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Nat Rev Chem. Author manuscript; available in PMC 2023 November 20.

Published in final edited form as:

Nat Rev Chem. 2023 April ; 7(4): 234–255. doi:10.1038/s41570-023-00468-z.

Nature-inspired protein ligation and its applications

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Abstract

The ability to manipulate the chemical composition of proteins and peptides has been central to the development of improved polypeptide-based therapeutics and has enabled researchers to address fundamental biological questions that would otherwise be out of reach. Protein ligation, in which two or more polypeptides are covalently linked, is a powerful strategy for generating semisynthetic products and for controlling polypeptide topology. However, specialized tools are required to efficiently forge a peptide bond in a chemoselective manner with fast kinetics and high yield. Fortunately, nature has addressed this challenge by evolving enzymatic mechanisms that can join polypeptides using a diverse set of chemical reactions. Here, we summarize how such nature-inspired protein ligation strategies have been repurposed as chemical biology tools that afford enhanced control over polypeptide composition.

Graphical Abstract

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Author contributions

The authors contributed equally to all aspects of the article.

Competing interests

The authors declare no competing interests.

Peer review information *Nature Reviews Chemistry* thanks Ashraf Brik, Anne Conibear and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.

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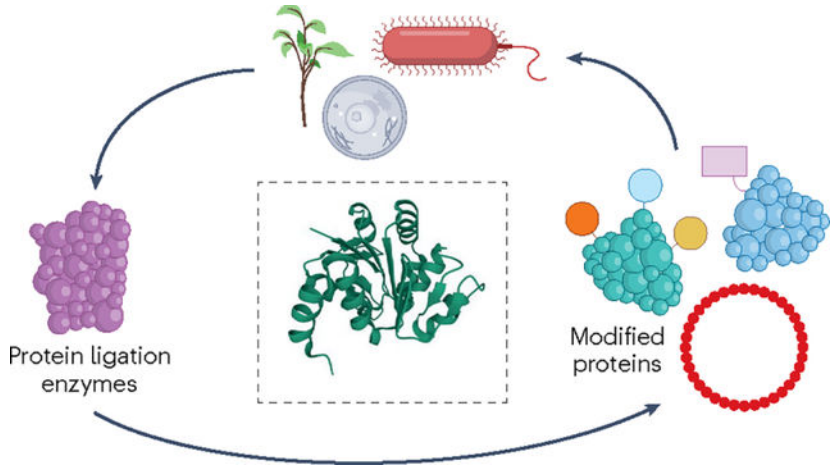

Introduction

Proteins and peptides carry out a vast number of biological functions that are ultimately dictated by their chemical composition. Owing to their involvement in most biological processes and the ever-increasing market for protein and peptide therapeutics[1], there is a great interest in strategies that can control polypeptide composition at levels that stretch beyond the linear, ribosomal assembly of genetically encoded natural amino acids. To this end, the ligation of two or more protein-based substrates is a facile approach that enables researchers to flexibly tailor the composition of proteins in various ways. Protein ligation strategies pave the way for protein semisynthesis, in which recombinantly produced proteins are fused with synthetic peptides, thereby expanding the chemical space that is available for (re)defining the primary structure of proteins. These strategies have been used to install a wide range of chemical probes into proteins[2], site-specifically incorporate post-translational modifications (PTMs) and conjugate cytotoxic drugs to antibodies[3,4]. In addition, protein ligation can be leveraged for segmental isotope labelling of proteins, thereby reducing the complexity of protein NMR spectra, which allows the structural characterization of protein domains that are otherwise inaccessible[5]. Ligation-based strategies have also proved useful for manipulating protein topology, as exemplified by the cyclization of polypeptides through intramolecular reactions, which can be used to generate stable and therapeutically relevant peptide libraries[6]. Moreover, polypeptide topologies can also be engineered through isopeptide bond formation between amino acid side chains. Evidently, the motivation for performing protein ligations can vary significantly, and it is therefore critical to choose a ligation platform that is best suited for the desired outcome (Fig. 1).

Successful ligation of polypeptides requires an efficient, chemoselective reaction that creates a specific amide bond, despite the presence of the ensemble of functional groups found in proteins. Chemical methods for protein ligation, of which native chemical ligation (NCL) and its extension expressed protein ligation (EPL), are the most common (Box 1), represent valuable platforms for generating semisynthetic, site-specifically modified proteins. However, these chemical approaches are ultimately limited by their biocompatibility and requirement for high concentrations of reactants. Alternatively,

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semisynthetic proteins, which are composed of recombinant and synthetic pieces, can be generated through non-ligation-based platforms, such as genetic code expansion (Box 1), bioorthogonal conjugation to reactive side chains[3,7] and post-translational mutagenesis[8,9], although all of these have inherent limitations. To develop novel strategies for protein ligation, inspiration has been drawn from natural enzyme-based approaches. Enzymes have long been known to be able to perform 'reverse proteolysis' and join two protein segments together[10]. Since this discovery, the toolbox for enzyme-mediated protein ligation has markedly expanded and now includes several powerful platforms that allow precise ligation of peptides and proteins under complex and dynamic conditions, such as living cells, at low reactant concentrations[2,3].

In this Review, we summarize the different classes of enzymes, which are grouped on the basis of the mechanism by which they facilitate amide bond formation, that are available to researchers for the generation of semisynthetic proteins in vitro and in cells. We highlight how these enzymes vary in key aspects of protein ligation to inform the selection of which strategy is best suited for a given task. These differences include how traceless the ligation is, that is, how substantial the 'ligation scar' that remains in the final product is, the selectivity and efficiency of the reaction, the type of bond that is created (native peptide bond or isopeptide bond), the synthetic availability of the reactants, and whether the platform is bioorthogonal. Moreover, we present selected examples of applications of these strategies together with additional sources for in-depth descriptions of each system. Finally, we emphasize how engineering of the naturally occurring enzymes has been critical for moving the field towards more efficient and expansive protein ligation.

Transpeptidase-based methods

Transpeptidases are a family of enzymes isolated from bacteria or plants that catalyse nucleophilic carbonyl substitution and transamination[11]. Transpeptidases perform their biochemical functions by initially cleaving an amide bond within a recognition sequence of the acyl donor using a catalytic cysteine residue. This generates an activated thioester intermediate that can then be attacked by an amine donor, restoring an amide bond and completing the transpeptidase cycle. Transpeptidases possess promiscuous enzymatic activities, including the cleavage of the D-alanyl–D-alanine bond and the subsequent crosslinkage of bacterial cell wall peptidoglycan (Fig. 2a) as well as peptide or protein macrocyclization. Consequently, transpeptidases are versatile scaffolds for developing protein ligases, and two major types of transpeptidases are widely used for protein ligation: sortases and asparaginyl endopeptidases (AEPs).

Sortases

Sortases are a highly ubiquitous group of transpeptidases found in Gram-positive bacteria that use their enzymatic activity to anchor various surface proteins to the peptidoglycan of the cell wall[12]. Of these, sortase A (SrtA), isolated from *Staphylococcus aureus*, has found widespread use for protein ligation[13]. In SrtA-mediated ligation, the catalytic cysteine attacks the scissile bond between threonine and glycine within a conserved amino acid sequence (R^1-LPXTG-R^2) to form a key thioester intermediate (R^1-LPXT-SrtA) and releases

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the by-product NH_2-GR^2 (Fig. 2b). The activated thioester then undergoes aminolysis by the N-terminal α-amine group of a donor (NH_2-GR^3), to generate a new protein sequence (R^1-LPXTG-R^3). This makes SrtA useful for both N-terminal and C-terminal protein modification. As the N-terminal residue of the amine donor must be glycine, the original LPXTG motif is regenerated in the ligated product. This enzymatic process, also referred to as the sortagging reaction, is Ca^{2+}-dependent owing to an allosteric Ca^{2+} binding site that stabilizes an otherwise disordered loop in SrtA[14]. Recombinant SrtA can be purified with high yields from *Escherichia coli* (typically >40 mg l^{-1}), cementing its utility as a chemical biology tool.

However, wild-type SrtA has relatively poor catalytic efficiency, even in the presence of Ca^{2+}, and the reaction requires high enzyme concentrations (0.1–1.0 molar ratios of SrtA:substrate) as well as long ligation times (more than 20 h). Moreover, the reversibility of SrtA-mediated transpeptidation restricts the overall yield of the ligated product, as the product (R^1-LPXT-GR^3) acts as a substrate for the reverse enzymatic cleavage and ligation (Fig. 2b). It has been shown that the addition of excess amine donor (NH_2-GR^3) or removal of the glycine by-product (NH_2-GR^2) can shift the equilibrium towards an increased yield of the desired product[15,16]. The yield can be further improved by using engineered depsipeptide substrates that mimic the glycine amide structure and release hydroxyacetyl by-products that cannot re-attack the thioester intermediate[17] (Fig. 2c). Although this strategy has only been successfully applied to N-terminal protein modification[18], an alternative approach has recently been developed in which SrtA uses a thioester substrate in an irreversible ligation reaction that is practical for both N-terminal and C-terminal protein modification[19] (Fig. 2c). Moreover, thioester-assisted ligation displays improved sequence tolerance, leaving only a single glycine residue as a ‘scar’ at the ligation site (Fig. 2d). Finally, StrA has also been engineered, for example, through directed evolution or rational design, to enhance its catalytic efficiency[20,21] and remove its Ca^{2+} dependency[22], thus improving its utility for protein ligation in living cells, which usually contain low concentrations of Ca^{2+}.

The chemoselectivity of SrtA is attributed to its recognition of the LPXTG motif, which serves as a conjugation tag for protein ligation. Even though this enzymatic ligation is not ‘traceless’, the modest size of the ‘ligation scar’ (LPXTG) can in some cases be introduced without remarkably affecting the functions of the ligated product protein substrates[15]. Still, several studies have used directed evolution of SrtA to broaden its substrate preference, thus increasing the flexibility of sortase-mediated protein ligation. By applying yeast display selection[20,23], error-prone PCR-based construction of random mutation libraries and structure-guided saturated mutagenesis[24], the catalytic efficiency of SrtA has been improved by increasing the substrate binding affinity for the mutants eSrtA[20,23] and 5M-D124G-Y187L-E189R (with three mutations relative to eSrtA)[24].

SrtA accepts any amine donor with an N-terminal glycine and therefore has limited selectivity in complex biological microenvironments. The combination of this low acyl donor selectivity and the requirement of high Ca^{2+} concentrations means that SrtA is mostly used in simpler systems with a single amine donor (with a N-terminal polyglycine sequence), including in vitro protein ligation[25], protein lipidation to facilitate membrane

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insertion[26], enzyme immobilization[27] and the homogeneous generation of antibody–drug conjugates[28]. Nevertheless, there are a few examples of SrtA-mediated ligation in eukaryotic cells[29], including a platform in which genetic code expansion facilitates sumoylation of an internal residue by SrtA[30].

Asparaginyl endopeptidases

AEPs are a subtype of cysteine proteases that cleave proteins at C-terminal asparagine or aspartic acid residues, forming a thioester intermediate that then undergoes hydrolysis or nucleophilic attack (for example, by a peptide α-amine) to yield the ligation product[31]. The result of AEP-mediated catalysis (hydrolysis or ligation) is therefore dependent on the concentration of nucleophiles in the microenvironment. In plants, AEPs play important roles in the maturation of seed storage proteins in the low pH environment of storage vacuoles[32]. To facilitate this, plant AEPs are expressed as zymogens that require low pH-induced autoactivation through the cleavage of N-terminal and C-terminal pro-domains[33]. The following examples, butelase-1 and *Oa*AEP1, are two representative AEPs that are widely applied in protein and/or peptide engineering.

Butelase-1.—Butelase-1 is a unique cysteine transpeptidase isolated from *Clitoria ternatea* seeds that acts as a cyclase in the biosynthesis of cyclotides, a family of cyclic, cysteine-rich peptides in plants. It exhibits little hydrolase activity but instead cleaves an Asn or Asp(Asx)–His–Val motif between Asx and His to form a reactive thioacyl–enzyme intermediate that can then be intercepted by the N-terminal α-amine of a peptide to eventually form a stable amide bond[34] (Fig. 2e). An important in vitro application of butelase-1 is to produce cyclic proteins and/or peptides through an intramolecular reaction driven by a nucleophilic attack by the N-terminal α-amine of the substrate on the thioester intermediate[35] (Fig. 2e). In comparison with wild-type SrtA [k_{cat}/K_M (catalytic constant/Michaelis constant) ≈200 l mol^{-1} s^{-1}][20,24], butelase-1 has a much higher catalytic activity, with k_{cat}/K_M values as high as 1,340,000 l mol^{-1} s^{-1} for medium-sized peptides. As a result, butelase-1-mediated ligations require only ~0.005 molar equivalents of the enzyme. Butelase-1 also possesses incredible cyclization rates that are >10,000 times faster than those of sortases[36]. Moreover, butelase-1 displays high catalytic promiscuity with negligible N-terminal sequence requirements for the acyl acceptor (Fig. 2e). This flexibility is highlighted by the fact that substrates consisting of D-amino acids (except for the P1 Asx residue) can also be ligated efficiently by butelase-1 (ref. [37]), thus enabling the efficient synthesis of D-amino-acid-containing peptide macrocycles[38]. Introduction of D-amino acids into cyclic peptides may largely improve the stability and pharmacokinetics of peptide drugs[39], making this an intriguing application of butelase-1-mediated ligation. This has been exemplified in the cyclization of sunflower trypsin inhibitor, the conotoxin MrlA and the antimicrobial θ-defensin[38].Conversely, the high sequence tolerance of butelase-1 prevents it from being used for chemoselective ligation in complex microenvironments.

