protein structure necessary to meet the objective function. RFdiffusion is a generative model that was applied to generate unconditional protein monomers and oligomers, scaffold the active site for an oxidoreductase, and design high-affinity binders to several clinically relevant protein targets (Torres et al., 2024; Watson et al., 2023). Another interesting generative model, Chroma, performs similar tasks to RFdiffusion with the addition of allowing natural text inputs (Ingraham et al., 2023). The recently released AlphaFold 3 also implements a generative approach and has been expanded to predict structures for proteins in complex with other proteins, ligands, and nucleic acids as well as for proteins with post-translational modifications (Abramson et al., 2024). Adaptation of generative models, such as AlphaFold, RFdiffusion, and Chroma, will enable the de novo design of novel conditional PPIs and can also be used to optimize existing PPI for new applications.

4. Outlook

Conditional control of protein interactions enables programmable assembly, colocalization, and spatial organization of proteins inside cells. Regulation of PPIs has been applied to finely regulate metabolic fluxes in microbial factories to improve resource allocation or limit the burden on the host. Additionally, these conditional PPIs can be utilized as living biosensors or mechanisms to activate targeted protein therapeutics. Conditional protein interactions have been engineered to respond to diverse inputs, including endogenous metabolites or mRNA transcripts or operator-supplied chemical ligands, light, and exogenous DNA. The stimuli-responsive protein domains are often application-agnostic and can be transferred to new protein-level regulatory strategies and hosts, providing a modular toolkit for protein engineers and synthetic biologists.

Thus far, most conditional PPIs have been adapted from natural sources, such as the rapamycin-induced dimerizers, light-responsive LOV domains, and CRISPR-Cas endonucleases, and further engineered to respond to new inputs by random mutagenesis or rational design. Additional efforts have been made to increase sensitivity, remove background interactions, and develop orthogonal protein pairings for existing systems. Identifying previously unknown natural proteins that respond to new stimuli continues rapidly due to advances in genomics and metagenomics that provide new source material for protein mining and screening (Medema et al., 2021).

However, with the recent advances in hyper-accurate protein structure prediction tools, such as AlphaFold and RF-diffusion, new PPIs can now be designed rationally to respond to novel inputs. An excellent example of the ability to use computational protein design for the development of a novel responsive PPI is the latching orthogonal cage-key (LOCKR) system in which a de novo-designed protein cage is latched by a helical peptide; addition of designed key peptide displaces and frees the latch peptide to unveil a binding epitope, localization tag, or degron tag (Langan et al., 2019). To assist users and accelerate the design of conditional protein interactions, we reviewed ancillary computational tools, such as databases, structure-similarity searches, ligand binding site identifiers, and docking tools. While these tools are powerful at sampling the protein space for potential options, there is a high failure rate, and high-throughput screening is often required to identify candidates that respond to the stimuli as desired. Continued advances in these computational tools and careful selection of screening conditions will likely yield improved efficacy in engineering novel responsive protein-level control architectures. The expansion of possible inputs for protein-level regulation empowers the generation of multi-level cellular regulation and diversifies the number of potential applications in improving biochemical production and human health.

Declaration of competing interest

The authors declare no competing interests.

Data availability

No data was used for the research described in the article.

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