Molecular Glues & Bifunctional Compounds: Therapeutic Modalities Based on Induced Proximity

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Introduction. This Perspective explores molecular glues and bifunctional compounds – proximity-inducing compounds – and offers a framework to understand and exploit their similarity to hot spots, missense mutations, and posttranslational modifications (PTMs).

Distinguishing Features and Overview. A defining feature of molecular glues, first identified in efforts to understand the mechanism of action of natural products FK506, cyclosporin, and rapamycin,[123] is their ability to induce ternary and higher-order complex formation **cooperatively**. The molecular glue first binds a presenter protein, creating a neo-protein with enhanced affinity for a target protein (Figure 1A, top). Cooperative binding is a key contributor to their high specificity. Having activity that is contingent on normally non-interacting partners being in the same location in the human body (organ, tissue, cell type of even subcellular locale) offers a new opportunity for selectivity, including tissue-targeted, small-molecule drug action.

Bifunctional compounds were conceived shortly after the discovery of molecular glues to simplify the identification of proximity-inducing agents. These two classes comprise Chemical Inducers of Proximity (CIPs).[4] Bifunctional compounds typically have a structural element that binds one protein, another element that binds a second protein and a connecting element that links the two binding elements (Figure 1A, bottom). The conceptual simplicity of their design is appealing, and they have already been shown to be applicable to at least a subset of the myriad functional activities exhibited by molecular glues, though their lack of cooperativity can create a dosing challenge since increasing concentration favors the inactive one-to-one complex (hook effect). Targeted protein degraders named PROTACs are a popular subset that induce proximal relationships of E3 ligases with neo-substrates to increase the rate of substrate ubiquitination.[5] With advances in screening, molecular glue degraders, which through Revlimid first emerged as effective drugs, are now a complementary focus of targeted protein degradation in drug discovery.

This Perspective pans out to take a wider lens view, providing a framework for understanding the relationship of proximity-inducing compounds to two familiar facets of biology – **missense mutations enshrined by natural selection** and **posttranslational modifications** (Figure 1B). It also highlights a wide array of cellular processes transcending targeted protein degradation and even proximity as the mode of action that can be commandeered with compounds that induce protein-protein associations. The activities of these compounds are shown to be well aligned with the blueprints for drug action emerging from human biology. Human genetics reveals mutations conferring risk or protection from disease. Mechanistic studies show these often gain a function, gain a protein interaction, are hypermorphs, etc. Examples herein reinforce that chemicals can be the same. Finally, novel rational approaches to discover either molecular glues or bifunctional compounds directly from compound libraries will be described.

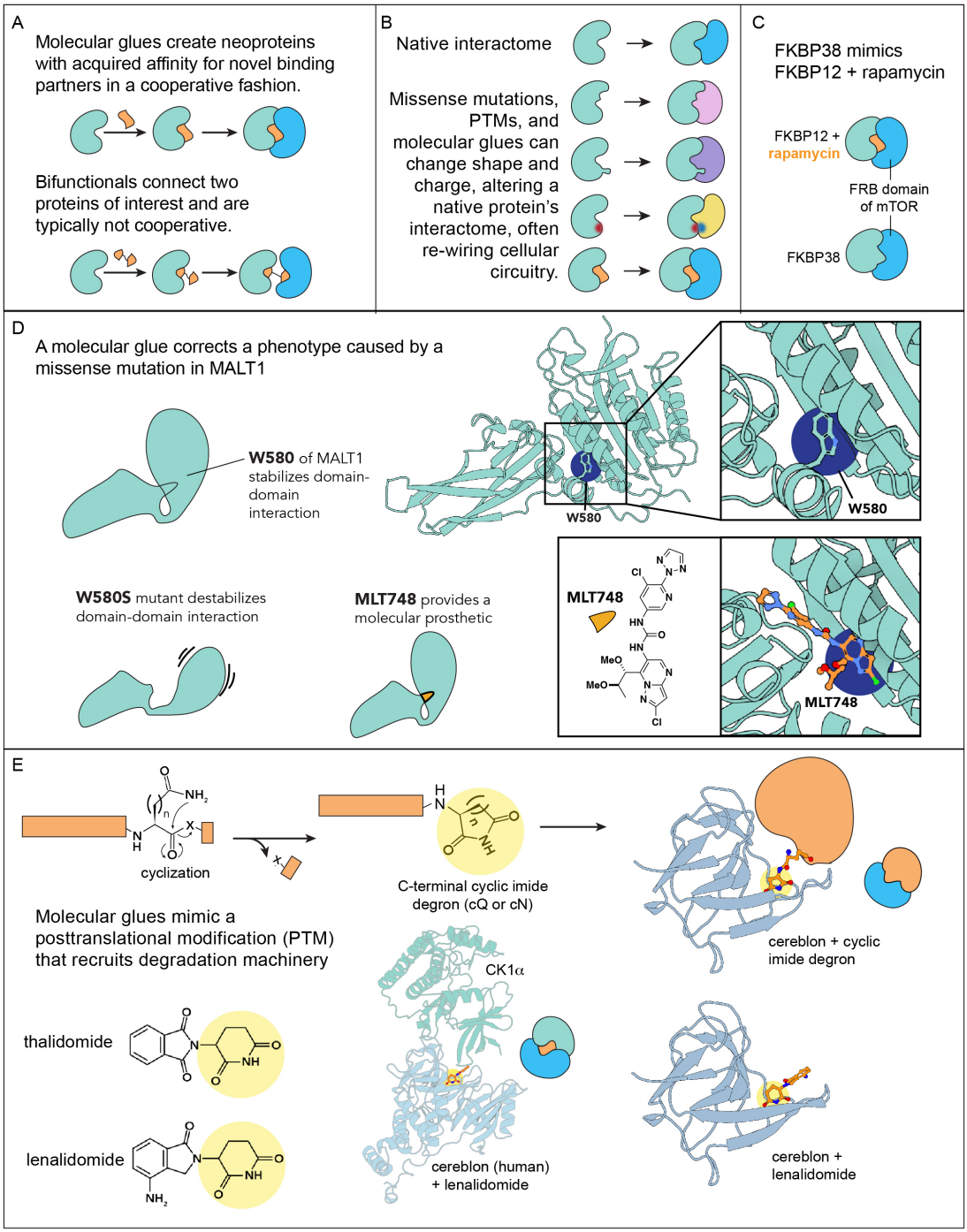

Figure1: Molecular glues are analogous to missense mutations and PTMs. D. PDB IDs: 3V55 (apoMALT1 334-719), 6H4A (MLT748-bound MALT1 329-728). E. PDB IDs: 8C3H (cereblon isoform 4 from Magnetospirillum Gryphiswaldense with degron), 4V30 (cereblon isoform 4 from Magnetospirillum Gryphiswaldense with lenalidomide), and 5FQD (human cereblon +lenalidomide + CK1α). 3D Protein Imager[4] was used to prepare protein structures.

