REVIEW SUMMARY

CHEMICAL BIOLOGY

Chemically induced proximity in biology and medicine

Benjamin Z. Stanton,* Emma J. Chory,* Gerald R. Crabtree†

BACKGROUND: Nature has evolved elegant mechanisms to regulate the physical distance between molecules, or proximity, for a wide variety of purposes. Whether it is activation of cell-membrane receptors, neuronal transmission across the synapse, or quorum sensing in bacterial biofilms, proximity is a ubiquitous regulatory mechanism in biology. Over the past two decades, chemically induced proximity has revealed that many essential features and processes, including protein structure, chromosomal architecture, chromatin accessibility, transcription, and cellular signaling, are governed by the proximity of molecules. We review the critical advances in chemical inducers of proximity (CIPs), which have informed active areas of research in biology ranging from basic advances to the development of cellular and molecular therapeutics.

ADVANCES: Until the 1990s, it was unclear whether proximity was sufficient to initiate signaling events or drive their effect on transcription. Synthetic small molecule–induced dimerization of the T cell receptor provided the first evidence that proximity could be used to understand signal transduction. A distinguishing feature of small-molecule induced-proximity systems (compared to canonical knockdown or knockout methods) is the ability to initiate a process midway and discern the ensuing order of events with precise temporal control. The rapid reversibility of induced proximity has enabled precise analysis of cellular and epigenetic memory and enabled the construction of synthetic regulatory circuits. Integration of CRISPR-Cas technologies into CIP strategies has broadened the scope of these techniques to study gene regulation on time scales of minutes, at any locus, in any genetic context. Furthermore, CIPs have been used to dissect the mechanisms governing seemingly well-understood processes, ranging from transport of proteins between the Golgi and endoplasmic reticulum to synaptic vesicle transmission. Recent advances in proximity-induced apoptosis, inhibition of aggregation, and selective degradation of endogenous proteins will likely yield new classes of drugs in the near future.

OUTLOOK: We review fundamental conceptual advances enabled by synthetic proximity as well as emerging CIP-based therapeutic approaches. Gene therapy with precise regulation and fully humanized systems are now possible. Integration of proximity-based apoptosis through caspase activation with chimeric antigen receptor (CAR) T cell therapies provides a safety switch, enabling mitigation of complications from engineered immune cells, such as graft-versus-host disease and B cell aplasia. Furthermore, this integration facilitates the potential for repopulation of a patient's cells after successful transplantation. With the recent approval of CTL019, a CAR T cell therapeutic from Novartis, integrated strategies involving the use of CIP-based safety switches are emerging. Innovative exemplars include BPX-601 (NCT02744287) and BPX-701 (NCT02743611), which are now in phase 1 clinical trials. By using a similar proximity-based approach, conditional small-molecule protein degraders are also expected to have broad clinical utility. This approach uses bifunctional small molecules to degrade pathogenic proteins by dimerizing with E3 ubiquitin ligases. Degradation-by-dimerization strategies are particularly groundbreaking, because they afford the ability to repurpose any chemical probe that binds tightly with its pathogenic protein but which may not have previously provided a direct therapeutic effect. We anticipate that the translation of CIP methodology through both humanized gene therapies and degradation-by-dimerization approaches will have far-reaching clinical impact. ■

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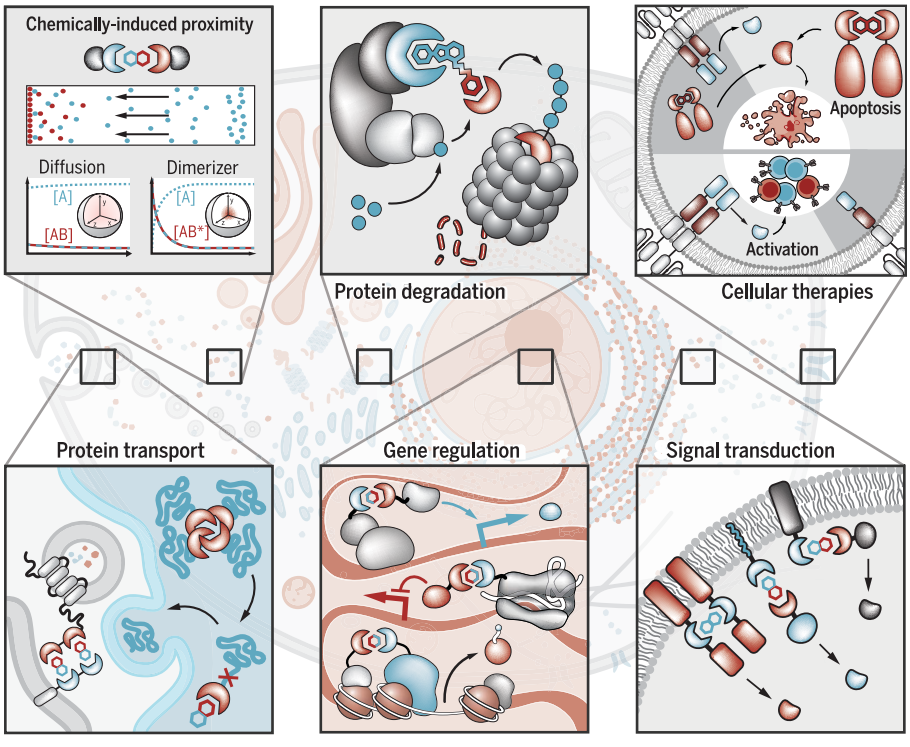

Chemically induced proximity. (Top) Left: Small molecules (hexagons) bind proteins of interest (crescents), dimerizing them to increase the effective molarity of reactions. [A] monomeric protein and [AB*] dimer concentrations; arrows, position coordinates. Middle: Synthetic dimerizers tag proteins (blue circles) for proteasomal degradation (red rods). Right: Homodimerizing molecules form kill switches for apoptosis. (Bottom) CIPs mimic cellular processes. Left: Protein transport mechanisms—nuclear import and export, membrane fusion, and protein folding. Middle: Regulation of gene activation by binding to DNA or chromatin (spheres with white strands), through recruitment of transcriptional activators or repressors (blue and red arrows). Right: Signal transduction pathways.

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Proximity, or the physical closeness of molecules, is a pervasive regulatory mechanism in biology. For example, most posttranslational modifications such as phosphorylation, methylation, and acetylation promote proximity of molecules to play deterministic roles in cellular processes. To understand the role of proximity in biologic mechanisms, chemical inducers of proximity (CIPs) were developed to synthetically model biologically regulated recruitment. Chemically induced proximity allows for precise temporal control of transcription, signaling cascades, chromatin regulation, protein folding, localization, and degradation, as well as a host of other biologic processes. A systematic analysis of CIPs in basic research, coupled with recent technological advances utilizing CRISPR, distinguishes roles of causality from coincidence and allows for mathematical modeling in synthetic biology. Recently, induced proximity has provided new avenues of gene therapy and emerging advances in cancer treatment.