The biochemical machinery of butelase-1 provides a route for the synthesis of protein thioesters, thereby enabling tandem chemoenzymatic ligations (for example, via NCL)[40]. Notably, another advantage of butelase-1 is that it does not rely on cofactors and is thus not limited by their availability, as in the case of the Ca^{2+}-dependent activity of SrtA.

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These advantages have enabled the use of butelase-1 for a number of applications besides cyclization, including engineering of the bacterial cell surface[41], production of peptide dendrimers[42] and high-yielding, N-terminal protein labelling using thiodepsipeptides as the acyl acceptor[43]. Importantly, SrtA and butelase-1 are orthogonal, and have been used for dual labelling of antibodies in one-pot reactions as well as C-to-C fusion of proteins[44]. However, the use of butelase-1 is not without drawbacks. Noticeably, the heterologous expression of recombinant butelase-1 has not been very successful so far, and most studies have been performed using the natural, plant-derived enzyme[45]. As a plant protein possessing three pairs of disulfide bonds, recombinant butelase-1 purified from *E. coli* or *Pichia pastorishas* exhibits undesirably low catalytic efficiency and yield, and thus the optimization of high-yield preparation of butelase-1 with excellent catalytic efficiency is still in demand[46]. This also means that engineering of butelase-1 has been limited. Similar to SrtA, butelase-1 suffers from the intrinsic reversibility of the transpeptidase reaction, which lowers the product yield, and an excess of substrate is required to reach yields of >50%. These obstacles largely limit the potential biotechnological applications of butelase-1 (ref.[16]).

***Oa*AEP1.—**In the search for butelase-1-like transpeptidases, the genomes of cyclotide-producing plants such as the Rubiaceae, Violaceae, Fabaceae, Solanaceae and Cucurbitaceae families have been mined to identify enzymes that can recognize and transform cyclotide pre-cursors containing a conserved C-terminal Asp or Asn residue. Among the identified AEPs, a promising alternative to butelase-1 is *Oa*AEP1, isolated from the plant *Oldenlandia affinis*[16]. Although *Oa*AEP1 is a less-active homologue of butelase-1, it is amenable to recombinant expression in *E. coli*, albeit at relatively modest levels (<2 mg l^{-1})[47]. *Oa*AEP1 can catalyse cyclization of a diverse range of substrates without the assistance of any cofactors and, similar to butelase-1, *Oa*AEP1 and its mutants (for example, E371V) have been widely used for macrocycle synthesis[48] as well as for protein modification, such as site-specific sequential protein labelling[49]. *Oa*AEP1 can also be used for ligating peptide–nucleic acid conjugates to proteins, thereby allowing erasable imaging of membrane proteins that rely on the sequential hybridization and removal of a fluorescent probe[50]. *Oa*AEP1 therefore represents an important platform for further evolution of AEP-based ligation strategies[31].

Protease-based methods

The major biological function of proteases is to cleave target protein substrates rather than facilitate transamination as seen for trans-peptidases. Proteases can be classified into broad groups on the basis of the nucleophilic residue that attacks the scissile bond in the substrate (serine proteases, cysteine proteases, threonine proteases, aspartic proteases, glutamic proteases, metalloproteases and asparagine peptide lyases)[51]. For protein ligation, serine proteases are of particular interest, as they can be engineered to catalyse protein and/or peptide ligation by favouring the ‘reverse proteolysis’ reaction. Here, the enzyme–substrate complex is resolved through aminolysis rather than hydrolysis, as the α-amine of a peptide donor serves as a nucleophile[52]. Most of these peptide ligases are derived from subtilisins (such as subtiligase, peptiligase and omniligase-1), which are secretory proteases

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found in soil bacteria[53]. These bona fide protein ligases accept a larger range of recognition motifs than the aforementioned transpeptidases, which makes them powerful and versatile tools for protein modification in vitro, but their lack of specificity does limit their potential for modifying proteins in complex microenvironments.

Subtiligases

The subtilase family of enzymes possesses an Asp–Ser–His catalytic triad and is the second largest serine protease family characterized to date, with more than 200 members identified. Subtilases are widespread and are found in eubacteria, archaebacteria, eukaryotes and even viruses[54].

Among all the subtilases, subtilisins, which are secretory proteins from soil bacteria with typical molecular weights of ~27 kDa, are well studied as protein ligase scaffolds[55]. One successful example is subtilisin BPN', isolated from *Bacillus amyloliquefaciens*. For most natural subtilisins, their peptide bond hydrolysis activity strongly dominates over the reverse peptide ligation activity. However, the introduction of two mutations (S221C and P225A) in subtilisin BPN' tunes the engineered enzyme (subtiligase) to efficiently catalyse ligation of a C-terminal peptide ester acyl donor and an N-terminal α-amine of a peptide or protein using Ca^{2+} as a cofactor[56,57]. The S221C mutation converts the enzyme from a serine into a cysteine protease that is able to form a thioacyl–enzyme tetrahedral intermediate instead of the original oxyester, thereby generating an intermediate that is more prone to aminolysis. The P225A mutation enhances the peptide ligase activity by two orders of magnitude by negating the steric crowding within the active site[58].

Unlike SrtA and butelase-1, subtiligase does not require a specific recognition motif at the substrate termini to catalyse ligation. Nevertheless, the residues on both sides of the ligation site greatly influence the catalytic performance of subtiligase, and the target is therefore usually modified to increase the yield. Notably, a protein and/or peptide ester substrate is needed in subtiligase-mediated ligation to serve as the acyl donor (Fig. 3a), necessitating that the N-terminal ligation partner is either entirely synthetic or has its C terminus functionalized. Subtiligase also requires a large excess of acyl acceptor to suppress hydrolysis. Moreover, subtiligases are typically expressed as pre-pro-proteins, in which the pre-sequence serves as a signal peptide for secretion and the pro-domain is required for folding of the functional mature enzyme before its autocatalytic removal[59].

After years of optimization and screening, functionally enhanced subtiligase variants have been successfully generated[60]. In one variant obtained through directed evolution[61], the pro-domain and calcium loop were deleted to circumvent the need for autocatalytic cleavage to generate the mature enzyme as well as the Ca^{2+} dependency. Similarly, a subtiligase variant termed stabiligase has been generated via the introduction of five stabilizing mutations (M50F, N76D, N109S, K213R and N218S) that enable protein ligation in the presence of denaturants such as SDS and guanidinium hydrochloride[62,63].

Owing to their high promiscuity, subtiligases have been widely used in protein chemistry. One important application is subtiligase-mediated EPL, in which the thioester substrate is usually synthesized from an intein-tagged recombinant protein through thiolysis[64]. Classical

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EPL is performed via NCL, meaning that an N-terminal cysteine in the amine donor is required. However, in subtiligase-mediated EPL, the cysteine residue is no longer needed because the transthioesterification and subsequent S→N-acyl shift of NCL are not required for the enzymatic ligation. This strategy has been used to efficiently produce C-terminally phosphorylated tumour suppressor protein PTEN (phosphatase and tensin homologue)[65]. A proteomics-based characterization of the specificity of 36 subtiligase variants has identified mutants with distinct reactivities that enable orthogonal N-terminal labelling of proteins with different N-terminal sequences[63]. By combining four of these mutants with broad N-terminal specificity together, the promiscuity of subtiligase-mediated labelling was exploited to enrich, and hence map, the cellular N-terminome.

Peptiligase and omniligase-1.—In the pursuit of improved peptide ligases, two-point mutations were introduced into an already heavily engineered subtiligase scaffold[66]. The resulting ligase, termed peptiligase, contains 18 stabilizing mutations as well as deletions of the pre-domain, pro-domain and calcium-binding domain. Peptiligase can catalyse ligations to extremely high yields (>98% yield in less than 1 h) using only a slight excess of one of the reagents, for example, 1.1–1.5 equivalents of acyl acceptor. Compared with other commonly used peptide ligases, peptiligase is thermostable (T_M = 66 °C) and functions well in the presence of organic solvents (up to 50 vol% *N, N*-dimethyl-formamide) as well as denaturants (2 M urea or guanidinium chloride), making it a particularly useful tool for the ligation of poorly soluble or folded proteins or peptides.

To gain a broader acyl acceptor substrate scope, peptiligase has been further engineered via site-directed mutagenesis[67]. One resulting enzyme is omniligase-1, which is useful for chemo-enzymatic peptide synthesis as well as for protein semisynthesis. Furthermore, omniligase-1 can catalyse the formation of head-to-tail macrocyclic products using substrates that are >300 residues long, and it has been applied in the gram-scale synthesis of cyclic peptides, making it viable for industrial-scale protein ligation[68,69]. In summary, both omniligase-1 and peptiligase-mediated coupling reactions are scalable and can be used as a versatile stand-alone technology, as well as in combination with chemical or intein-based protein ligation methodologies[67].

Trypsiligase

Trypsin is a serine protease belonging to the PA (proteases of mixed nucleophile, superfamily A) clan superfamily and is one of the most widely used enzymes in proteomics research[70]. Trypsin is found in the digestive system of many vertebrates, where it hydrolyses proteins by cleaving peptide bonds at the carboxyl side of unmodified lysine and arginine residues[71]. Although the potential use of trypsin variants for peptide and protein synthesis has been known for decades[71], extensive protein engineering was required before trypsin-derived ligases became an integral part of the protein semisynthesis toolbox.

One of the most successful and widely used trypsin variants is trypsiligase, a rationally designed quadruple mutant (K60E, N143H, E151H and D189K) of anionic rat trypsin II (ref. [72]). Trypsiligase has a high ligation rate and specifically cleaves the tripeptide motif Y–*RH*, followed by transpeptidation of the acyl to an α-amine with an N-terminal

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RH motif (Fig. 3b). This means that trypsiligase catalyses N-terminal modification that only leaves a small, two-residue (*RH*) 'ligation scar'. However, as with all transpeptidase reactions, trypsiligase-catalysed ligation suffers from lower yields caused by the reversibility of the reaction and competing hydrolysis of the acyl– enzyme intermediate. Additionally, trypsiligase adopts a zymogenlike conformation, meaning that both the Y–*RH* tripeptide motif and its cofactor Zn^{2+} are required to induce its ligation activity[73]. This unique biochemistry minimizes proteolytic side reactions, enabling trypsiligase to mediate N-terminal labelling with a substrate mimetic as the acyl donor[74] (Fig. 3b), as well as C-terminal labelling with synthetic moieties[75], including click handles that allow further derivatization[73]. Intriguingly, only 0.5% of all proteins in the SwissProt database contain the Y–*RH* recognition motif despite its small size[45], thereby allowing Y–*RH* to serve as a useful tag for site-specific modification of either the N-terminal or C-terminal region of target proteins in trypsiligase-mediated ligations (Fig. 3b).

Macrocyclases from microbial biosynthetic pathways

In the biosynthetic pathways of microbial polypeptide metabolites, some macrocyclases have evolved dual functions and perform both proteolysis and macrolactonization[76–79]. These enzymes have been discovered in the biosynthesis of cyanobactins, a family of ribosomal cyclic peptides produced by cyanobacteria[80].

One particularly well-studied example is PatG, a macrocyclase involved in the biosynthesis of patellamide. The PatG-like macrocyclases contain an Asp–His–Ser catalytic triad, enabling them to catalyse proteolytic cleavage of a C-terminal recognition sequence, termed the 'follower peptide', in tandem with peptide macrocyclization[81] (Fig. 3c). In contrast to other protease-derived protein ligases, PatG-like enzymes have a narrower substrate scope, limited to peptides that have been post-translationally modified by heterocyclization of cysteine, serine or threonine to form thiazole, thiazoline or oxazoline residues. Although this equips PatG with a high degree of specificity, it also necessitates the synthesis of non-standard proteogenic substrates for ligation[82]. Structural biology studies indicate that PatG enzymes contain a conserved helix–loop–helix insertion that may prevent the acyl–enzyme intermediate from being attacked by a water molecule[81], thus preventing hydrolysed by-products. Interestingly, deletion of this segment results in a PatG variant with maintained protease activity that no longer catalyses peptide macrocyclization[83,84]. Although useful in producing peptide macrocycles, further engineering and optimization are required to make PatG a more widely applied molecular tool, as illustrated by efforts that circumvent the need for a C-terminal proline/thiazoline (for example, $PagG^{mac}$-Cys275Ala)[85,86].