Beginning with the basics. When small molecules bind dynamic proteins, they change their biophysical properties – we can think of a small molecule–protein complex as a neo-protein. These altered biophysical features have functional consequences, often by changing the target's

interactome. Consequently, these compounds can stabilize, destabilize, degrade, move the protein from one cellular compartment to another, increase or decrease its activity, induce targeted posttranslational modification, and restrict whole-body distribution, among others. Molecular glues such as rapamycin and FK506 exemplify the latter two examples; through a glue mechanism rapamycin induces a single serine dephosphorylation of its target mTOR[6] and both rapamycin and FK506 are concentrated in enucleated red blood cells,[7] which have high concentration of their presenter protein FKBP12.[8]

Nature induces protein–protein interactions in a variety of ways that are reminiscent of molecular glues. For example, molecular glues share features in common with missense mutations under natural selection. Changes in amino acid sidechains in proteins often alter interactomes and induce neo-protein interactions in a functional way (Figure 1B).

Jim Wells taught us a foundational principle in chemical biology; namely, that small molecules bind proteins at specific locations normally involved in protein–protein interactions or catalytic activities named hotspots.[9] Binding these regions has functional consequences because they normally interact with function-conferring macromolecules or substrates. Although mutations arise largely randomly throughout a genome, missense mutations *under natural selection* are observed to emerge most commonly at functional sites. These mutations are often found in or near hotspots, where the resultant new amino acid residue is analogous to a small molecule binding the native form. Think of the molecular glue as a nonnatural mimic of an amino acid sidechain (Figure 1B). An example that reinforces this notion comes from the fact that FKBP12 binds mTOR only in the presence of natural product molecular glue rapamycin, whereas the paralogous FKBP38 binds mTOR directly, without assistance from a molecular glue (Figure 1C).[10]

This point was reinforced further by research from Haian Fu and his team at Emory University.[11] This team looked systematically at changes in interactomes resulting from somatic missense mutations in oncogenes. Their resulting protein-interactome maps revealed that missense mutations frequently induce functionally relevant neo-protein interactions. The resulting amino acid changes are acting functionally as molecular glues, often rewiring cellular circuitry.

Since both missense mutations and molecular glues can enact similar functions, it makes sense that molecular glues can complement missense mutations, providing for example “molecular prosthetics” as novel modalities in medicine. A neurodevelopmental disorder resulting from a disease-causing W580S missense mutation in MALT1 is illustrative (Figure 1D).[12] W580 stabilizes a crucial intraprotein domain–domain interaction in MALT1. Correcting the missense mutation began with the observation that a MALT1 inhibitor, MLT748, binds to wild type MALT1 with its diazaindole situated precisely where the indole ring of tryptophan 580 naturally resides, displacing its indole ring. W580S creates a pocket lacking the indole side chain that confers the crucial domain-domain stabilization in wild-type cells. MLT748 fills this pocket and corrects the disease phenotype in cells derived from patients homozygous for W580S. This example reinforces the similarity of amino acid sidechains and small-molecule binders and illustrates the concomitant emergence of a missense mutation and a small molecule that binds in the same hot spot. This gain-of-function corrector also distinguishes the analogy of molecular glues to missense mutations from the traditional analogy in chemical genetics likening small-molecule inhibitors to genetic knockouts made in the laboratory.

Post-translational modifications (PTMs) such as phosphorylations yielding phosphate groups on serines, threonines, and tyrosines, or acetylations and methylations yielding acetyl or methyl groups on lysines also function in a manner analogous to molecular glues. These PTMs create neo-binding and docking sites for proteins, which is essential in signal transduction for information

transfer. For example, recruitment of an SH2 domain-containing lipid kinase to a phosphorylated intracellular receptor tail places the kinase near its phospholipid substrates in the plasma membrane, increasing effective molarity and the rate of the phosphotransfer reaction. PTMs work through the same mechanism in nuclear signal transduction. For example, the acetylation of lysines on histone tails creates a docking site for bromodomain-containing enzymes involved in nuclear signaling such as nucleosome-remodeling enzymes. This places the latter in proximity with their substrate so that chemistry can occur more efficiently.[13] Similarly, methylation of lysines on histone tails creates a docking site for chromodomain-containing enzymes with subsequent proximity-based functional consequences in gene expression. Like molecular glues, PTMs induce proximity. Analogies can be found in synthetic PHICs[14] and PHORCs[15], which are bifunctionals that, like PTMs, hold substrates in proximity to kinases and phosphatases.

Bifunctional compounds were originally conceived to induce neo-substrate interactions of fusion protein targets with enzymes and were shown to be highly effective at increasing the rate of post-translational modifications in cells with a plethora of cellular consequences.[16] Molecular glues also perform this function, as evidenced by lenalidomide and thalidomide, which mimic a PTM that marks proteins for cellular degradation (the C-terminal cyclic imide "degron", Figure 1E).[17] Cellular proteins displaying the imide degron bind cereblon, delivering themselves to the E3 ligase enzyme complex resulting in ubiquitination and degradation. Similarly, lenalidomide and thalidomide molecular glues bind their presenter protein cereblon at the degron-binding site. The cereblon–lenalidomide composite surface then recruits neo-substrates to the cereblon E3-ligase complex. Overall, these Revlimid-related molecular glues increase the rate of ubiquitination of neo-substrates, leading to their cellular degradation through the ubiquitin-targeted proteasome.

A third analogy involves ubiquitous **scaffold proteins**, including the cytokine interferon (IFN) or the platelet-derived growth factor (PDGF). Studies of signal transduction revealed that membrane cytokine or tyrosine kinase receptors can be activated by extracellular proteins. IFN and PDGF function as scaffolding proteins, recruiting a substrate to an enzyme, in this case promoting a trans phosphorylation and creating novel phosphotyrosine docking sites. By this mechanism of induced protein associations, the rate of intracellular chemistry is substantially increased through an extracellular binding event, without the extracellular scaffold protein entering cells. An example that provided the origins of modern targeted protein degradation is the papilloma virus E6 protein, which promotes targeted protein degradation. This scaffold protein functions to bridge key host factors p53 (directly) and Rb (indirectly) to a host E3 ligase protein E6AP (aka UBE3A) by ternary complex formation, which results in the ubiquitination and proteasome-dependent degradation of the tumor suppressors. In this way, the virus achieves targeted degradation of p53 and Rb, thereby promoting oncogenesis. The viral protein E6 functions like a bifunctional or molecular glue degrader.