Biochemical processes are often regulated by the physical distance, or proximity, between molecules to initiate an effect. Proximity plays both a ubiquitous and essential role in biology, whether it relates to individual cells, as in confining enzymes within densely packed organelles, or whole populations, as with quorum sensing in bacteria. The importance of utilizing small molecules to induce proximity of proteins was recognized upon the discovery that the Src homology 2 (SH2) domain of tyrosine kinases mediates signal transduction by binding phosphotyrosine in the absence of catalysis (*1*). Later research showed that acetylation, methylation, ubiquitination, and a host of other transient or stable protein modifications recruit proteins that influence many processes, such as gene regulation and protein degradation. The realization that these changes in localization could produce distinct cell-fate decisions led to a fundamental question, "How does a quantitative change in localization produce discrete biologic responses?" The answer appears to lie in the simple fact that the probability of an effective collision between two molecules is a third-order function of distance (*2*). This simple relation allows steep concentration gradients to produce qualitative changes, such as cell lineage commitment. Yet, mechanisms other than proximity, like allostery, might mediate these biologic responses. How does one separate the consequences of these processes?

The effects of proximity were first distinguished from allosteric or alternative effects by the synthesis of a bivalent molecule, FK1012, that bound its ligands with no detectable allosteric changes. The nontoxic molecule simultaneously binds two FK binding proteins (FKBPs), each of which is a 108–amino acid prolyl isomerase. FK1012 was first used to homodimerize the intracellular domain of the T cell receptor (TCR) zeta chain (Fig. 1), producing signaling events that reproduce transmembrane signaling by the TCR (*3*). This first demonstration that chemically induced proximity (also referred to as chemically induced dimerization) could activate signaling was followed by similar approaches with Ras signaling (*4*), death receptor signaling (*5*), and transcriptional activation (*6*), among others. Each case supported a causative role of simple proximity in qualitative cellular changes. Although the role of proximity in the absence of allostery is still debated (*7*), we will focus this review on the emerging use of induced proximity with small molecules in resolving complex biologic questions and designing new therapeutic strategies.

Tool kits to explore proximity in biology

The first chemical inducer of proximity (CIP), FK1012 (*3*), a homodimer of FK506, was followed by many others (Fig. 1). These molecules have the common feature of binding two peptide tags on either side of each molecule. Given that induced proximity is observed within minutes, one can study the immediate, primary effects of activating a specific molecule without concern for delayed toxic effects of the dimerizer on proliferation, transcription, or other much slower processes. Often these molecules are naturally occurring and illustrate how biology regulates proximity to its own benefit. For example, FK506 binds FKBP12 on one of its sides and calcineurin, a phosphatase essential for immunologic activation, on the other side (Fig. 1), illustrating how induced proximity and inhibition of calcineurin by FKBP12 functions in immunosuppression. Other examples of naturally induced proximity include cyclosporine A (Fig. 1), which also inhibits calcineurin by recruiting cyclophilin to its active site (*8*), thereby inhibiting phosphatase activity and nuclear localization of NFATc (nuclear factor of activated T cells) family members (*9*). Rapamycin, an immunosuppressant structurally related to FK506, also shares the primary target FKBP and acts through formation of a ternary complex (Fig. 1) with the FKBP12-rapamycin-binding (FRB) domain of target of rapamycin (TOR) kinases (*10*, *11*).

In plants, induced proximity with abscisic acid (Fig. 1) blocks germination and also induces leaves to abscise in the fall. It functions by inducing proximity of the monomeric receptor Pyl to the protein phosphatase ABI1 (*12*, *13*). This molecule is present at high concentrations in our diets and is not toxic in humans. Similarly, gibberellin (Fig. 1), which promotes germination and stem elongation in plants, functions by induced proximity of the receptor GID1 and hormone GA1 (*14*).

Dynamics of chemically induced proximity

Over the past 20 years, CIP technology has advanced from its origins to afford methods to understand signaling, transcription, and protein localization on rapid time scales. Much of the progress hinges on the ability to initiate biologic processes midpathway in vivo, such as downstream of a signal-activation event, and then discern the order of reactions after induced activation. The power of this approach arises from the fact that temporally ordering events places rigorous limits on causality.

Paradoxically, the responses from chemically induced proximity are often more robust than those from rigid protein fusions, especially in cases where a protein fusion can result in steric hindrances that prevent functionality. Furthermore, chemically induced proximity provides minute-by-minute kinetic analysis, allowing precise mathematical modeling. The fundamental concept of effective molarity—that a localized concentration within solution may differ from the bulk concentration—underlies the rationale and practicality of using chemically induced proximity to study complex biologic mechanisms. Proximity becomes a critical regulator of cellular processes by the fact that the probability of an effective interaction between two molecules is a function of the distance between them. This phenomenon can be observed by considering the scaling relationships between physical distance and reaction probability. In most relevant cases, reaction rate scales with concentration (the inverse cubed root of particle density), which scales with mean interparticle distance, i.e., the closeness of molecules (*2*).

The contributions of effective molarity are readily observed in natural processes such as protein compartmentalization within organelles, membrane localization, and protein scaffolds. Molecular

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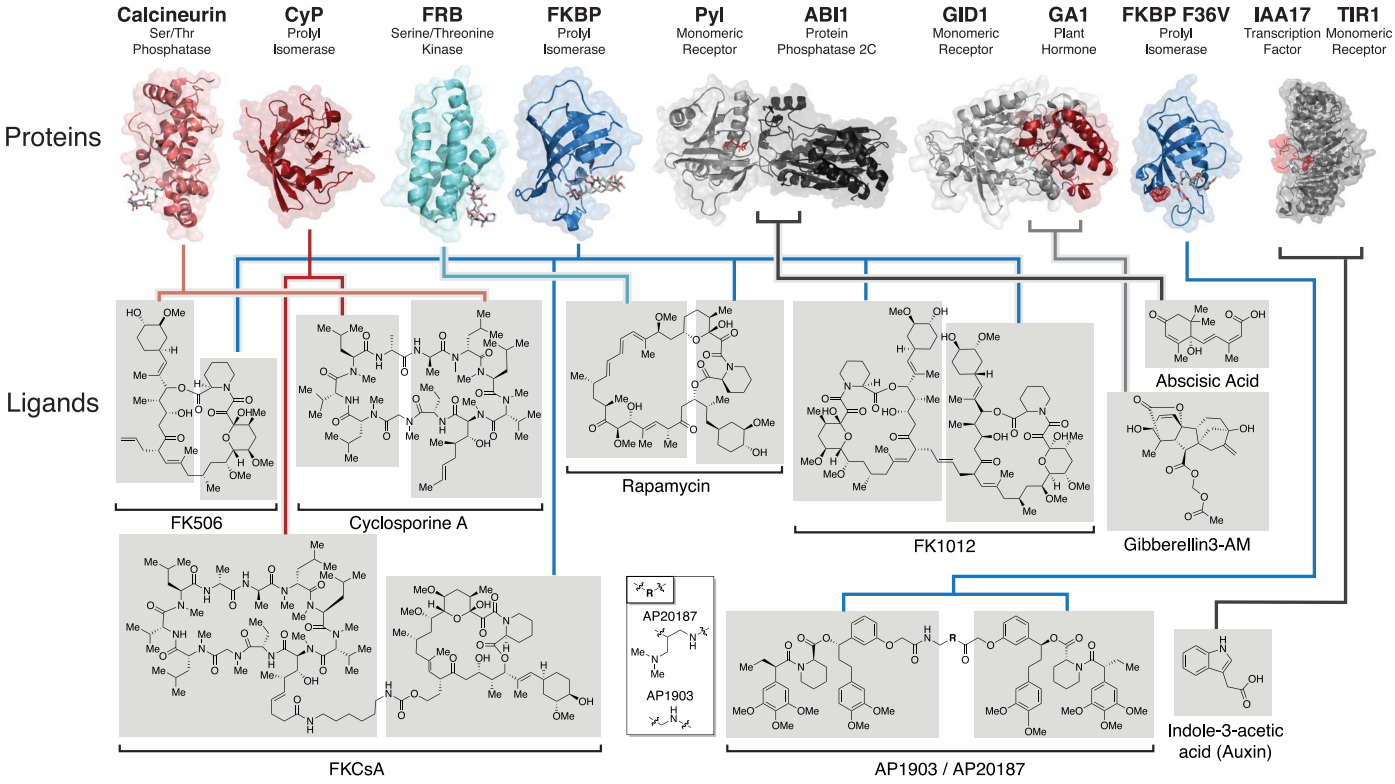