Another subtype of dual-functional macrocyclases are TsrI-like enzymes, which are involved in the biosynthetic pathway of bicyclic thiopeptides and play essential roles in the construction of the molecular structure of thiostrepton. TsrI belongs to the α/β-hydrolase fold enzyme family and possesses a Ser–His–Asp catalytic triad. Similar to PatG, TsrI catalyses proteolytic cleavage followed by macrocylization. However, unlike PatG, TsrI-mediated macrocylization occurs through a unique epoxide ring-opening reaction following cleavage of the N-terminal leader peptide[87] (Fig. 3c). Although previous studies have shown that TsrI-like enzymes can tolerate amino acid substitution in the sequences of the leader

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and core peptides, this family of enzymes has not been commonly used in protein or peptide chemistry as they require highly modified substrates[87].

Transglutaminase-mediated methods

Unlike the enzymes mentioned previously that specifically catalyse ligations between the N-terminal α-amino groups and C-terminal carboxyl groups of peptides or proteins, transglutaminases (TGMs) are a type of naturally occurring protein ligase that non-specifically catalyses isopeptide bond formation using the side chain amide group of glutamine residues[88]. An aminolysis reaction between the glutamine γ-carboxamide and the ε-amino group of lysine results in the formation of covalent crosslinks that bind the proteins together (Fig. 4). The food industry employs TGMs to crosslink pieces of meat; hence, TGMs are also referred to as 'meat glue'[89]. TGMs are found in most domains of life including animals, plants and microorganisms. Mammalian TGMs (for example, TGM2) require Ca^{2+} as a cofactor, whereas those isolated from bacteria (such as *Streptomyces mobaraensis*) are calcium-independent enzymes. TGMs have a very broad natural substrate scope ranging from cytoplasmic proteins to histones[90]. Because TGMs do not have any sequence selectivity, they have limited use for in vivo and in cellulo applications for protein ligation[91].

Although TGMs are unable to catalyse the formation of peptide bonds, constructing isopeptide bonds can be useful for producing side chain-conjugated proteins. For example, *S. mobaraensis* TGM is a versatile tool for manufacturing antibody–drug conjugates, such as human IgG1 derivatives[92]. Here, either lysine or glutamine residues within the antibody are used to create the conjugate, and the drug molecule is designed with a carboxamide or primary amine as the reactive group to target the complementary residue (or residues).

Molecular superglue-mediated methods

The emergence of 'molecular superglue' techniques has provided new protein and peptide ligation strategies, such as the SpyTag–SpyCatcher and SnoopTag–SnoopCatcher reactive pairs[93,94]. These techniques rely on the formation of an isopeptide bond between aspartate (or glutamate) and lysine residues by spontaneous condensation[95]. The reaction was initially discovered in Gram-positive bacteria (for example, *Streptococcus pyogenes*) where it serves to stabilize extracellular proteins such as Spy0128 (refs. [96,97]). In the invasive strains of *S. pyogenes*, the second immunoglobulin-like collagen adhesion domain (CnaB2) from the fibronectin-binding protein FbaB contains a single isopeptide bond that is autocatalytically formed, stabilizing the protein and extending its half-life and durability. To take advantage of this unique biochemical arrangement, CnaB2 has been split and engineered to produce a 13-amino acid peptide tag (SpyTag) and a 138-amino acid protein partner (SpyCatcher). The SpyTag and SpyCatcher associate with nanomolar affinity and are able to ligate two protein segments in vitro or in vivo[93] (Fig. 5a). Recently, the SpyTag–SpyCatcher pair was used to enforce asymmetry on nucleosome core particles, which represent the fundamental unit of chromatin, by covalently linking two different variants of a given histone subtype (in this case, H3) during in vitro reconstitution, thus generating a more physiologically relevant form of the complex[98]. Moreover, SpyTag–SpyCatcher has been used to overcome the low

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catalytic activity of SrtA by covalently linking the enzyme to its substrate in the so-called 'proximity-based sortase-mediated ligation'[99].

A similar splitting and engineering strategy has been used for the adhesion protein RrgA from *S. pneumoniae*, yielding the peptide SnoopTag (12 amino acids) and its protein partner SnoopCatcher (112 amino acids) (Fig. 5b). The condensation of SnoopTag and SnoopCatcher occurs in near quantitative yields and does not crossreact with SpyTag–SpyCatcher, enabling bioorthogonal protein labelling using these protein pairs[94,100]. In a similar Tag–Catcher system, the CnaB protein from *Streptococcus dysgalactiae* has been split and engineered into the SdyTag–SdyCatcher pair, a homologue of SpyTag–SpyCatcher[101].

The molecular superglue principle has been further developed to generate 'peptide–peptide staplers', an alternative approach for efficient protein ligation both in vivo and in vitro[102]. In this unique ligation reaction, the CnaB2 protein is divided into three components by further splitting the SpyCatcher piece at a solvent-exposed second loop region to yield SpyStapler and BDTag. Although SpyStapler is intrinsically disordered on its own, it forms a stably folded structure in the presence of SpyTag and BDTag, and a glutamate residue in SpyStapler is critical for isopeptide bond formation between SpyTag and BDTag. (Fig. 5c). By expanding the Tag–Catcher platform with a third component, the level of spatiotemporal control over covalent coupling may be improved by controlling the localization or expression of SpyStapler[102]. Although these systems are far from traceless and involve fairly large tags, they are promising bioorthogonal protein ligation tools for generating intramolecularly and intermolecularly crosslinked structures. As such, these techniques have been employed for tracking the dynamics of a membrane protein during cell division in *E. coli*[103], increasing the thermostability of luciferase by cyclization[104] and creating artificial protein structures of different topologies[105].

Ubiquitin ligase-based ligation strategies

Ubiquitin (Ub) is a small protein of 76 amino acids, which is highly conserved from yeast to humans, that can be covalently linked to the lysine residues of target proteins to signal their degradation by the 26S proteasome or to modify their function or localization[106]. A set of three enzymes (E1, E2 and E3) catalyses the ligation of Ub to the ε-amino group of lysine (Fig. 6A) to generate a branched protein structure through an isopeptide bond. The Ub-ligating enzymes are referred to as E3s and operate in conjunction with an E1 Ub-activating enzyme and an E2 Ub-conjugating enzyme[107]. E1 enzymes require ATP as a cofactor to activate Ub and generate a thioester complex that is subsequently transferred to an E2 enzyme. Most commonly, the E2 enzyme then interacts with an E3 enzyme, enabling Ub to be transferred to the target protein substrate. This process can occur multiple times on a single substrate to eventually generate a polyubiquitylated product[108]. The E3 ligases are often multisubunit protein complexes, and they control the specificity of ubiquitylation by directing the PTM to specific substrate proteins.

The unique biochemical features of ubiquitylation have been used to turn Ub into a degradation tag for selectively downregulating cellular levels of target proteins. Such proteolysis targeting chimeras (PRO-TACs) have emerged as a powerful tool for studies

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of protein function as well as for drug development. However, as protein ubiquitylation plays essential roles in cellular signal transduction, especially protein degradation, E3 Ub ligases have not found use in protein semisynthesis or engineering owing to the risk of off-target activity that could perturb cellular function. In addition to Ub, there are multiple Ub-like (UBL) modifiers that are structurally similar to Ub and possess a conserved C-terminal glycine residue, which facilitates the condensation with lysine residues in substrate proteins[109]. Of the eight families of UBL modifiers that are conjugated to protein substrates[109], only the machinery installing SUMO (small UBL modifier) has served as a starting point for the development of protein ligation strategies so far[110,111].

The E2 conjugase of SUMO, Ubc9, has been used for the site-specific attachment of biochemical probes, one-pot dual labelling in combination with either SpyTag–SpyCatcher or the sortase variant $SrtA^{7M}$ and conjugation of wild-type Ub and ISG15 to recombinant target proteins[112]. This strategy, termed lysine acylation using conjugating enzymes (LACE), bypasses the need for E1 and E3 enzymes and enables isopeptide bond formation using just Ubc9. In this reaction, a short genetically encoded tag (LKSE or IKXE) is required in the substrate proteins or peptides to act as an acyl acceptor, whereas the acyl donors are thioesters possessing a C-terminal LRLRGG sequence that can be further activated by Ubc9 (Fig. 6Ba). In this way, lysine acylation using conjugating enzymes permits site-specific modification of internal lysine residues through the introduction of a small ‘ligation scar’. Additionally, Ubc9 can also use the non-activated SUMO3 as a substrate in combination with the E1 enzyme and ATP cofactor[112] (Fig. 6Bb). Because the loading of the thioester onto Ubc9 is rate-limiting, a recent method used an engineered E1 enzyme to speed up the formation of the Ubc9–thioester intermediate[113] (Fig. 6Bc). Importantly, this strategy also circumvents the need for synthetic thioesters, thus enabling conjugation of non-activated Ub (Fig. 6Bd).

Intein-based methods and applications

Although the field of transpeptidase-mediated and protein ligase-mediated protein ligation is rapidly evolving, these strategies have found limited use in complex biological systems. The specificity of these enzymes is dictated by recognition sequences that are retained as ‘ligation scars’ in the product, preventing protein ligation from being simultaneously highly specific and traceless.

Inteins (intervening proteins) can overcome these issues by relying on high-affinity, protein–protein interactions rather than primary sequence motifs for nearly traceless and chemoselective ligation. Additionally, intein-mediated ligation is irreversible and can thus reach high yields. More than 1,000 intein-encoding genes have now been identified in unicellular organisms from all three domains of life: Bacteria, Archaea and Eukarya[114,115]. Inteins self-excise from their host proteins, thereby ligating the flanking polypeptides (exteins) together through a native peptide bond (Fig. 7a). Inteins can therefore be viewed as single-turnover enzymes that break two amide bonds to form a single new bond[116]. Contiguous inteins are produced as single polypeptide chains and can control the reassembly, and hence function, of the naturally or artificially split proteins in which they are embedded. Importantly, inteins themselves can be split into two discrete fragments

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that facilitate protein *trans*-splicing (PTS) upon association (Fig. 7b). Such split inteins are powerful tools for the bioorthogonal production of semisynthetic, site-specifically modified proteins through fragment condensation. Here, we focus on major developments in intein technology that have moved the field towards control of the chemical composition of proteins in complex biological systems. Other parts of the intein toolbox have been excellently reviewed elsewhere[2,117,118].

The mechanism of intein-mediated protein ligation

Inteins catalyse protein splicing through a series of nucleophilic attacks that are supported by conformational changes in intein structure[119,120]. Although the precise splicing mechanism may vary, most inteins use a four-step reaction sequence that relies on a few conserved residues (Fig. 7a–c). First, the N-terminal nucleophile of the intein (Cys or Ser) attacks the carbonyl carbon of the adjacent amide bond, inducing a reversible NS/O acyl shift that generates a linear intermediate. To favour (thio)ester formation, inteins may distort the scissile bond and/or increase the nucleophilicity of the position 1 Cys or Ser[121–124]. Second, the N-extein is transferred to the C-extein through a trans(thio)esterification step mediated by the +1 nucleophile of the C-extein, resulting in a branched intermediate. The +1 nucleophile, which is the N-terminal residue of the C-extein, can be Cys, Ser or Thr (Fig. 7a). Third, the irreversible cyclization of a conserved, C-terminal Asn resolves the branched intermediate and excises the intein as a succinimide[125]. This is the rate-limiting step of protein splicing, which is accelerated by conformational changes associated with the formation of the branched intermediate[126–129]. Lastly, the exteins undergo a spontaneous S/O→N acyl shift to restore a native peptide bond and complete protein ligation. Along its reaction path, protein splicing competes with side reactions that lead to N-terminal or C-terminal cleavage through hydrolysis of the (thio)ester intermediates[130]. Inteins generally facilitate efficient protein splicing when situated within their native extein contexts, but the risk of side reactions increases when the coordination of the reaction steps is perturbed by, for example, splicing of non-native exteins[126,131]. Thus, as discussed subsequently, increasing the extein tolerance of inteins will decrease the level of side product formed during splicing.

PTS follows the same overall mechanism as canonical inteins but depends on initial association of the split-intein fragments Int^N and Int^C (Fig. 7b). The split fragments are largely disordered and can refold into a functional intein by a two-step, association–collapse sequence into an intertwined, stable complex[132–134]. Split inteins can either be naturally occurring or artificially split versions of contiguous inteins, but the natural split inteins often display superior properties and associate more strongly with K_D values in the low nanomolar range[132,135], whereas the K_D value of artificially split inteins can be in the low micromolar range[136]. Furthermore, the kinetics of split-intein association and the subsequent folding are fast and do not limit the rate of PTS[137]. Consequently, efficient PTS can be achieved using even low concentrations of the reactants.

The ever-expanding intein toolbox

Despite the great potential of intein-based protein modification, its application comes with a set of challenges, including poor intein fragment solubilities, slow splicing rates, strong

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extein dependencies and the size of split inteins making them practically inaccessible to solid-phase peptide synthesis (SPPS). Although these issues remain relevant, tremendous strides have been made to lower these barriers through protein engineering and continued characterization of new naturally occurring intein pairs.