Intramolecular glues. To get the most out of this promising therapeutic modality, it is useful to think of molecular glues more expansively. For instance, compounds that connect distinct domains of target proteins, like in the example of MALT1 above, and alter their dynamic properties are a promising class of emerging drugs that can be also considered molecular glues. These compounds bind a protein hot spot in a manner that induces or stabilizes otherwise dynamic intramolecular or intra-complex interactions. These indirect effects on function underlie an important element of the "binder first, function later" approach to drug discovery.[18]

Recent examples show the power of intramolecular glues stabilizing *inactive* conformations. A team at Novartis discovered NP3-146 and showed that by binding four distinct domains of the NLRP3 inflammasome, the compound locks the inflammasome into an inactive conformation (Figure 2A).[19]

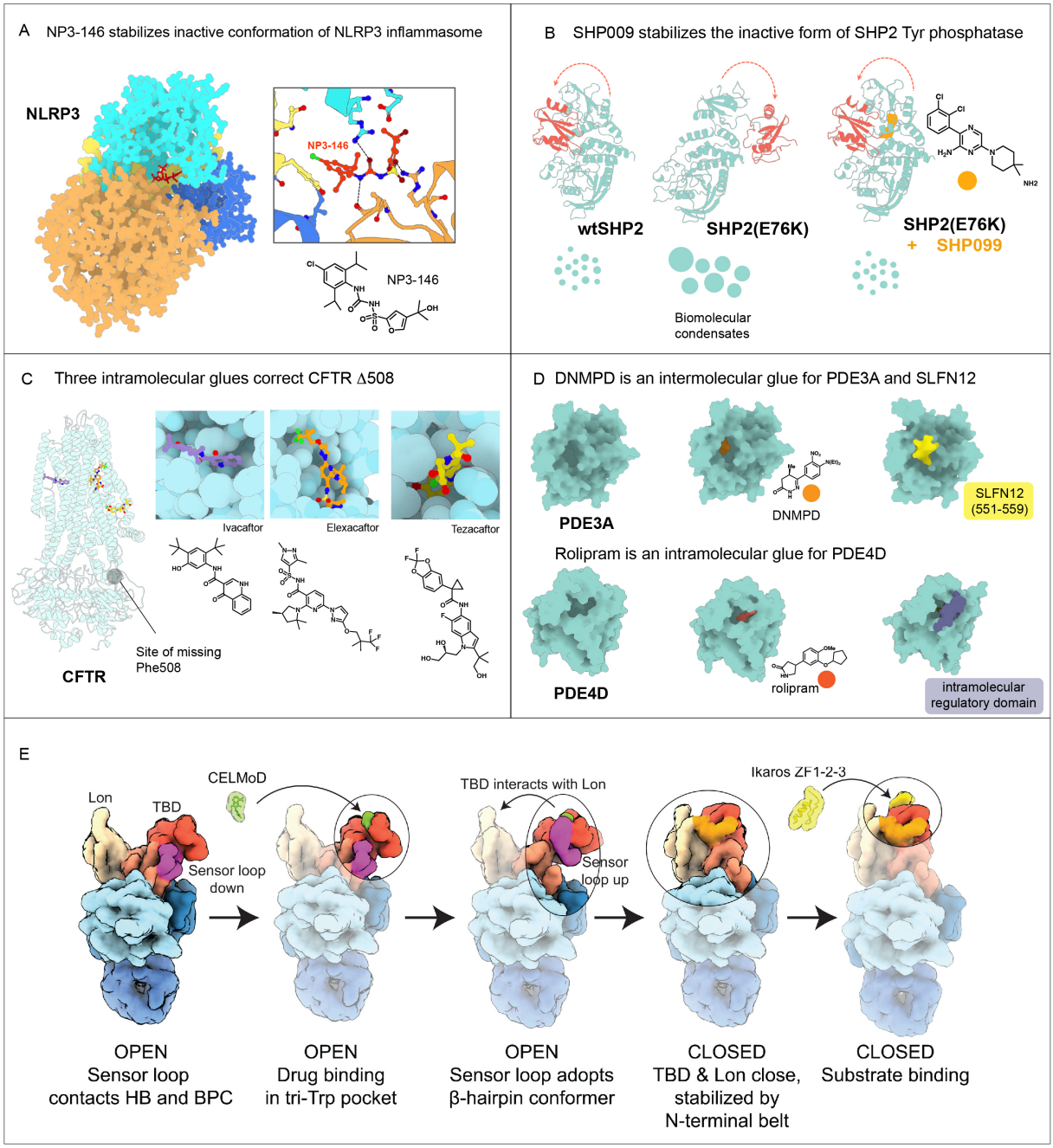

Figure 2: Intramolecular molecular glues are a promising therapeutic modality. A. PDB ID: 7ALV B. PDB IDs: 4DGP (wtSHP2), 6CRF (SHP2(E76K), and 6CMS (SHP2(E76K) with SHP099). C. PDB ID: 8EIQ D. PDB IDs: 3SL3 (apoPDE4D2), 3G4K (PDE4D with rolipram), 3G4G (PDE4D with regulatory domain and D155871 inhibitor). Rolipram structure does not include regulatory domain but was shown to recruit it similarly to D155871. E. from: Molecular glue CELMoD compounds are regulators of cereblon conformation. Edmond R. Watson, Scott Novick, Mary E. Matyskiela, Philip P. Chamberlain, et al. Science, 2022, 378, 6619. Reprinted with permission from AAAS. Otherwise, 3D Protein Imager[4] was used to prepare protein structures.

Like MLT748 above, another stabilizing intramolecular glue, SHP099, corrects for somatic oncogenic missense mutations of the challenging SHP2 tyrosine phosphatase. The mutations prevent the N-terminal SH2 domain from auto-inhibiting its enzymatic active site.[202122] Cong Liu,

Jidong Zhu, et al. of Shanghai Institute of Organic Chemistry found that these constitutively active oncogenic forms separate into a liquid–liquid phase-separated condensate, but SHP099 reglues the repressive N-terminal domain into the active site and liberates the enzyme from biomolecular condensates (Figure 2B).[23]

Over 30 years after Vertex Pharmaceutics was founded on the promise of the molecular glue FK506, researchers there discovered synergistic molecular glues as treatment for cystic fibrosis (CF). The correctors tezacaftor and elexacaftor are intramolecular glues that move the CF-causing mutant channel protein CFTR(ΔPhe508) to its proper cellular location while the potentiator ivacaftor is an intramolecular glue that increases its activity (Figure 2C).[24] These three drugs synergistically bind the same protein – each addressing a functional shortcoming of the primary disease allele of cystic fibrosis. Analogous to the MALT1 example above, this 3-component molecular prosthesis (Trikafta) rearranges domains to fill the crevice created by the "missing" phenylalanine in CFTR(ΔPhe508).