Fig. 1. The evolution of systems for CIPs. Protein targets and chemical ligands are shown for CIP systems. Proteins are represented as ribbon diagrams from available crystal structures, with endogenous monomeric functions indicated. Chemical ligands are represented in bound conformations docked with protein targets and also represented separately as individual structures. Gray shading of the chemical structures is divided to annotate specific structural moieties associated with molecular recognition of annotated protein targets. CIP systems are represented from left to right in approximate order of development. Me, methyl group; R, linker moiety.

scaffolds increase effective molarity in biochemical processes such as transcription, translation, and biosynthetic pathways. Protein scaffolds can enhance the speed of enzymatic reactions by several thousandfold (*15*, *16*). Organelles sequester critical reaction components through compartmentalization to increase the effective molarity of relevant substrates.

Previously, mathematical models of reaction-diffusion systems have been used to describe dynamic biologic processes that correspond to a change in concentration with respect to space, time, and changing substances (reactions). Some examples of reaction-diffusion models in biology include those that explain the improved enzyme catalytic efficiency resulting from compartmentalization (*17*) and those that describe the improved kinetics of push-pull networks in which two enzymes control signal transduction pathways in an antagonistic manner (*18*). Similarly, the effective concentration increase at a CIP recruitment site can be understood by considering any dimerization event as a reaction occurring in a classic reaction-diffusion system (Fig. 2A) (because equilibrium models cannot describe steep concentration gradients). In a chemically induced–proximity event, one member of a ternary complex, [A], is freely diffusing, while the other, [B], is localized (at the cell membrane, on chromatin, etc.). The addition of a chemical dimerizer creates a concentration gradient of the complete complex, [AB*], with a maximum concentration at the recruitment site (Fig. 2A).

The reaction-diffusion equation is as follows:

$$\frac{\partial u(x,t)}{\partial t} = D\frac{\partial^2 u(x,t)}{\partial x^2} + ku(x,t)$$

From Fick's laws of diffusion, the flux of the substance at position (x) is proportional to the concentration gradient, and the change in concentration with respect to time ($\partial u/\partial t$) is related through the differential equation $\frac{\partial^2 u}{\partial x^2}$. The rate of concentration changes ($\partial u/\partial t$) is also impacted by the reaction rate, $ku(x, t)$. In the absence of a chemical dimerizer, dimerizing proteins [A] and [B] have little-to-no binding affinity for each other (Fig. 2B). Although they freely collide, their rate of diffusion dominates over the binding rate. By contrast, in the presence of a CIP (Fig. 2B), reaction is faster than diffusion. As a result, the concentration of bound [AB*] near the recruitment site is far greater than the freely diffusing condition, which creates a virtual cloud of molecules to amplify the effects of proximity while relieving steric constraints.

Although it is tempting to solely credit the superiority of chemical dimerization to pure kinetics, the thermodynamic contributions of the system should not be understated (Fig. 2C). By increasing the effective molarity of a substrate, the cell is relieving the cost of translational (x, y, z) entropy (Fig. 2C). The use of a CIP minimizes the relative configurational entropy of the system by reducing the possible collision angles relative to freely diffusing molecules. As a result, the CIP provides both kinetic and thermodynamic advantages by increasing the probability of interactions through effective concentration and by minimizing translational or rotational entropy.

Despite the early characterization of binary-complex equilibria in 1916 by Irving Langmuir (*19*), a mathematical description of a three-body system, for example, FKBP-rapamycin-FRB, in equilibria (Fig. 2D) has only recently been described (*20*). Spiegel and others developed a mathematical framework for ternary-complex formation that used measurable parameters (analogous to total concentration and dissociation constants) to define the maximum concentration of dimerized complexes, $[AB^*]_{max}$ (*20*). Their framework was extended to several biologic systems and cooperative ternary complexes, including the TCR. For systems such as FKBP-rapamycin-FRB, where the dissociation constant K_d values have been rigorously defined (*21*), more complete descriptions of the kinetics of ternary systems may prove useful when characterizing the fundamental processes governed by proximity.

Using induced proximity to explore biologic mechanisms: Biologic mimicry

Arguably the major contribution of chemically induced proximity is the ability to rapidly initiate

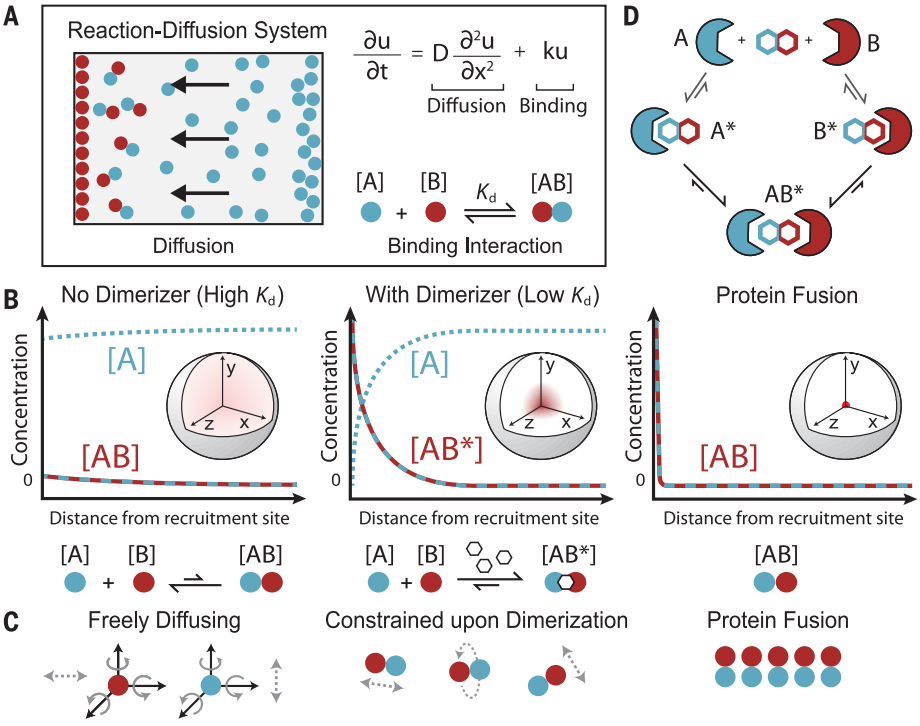

Fig. 2. Modeling reaction kinetics associated with systems of induced proximity. (**A**) The differential concentration with respect to time is explained by the changes in the rate of diffusion and the binding kinetics of the dimerizer system. (**B**) Changes in concentrations of monomeric and dimeric complexes are dependent on rate of reaction and rate of diffusion, defined by the distance to the site of recruitment. With no chemical induction or high K_d, formation of ternary complexes is determined by the rate of diffusion, as $ku(x, t)$ approaches zero. With chemical induction and low K_d, induced ternary-complex formation is strongly dependent on the rate of the reaction $ku(x,t)$, which dominates the rate of diffusion. Direct-fusion systems are exclusively localized at the site of recruitment as the reaction rate approaches infinity and dominates the rate of diffusion. In protein-dimerizer interactions, these complexes are designated by *. (**C**) Thermodynamic contributions to chemically induced dimerization include minimizing translational and rotational entropy. Multistate binding equilibria associated with initial binding of a bifunctional dimerizer molecule (hexagons) to respective targets by forming unstable binary complexes that form composite surfaces and rapidly assemble ternary complexes. Arrows show direction of movement or rotation. (**D**) Kinetics of ternary-complex assembly can be described by three-body binding equilibria.

and track the minute-by-minute consequences of a biochemical process in living cells. This allows precise kinetic studies, construction of synthetic regulatory circuits, and analysis of cellular memory and places stringent limitations on causality within genetic and biochemical networks.