The capacity to carry out efficient protein splicing is considered the only evolutionary pressure exerted on inteins. Because natural selection has only occurred within their native extein context, inteins are often highly specialized, and many contiguous inteins splice inefficiently with half-lives of several hours when tasked with ligating non-native extein sequences[120,126]. The first naturally split intein to be identified, the *Ssp* DnaE intein from *Synechocystis* sp. PCC6803, splices with a half-life of 75 min at 30 °C[138], which is considerably slower than many biological processes. However, nature has also developed ultrafast inteins, including the well-characterized *Npu* DnaE intein, identified from *Nostoc punctiforme* PCC73102, which splices with a half-life of 63 s at 37 °C[139]. Fast inteins are widespread in cyanobacteria[138] and have enabled the generation of a consensus fast (Cfa) intein that splices with an improved half-life of 20 s at 30 °C and display enhanced protein stability[140]. Additionally, even faster intein pairs have been identified from metagenomic data, including Gp41–1, which remains the fastest intein to date with a half-life of ~5 s at 37 °C[141]. Examination of data from a saline lake in Antarctica identified the AceL–TerL intein, which splices efficiently at 8 °C ($t_{1/2}$ ~ 7 min)[142], paving the way for efficient protein labelling at low temperatures.

Traceless PTS requires inteins to accommodate the sequences of any protein of interest (POI) as exteins. However, inteins are not inherently promiscuous, as the +1 nucleophile is essential for the initial (thio) esterification step that generates the linear (thio)ester intermediate. Furthermore, the splicing efficiency depends on the nature of the C-terminal and N-terminal residues in the N-extein and C-extein, respectively[127,130,143], and it has often been necessary to insert three to five residues of the native extein sequences into target proteins to promote splicing. In particular, splicing is highly sensitive to the identity of the residues at the +2 and +3 positions. Owing to this extein dependency, protein engineering has been used to alter the extein preference of the chimeric *Npu* $DnaE^N$ + *Ssp* $DnaE^C$ pair[144], *Ssp* DnaB (yielding the M86 intein)[145] and the *Pho* RadA intein[146]. Furthermore, the splicing capacity of the widely used DnaE inteins depends strongly on the presence of a large hydrophobic +2 residue[147–149], a requirement that can be alleviated by introducing three mutations that increase the promiscuity of both *Npu* DnaE and Cfa[150]. Finally, different intein pairs will naturally have varying extein preferences, as they have adapted to different host proteins[146,151], suggesting that the continuous characterization of new inteins will further increase the extein tolerance of the intein toolbox at large.

A key application of PTS is the generation of semisynthetic proteins by fusing one split intein to a truncated protein and ligating synthetic cargo to the other (Fig. 8a). Hence, the size of the intein–synthetic cargo fusion should ideally be compatible with standard SPPS. Most naturally split inteins are split at a canonical site that produces Int^N and Int^C fragments of approximately 100 and 35 residues, respectively[2]. This means that the Int^N is well beyond the reach of SPPS, whereas Int^C is near the feasible limit of SPPS, leaving little room for the addition of synthetic cargo. Thus, it has been a priority to

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search for novel split sites that reduce intein fragment size while preserving efficient PTS kinetics. The feasibility of N-terminal modification, in which Int^N carries the synthetic cargo (Fig. 8a), has been increased by identifying atypically split inteins from metagenomic data[142,152,153]. The Int^N of these inteins is significantly shorter, with the shortest being only 15 residues[153]. Interestingly, a consensus atypical split intein (Cat) was found to have a high C-extein tolerance[134], highlighting that the location of the split site may be an underappreciated strategy for relaxing extein dependency. The Int^N size has been further reduced to 11–12 residues using artificial split sites[154,155]; however, this does compromise splicing efficiency[156]. Moreover, artificially split-intein pairs often suffer from decreased binding affinities, requiring intein fragments to be fused to a pair of high-affinity interacting modules to achieve efficient PTS[136,157]. To support C-terminal modification, Int^C fragments have been shortened to only five and six residues without a significant loss of splicing efficiency[156,158]. Recently, Thompson et al.[159] developed a one-pot strategy termed transpeptidase-assisted intein ligation (TAIL) for making semisynthetic proteins in which SrtA-mediated ligation is combined with PTS (Fig. 8b). By using SrtA for assembling an active split intein, TAIL reduces the size of the synthetic intein to seven residues and is applicable to N-terminal and C-terminal protein modification.

Conditional protein splicing

The spontaneous nature of protein splicing makes naturally occurring inteins unattractive for examining biological processes that require strict temporal and/or spatial control. Therefore, several conditional protein splicing (CPS) techniques have been developed, which allow control of intein activity by extrinsic cues, that can be grouped based on the nature of the trigger (Fig. 9).

CPS can be induced using small molecules that promote binding, and hence splicing, of weakly associating split inteins. The first example of such proximity-induced CPS was based on fusing Int^N and Int^C of the *Sce* VMA intein from *Saccharomyces cerevisiae* to the 12-kDa FK506-binding protein (FKBP12) and FKBP12-rapamycin binding domain, respectively[160] (Fig. 9Aa). The resulting rapamycin-dependent system has been used for in vitro kinase activation[161], three-piece ligation[148], in cellulo splicing[162–164] and CPS in live *Drosophila melanogaster*[165]. Additionally, rapamycin can also act as an off switch in a system that relies on a homodimerizing mutant of FKBP12 (ref. [166]) (Fig. 9Ab). Contiguous intein-based CPS can similarly be achieved by coupling the conformational changes of hormone receptors upon ligand binding to splicing. Insertion of the ligand binding domain of the human oestrogen receptor into the *Mtu* RecA intein enables 4-hydroxy tamoxifen to trigger splicing in yeast[167], and this system has been further optimized for CPS in human cells[168–171] (Fig. 9Ac). Noticeably, this intein has been shown to improve the genome-editing specificity of Cas9 by restricting Cas9 activity to the window of 4-HT treatment, thereby minimizing off-target cleavage[171]. Moreover, conceptually similar CPS systems that rely on the thyroid hormone receptor have been developed, enabling oestrogen to act as both an on switch and an off switch[172].

PTS can also be controlled using photosensitive strategies. In the most straightforward of such strategies, PTS is inhibited by a photocaged nucleophile analogue at position

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1 (Fig. 9Ba), making splicing contingent on light-induced deprotection[173,174]. Similarly, splicing can be obstructed by incorporation of *ortho*-nitrobenzyl-tyrosine at the position of a conserved Phe residue in the Gp41–1 and M86 inteins[175,176] (Fig. 9Bb). Backbone-modifying strategies also allow photoinducible PTS, as two photocaged glycine residues can prevent an Int^C fragment from attaining its splicing-competent conformation before protecting group removal[177] (Fig. 9Bc). Similarly, a kink can be introduced in the intein backbone using an O-acyl linkage, and PTS is induced by deprotection of a photocleavable α-amino group and a subsequent O→N acyl migration that restores the native intein structure[178,179] (Fig. 9Bd). By substituting the α-amino-protecting group to a protease cleavage site, this strategy also enables CPS to be triggered by proteolysis[178] (Fig. 9Ca). Protease-regulated splicing can also be realized by fusing each split intein to a segment of their cognate intein partner using a protease cleavage site-containing linker. The resulting 'caged', inactive inteins are only able to associate and splice, following their proteolytic liberation[180] (Fig. 9Cb). These auto-inhibited inteins have also been combined with the proximity-induced FKBP12–FRP system to allow rapamycin-triggered splicing[181] (Fig. 9Ac).

Light-triggered CPS can also rely on conformational changes in protein domains rather than removal of photolabile groups (Fig. 9D). The light, oxygen or voltage domain 2 of a photoreceptor from *Avena sativa* undergoes a flavin-dependent conformational change upon illumination, which has been used to generate *trans* CPS systems using split inteins (Fig. 9Da) as well as *cis*-CPS systems in mammalian cells[182,183]. Furthermore, a photosensitive, proximity-induced CPS platform has been developed by fusing *Sce* VMA split fragments to phytochrome B and transcription factor phytochrome-interacting factor 3 from *Arabidopsis thaliana*. When the phycocyanobilin chromophore is available, these proteins associate upon illumination at 660 nm, whereas 750-nm light leads to their dissociation, thereby enabling light to act as an on–off switch of PTS activity[184] (Fig. 9Db).

In vivo applications of protein splicing

Intein-mediated protein ligation has found use for the in vitro production of bioconjugates[117], isotopic labelling of protein segments for NMR studies[151] and bypassing the packaging constraints of adeno-associated virus-based delivery[185]. In addition, split inteins are powerful tools for N–C cyclization of polypeptides through intramolecular splicing[186], a reaction that is used in split-intein circular ligation of peptides and proteins (SICLOPPS) to make genetically encoded libraries of cyclic polypeptides[6,187]. Although these applications constitute key aspects of intein technology, the ability to tailor protein composition in complex microenvironments by generating semisynthetic proteins with temporal and/or spatial control still represents a key goal in protein ligation. Here, we focus on selected examples that have advanced the field of intein-mediated protein ligation in cellular contexts. However, more examples exist and have been reviewed elsewhere[2,3,117].

To modify proteins in cells, one split intein carrying a synthetic cargo is delivered to cells expressing a POI fused to the other split-intein fragment (Fig. 10Aa). Thus, PTS will result in a full-length protein containing the modification (or modifications) defined by the delivered, semisynthetic cargo. Using this general strategy, in cellulo protein semi-

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synthesis has been used to install protein tags[188], biotin[150,159], fluorophores[157,159,189–191] and quantum dots[192] into various proteins, allowing them to be probed within their native cellular environment.

The extracellular part of membrane proteins can also be modified using PTS[189,193] (Fig. 10a,b). Cysteine-containing inteins are redox-sensitive, which complicates PTS in oxidizing environments, such as the extracellular space. Therefore, an artificially split, cysteinefree intein has been developed by engineering a split intein from an *Aeromonas* bacteriophage[136]. One drawback of PTS-based modification is that it is limited to modification of protein termini; to modify central protein segments, two (or more) orthogonal intein pairs are required to perform tandem PTS[148,194,195] (Fig. 8a). For this, a central, synthetic segment of a POI is fused to an N-terminal Int^{C}_{1} and a C-terminal Int^{N}_{2}, thus enabling reconstitution of the full-length protein through PTS with the N-terminal part of the POI fused to Int^{N}_{1} and the C-terminal protein segment fused to Int^{C}_{2}. Tandem PTS was recently extended to eukaryotic cells by modifying central residues in membrane proteins in *Xenopus laevis* oocytes and green fluorescent protein in HEK293 cells[196]. Noticeably, a library of 15 mutually orthogonal intein pairs has recently been established, thereby expanding the selection of inteins that are suitable for tandem PTS[197].

Intein-based protein semisynthesis in cells has mostly been leveraged for proof-of-principle studies. The field of histone PTMs is an exception to this, as PTS is finding increased use for tailoring the chemical composition of chromatin. Chromatin, which is the physiologically relevant form of DNA in eukaryotes, is a complex comprising histone proteins bound to genomic DNA, and the compaction state of chromatin is key for the regulation of all DNA-templated events, including replication and transcription. Histones can be extensively modified, and the combined pattern of histone PTMs, the so-called histone code[198], is read in a way that directly impacts chromatin compaction and hence cellular function. To uncover a ‘rosetta stone’ for the histone code, it is thus crucial to be able to control histone composition in cells to probe the role of individual PTMs and combinations thereof.

In the first example of split-intein-based, chromatin modification in the nucleus, Ub was installed at position K120 in histone H2b, following the general strategy outlined in Fig. 10a. A C-terminally truncated version of H2B fused to Int^{N} (H2B (1–116)–Int^{N}) was first expressed in cells. Nuclei were isolated from these cells and incubated with the cognate Int^{C} fused to the synthetic, missing piece of H2B carrying the PTM (Int^{C}–H2B(117–125)–K120Ub). Upon protein splicing, full-length H2B carrying K120Ub was generated, which showed that the site-specific installation of this mark promotes H3K79 methylation[199,200] (Fig. 10c). This proved that ligation-based protein semisynthesis can be used to dissect the relationship between histone chromatin composition and function, and a similar strategy has also been adopted for modifying the N terminus of histone H3 (refs. [150,201]). Importantly, intein-driven chromatin modification can be extended from in nucleo to living cells by conjugating the semisynthetic cargo to a cell-penetrating peptide[199] or by delivering it using electroporation[202] (Fig. 10a,c).