The phosphodiesterase family of proteins further illustrates the similarity of inter- and intramolecular glues. When the small molecule DNMPD binds a hot spot on PDE3A, the resulting composite surface – analogous to a genetic neomorph – recruits the ribonuclease SLFN12 (Figure 2D top).[25] This neo-protein interaction increases SLFN12 ribonuclease activity, leading to cytotoxic effects in some cancer cells. The antidepressant rolipram binds an analogous hot spot in PDE4D, but this composite surface – analogous to a genetic hypomorph – recruits a remote domain of the same protein, locking PDE4D in an inactive conformation (Figure 2D bottom).[26]

While the phosphodiesterase family of proteins illustrate both inter- and intramolecular proximity-inducing events, a detailed study of the cereblon presenter protein highlights that a glue may be required to use both modalities to achieve a functional outcome – targeted protein degradation in this instance. Effective molecular glue degraders first function intramolecularly, gluing two domains of cereblon. It is this feature that presents a surface capable of gluing a neo-substrate to the altered cereblon and its associated E3 ligase complex (Figure 2E).[27]

Lessons from the past for modern molecular glues. The first use of molecular glues in medicine – before it was even known they were glues – was with the natural products cyclosporin and FK506, to be followed later by rapamycin when all three had proved to work through a molecular glue mechanism. The first clue that FK506 and rapamycin were molecular glues was that even though they both inhibited the peptidyl-prolyl isomerase activity of FKBP12, they had distinct functional outcomes. By synthesizing the common FKBP12-binding portion of these compounds, my lab showed that inhibition of FKBP12 wasn't sufficient to carry out their biological functions yet 506BD was able to block the actions of both FK506 and rapamycin (Figure 3A).[28] Further studies showed that FK506 first binds FKBP12 as a presenter protein. FK506 and cyclosporine, despite their distinct structures, both bind the target protein phosphatase calcineurin (aka PP2B); however, cyclosporin uses cyclophilin as a presenter protein (Figure 3B).[1] Neither the small molecule nor the presenter protein has appreciable affinity for calcineurin, but complexes thereof bind their target calcineurin cooperatively and with high affinity. These findings enabled the use of FK506 and cyclosporin to probe and illuminate the mechanism of the calcium–calcineurin–NFAT signaling pathway.[29] Molecular glues became a new modality – not a simple small molecule or protein, but a combination of the two. The term molecular glue was drawn from the analogy to MHC–antigenic peptide complexes binding distinct T-Cell Receptors (TCRs).[30] Different peptides direct the same MHC (presenter) protein to an array of TCRs. Likewise, different small molecules, following binding to FKBP12, target an array of intracellular proteins (discussed below).

An unusual feature of FK506 and cyclosporin is that rather than bind the enzyme's active site, the complexes formed with their respective presenter proteins bind an exosite and thereby block the entry portal of a subset of substrates (Figure 3B). This mechanism achieves "substrate-selective inhibition" of a phosphatase.

A similar effect was revealed when mTOR was identified as the target of rapamycin using a similar affinity-based approach.[23] Structural studies by Jon Clardy and Nikola Pavletich revealed that by binding the FRB domain, the molecular glue complex again sterically blocks the entry portal for some, but not all, mTOR substrates (Figure 3C).[313233] Like calcineurin, this binding mode explains rapamycin's substrate-selective inhibitory properties.

The mTOR discovery opened the door to selective hetero association of fusion proteins by pairing one with FKBP12 and another with the FRB of mTOR (discussed below). Specificity for the FRB fusion over endogenous mTOR was engineered using a "bump-hole" concept developed earlier by Peter Belshaw for cyclosporin.[33] A strategically placed methallyl substituent "bump" on rapamycin was coupled with a complementary mutant "hole" of mTOR's FRB.[34] These and other related systems have been used to alter cellular circuitry by inducing a wide range of protein–protein associations, including activation and repression of targeted genes and signaling pathways (e.g., PDGFR, EGFR, Insulin Receptor, TGFβ Receptor, Fas Receptor (Figure 3D), among others) in cells and animals.[35] Gene expression and repression, chromatin remodeling and targeted chromatin modifications, and targeted protein localization-correction and protein degradation were also achieved. These demonstrations using fusion proteins (e.g., Figure 3D) suggested many opportunities for rewiring cellular circuitries with molecular glues and bifunctional compounds inducing associations of native proteins; indeed, many of these have by now been realized.