Induced proximity in signal transduction and transcription

Chemically induced proximity fueled conceptual advances in understanding signaling, including the role of proximity, the ordering of biochemical events, and the intersection with transcription. By using molecules that did not show allosteric effects upon binding their ligands by crystallography, it was found that receptor signaling could be induced by chemically mediated proximity (Fig. 3A), initially at the TCR (*3*) and later for a host of different receptors. Dimerizing TCR-signaling components at the membrane with FK1012 revealed that dimerization was sufficient to initiate downstream TCR signaling events (*22*, *23*). In addition, recruiting the guanine nucleotide exchange factor Sos to the membrane revealed that Sos proximity could induce Ras signaling and that a major role of the linking molecule Grb-2 was to increase effective local concentration (*24*).

Temporal analysis of the biochemical consequences of proximity defined the order of complex signaling mechanisms using both linear and parallel steps in a pathway. Membrane-induced proximity of zeta chain–associated protein kinase (ZAP70) helped place its function in the TCR signaling cascade, as did similar approaches for signal components downstream of death and growth factor receptors (*25*). Activation of individual signaling molecules, not possible with ligands that induce several pathways, unveiled the "AND gate" function of several transcription factors, including NFAT, meaning that two signaling inputs are required for a robust transcriptional output. For example, isolated Ras activation could not activate NFAT nor could isolated Ca^{2+} signaling. Simultaneous Ras and Ca^{2+} signals were essential for NFAT-dependent transcription and provided a check on inappropriate gene activation (*24*).

The role of proximity in transcriptional regulation became clear with the early understanding of the spatial organization of promoters and the proteins bound by them. However, chemically induced dimerization allowed the examination of the in vivo kinetics in yeast, flies, and mammals. Chemical recruitment of transcriptional activators led to the finding that transcriptional activation can be accomplished through proximity on time scales of minutes, rather than hours or days (Fig. 3B) (*6*), helping investigators to temporally order events in the complex sequence leading to transcriptional activation.

An important feature of a CIP is its rapid reversibility (by small-molecule washout with competitive inhibitors), which enables the study of molecular memory in cells. To enhance reversibility, nontoxic FK506 analogs were developed to competitively wash out synthetic dimerizers. The first competitive inhibitor of dimerization (FK506M) (*3*) was used to demonstrate that dimerizer-mediated transcription was rapidly reversible and induced no stable memory (Fig. 3B). However, developing a system for carrying out order-of-addition and co-occupancy of activators and repressors lead to the discovery that transcription could persist in certain contexts in yeast even after the activator was released (*26*). This indicated that memory was hardwired into these systems, by virtue of repressor resistance. More-refined analysis of transcriptional memory emerged from later studies of epigenetic regulators (see section "Chromatin regulation" below and Fig. 3E).

Induced proximity has also been pivotal for understanding the kinetics of transcriptional regulation in individual cells. In both yeast and human cells, transcription at a single allele was induced in an all-or-none quantal manner (*6*, *27*).

CRISPR and CIP-regulated transcription

Recent advances with CRISPR-Cas9 (*28*) have ushered in a new era of CIP transcriptional regulation (*29*, *30*). Zhang and others developed a rapamycin-inducible assembly of enzymatically dead Cas9 (dCas9) and locus-specific guide RNAs (Fig. 3B) (*31*). Recently, other dCas9 fusions (Fig. 3B) were found to be highly compatible with a variety of CIP systems (*32*, *33*).

The dCas9-based dimerizer systems allow combinatorial recruitment as well as ordered recruitment of activators and repressors, which have enabled studies of synergy and antagonism. For example, proximity-induced formation of repressive transcriptional states by recruiting a KRAB repression domain had a deterministic silencing effect on transcription, even with co-recruitment of an activator (*32*).

Protein folding and localization

Regulated compartmentalization of molecules is a common biological process easily mimicked by CIPs. Schreiber and others used a synthetic heterodimer, FKCsA (Fig. 1) (*34*), which targeted