One drawback of these studies is that chromatin is modified across the entire genome, whereas endogenous chromatin is organized into spatially discrete regions. In a step towards

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achieving genome specificity, inteins have been used to modify dead Cas9 (dCas9) in the cell medium with synthetic ligands that recruit epigenetic modifiers. Subsequently, cationic lipid-mediated transfection was used to deliver the semisynthetic dCas9 into the cells, thereby recruiting the target enzymes to the DNA sequences of interest[203] (Fig. 10d). In addition, the specificity of dCas9 has been combined with a rapamycin-induced CPS platform. The histone acetyltransferase p300 was split at a site that resulted in two non-functional pieces that were each fused to one member of a split-intein pair. Furthermore, the N-terminal p300 fragment was fused to dCas9 to direct the construct to the loci of interest. By adding rapamycin to cells expressing these constructs, it was possible to reconstitute functional p300 to drive transcription at the reporter sequence targeted by dCas9 (ref. [181]) (Fig. 10e). Going forward, it is interesting to see whether CPS-based, genome-targeted strategies in combination with in situ protein semisynthesis will enable site-specific histone modification in a temporally and spatially controlled reaction.

Summary and outlook

Nature has provided multiple routes for enzymatically forming amide bonds between two distinct polypeptides. The past decades have seen impressive advances towards repurposing protein ligation strategies for unprecedented control over protein composition. At the forefront of these efforts have been the continued discovery of novel enzymes as well as the improvement of existing enzymes through protein engineering. With this expanded toolbox at hand, many proteins are now amenable substrates for protein ligation. In this Review, we summarize the mechanisms of various enzyme-mediated protein ligation technologies and use this knowledge to highlight the advantages and disadvantages that come with each approach. In particular, there is a general juxtaposition between the need for a chemoselective and site-selective reaction and the pursuit of traceless ligation. This is exemplified by butelase-1 facilitating efficient and promiscuous ligation that leaves as little as a single residue behind in the ligated product, whereas the more selective SrtA-mediated ligation creates a pentapeptide ‘scar’. A similar choice between selectivity and tracelessness is seen for the isopeptide-generating enzymes, as TGM2-based modification is highly promiscuous and traceless, whereas the molecular superglues are highly specific but require the POI to be fused to large tags. Moreover, additional features of the different ligation strategies, including catalytic efficiency, enzyme availability and reaction conditions, should all be taken into consideration before committing to a protein ligation strategy.

The manipulation of protein composition in cellular environments remains a major goal in protein ligation, with the applicability of many enzymes restricted by their lack of chemoselectivity and/or need for high reactant concentrations or chemically modified reactants to drive reversible reactions towards higher yields. Moving forward, these issues could partly be mitigated by developing systems in which protein ligation is temporally and spatially controlled by exogenous triggers, thereby tightly regulating an otherwise promiscuous protein modification reaction. Currently, only SrtA, inteins and molecular superglues have been applied in cells, whereas intein-based strategies remain the only feasible route for general in situ protein semisynthesis, which is still considered a technically demanding feat. One obstacle that reduces the efficiency of in vivo ligations is the cell membrane itself, as delivery of the synthetic cargo is a considerable challenge. Fortunately,

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much progress is being made in cellular delivery techniques[204], including the use of nanoparticle technology[205]. As these delivery platforms become more routine, we expect the entry barrier for in vivo protein ligation to become lower. Considering the recent maturation of intein-based techniques, the field seems primed for a shift from method development towards probing the role of protein composition and modification in complex biological processes. Nevertheless, there is still room for continued methodological development, as the generation of semisynthetic integral membrane proteins with high yields remains out of reach. It is similarly unfeasible to use inteins to install multiple modifications within a protein segment that are located too far apart in the primary sequence to be covered by a single synthetic piece, highlighting that no one ligation platform is likely to be able to solve all protein modification challenges.

The emergence of novel platforms that harness the benefits of two (or more) protein manipulation techniques thus constitutes an intriguing development in the field. The recently developed TAIL strategy[159] combines the advantageous features of two different ligation strategies, by exploiting the short, and hence synthetically accessible, recognition sequence of SrtA and the irreversibility of intein-based PTS. Similarly, the ability of genetic code expansion to site specifically install unnatural amino acids, which allow for bioorthogonal chemical reactions, at any position along the entire protein sequence has been combined with SrtA-mediated protein ligation to modify proteins with Ub and SUMO[30]. This method was recently expanded further to use *Oa*AEP1-based ligation to modify internal UAAs introduced by genetic code expansion with biophysical probes and Ub[206]. Thus, continued innovation that allows the best of two (or more) protein engineering platforms to be merged is likely to improve our control of protein composition in the future. Although protein ligation is still not a straightforward task, there is now a diverse set of well-established platforms available. This paves the way for researchers to aptly transcend the chemical space afforded to proteins by the genetic code in pursuit of semisynthetic proteins as well as novel protein and peptide conjugates.

Acknowledgements

The authors thank M. Luo for critical reading of the manuscript. Work in the David Laboratory is supported by the Josie Robertson Foundation, the Pershing Square Sohn Cancer Research Alliance, the NIH (CCSG core grant P30 CA008748, MSK SPORE P50 CA192937, R21 DA044767 and R35 GM138386), the Parker Institute for Cancer Immunotherapy and the Anna Fuller Trust. In addition, the David Laboratory is supported by the William H. Goodwin and Alice Goodwin Commonwealth Foundation for Cancer Research and by the Center for Experimental Therapeutics at MSKCC. R.P. is supported by the Novo Nordisk Foundation grant NNF20OC0061064. The Zheng laboratory is supported by OSUCCC startup funds.

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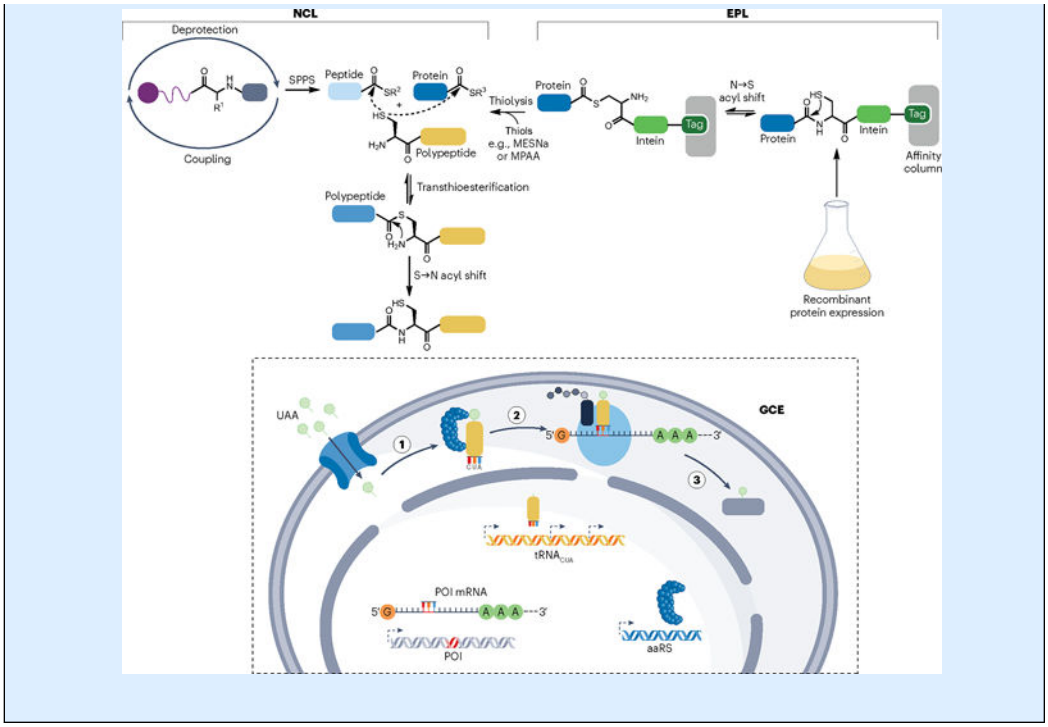
Box 1

Alternative strategies for generating semisynthetic polypeptides

Native chemical ligation (NCL) joins unprotected polypeptides through condensation of a C-terminal thioester and an N-terminal cysteine by a mechanism that is reminiscent of intein-based ligation[207]. NCL is performed under aqueous conditions at neutral pH and has been instrumental for incorporating synthetic moieties into polypeptides. However, to routinely generate larger, semisynthetic proteins, at least one fragment must first be recombinantly produced. Although there are several routes for producing recombinant proteins with N-terminal cysteines[2], facile production of C-terminal thioesters requires assistance from nature. By fusing a protein of interest (POI) to an intein variant that arrests splicing at the thioester intermediate level[208], an NCL-suitable α-thioester can be released through thiolysis. The expressed protein ligation (EPL) strategy overcomes the size limitation for C-terminal modification and has been widely used since its conception[209]. Importantly, the use of multiple sequential ligation steps, including both NCL-based and EPL-based reactions, further increases the size of the semisynthetic product and allows internal residues to be modified[210–213]. Nevertheless, the applicability of NCL and EPL is still restricted by the fact that millimolar reactant concentrations are needed to drive the rate-limiting transthioesterification step to promote spontaneous amide bond formation over thioester hydrolysis. This prevents these ligation strategies from being used in cells where there are high concentrations of thiols.

Synthetic moieties can also be introduced site specifically into proteins by forcing the translational machinery to accommodate unnatural amino acids (UAAs) through genetic code expansion (GCE). This strategy classically overwrites the natural decoding of an mRNA triplet, often the amber codon, by ectopic expression of an orthogonal aminoacyl-tRNA synthetase and a modified tRNA that recognizes the specific triplet within the mRNA of interest[214]. GCE has the advantage that it can readily target the entire protein sequence, whereas ligation-based strategies require multiple ligations to extend beyond the termini. Conversely, although ligation of synthetic peptides unlocks the door to the entire chemical space available through solid-phase peptide synthesis, GCE requires an aminoacyl-tRNA synthetase and tRNA pair for each UAA. Furthermore, it remains challenging to install multiple UAAs by GCE, despite novel approaches relying on quadruplet codons[215] or even entirely synthetic genomes[216]. Thus, NCL, EPL and GCE are invaluable platforms for protein manipulation that are constantly undergoing further developments and aptly complement enzyme-based strategies. MESNa, sodium 2-mercaptoethane sulfonate; MPAA, 4-mercaptophenylacetic acid; aaRS, aminoacyl-tRNA synthetase; SPPS, solid-phase peptide synthesis.



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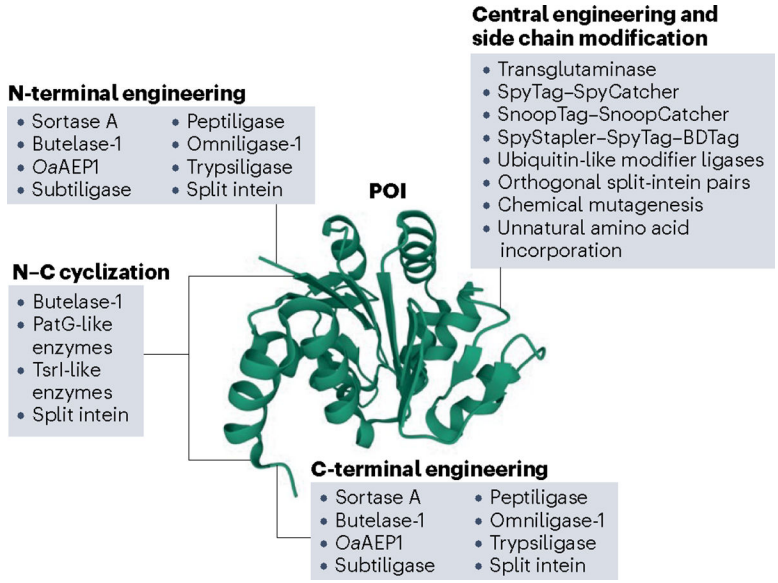

Fig. 1 |. An overview of the enzymatic toolbox for protein ligation-mediated polypeptide engineering covered in this Review.

There are multiple enzyme-based platforms available for modifying a protein of interest (POI). As all ligation strategies are not created equal, they have distinct advantages and disadvantages. Different enzymes allow access to different segments of the POI. These enzymes also differ in the mechanism by which they catalyse amide bond formation, as N–C cyclization requires intramolecular ligation, whereas N- and C-terminal engineering is achieved by intermolecular reactions. Other platforms modify side chains by generating isopeptide bonds. Two fundamental questions for any protein ligation endeavour are thus, where in the POI the modifications will be introduced and what the nature of the resulting bond should be. Human DJ-1 is shown as a model protein, Protein Data Bank (PDB): 1PDV.