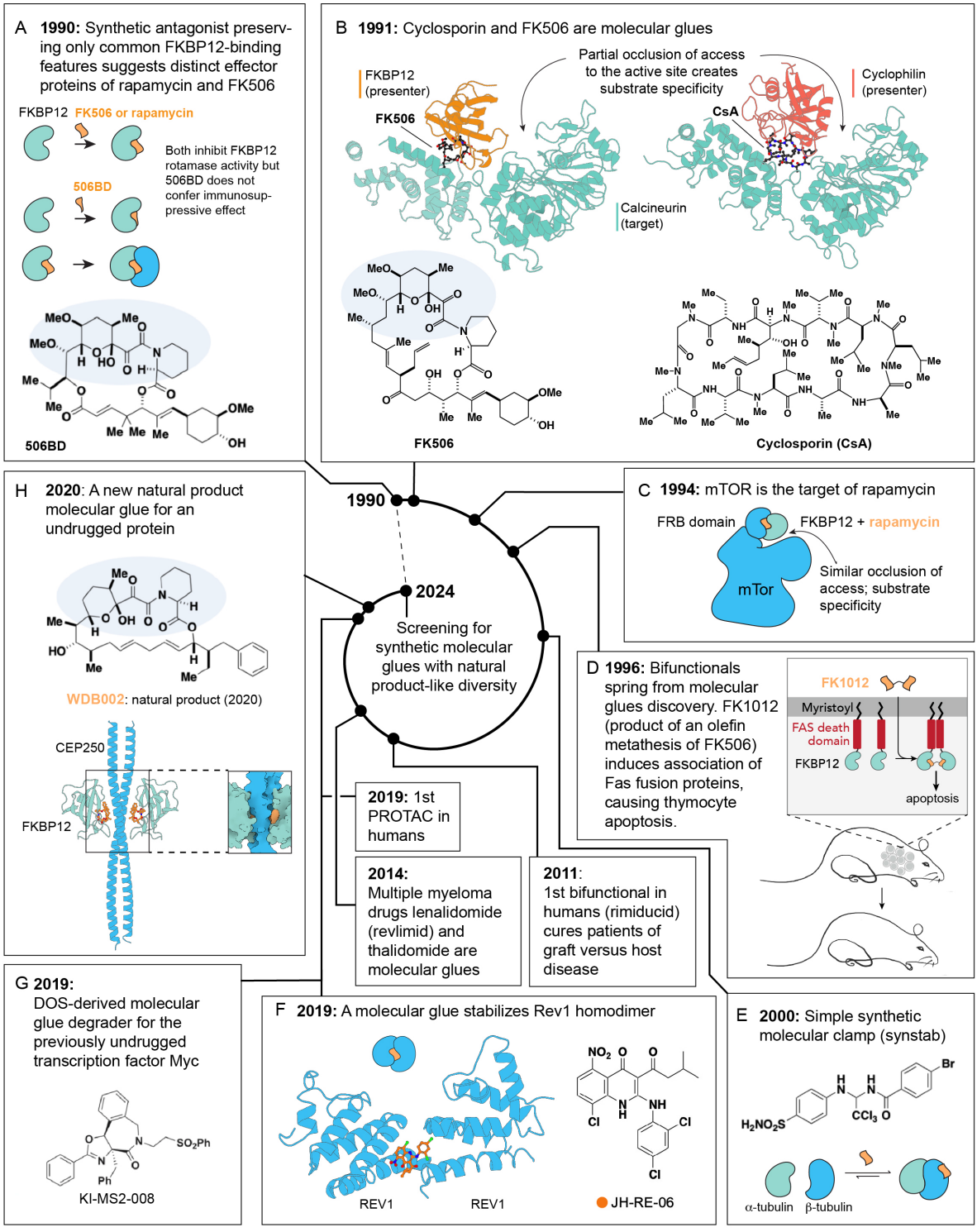

Figure 3: From natural products to bifunctionals and back to natural products and simple synthetic compounds. B. PDB IDs: 6TZ7 (FK506 complex) and 1MF8 (CsA complex) F. PDB ID: 6C8C H. PDB ID: 6OQA 3D Protein Imager[4] was used to prepare protein structures.

Bifunctional compounds. Following the early 1990s discoveries that certain natural products behaved as molecular glues, members of Jerry Crabtree's lab at Stanford and my lab at Harvard envisioned together how similar molecules might prove such proximity effects a generalizable principle in biology; thus far only serendipity had surfaced them. We imagined they would be hard to uncover broadly. A simple "work-around" was conceived – find small-molecule binders to two proteins of interest and then connect them with a chemical linker. As studies of intracellular signal transduction were illuminating the roles of posttranslational modifications, we were initially interested in inducing proximity of enzyme–neo-substrate pairs such as kinases and E3 ligases with substrates of our choosing. Those interests quickly expanded. For example, when chromatin PTMs were recognized as playing a role in nuclear signaling by induced proximity,[13] we became interested in the recruitment of chromatin-modifying enzymes, and activation and repressor domains to genomic loci via transcription factors. To explore the breadth of biology that can be controlled by this approach, we started by creating genetic fusions of signaling proteins with domains such as FKBP12 and FRB of mTOR that bind CIPs. We could then bring together nearly any two proteins of our choosing.

An early example of a bifunctional compound that induces fusion protein–protein interactions is FK1012 – synthesized by an olefin metathesis reaction linking two FK506 molecules. This compound no longer binds calcineurin but instead binds two FKBP12 proteins as shown by X-ray crystallography.[36] By fusing FKBP12 to intracellular proteins, especially pairs of proteins creating neo-substrate–enzyme relationships, this single bifunctional compound induced novel chemistry on target proteins with generality leading to a breadth of outcomes.

As an example of achieving temporal and spatial control of biology with bifunctionals, FK1012 and equivalents induced association of the cytoplasmic tail (death domain) of the FAS receptor fused to myristoylated FKBP12, triggering the extrinsic apoptotic pathway (Figure 3D). A myristoylation sequence precluded the need for native extracellular and transmembrane domains, isolating the biological effect to simple proximity. Placing expression of the fusion protein under the control of an Lck promoter in transgenic mice enabled targeted ablation of CD4+/CD8+ thymocytes.[37]

This system would also prove to be effective in humans 18 years later in a therapeutic context, in which a related fusion was expressed in donor hematopoietic stem cells prior to bone marrow transplantation. Five leukemia patients who developed graft vs. host disease (GVHD) from transplantation were treated with the related bifunctional compound rimiducid, curing them of their GVHD.[38] To negate binding to host FKBP12, selectivity was engineered through a Phe36Val "hole" mutation in FKBP12 that has complementarity to a chemical "bump" on each end of the bifunctional rimiducid.

Bifunctional CIPs were widely adopted by the scientific community, resulting in over 1,000 research papers, many of which stem from small-molecule kits that were made freely available to over 1,200 labs by Ariad Pharmaceuticals. Optogenetics is fundamentally related where a photon rather than a small molecule is used to induce protein associations (via fusions of signaling domains to, for example, the light-sensitive cryptochrome-2 protein).[39] The stunning generality of this system's ability to recapitulate biology showed just how much of it is governed by proximity.

Combining bifunctional compounds with genetic fusion proteins clearly illustrated the broad scope of biology accessible to this strategy and continue to inspire new areas of research and therapeutic potential. For example, a bifunctional CIP can recruit an activation domain to a transcriptional repressor and thereby alter transcriptional circuitry. This area alone seems rich with future applications and may have novel therapeutic applications when applied to native

proteins. New genetic tools are leading synthetic biologists to change cell circuitry by genome editing, while molecular glues and bifunctional compounds allow us to imagine a future where cellular circuitry can be rewired at the level of proteins.

Despite the wealth of knowledge obtained from genetic fusions, the next step was to extend it to native proteins to probe them in a more physiologically relevant manner and to preclude the need for genetic modification so that their therapeutic promise could be more fully realized.