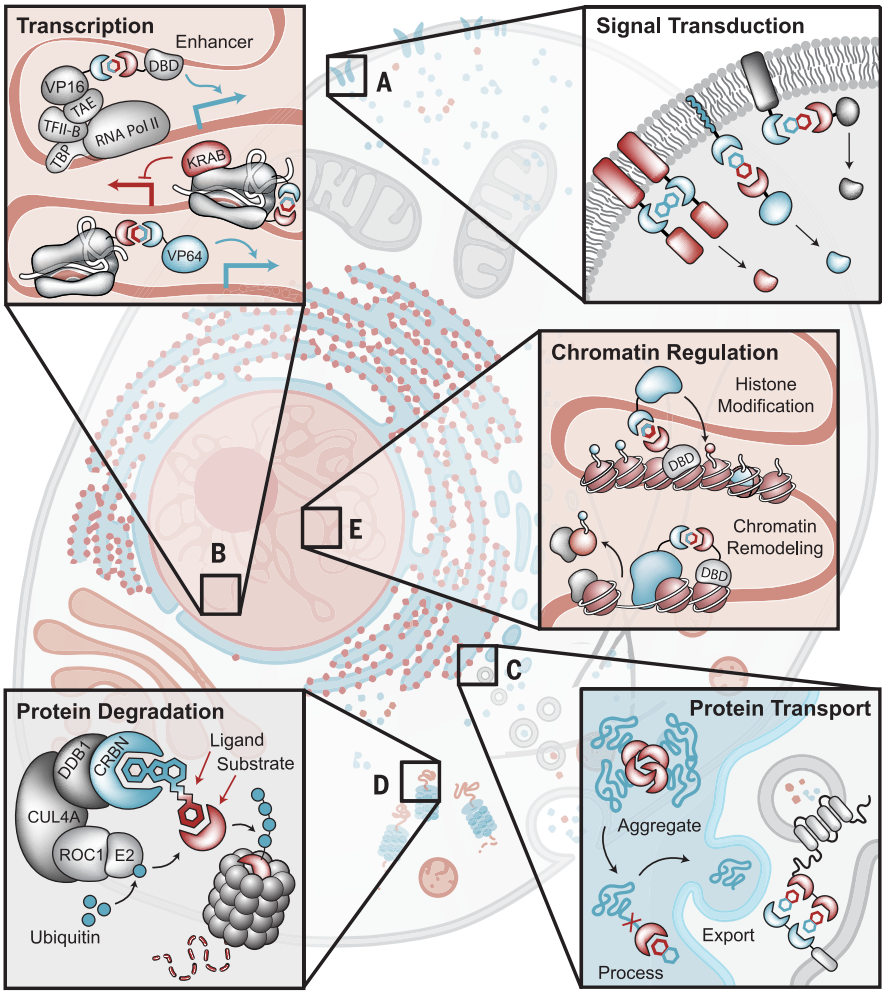

Fig. 3. Chemical induction of proximity is sufficient for the regulation of diverse cellular processes. Induced proximity has been shown to regulate initiation of transcription, signaling cascades, chromatin dynamics, proteasomal degradation, and subcellular localization. (**A**) Induced proximity has been systematically explored to bypass T cell antigen receptor activation and for synthetic induction of a variety of signaling cascades. (**B**) CIPs have been developed for rapid induction of transcriptional activation (VP16) and repression (KRAB or HP1) using DNA binding domains (DBDs) as well as CIP of split–CRISPR-Cas proteins or CIP recruitment of activators or repressors through CRISPR-Cas9 systems. (**C**) CIP has been used for rapid protein localization, including nuclear import and export, localization to components of the secretory pathway, synaptic vesicles, and mitochondria. (**D**) Rapid proximity-based protein degradation is achieved through bifunctional molecule–mediated recruitment of E3 ubiquitin ligase complexes (complex composed of E2, ROC1, CUL4A, DDB1, and CRBN). With a related approach, auxin can induce ubiquitin-mediated degradation through recruitment of the TIR1-Cul1 complex. (**E**) Induced proximity has been used for rapid induction of activated chromatin states through recruitment of ATP-dependent remodeling complexes and induction of repressive chromatin states through HP1-mediated heterochromatin formation.

FKBP and prolyl isomerase CyP without binding calcineurin, to rapidly induce nuclear translocation of CyP-tagged green fluorescent protein (GFP) with a nuclear-localized NLS-FKBP. A CIP was also used to rapidly export proteins from the nucleus, thereby reversibly inactivating them (*35*). Furthermore, this approach proved highly effective in yeast for inactivating nuclear proteins by shuttling them out of the nucleus using the "anchor-away" system (*36*–*39*). Anchor away is now frequently used to rapidly inactivate and reactivate nuclear proteins to understand their direct actions.

By expanding CIP localization beyond the nucleus to other organelles, Rivera and colleagues sought to activate specific secretory pathways in the endoplasmic reticulum (ER) for therapeutic purposes, building upon two critical advances (*40*). First, a multimer-forming, conditional aggregator of FKBP12(F36M) was found to be retained in the ER (Fig. 3C) in the absence of chemical ligands (AP22542, APAP21998). Second, a Golgi-specific protease (furin) was harnessed to target a cleavage site (FCS) engineered into fusion proteins with human growth hormone or insulin. By coupling these two advances, it was demonstrated that a CIP could both simultaneously cleave FKBP and, by resolving the aggregation, induce protein secretion (*40*). Furthermore, these ligands induced insulin secretion in hyperglycemic, FKBP(F36M)-FCS-insulin transgenic mice. This study showcased the clinical potential of CIPs for gene therapy applications, in addition to providing new insights into the secretory pathway.

Chemical dimerizers were further utilized to investigate how Golgi membranes associate with the ER during cell division (*41*). To investigate secretory mechanisms of Golgi-ER interaction during the cell cycle, the authors expressed fusions of FKBP-GFP with sialyltransferase (ST; Golgi specific), and FRAP-HA with the human invariant-chain protein (li; ER specific) (*41*). When coexpressed in COS-7 cells, the proteins remained associated within their respective cellular compartments as monomers, even upon the addition of chemical dimerizer. Notably, when treated with brefeldin A, a small molecule that induces Golgi-ER membrane fusion, the authors observed rapamycin-dependent colocalization of ST and li. This unexpected finding, that the Golgi and ER exhibit spatial independence during cell division, demonstrates how CIPs continue to reveal previously unknown aspects of seemingly well-characterized biologic mechanisms.

Svoboda and others developed an ingenious CIP approach to tether synaptic vesicle proteins. This allowed for inducible and reversible activation of synaptic transmission in neurons (Fig. 3C) (*42*). In motor neurons expressing vesicle-associated membrane protein (VAMP) or synaptobrevin-FKBP(F36V), the authors inhibited 50 to 100% of synaptic transmission in minutes using the synthetic dimerizer AP20187 (Fig. 1). This system was extended to both cultured neurons ex vivo and motor neurons in vivo. Furthermore, in Purkinje neurons of living mice, dimerization of VAMP–Syb-FKBP(F36V) with AP20187 induced functional ataxia during learned balancing tasks (*42*). This study highlights the potential for CIPs in understanding neuron function and complements invasive optogenetic systems.

The mechanism of Ca^{2+} entry was probed by Lewis and others through stromal interaction molecule 1 (STIM1) oligomerization (*43*). The authors demonstrated that the rapalogue AP21967 could rapidly oligomerize STIM1-FKBP and FRB-STIM1 and localize the complex to the cell periphery. This activated CRAC (Ca^{2+} release–activated Ca^{2+}) channel currents and revealed that induced oligomerization of STIM1 was sufficient for calcium entry via CRAC channels.

The expanded chemical repertoire (including new orthogonal rapalogs that were specifically tuned to FRB mutants) heavily influenced the multiplex ability of subcellular-localization studies (*44*). First, a triple-mutant FRB [residue Lys2095→Pro2095 (Lys2095Pro), Thr2098Leu, Trp2101Phe], denoted FRB*, was developed to selectively form a ternary complex with FKBP and C20-methallylrapamycin (C20-MaRap) (*45*). Through utilization of a CIP transcriptional-reporter screen, it was found that by introducing rapalogs and respective FRB mutants, precise