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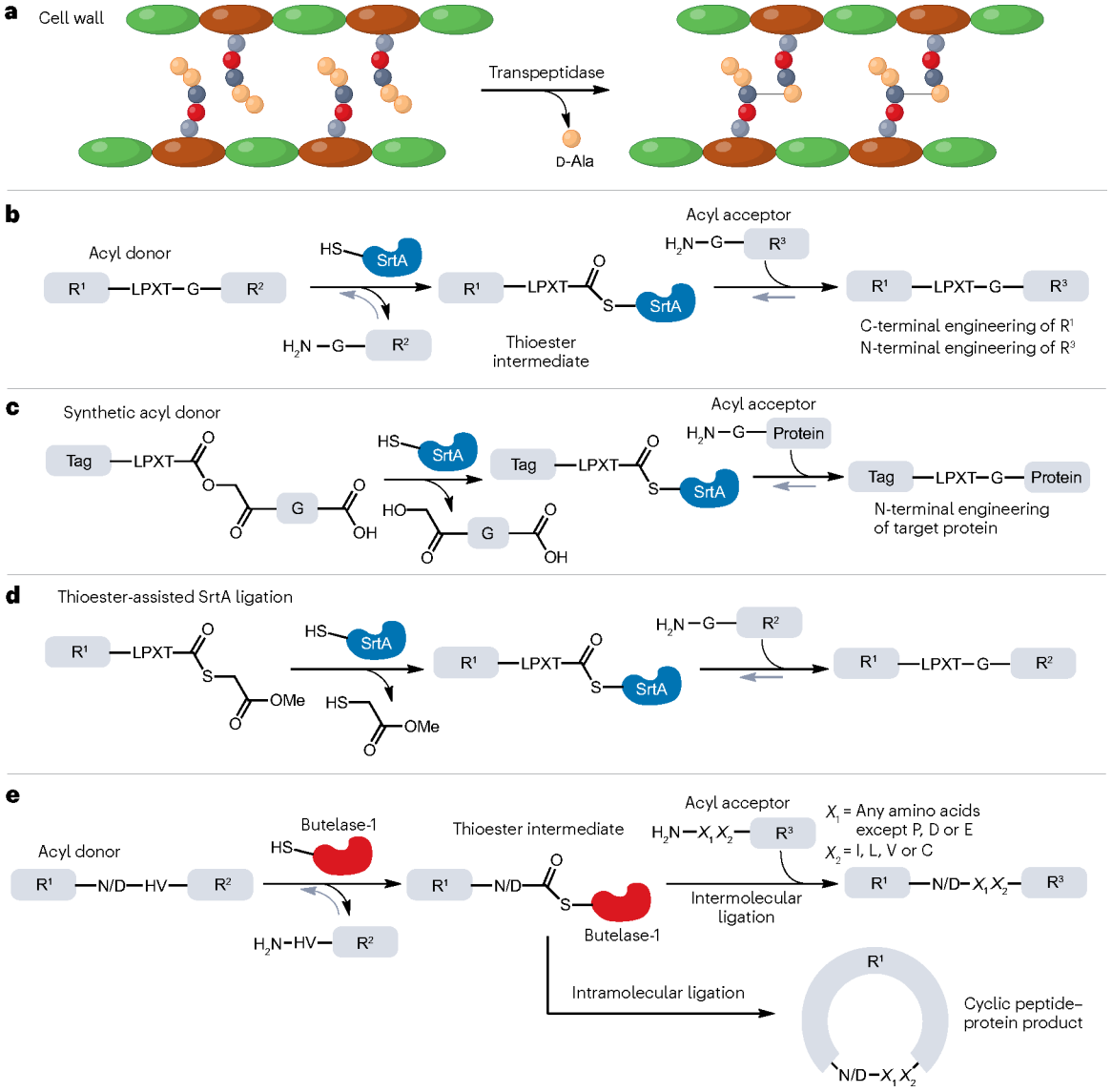

Fig. 2 |. Biochemistry of protein and peptide ligations mediated by sortase and butelase-1.

a, The biological function of transpeptidase-mediated peptide ligation is to covalently modify the cell wall of bacteria and plants. Shown here, the crosslinking of peptidoglycan by the D-alanyl–D-alanine-transpeptidase. **b**, Biochemical mechanism of sortase A (SrtA)-catalysed reversible ligation and its application in protein N- and C-terminal engineering (sortagging). The LPXT-G sequence is recognized and cleaved by the enzyme SrtA. R^1 and R^2 are peptide or protein sequences. **c**, Irreversible protein–peptide ligations mediated by SrtA with rationally designed synthetic or semisynthetic acyl donors, where the 'G' residue in the recognition sequence 'LPXT-G' is replaced by synthetic moieties. 'Tag' could be synthetic fluorescent molecules or specific peptide sequences for imaging, enrichment and tracking of the products after ligation. R^1 and R^2 are peptide or protein sequences. **d**, As in panel **c**, irreversible SrtA-based ligation can be achieved using thioesters to modify the N-terminal part of the protein of interest. **e**, Biochemistry and application of protein ligation by butelase-1, which proceeds through formation of a key thioester intermediate.

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The N/D–HV sequence can be recognized and cleaved by butelase-1 to generate a thioester intermediate, which can be resolved to yield a linear or cyclic product. R^1, R^2 and R^3 are peptide or protein sequences. X_1 and X_2 are specific amino acid residues that can be recognized by butelase-1 in this ligation reaction: X_1 can be any amino acid except P, D or E; X_2 can be I, L, V or C.

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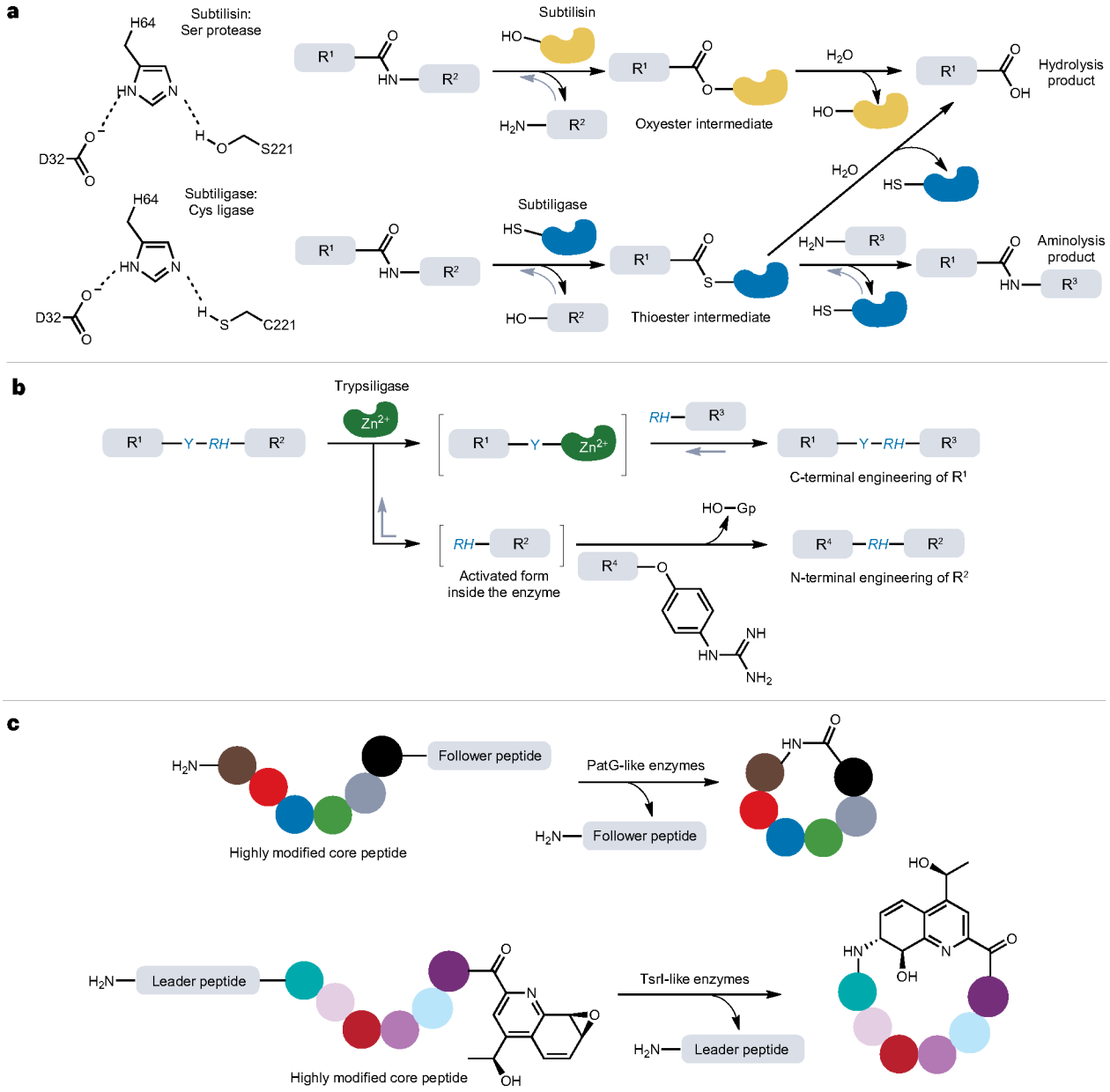

Fig. 3 |. Biochemistry of protease-based protein and peptide ligations.

a, Biochemical mechanisms of subtilisin and subtiligase-mediated protein and peptide ligation. The catalytic triad of subtilisin is Asp–His–Ser (D32–H64–S221) and that of subtiligases is Asp–His–Cys (D32–H64–C221). For subtiligase, aminolysis is favoured over hydrolysis, thereby enabling the enzyme to support protein ligation. **b**, Trypsiligase-mediated protein ligation and its applications. The Y–*RH* sequence can be recognized and cleaved by trypsiligase. Guanidinophenyl (Gp) is a good leaving group that activates the C terminus of acyl donor, R^4. R^1–R^4 denotes peptide or protein sequences throughout (**a**,**b**). **c**, Macrolactonization catalysed by protease-derived macrocyclases, PatG and TsrI, involved in microbial biosynthetic pathways. These enzymes catalyse not only the removal of signal peptides (such as leader or follower peptides) but also the intramolecular peptide

bond formation between the N and C termini, as in the case of peptide macrolactonization. The coloured circles represent individual amino acid residues after post-translational modifications.

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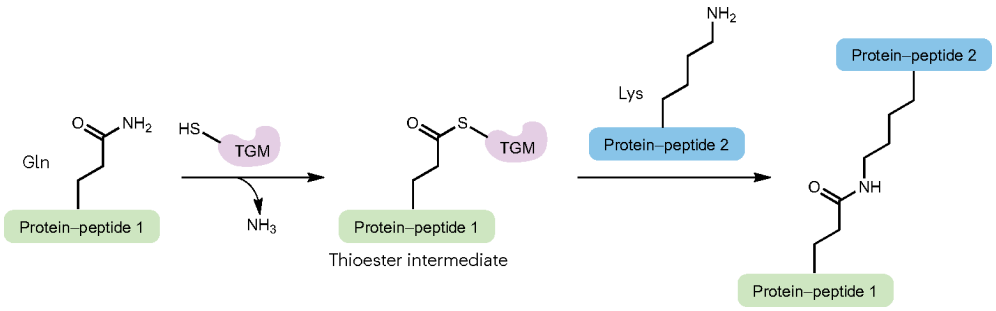

Fig. 4 |. Biochemistry of the isopeptide bond formation mediated by transglutaminases.
The isopeptide bond is formed between Gln and Lys residues of the protein or peptide substrates. A Cys in transglutaminases (TGMs) is the catalytic residue for this reaction, which proceeds through a reactive thioester intermediate. Ammonia is the by-product of this enzymatic ligation.

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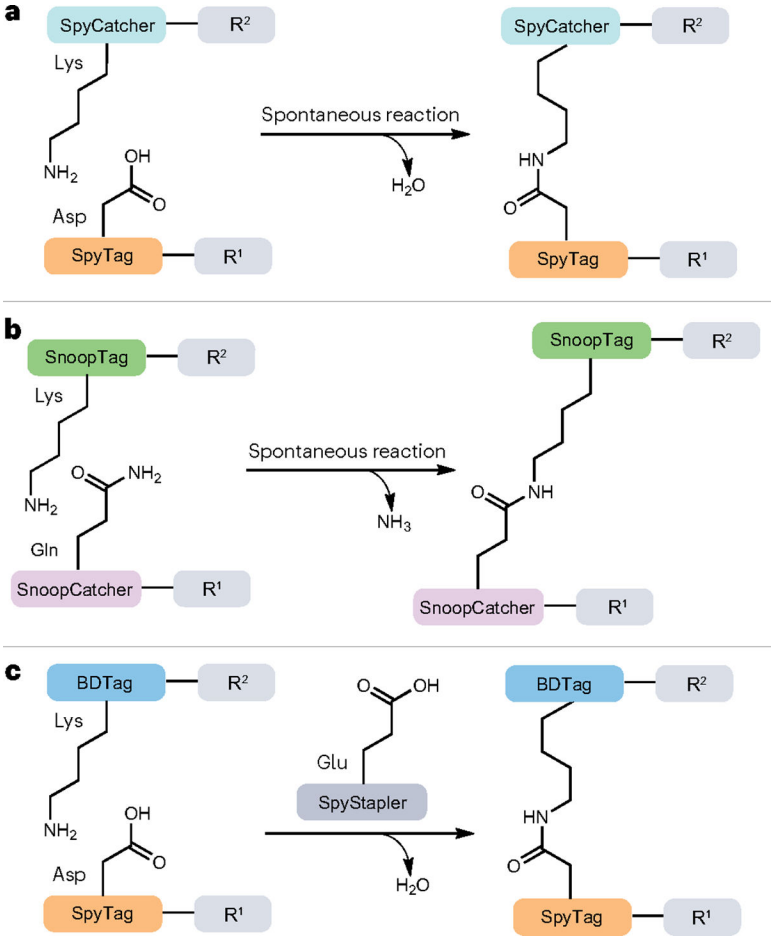

Fig. 5 |. Molecular superglue-mediated isopeptide bond formation.

a–**c**, Spontaneous ligation reactions mediated by SpyCatcher–SpyTag (**a**), SnoopCatcher–SnoopTag (**b**) and SpyStapler–SpyTag–BDTag (**c**) (also known as ‘peptide–peptide staplers’) systems. In the SpyStapler–SpyTag–BDTag system, the second immunoglobulin-like collagen adhesin domain (CnaB2) is divided into three components by further splitting SpyCatcher to yield an intrinsically disordered protein, SpyStapler, which can form a stably folded structure in the presence of SpyTag and BDTag to facilitate the formation of an isopeptide bond between them. R^1 and R^2 stand for peptide–protein sequences. Water (**a**,**c**) and ammonia (**b**) are the by-products of this type of enzymatic ligation.