A hybrid example is seen in the dTAG targeted protein degradation system developed by Jay Bradner and Nathanael Gray.[40] The dTAG reagent is a bifunctional compound having a cereblon binder connected to a bump–hole variant of the FKBP12 system. The dTAG system and several newer variants require tagging a target protein or library of proteins with the mutant FKBP12 (or equivalent), but then exploits the endogenous cereblon protein and its E3 ligase complex to target ubiquitination of proteins. The bifunctional degraders named PROTACs completely depart from genetic fusions, binding endogenous targets and E3 ligase complexes. Craig Crews and Ray Deshaies, Jay Bradner, and others have made advances in targeted protein degradation using bifunctionals.[41] LYTACs, AbTACs, and others are variations that achieve targeted protein degradation of extracellular and cell-surface proteins.[42] Returning to promising new avenues outside of targeted protein degradation, two recent examples show the use of bifunctional compounds to rewire transcriptional circuitry.[43],[44]

Induced protein associations are a more common phenomenon than originally imagined. Just ten years after the discovery of molecular glues, a small-molecule screen led to the identification of a simple synthetic compound named synthetic stabilizer-A (synstab-A) that stabilizes the association of proteins that normally interact dynamically in cells – in this case alpha and beta tubulin (Figure 3E).[45] This same "molecular clamp" activity underpins the therapeutic value of complicated natural products such as taxol, discodermolide, and epothilone, yet synstab-A is not the product of natural selection. Studies over the intervening 20 years have shown again and again that molecular glues are ubiquitous, possibly more often the norm than the exception, and likely more accessible than often sought-after disruptors of protein–protein interactions. The molecular glue and clamp features of many compounds remained opaque because we failed to look for them.

Synstab-A also hinted at what has become evident over the years – molecular glues and clamps can be structurally simple, even lower molecular weight than conventional drugs. Perhaps the scientific community was thrown off by the complex structures of natural product glues. Perhaps our intuition about the need for extensive contact surfaces would have been reconsidered had we considered the analogy of missense mutations and PTMs in hot spots. Nevertheless, we now know that highly effective molecular glues, behaving analogously to sidechain alterations, are frequently low molecular weight and structurally simple.[46] The illumination of the molecular mechanism-of-action of the simple myeloma drug lenalidomide/Revlimid and its structural analogs such as thalidomide further reinforced this notion.[47]

Undrugged protein targets. In the 1980s, both phosphatases and kinases were in the "undrugged" category, which today of course is hard to imagine given the plethora of protein kinase-targeting drugs. Cyclosporin, marketed as Sandimmune, was already a blockbuster drug, so the discovery of its mechanism immediately disproved the hypothesis of protein phosphatase undruggability. Shortly thereafter, rapamycin, as sirolimus, was approved as the first protein kinase-targeting drug. Ironically, most subsequently developed protein-kinase inhibitors bind the conserved ATP-binding pocket, which was the basis for skepticism that kinase inhibitors could be safe and selective; indeed, it might be time to rethink protein kinase inhibition more broadly

through the molecular glue mechanism, particularly in terms of its ability to achieve substrate-selective inhibition.

The principles outlined thus far have been reinforced with increasing frequency in recent years by many novel discoveries. Pei Zhou at Duke University found that JH-RE-06 inhibits the non-canonical DNA polymerase zeta by prying the REV1 protein from its ternary complex with REV7 and POL zeta and into a glued REV–REV1 complex (Figure 3F).[48] Consequently, JH-RE-06 inhibits the polymerase in cells.

Angela Koehler boldly sought to drug the more recently declared undruggable transcription factors, which led her lab to discover a diversity-oriented synthesis-derived molecular-glue mechanism of MYC inhibition (Figure 3G). The mechanism uncovered by the MIT team is striking as a means for small molecule-induced degradation – here KI-MS2-008 pries MAX away from the MYC–MAX heterodimer and into a glued MAX–MAX complex, thereby leaving a destabilized MYC more susceptible to cellular degradation.[49]

Coming full circle, a third natural product molecular glue using FKBP12 as its presenter protein through a structure reminiscent of 506BD is WBD002, whose complex binds the otherwise featureless coiled coil secondary structure of CEP250 (Figure 3H).[50] The structure of the ternary complex reinforces that cooperative binding of molecular glues may be a general mechanism to target challenging protein targets, and that the presenter protein–small molecule complex should be considered a modality distinct from traditional small-molecule or protein therapeutics.

In the same vein, Jun Liu's group at Johns Hopkins synthesized 45,000 natural product-inspired "rapafucins" – compounds incorporating the FKBP12-binding domain of early probe compound 506BD but replacing its linking connector with combinations of peptides and peptoids. Unlike typical acyclic bifunctional linkers, these elaborations are displayed as macrocycles. Reinforcing the synthetic availability of molecular glues, his team used this library to discover novel FKBP12-dependent proximity inducers that bind challenging targets such as the transmembrane channel protein SLC29, which is targeted by FKBP12–rapadocin.[51]

Targeted protein kinase degradation has also been achieved by the simple EGFR inhibitors erlotinib and gefitinib. Indeed, simple enzyme inhibitors can lead to targeted protein kinase degradation far more broadly than originally imagined.[52]

These and many other studies, too numerous to list here, show that molecular glues can have simple or complex structures, function inter- and/or intramolecularly, be natural or synthetic, and are far more ubiquitous than previously recognized. The field has come full circle through bifunctionals and back to molecular glues such as the iMiDs, DNMDP, WBD002, and many more. Only now, we have ways of discovering them that do not rely on serendipity.

Novel approaches to the discovery of molecular glues and bifunctional compounds. To extend the impact of proximity-inducing compounds in the future, the field will benefit from methods to induce the association of preselected presenter and target proteins. Target proteins are typically identified based on their potential for therapeutic intervention. Advances in human biology are increasingly offering confidence that target engagement will confer the intended medical benefits with a necessary margin of safety. Understanding disease mechanism is enabling blueprints for the often-novel activities – beyond simply inhibition – that drugs should confer on their targets, as many of the examples presented in this Perspective illustrate. Presenter proteins can be selected with the novel mechanisms in mind – enabling otherwise challenging modulations of target protein activities, including their activation, changes in cellular localization,

substrate-selective inhibition, and degradation, among others. Presenter proteins also promise to reprogram cell circuitry; for example, by recruiting activation or repressor domains[44] or nuclear localization sequences[43] to transcriptional factors. Another promising avenue for presenter proteins is to mitigate on-target, off-tissue toxicity by providing a contingency for target modulation – a circulating glue will only engage its target if the pre-selected presenter protein is present in the disease tissue.