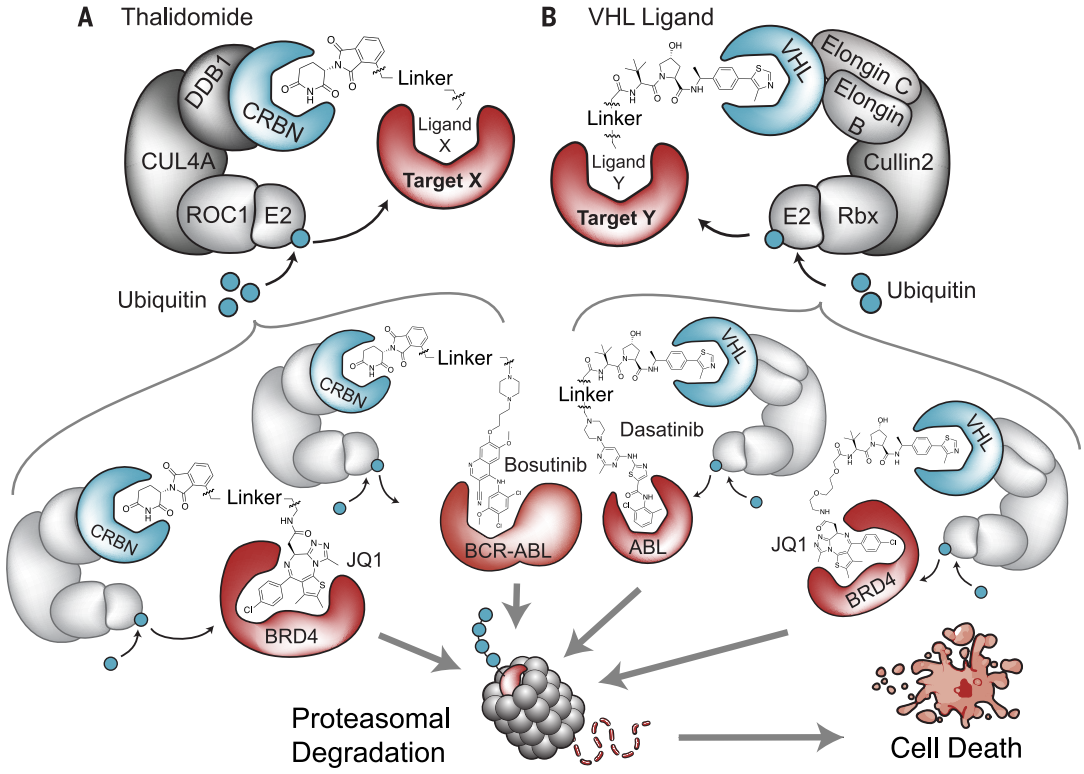

Fig. 4. Ubiquitin ligase complexes rapidly degrade oncogenic protein targets. (**A**) The CBRN-CUL4A ubiquitin ligase complex can be recruited to BCR-ABL and BRD4 with thalidomide conjugated to bosutinib and JQ1, respectively. Induced proximity rapidly degrades these targets, which are known to drive chronic myelogenous leukemia (BCR-ABL) and acute myeloid leukemia (BRD4). (**B**) The VHL ligand fused to desatinib or JQ1 can efficiently recruit the VHL-Cullin2 ubiquitin ligase complex to ABL and BRD4, respectively. In each case, rapid degradation of the oncogenic targets results in inhibition of cancer growth.

measurements could be made to define new patterns of specificity. By using these new orthogonal dimerizers, simultaneous expression of nuclear exporting FRB*(LT) and nuclear localizing FRB* (LW) provided a platform for push-pull control of FKBP–GSK3β (glycogen synthase kinase 3β), which was modulated by treatment with the appropriate orthogonal rapalog (*44*).

Modulating protein structure is another critical component of posttranslational modification that has been explored through proximity-based approaches. Muir and others developed a facile approach to conditional protein structural variation. Using rapamycin, they were able to mimic natural protein splicing with proximity-based intein cleavage (*46*).

Recently, Ballister and colleagues developed a clever light-induced proximity system—defining a photon as the smallest dimerizer (*47*). Modifying a previous bifunctional *bis*-methotrexate dimerization system (*48*), the authors labeled the dihydrofolate reductase (DHFR) ligand with a photocleavable moiety, which blocked the requisite DHFR-interacting surfaces in the absence of irradiation. Tagging the photocleavable moiety with a HaloTag linker formed an irreversible adduct with a Haloenzyme allowing for selective, light-inducible Halo-tagging and subcellular relocalization of DHFR upon irradiation. Furthermore, photoinduced DHFR relocalization was extended to the centromere, kinetochore, centrosome, and mitochondria with CENP-Halo, Nuf2-Halo, AKAP9-Halo, and ActA-Halo, respectively.

Protein degradation

Loss-of-function studies have been the mainstay of genetics but are plagued by the slow loss of protein, allowing the accumulation of compensatory and indirect responses clouding mechanistic interpretation. CIP-regulated protein stability was developed to circumvent these classic problems. By using C20-MaRap, it was determined that the stability of FRB* fusions (*44*, *45*) was dependent on formation of the FRB*–C20-MaRap–FKBP ternary complex. To investigate the function of GSK3β in developing mice, FRB* was knocked into the endogenous GSK3β gene. Because expressed GSK3β could only be stabilized in the presence of C20-MaRap and was otherwise degraded, dosing for short periods allowed the authors to define separate, discrete periods of development during which the gene executed its function in skeletogenesis and palate development (*49*).

Wandless and others found that double-mutant FKBP(Phe36Val, Leu106Pro) could also be used as a conditionally stabilizing allele, which allowed for rapid in vitro degradation of target proteins (*21*). The synthetic dimerizer (Shield-1) used in these studies was degraded in minutes in cell culture, allowing rapid reversal of the reaction.

To regulate proteasome-mediated degradation, CIP systems were developed to target chimeric E3 ligase complexes. The TIR1 receptor–auxin (Fig. 1) degradation pathway in *Arabidopsis*, which utilizes the dimerizer indole-3-acetic acid (IAA; auxin), induces dimerization of the TIR1-SCF E3 ubiquitin ligase complex with an auxin-inducible degron (AID) (*50*, *51*). In nonplant systems, TIR1 was successfully reconstituted into endogenous E3 ligase complexes to selectively recruit AID-fusion proteins to the Cul1 complex (*50*). This resulted in auxin-mediated ubiquitination of the AID fusion and rapid proteasomal degradation (Fig. 3D). Later, a short 44–amino acid tag referred to as IAA17 (AID*) was developed to expand the utility of the auxin system (*52*). The auxin-degron (AID*) system has been used to regulate kinases and essential genes that lack selective inhibitors. The essential Plk4 kinase, which is associated with tumor suppression (*53*, *54*), was degraded with AID-fusion transgenes (*55*) and homozygous knockins (*56*) to reveal that Plk4 positively regulates centriole duplication in a reversible and dosage-dependent manner. The auxin system has enabled rapid degradation of a wide variety of targets across many species (*55*, *57*–*59*). Recently, auxin-mediated degradation of the transcription factor CTCF (*60*) has differentiated its roles in local topologically associating domain (TAD) structure from chromosomal-compartment architecture.

Chromatin regulation

The immense complexity of chromatin, with its many developmentally specific histone modifications, topology, long-range interactions, variegated DNA methylation, and uncharacterized chromatin components, has proven to be a formidable target for investigation. The limitations encountered in formation of chromatin in vitro have become apparent (*61*). To circumvent these challenges, a CIP technique (CiA, chromatin in vivo assay) was developed to study chromatin in all its topological, biochemical, and developmental diversity (*62*). With CiA, one can "chemically pipette" a chromatin regulator of interest into essentially any locus in the genome of any cell type (Fig. 3E).

The CiA system was first used to study chromatin-based memory. Recruiting heterochromatin protein 1α (HP1α) to the active *Oct4* (transcription factor) locus in mouse embryonic stem cells (ESCs), resulted in an expanding domain of repression