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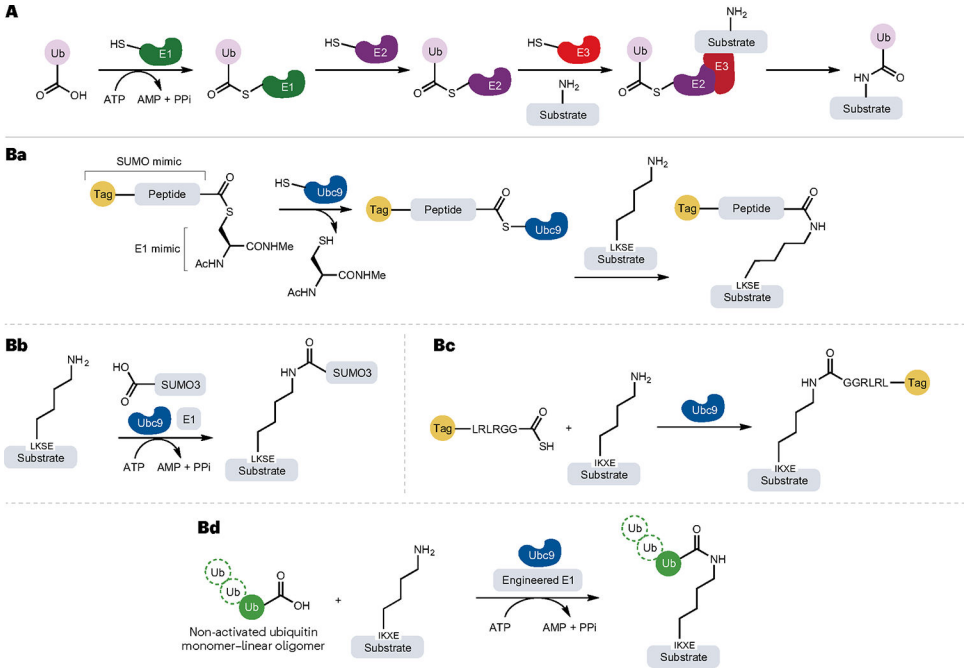

Fig. 6 |. Ubiquitin and ubiquitin-like modifier ligase-mediated isopeptide bond formation and its applications.

A, General mechanism of protein ubiquitylation (isopeptide bond formation) catalysed by E1, E2 and E3 enzymes. **Ba,Bc,** Site-specific modification and ubiquitylation of target proteins containing synthetic or genetically encoded tags by the LACE platform (lysine acylation using conjugating enzymes). A minimal genetically encoded tag (LKSE or IKXE) in the substrate proteins or peptides acts as acyl acceptor, whereas a peptide sequence (LRLRGG) mimicking the C terminus of ubiquitin (Ub) functions as an acyl donor when functionalized as a thioester. Thus, the thioester acyl donor mimics the structure of the small Ub-like modifier (SUMO)–E1 complex. Using thioesters and Ub-conjugating enzyme E2 (Ubc9), proteins can be modified with chemical probes and even small proteins. **Bb**, Non-activated SUMO3 can be site specifically installed at the LACE tag by the combined action of Ubc9 and an E1 enzyme in the presence of the cofactor ATP, which is hydrolysed to AMP and two molecules of phosphate (PPi). **Bd**, The development of a chimeric E1 enzyme enables conjugation of non-activated Ub. Ac, acetyl group; Me, methyl group.

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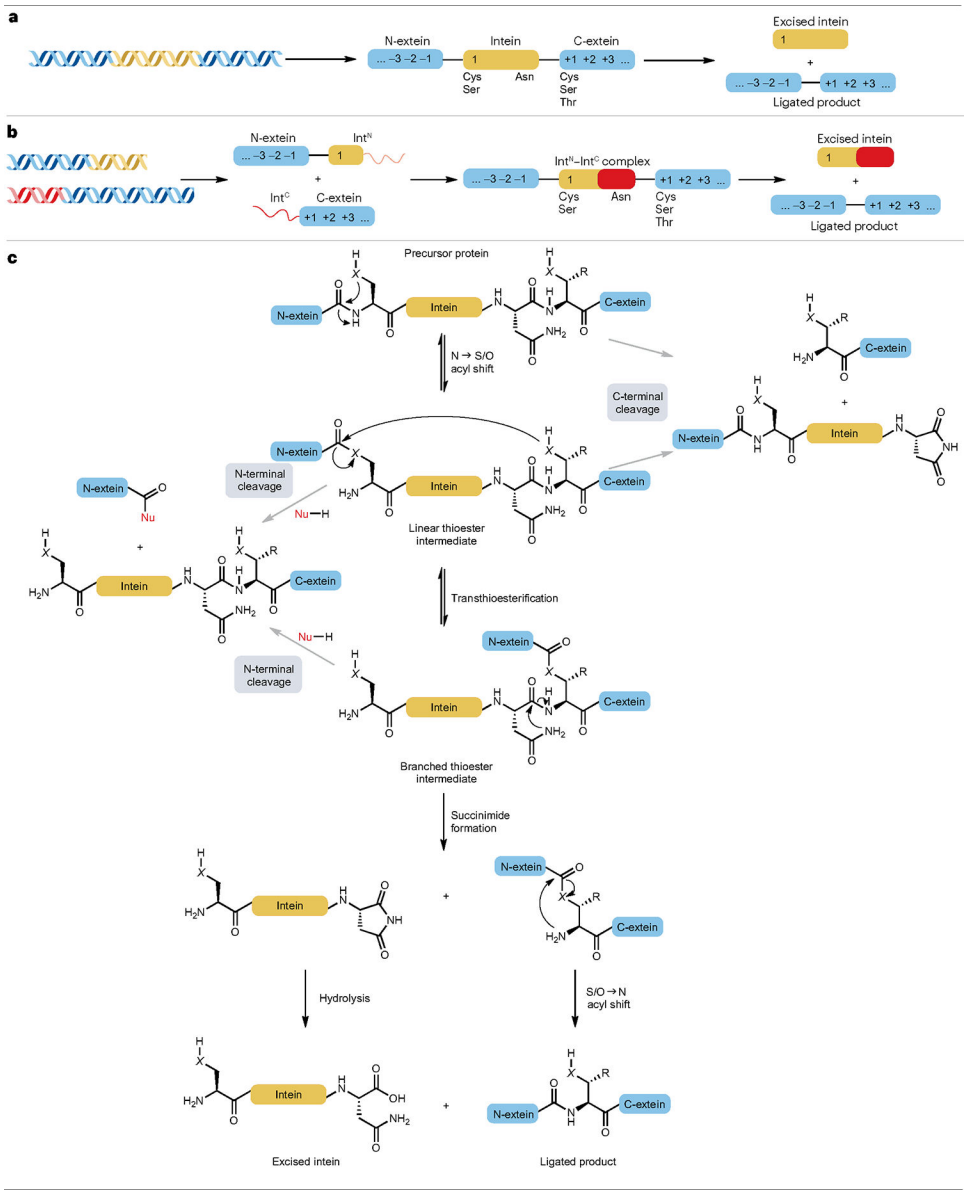

Fig. 7 |. Mechanism of intein-mediated protein ligation.

a, Contiguous inteins are produced as single proteins flanked by two extein sequences. The numbering highlights the nomenclature used to denote key amino acid positions in inteins and exteins together with the type of amino acids found at the indicated positions. **b**, Split inteins are generated as discrete polypeptides that associate to facilitate ligation of the flanking extein sequences. Split inteins contain disordered regions that fold into a splicing-competent complex upon association to carry out protein *trans*-splicing. The canonical intein split sites yield larger N-terminal split-intein fragments (Int^N) and smaller C-terminal split-intein fragments (Int^C). **c**, Mechanism of intein-based splicing. Efficient splicing competes with premature cleavage reactions in which the thioester intermediates are intercepted by a nucleophile (Nu-H, usually H_2O). *X* designates oxygen for inteins with a Ser residue at position 1 or sulfur for inteins that use Cys as their nucleophile.

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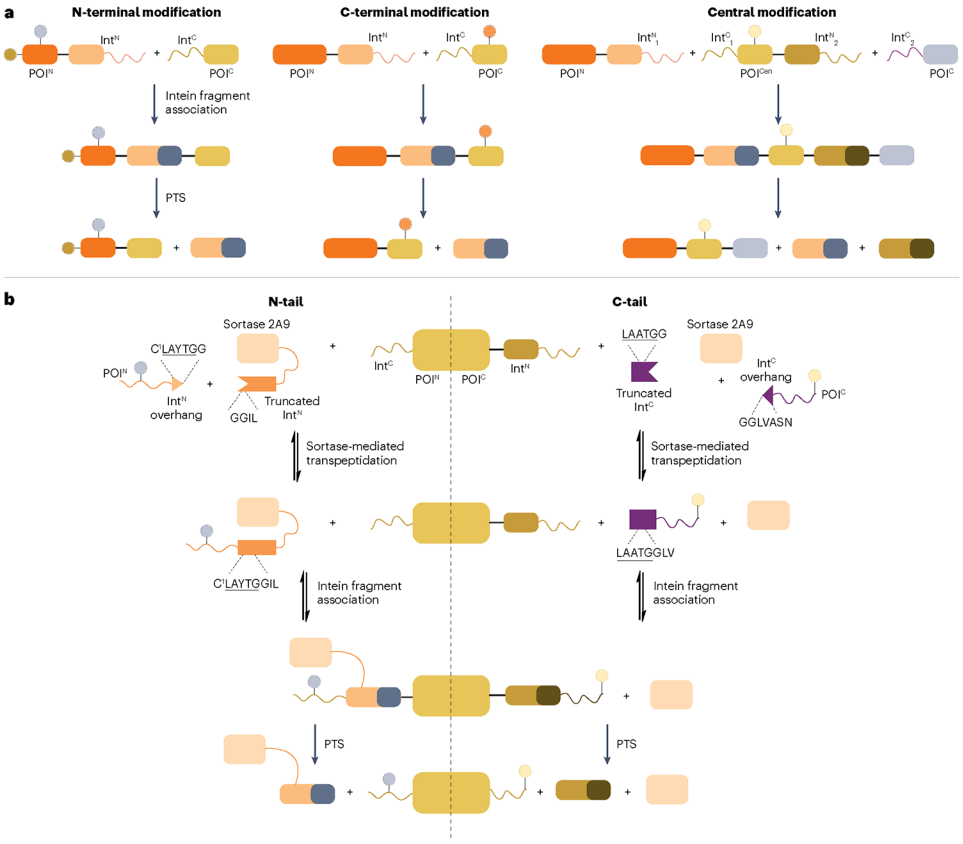

Fig. 8 |. Split intein-based strategies for generating semisynthetic proteins.

a, Proteins can be site-specifically modified at their termini by splitting them into two separate pieces, each fused to a split-intein fragment. For N-terminal modification, a short, synthetic N-terminal segment of the protein of interest (POI) (POI^N) is fused to the N-terminal split intein fragment (Int^N), whereas the recombinantly expressed C-terminal POI fragment (POI^C) is fused to the C-terminal split-intein fragment (Int^C). Upon association and protein *trans*-splicing (PTS), an N-terminally modified POI is produced. Similarly, C-terminal modification is performed by making the Int^C–POI^C complex synthetically. To modify a central segment of a POI (POI^{Cen}), orthogonal intein pairs are used in a tandem protein splicing scheme in which the POI^{Cen} is synthetic and fused to intein fragments at both termini. Int^N_1 and Int^C_1 as well as Int^N_2 and Int^C_2 represent two pairs of orthogonal split inteins. **b**, Transpeptidase-assisted intein ligation (TAIL) enables N-terminal (N-tail) and C-terminal (C-tail) modification of proteins by combining sortase- and intein-mediated protein ligation. Split inteins are further split into small overhangs (Int^N overhang and Int^C overhang) containing sortase recognition sequences LAYTG or LAATG and truncated, inactive inteins. The reversible, sortase-mediated transpeptidation step generates active split inteins carrying synthetic cargo, which associate with their split-intein partner for protein semisynthesis through irreversible PTS. Note that sortase and the truncated Int^N are fused in N-tail to increase the reaction rate and thereby suppress premature cleavage of Int^C–POI^N complex.