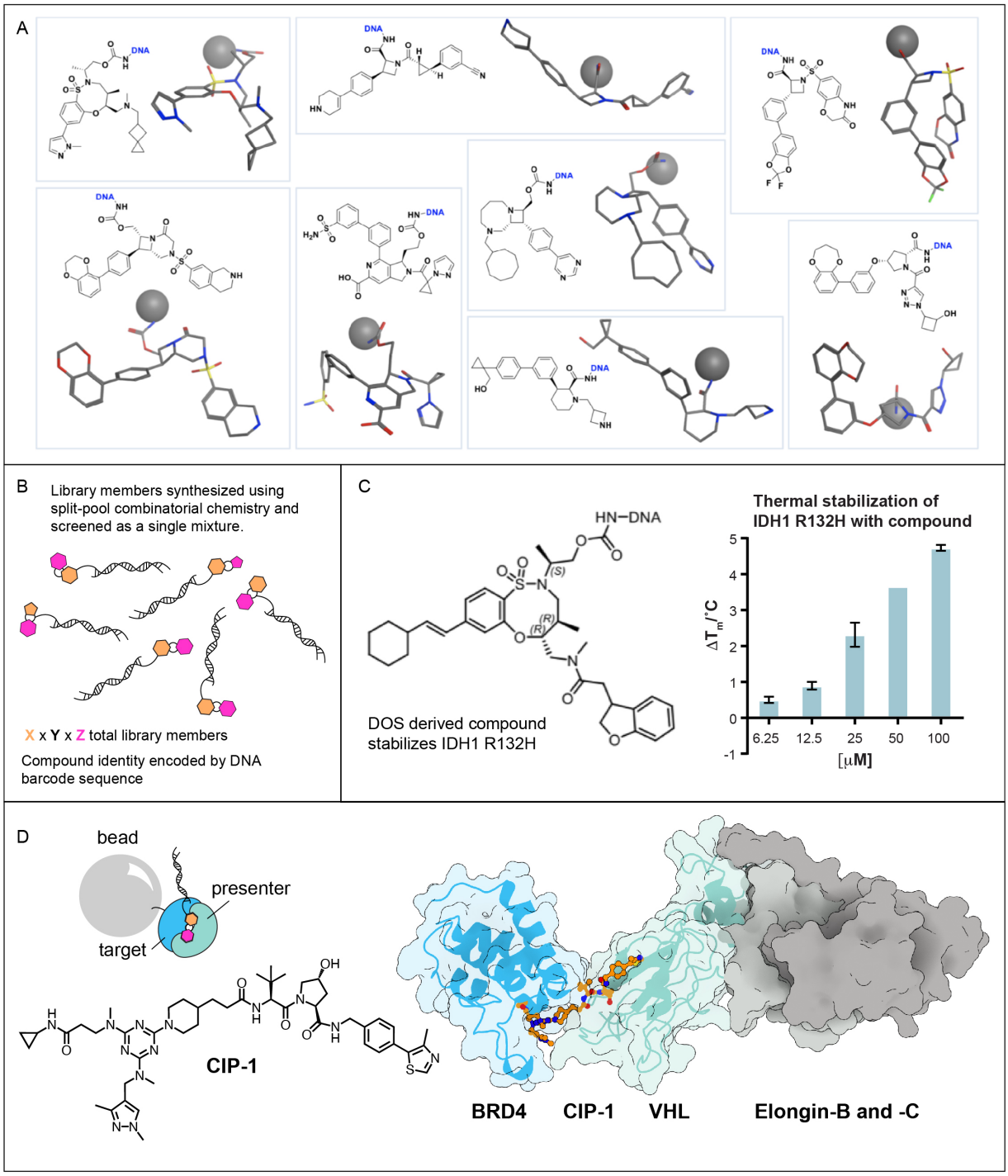

Figure 4: Diversity-oriented synthesis (DOS) and DNA-encoded library (DEL) screening moves discovery beyond serendipity. D. PDB ID: 8EWV 3D Protein Imager[4] was used to prepare protein structures.

Diversity-oriented synthesis (DOS) has been used extensively in cell-based phenotypic screens resulting in the discoveries of compounds having novel mechanisms-of-action and selectivity.[53] Combining the power of DOS with that of DNA-encoded libraries (DEL)[54] creates opportunities to uncover new molecular glues and bifunctionals using biochemical screens of pre-selected proteins. But extending this synthesis strategy to DEL syntheses requires innovating DNA-compatible organic reactions[55] or identifying novel strategic considerations for building DELs.[56] An example of the latter rethinks the roles of skeletons and appendages in DELs. DELs are often constructed using split–pool DNA barcoding of a single skeleton with collections of appendages attached at three distinct sites. An alternative approach views the skeleton itself as a diversification element.[5758] A large collection of compounds having diverse skeletons with two orthogonal attachment sites was synthesized using the DOS strategy with two appendage diversification steps performed subsequently (Figure 4B).[59] Affinity-based screens of this library yielded novel binders, including one that clamps a protein–protein interface of the oncogenic form of IDH1 (R132H). This binder-first approach directly yielded a potent molecular clamp that reinforces protein–protein interactions as inferred from thermal stabilization measurements (Figure 4C).

DELs can now be screened using pre-selected presenter and target proteins to discover either bifunctional compounds or molecular glues rationally by simply analyzing the barcode enrichments in different ways. These screens are performed using target proteins attached to magnetic beads and presenter proteins in solution incubated stoichiometrically with the entire barcoded compound library.[606162] Early studies of this approach using bromodomain family members including BRD4 and the VHL–elongin C–elongin B complex (VCB) show that the traditional approach of comparing barcodes enriched in the presence of immobilized target plus free presenter *vs.* beads alone leads to bifunctional compounds that induce proximal relationships of presenters and targets in cells and can have functional consequences such as target protein degradation (Figure 4D).[62]