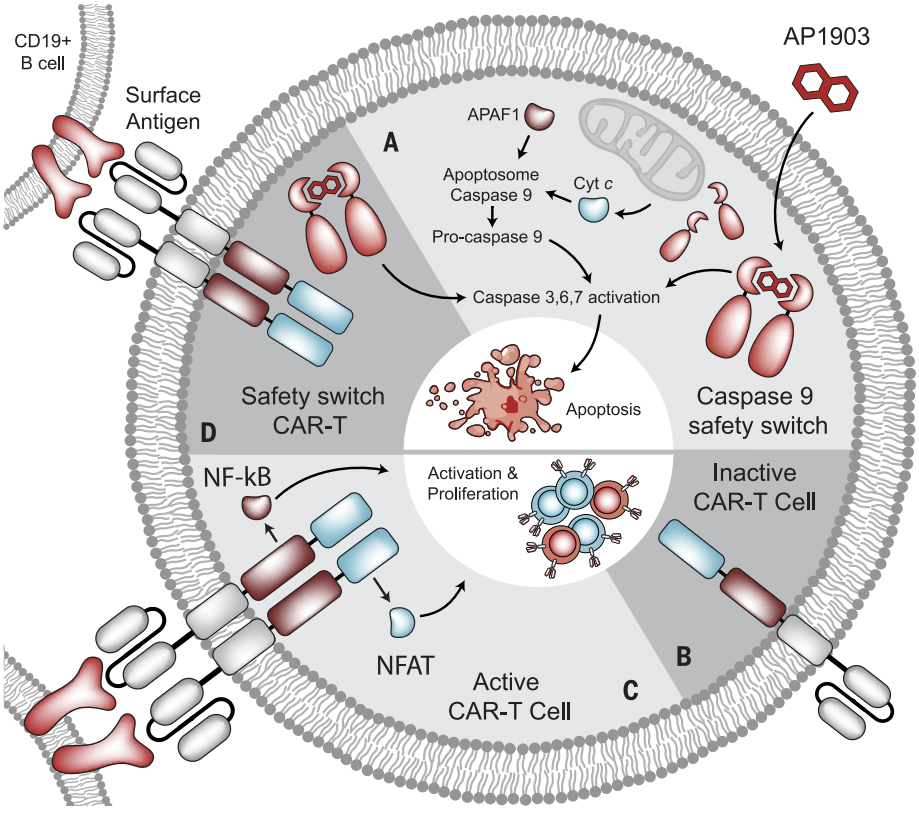

Fig. 5. CAR T cell therapeutic applications of CIPs. (**A**) Engineered safety switches using ATTAC systems with AP1903. (**B**) An inactive engineered CAR T cell receptor and (**C**) an active engineered CAR T cell receptor binding its cognate antigen. (**D**) AP1903-induced caspase dimerization and activation allows for rapid apoptosis of CAR T cells to prevent complications that may arise from transplant.

that silenced Oct4. By washing out the CIP, it was observed that H3K9me3 (trimethylated histone H3 on lysine 9) islands are stable for days after rapamycin washout but that the effect could be rapidly reversed by initiating transcription by plant hormone abscisic acid (ABA)–mediated recruitment of the transcriptional factor VP-16 (*62*). Furthermore, the ability to rapidly control H3K9me3-based heterochromatin permitted mathematical modeling, which put forth a "balanced intrinsic reaction rate" model for the propagation of H3K9me3 repression on the basis of kinetic parameters (*62*, *63*). In this model, rates of addition and removal determine the propagation rate of H3K9me3 and accurately predict 99% of the H3K9 domains over the murine ESC genome.

One of the most persistent problems in epigenetics has been understanding the placement and stability of polycomb repressive complexes (PRCs). In 1988, mutations in the *Brahma* gene, which encodes an adenosine triphosphate (ATP)–dependent chromatin remodeler, were found to repress mutations in the PRC1 complex, indicating that these two chromatin regulators opposed one another (*64*). The mechanism was elusive because of the inability to form PRC-repressed heterochromatin in vitro. To understand this problem, the SWI/SNF (switch-sucrose nonfermentable) or BAF (Brahma/Brg associated factor) complex was recruited to a polycomb-repressed promoter with the CiA system (*65*, *66*). PRC1 eviction occurred in minutes, followed by PRC2 eviction. The rapid action of BAF complexes led to the finding that they directly bind and release PRC1 by an ATP-dependent mechanism (*65*, *66*). Furthermore, heterozygous expression of cancer mutations of Brg, the regulatory adenosine triphosphatase (ATPase) of BAF, lead to polycomb accumulation, extending the CiA results to the genome. New CIP-dCas9 systems for manipulating chromatin architecture (*33*, *67*) will likely prove critical in uncovering chromatin regulatory mechanisms in a host of genomic contexts.

CIPs provided additional insights into the dissolution and formation of heterochromatin by the observation that recruitment of the BAF complex lead to accumulation of TopoIIα binding at the recruitment site, as suggested from an earlier study (*68*). Unexpectedly, TopoIIα function was found to be essential for both the dissolution of heterochromatin after recruiting BAF and the formation of heterochromatin after releasing BAF (*69*). Remarkably, the strand-cleaved reaction intermediate was found at the precise time and position of heterochromatin formation and dissolution. These studies, using rapid reversible co-recruitment of TopoIIα and BAF, indicated that decatenation is essential for the regulation of heterochromatin.

Chromosomal dynamics

To assess the mechanisms of DNA association with the cohesin complex during cell division, Nasmyth and others used dimerizers to "lock" the Smc1-Smc3 complex in place during specific windows of the cell cycle in yeast (*70*). By releasing yeast from G_1 (prereplicative phase) arrest, with or without the conditional dimerization of Smc1 and Smc3, it was discovered that the Smc1-Smc3 complex must open during mitosis and that this was necessary for chromatid cohesion.

A similar CIP-based approach was used to understand the role of Scc1 in sister chromatin association. Relocalization and inhibition of Scc1-FRB by rapamycin with ribosomal protein anchor RPL13A-FKBP demonstrated that 30% of sister chromatid association was disrupted by anchoring away Scc1 in yeast (*36*).

Induced proximity in medicine

Degrading or inactivating pathogenic proteins

The above studies, directed primarily at dissecting biologic mechanisms, demonstrate how widely this methodology can be used. However, the application to the treatment of disease had been hampered by the requirement that proteins must be tagged with dimerizing peptides. Graef and others reasoned that induced proximity of endogenous, unmodified proteins would be widely useful. To make a potential therapeutic for Alzheimer's disease, they synthesized a molecule that bound both FKBP (SLF, synthetic ligand of FKBP) and the pathogenic β-amyloid (Aβ) peptide (CR, Congo red) (*71*). Their two-sided molecule (SLF-CR) showed activity in in vitro assays of Aβ aggregation but was too toxic to be used as a therapeutic. A similar approach also extended the half-life of an HIV protease inhibitor by causing it to remain intracellular and protected (*72*).

Although neither of these bifunctional molecules had good pharmacologic characteristics, this conceptual advance precipitated a wave of efforts to extend proximity-inducing molecules to many other medical problems. One of the first of these was designed to stabilize the pathogenic aggregation of proteins. Transthyretin (TTR) can produce aggregating amyloid fibrils and causes amyloidoses, including cardiomyopathies such as senile system amyloidoses, familial amyloid cardiomyopathy, and familial amyloid polyneuropathy. The development of bifunctional stabilizers of TTR, such as AG10 by Graef and others (*73*), provides a promising candidate in preventing the progression of diseases associated with amyloid aggregation.

In many diseases, pathogenic proteins arise from mutation, recombination, or stable allosteric modification. What if these culprits could be degraded by induced proximity? In 2010, CRBN, a component of the DDBI-CRBN E3 ubiquitin ligase complex (Fig. 3D), was found to be the primary molecular target of thalidomide and related molecules (IMiDs, immunomodulatory

drugs) (74). Several years later, elegant structural studies for IKAROS family transcription factors (IKZF1, IKZF3) indicated that ubiquitin binding and targeting was IMiD dependent (75). These insights, coupled with the knowledge that thalidomide differed from analogs lenalidomide and pomalidomide through a single C-4 aniline substitution (75), provided a clear path to conjugate new ligands for the purposes of proximity-based protein degradation.