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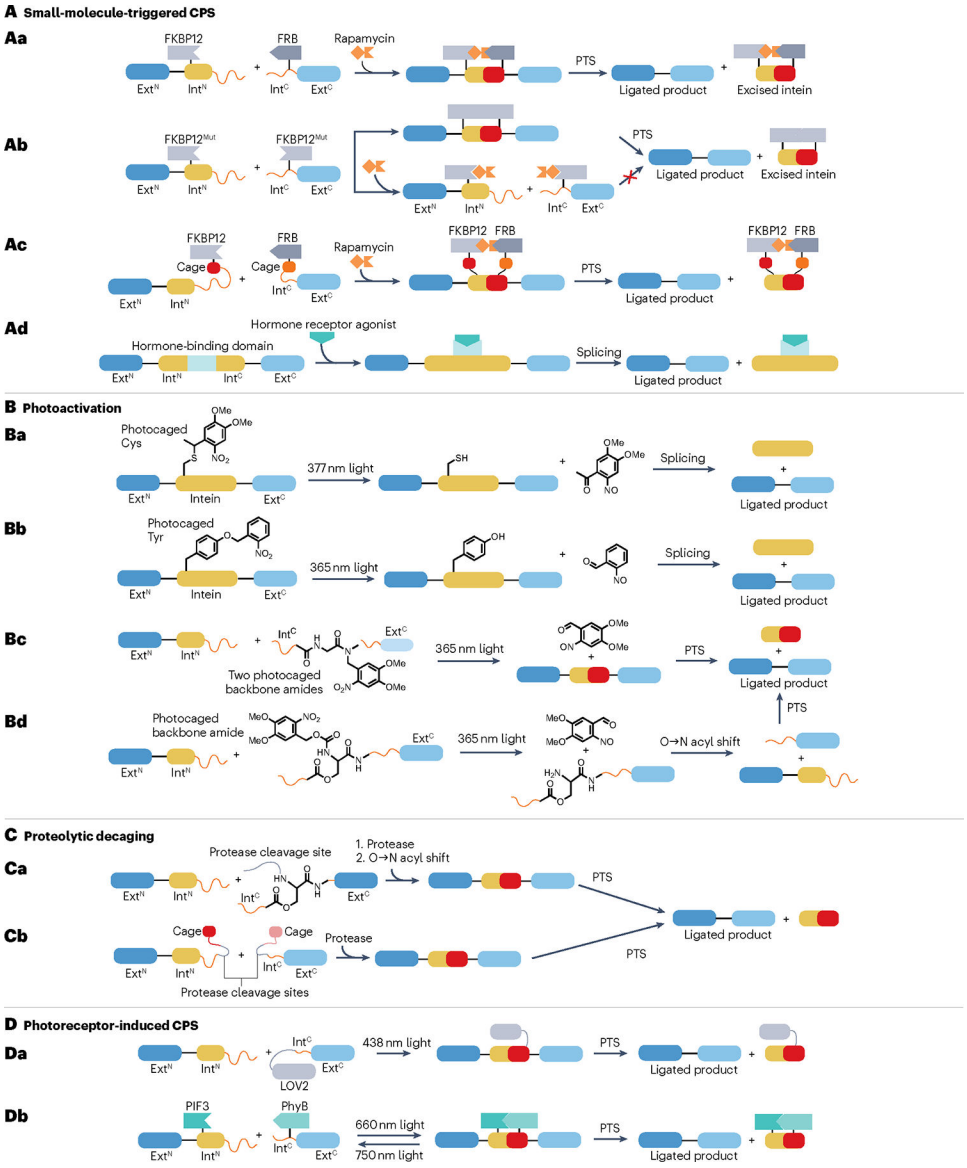

Fig. 9 |. Strategies for conditional protein splicing (CPS).

A, Small-molecule-triggered conditional protein splicing (CPS). **Aa**, The association of split inteins is controlled through the rapamycin-dependent interaction of the 12-kDa FK506-binding protein (FKBP12) and FKBP12-rapamycin binding (FRB) domain. By linking the N-terminal extein (Ext^N) to a fusion of the N-terminal split intein (Int^N) and FKBP12 protein, protein *trans*-splicing (PTS) occurs when rapamycin brings the construct into proximity of a fusion of the C-terminal split intein (Int^C), FRB and the C-terminal extein (Ext^C). **Ab**, Using a homodimerizing mutant of FKBP12, rapamycin can turn PTS off by disrupting the interaction between the FKBP12 domains. **Ac**, PTS is inhibited by the introduction of ‘cages’ that prevent split-intein association by binding to their cognate intein fragments. As rapamycin brings the constructs together, these cages are displaced upon binding of the split-intein fragments, thus resulting in PTS. **Ad**, Contiguous inteins carrying insertions of binding domains from hormone receptors can be triggered by the hormones

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4-hydroxy tamoxifen and oestrogen. **B**, Photoactivated CPS. Splicing can be controlled by installing photoremovable protecting groups on side chains of either Cys (**Ba**) or Tyr (**Bb**) residues that are key for splicing. **Bc**, Insertion of photoremovable protecting groups on the backbone amides of two Gly residues prevents proper folding of the split-intein fragment. Upon removal of these photocages, the split-intein refolds and associates with its intein partner to facilitate PTS. **Bd**, An O-acyl linkage on a backbone amide introduces a kink in the protein that prevents PTS. The protecting group can be removed by light, inducing an O→N acyl shift to restore the backbone conformation and allow splicing to occur. **C**, CPS through proteolytic decaging. **Ca**, A kink introduced into the intein backbone can be resolved by removing the protecting group through proteolysis. The subsequent O→N acyl shift occurs similarly to that in panel **Bd**. **Cb**, Inhibitory cages (as in panel **Ac**) are fused to the intein fragments through linkers that contain a protease cleavage site, and proteolytic removal of these cages therefore triggers PTS. **D**, Photoreceptor-mediated CPS. **Da**, The Int^C is fused to the light, oxygen or voltage domain 2 (LOV2), which undergoes conformational changes upon illumination to facilitate PTS. **Db**, The interaction between transcription factor phytochrome-interacting factor 3 (PIF3) and phytochrome B (PhyB) can be modulated by light at different wavelengths, thereby enabling CPS to be turned on and off.

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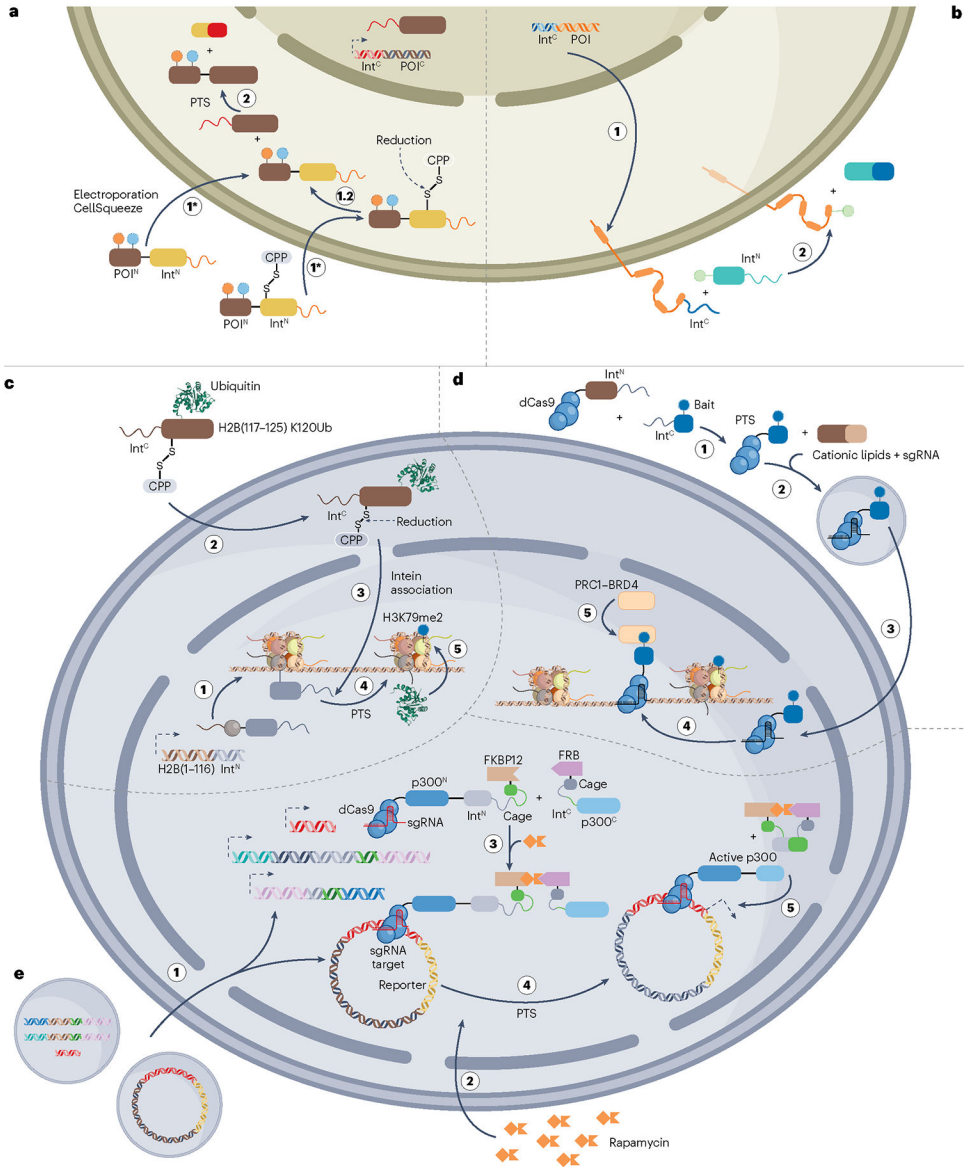

Fig. 10 |. Cellular protein manipulation strategies using inteins.

a, Split-intein-based modification of proteins of interest (POIs) in vivo. To modify intracellular POIs at their N terminus using protein *trans*-splicing (PTS), two constructs are designed: (1) the larger, C-terminal segment of the POI (POI^C) fused to the C-terminal split-intein fragment (Int^C) and (2) the synthetic, N-terminal segment of the POI (POI^N) carrying the modification (or modifications) of choice fused to the N-terminal split-intein fragment (Int^N). The cells are transfected to recombinantly express the Int^C–POI^C construct, whereas the semisynthetic piece can be delivered by electroporation, CellSqueeze or conjugation of the cargo to a cell-penetrating peptide (CPP). For CPP-based delivery, the CPP is often conjugated to Int^N through a disulfide bond that is reduced in the cytoplasm upon cell entry. The modified POI is then generated by PTS as the Int^N–POI^N and Int^C–POI^C constructs associate within the cell. Although this figure only depicts N-terminal POI modification, this strategy is also applicable to C-terminal modification (**c**). **b,** PTS-based modification

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of the extracellular part of membrane proteins can be achieved using a strategy similar to that in panel **a**. However, as splicing occurs at the cell surface, such modifications are more straightforward than those targeting intracellular proteins. **c**, Site-specific modification of histones. To install ubiquitin (Ub) at lysine 120 of histone H2B, cells are transfected with a plasmid encoding a truncated segment of H2B (residues 1–116) fused to Int^N. By delivering the missing piece of H2B (residues 117–125) carrying the K120Ub modification fused to Int^C using a CPP, the PTM is site specifically installed in the full-length H2B protein. The control of H2B composition afforded by this strategy highlighted that H2B K120Ub stimulates writing of another histone PTM, namely, H3K79me2. **d**, Locus-specific recruitment of epigenetic regulators. PTS was used to fuse a synthetic bait moiety to catalytically dead Cas9 (dCas9) in the culture medium. The semisynthetic dCas9 was then delivered to the cell together with a single guide RNA (sgRNA) using cationic lipid transfection. In the nucleus, the sgRNA guides the dCas9–bait complex to the DNA sequences of interest, leading to specific recruitment of either of the epigenetic modulators polycomb repressive complex 1 (PRC1) or bromodomain-containing protein 4 (BRD4) depending on the choice of synthetic bait. **e**, Genome-specific activation of the histone acetyltransferase p300 by conditional protein splicing. p300 was split into inactive N-terminal ($p300^N$) and C-terminal ($p300^C$) fragments. The $p300^N$ fragment was fused to dCas9 and a caged, N-terminal split-intein fragment linked to the 12-kDa FK506-binding protein (FKBP12) domain. Similarly, $p300^C$ was fused to the cognate, caged Int^C linked to the FKBP12-rapamycin binding (FRB) domain. In cells that express these two constructs together with sgRNAs of interest, p300 activity can be reconstituted at specific genomic regions by rapamycin-triggered conditional protein splicing. This was used to selectively enhance transcription of a reporter gene that was targeted by the sgRNA.