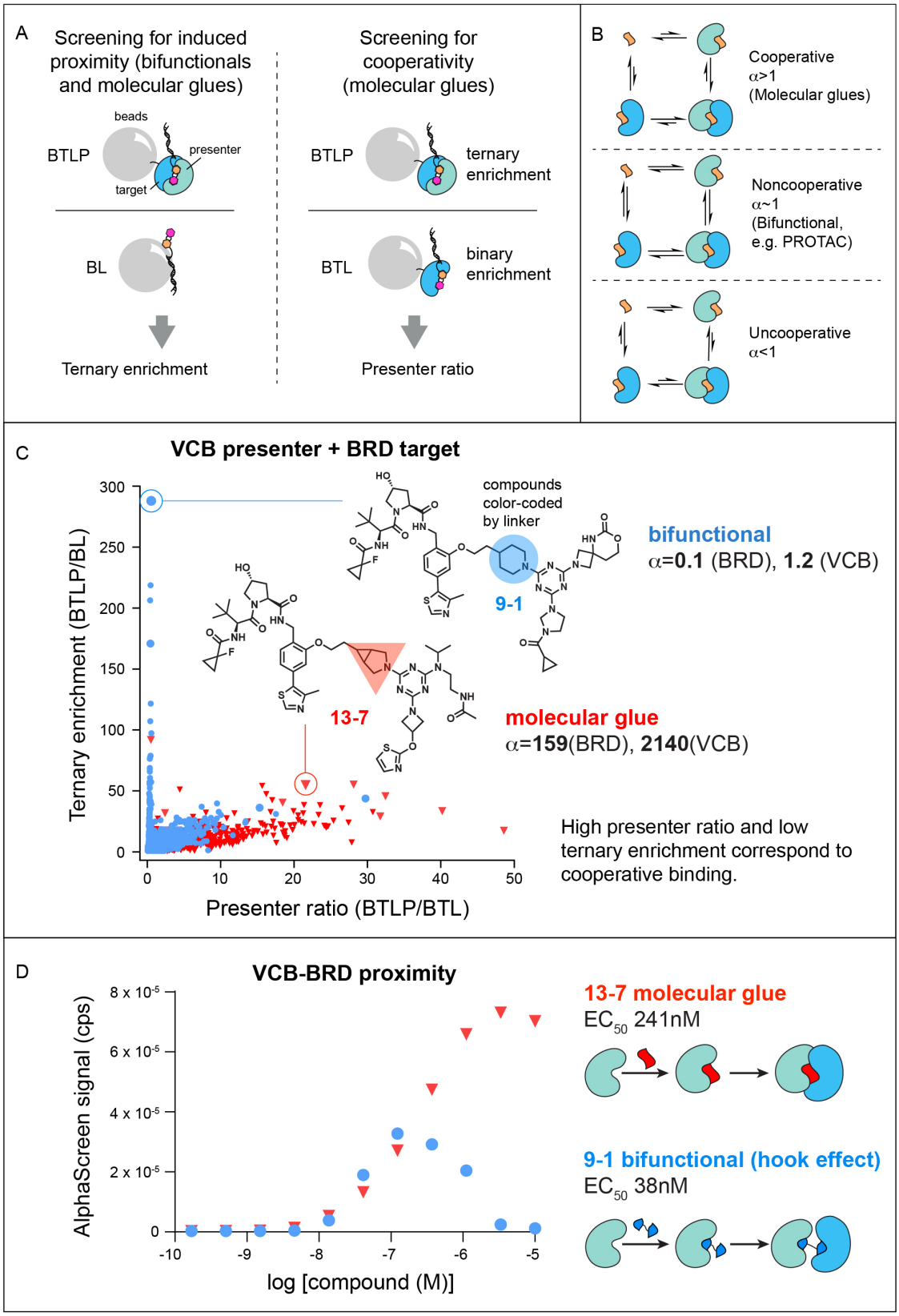

Figure 5: Screening for novel, cooperative, molecular glues

In contrast to this high "ternary enrichment" (ratio of sequenced barcodes seen with BTLP vs BL where B = magnetic Beads, T = Target protein; L = DNA-encoded Library, P = Presenter protein; in analogy to the "binary enrichment" measured in standard DEL screens), comparing barcodes enriched using an immobilized BRD9 target and free VCB presenter *vs.* bead-immobilized target alone (high "presenter ratio"; BTLP vs. BTL, or the ratio of ternary enrichment/binary enrichment) can point to molecular glues that induce proximity through cooperative binding (Figure 5A).[61] This is a satisfying finding since comparative enrichment of target + presenter vs. target alone normalizes for non-specific binders but also, on first principles, favors barcodes associated with compounds that gains presenter-dependent strength in target binding, i.e., cooperative binding (Figure 5B). Molecular glues have been called matchmakers,[63] an apt description of bifunctional compounds that reflect a marriage of convenience, whereas cooperativity-inducing molecular glues behave more like Cupid's arrow, creating novel *affinity* of the presenter protein for its target.

These screens offer a rational approach to the discovery of either bifunctional compounds or molecular glues directly. Traditional bifunctional compound discovery can be achieved by sequential identification of binders and a connector, but the CIP-DEL approach combines these in a single step. Rationally designing cooperative binding in ternary complex formation seems beyond current capabilities, even with computer assistance. The subtle structural distinctions between bifunctional inducers of proximity of the VCB presenter and BRD9 target (from searching for high ternary enrichment) vs. a highly cooperative molecular glue (from searching for high presenter ratios) reinforce this notion (Figure 5C). These structural changes and their functional consequences (highly cooperative interactions – α = 159 (BRD9) and 2140 (VCB) and mitigation of the hook effect in cells) could not be predicted in advance yet are uncovered directly by rational screening (Figure 5D).[61]

Summary and Future directions: Precision medicine "in the right location". This Perspective has emphasized a new modality in drug discovery. Proximity-inducing molecular glues and bifunctional compounds can modulate challenging therapeutic targets in novel ways – stabilizing, degrading, translocating, activating, rewiring transcriptional circuitries, and inducing high effective molarities of enzyme–neo-substrate pairs to accelerate chemical modifications such as phosphorylation and ubiquitination. Advances in synthesis and screening techniques offer the promise of cooperativity-dependent levels of specificity not generally achieved with traditional small-molecule drugs.

The logic underlying this modality stitches together four concepts highlighted in this Perspective: 1) Small molecules bind hot spots on proteins, and these hot spots are often the sites of functional protein–protein interactions. 2) Small-molecule binders often alter interactomes of their protein targets or alter the dynamic properties of proteins by gluing distinct domains intramolecularly. 3) Proximity-inducing compounds mimic missense mutations, which tend to emerge in or near hot spots and can alter the interactome of the mutant paralog. 4) Proximity-inducing compounds mimic posttranslational modifications, thus hijacking native biological systems to induce specific outcomes with temporal and sometimes even spatial control.

Whereas the ubiquity of molecular glues is only now becoming apparent, these compounds have largely to-date been found serendipitously – usually following mechanism-of-action studies of compounds discovered in phenotypic assays. However, new methods that enable the identification of bifunctional compounds and molecular glues that act on pre-selected presenter and target proteins open new avenues for exploration. For precision medicine, there may be a unique opportunity to extend to "the right drug for the right patient at the right time – and in the

right tissue". There is still much work to be done to realize this avenue and others afforded by this new modality.

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Declaration of Interests. S.L.S. is a shareholder and advises Magnet Biomedicine and is the Founding C.E.O. of Arena BioWorks, LLC.

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