To investigate the potential applications of CRBN-IMiDs, the Bradner and Crews labs developed strategies to conjugate BRD4-targeting cell-permeable small molecule JQ1 (76) with thalidomide and investigated its potential as an inducible proximity-based ubiquitinase (Fig. 4) (77, 78). These studies were precipitated by the observation that IMiDs could bind directly to CRBN without inhibiting the associated ubiquitin ligase complex. In the first study, the phthalimide-JQ1 conjugate (dBET1) induced ubiquitin-mediated degradation of BRD4 on a time scale of hours, with a mechanism analogous to auxin-based degradation systems (without requiring genetic manipulations). The same C-4 phthalimide linkage was used to construct thalidomide-SLF conjugates (d-FKBP-1) for rapid and selective ubiquitin-mediated degradation of FKBP12. This demonstrated that these bifunctional conjugates were highly selective and had activity in human cells. By using a longer polyethylene glycol (PEG)–based linker attached to phthalimide, Crews and others simultaneously found that BRD4 could be degraded using both CRBN and von Hippel–Lindau tumor suppressor (VHL) ubiquitin ligase–targeting ligands with their strategy termed proteolysis targeting chimeras (PROTACs) (Fig. 4). Their bifunctional PROTACs were capable of rapidly recruiting the VHL-associated ubiquitin ligase complexes by using ligands specific to both estrogen-related receptor alpha (ERRα) and the serine-threonine protein kinase RIPK2 with notably high selectivity and activity in live mice (79).

In an important follow-up study, Bradner and others identified ENL, bearing a YEATS acetylated-lysine reader domain, as the product of an essential gene in a human acute myeloid leukemia (AML) model system with a mixed-lineage leukemia (MLL)–AF4 translocation (MV4;11) (80). To understand the function of ENL as a potential driver in AML, the authors expressed the ENL-FKBP12(F36V) protein in an ENL-deficient ($ENL^{-/-}$) MV4;11 cell line and used SLF (with no calcineurin or mTOR inhibition) conjugated to phthalimide (dTAG-13). Selective degradation of ENL with dTAG-13 resulted in decreased expression of AML drivers, including MYC, HOXA10, and MYB, and substantial reduction in elongation factors AFF9 and CDK9. This revealed that ENL may drive leukemogenesis through binding and elongation of canonical AML targets and demonstrated a creative use of degrading-CIPs with therapeutic potential.

Inhibition of BRD4, which results in repression of c-Myc activity, has been proposed as a therapeutic strategy in a host of diseases, including AML, acute lymphoblastic leukemia, NUT midline carcinoma, and HIV (76, 81, 82). Concomitantly, Crews and others, aware of the limitations of peptide-based degradation strategies, independently identified potent, small molecules targeting VHL E3 ubiquitin ligase (83). By utilizing this VHL-targeting strategy, dimeric ligands have selectively degraded ERRα RIPK2, which is involved in nuclear factor κB (NF-κB) and mitogen-activated protein kinase (MAPK) activation, and BRD4 (79, 84, 85). Furthermore, proximity-induced degradation provides a platform for rapid iteration through combination. Crews and others recently demonstrated that by varying the E3 ligase target and the functional protein-targeting warhead, the selectivity of known tyrosine kinase inhibitors (TKIs) could be improved (85). By modulating known TKIs—imatinib, dasatinib, or bosutinib—and the E3 ligase target, these small molecules degraded both c-ABL and the oncogenic BCR-ABL (Fig. 4), with varying degrees of specificity (85). Even though TKIs have proven to be immensely successful in the treatment of chronic myelogenous leukemia, it remains a lifelong condition. One hypothesis for persistent leukemic cells is that the pathogenesis is not entirely dependent on kinase activity and that BCR-ABL may play a scaffolding role in signaling (86). As such, proximity-induced degradation may not only provide a mechanism for inhibition of oncogenic proteins [with efficacy comparable to RNA interference (RNAi) or CRISPR, but without the immunogenicity of Cas9] but may also provide cures for diseases for which present catalytic inhibitors are inadequate. This strategy may revive the imperfect chemical probes that bound the protein of interest but failed to deliver cures for critical therapeutic targets.

Induced proximity in cellular therapies

Gene therapies require the delivery of precise amounts of therapeutic proteins at specific times as well as a humanized system to prevent immune rejection of the engineered cells. On the basis of earlier FK1012-mediated transcriptional activation studies (6), Clackson and others developed a completely humanized delivery system that provides long-term, regulated expression in primates (87–89). A major challenge for any method of regulated gene expression is the steep dose-response curve induced by rapamycin. The use of nontoxic dimerizers such as abscisic acid provide a more graded dose response (13) and could be useful for precise dosage control.

Certain therapeutic strategies require removal of pathogenic cell types. Early studies demonstrated that dimerizing the intracellular domain of the Fas receptor or other death-signaling molecules could accomplish this goal (90–93). By using a technique called apoptosis through targeted activation of caspase 8 (ATTAC) (Fig. 5A), an animal model was developed to study obesity and glucose-stimulated insulin secretion. This CIP "suicide-switch" strategy was extended to a senescent cell–clearing mouse to study age-related pathologies (94). Baker and colleagues observed that CIP-mediated clearance of senescent cells extended health and life span of normal tissues. Furthermore, with precise temporal control, the ATTAC system attenuated the progression of age-related diseases (94, 95).

Cellular therapies remain one of the most hopeful strategies; however, concerns remain around potential off-target damage or possible malignancy that may result from genomic integration of an introduced gene. Although allogenic transplantation of hematopoietic stem cells is an effective leukemia treatment, positive benefits of this therapy are often counteracted by graft-versus-host disease (GVHD). To circumvent this, a CIP "safety switch" was developed to selectively induce apoptosis in hematopoietic transplants in the case that severe GVHD arises in patients (96). In a small clinical trial, patients with GVHD were treated with AP1903 (Fig. 1), a bioinert analog of FK1012 (97). AP1903 selectively eliminated 90% of the modified T cells within 30 min and eliminated GVHD without recurrence (96). Subsequent studies further demonstrated the usefulness of this safety switch in long-term GVHD complications (98, 99).

CIP safety switches may also prove to be a promising strategy for mitigating side effects of cancer immunotherapy treatments. In a recent study in humanized mice, T cells were simultaneously modified with a chimeric antigen receptor (CAR) and the iCaspase9 safety switch (Fig. 5, B to D). Despite the effectiveness of CAR T cell therapies for treating B cell malignancies (including acute lymphoblastic leukemia and lymphomas) (100, 101), possible side effects can be severe. Treating mice with a CD19–FKBP-iCaspase9 T cell therapy provided two advantages. First, induced dimerization of the caspase 9 protein provides a built-in temporally controlled mechanism to ablate harsh side effects in patients, such as cytokine release syndrome or B cell aplasia. Second, selective apoptosis provides a mechanism to eliminate transplanted T cells in a controlled manner that allows for patient-specific responses in the clinic, along with the ability to repopulate a patient's own immunity (102).

Summary

The application of chemically induced proximity to elucidate biologic mechanisms continues to grow with recent advances in understanding epigenetic regulation, chromosomal dynamics, and topology. However, the use of this mechanism in treatment of disease is still in its embryonic form. Bifunctional molecules that use induced proximity for the elimination of pathogenic proteins and aggregated proteins and to control subcellular localization are likely to make a substantial impact on the treatment of disease in the near future. The development of totally humanized systems for gene and cellular therapy is now under clinical investigation and showing promise. Small molecules that capture the universal biologic regulatory mechanism of induced proximity will likely have many other unanticipated uses and provide a playground for our imaginations.

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Chemically induced proximity in biology and medicine

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Regulating molecule proximity

The physical distance, or proximity, between molecules often directs biological events. The development of membrane-permeable small molecules that reversibly regulate proximity has enabled advances in fields such as synthetic biology, signal transduction, transcription, protein degradation, epigenetic memory, and chromatin dynamics. This "induced proximity" can also be applied to the development of new therapeutics. Stanton *et al.* review the wide range of advances and speculate on future applications of this fundamental approach.

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