Trends in Biotechnology

CellPress
REVIEWS

Opinion

Targeting the Post-translational Proteome with Intrabodies

Antonino Cattaneo[1,*] and Michele Chirichella[1,2]

The complexity of the proteome exceeds that of the genome. Post-translational modifications (PTMs) and conformational changes of proteins trigger new molecular interactions whose systematic elucidation is hampered by the lack of specific tools. PTMs are particularly relevant for epigenetic regulation of gene expression; a field of translational interest. However, state-of-the-art inhibitors used in epigenetic studies and therapies target modifier enzymes such as acetylases and deacetylases, rather than a single PTM protein *per se*. The systematic development of anti-PTM intrabodies, which allow targeting of intracellular proteins in the context of living cells, will help reaching a new level of precision and specificity in the description of epigenetics, paving the way to new therapeutic opportunities.

Importance of Targeting Post-translational Modifications

PTMs modulate the activity of most eukaryotic proteins [1–3] and represent an untapped source of targets of biological and therapeutic potential. Indeed, PTMs such as phosphorylation and acetylation are fundamental regulators of the cell cycle [4], transduction signal cascades [4], and chromatin organization [5]. PTMs also determine different protein conformations and allosteric interactions of physiological and pathological relevance [6,7]. While the analysis of these PTMs by proteomic techniques has made substantial progress, the functional study and validation of these targets present formidable challenges, and can only be indirect. Indeed, PTMs cannot be selectively inhibited by nucleic-acid-based interference approaches because they interfere with all variants of a protein simultaneously. Therefore, developing compounds that can selectively target PTM-proteins, as opposed to inhibiting the modifying enzymes, represents a 'holy grail' of drug development in different fields, including epigenetic therapeutics, cancer, and neurodegenerative diseases. Despite the richness and potential of this class of targets, no FDA-approved drug targets a PTM directly [8,9], mainly due to the difficulties in systematically and generally designing or selecting such kind of inhibitors.

A deeper knowledge of PTM-mediated pathways is necessary to validate PTMs as new therapeutic targets. Nevertheless, this would require a selective neutralization (or ablation) of a specific PTM form of a protein, without interfering with the unmodified (or differently modified) version of the same protein. This is well exemplified by acetylation, which plays a fundamental role in the epigenetic regulation of gene expression [10]. Current state-of-the-art methods to study cellular mechanisms regulated by acetylation use broad inhibitors of histone acetyl transferases (HATs) [11,12], histone deacetylases (HDACs) [13,14], and bromodomain-containing transcription factors [bromodomain and extra terminal motif (BET) proteins] [15,16]; each of which modifies or interacts with several distinct proteins at different aminoacidic positions. Thus, epigenetic modifiers can be described as 'writers', 'readers', or 'erasers' [17]. Each of these modifiers acts on many different substrates we can define as **epigenetic words** (see Glossary). All currently available inhibitors of these epigenetic modifiers will inhibit their action on many different words simultaneously.

Highlights

PTMs represent a source of targets with biological and therapeutic potential.

No FDA-approved drug targets a PTM directly due to the difficulties in designing or selecting such an inhibitor.

Anti-PTM intrabodies targeting native proteins can achieve PTM-selective neutralization in eukaryotic cells via PISA technology. PISA streamlines the selection of intracellular anti-PTM binders.

Coupling PTM-oriented targeting technologies with CRISPR/Cas9 could restrict intrabody-mediated knock down of a PTM involved in chromatin remodeling to a specific genomic locus.

Tools for inhibiting modified residues on native proteins will help to discover new molecular interactions and validate therapeutic targets, increasing understanding of cellular networks and diseases.

PISA antibodies will provide new therapeutic tools to interfere selectively with PTMs.

[1]Bio@SNS Laboratory, Scuola Normale Superiore, Piazza dei Cavalieri, 7 - 56126 Pisa, Italy
[2]Current address: Institute for Research in Biomedicine, Università della Svizzera italiana (USI), Via Vincenzo Vela 6, CH-6500 Bellinzona, Switzerland

*Correspondence:
antonino.cattaneo@sns.it (A. Cattaneo).

 https://doi.org/10.1016/j.tibtech.2018.11.009

Therefore, the information gathered with these inhibitors is biased by the pleiotropic effects of the targeted enzymes and limits their uses for target validation and therapeutic development (Figure 1). Methods to target epigenetic words like single PTMs are therefore needed and would represent a breakthrough. Recent work has shown how to achieve more precise locus-specific histone deacetylation using a synthetic CRISPR/Cas9-based HDAC [18], or how to design screening schemes to discover small molecules specifically inhibiting PTM-modifying enzymes [19–21]. Nevertheless, none of these technologies can interfere precisely with single-acetylated residues to study the effects of a single-modified PTM amino acid on cell function.

In principle, it is possible to derive antibody domains that specifically recognize PTMs on desired protein targets [22]. Such recombinant antibody domains could then be used as intracellular antibodies (**intrabodies**) and exploited to achieve PTM-selective interference [23]. Indeed, intrabodies represent a class of intracellularly stable molecules able to target and neutralize intracellular proteins in mammalian cells [24]. However, it is not currently possible to streamline the isolation of antibodies against native PTM-proteins, for PTM-specific intracellular inhibition. The main hurdles are, first, that antibodies selected *in vitro* (e.g., with phage display) are not validated to function inside cells, and second, it is currently difficult to purify a native protein harboring a target PTM for downstream antibody derivation.

In this opinion article, we discuss the importance of targeting the **post-translational proteome**, the difficulties of this task, and also its opportunities. We discuss recent advances showing how intrabodies against PTM epitopes in the context of their native protein counterpart can be selected and how the selected antibody domains can be exploited to achieve PTM-selective interference in mammalian cells.

Post-translational Selective Interference: 'Cutting the Edge' of Individual-PTM Epitopes in Native Proteins

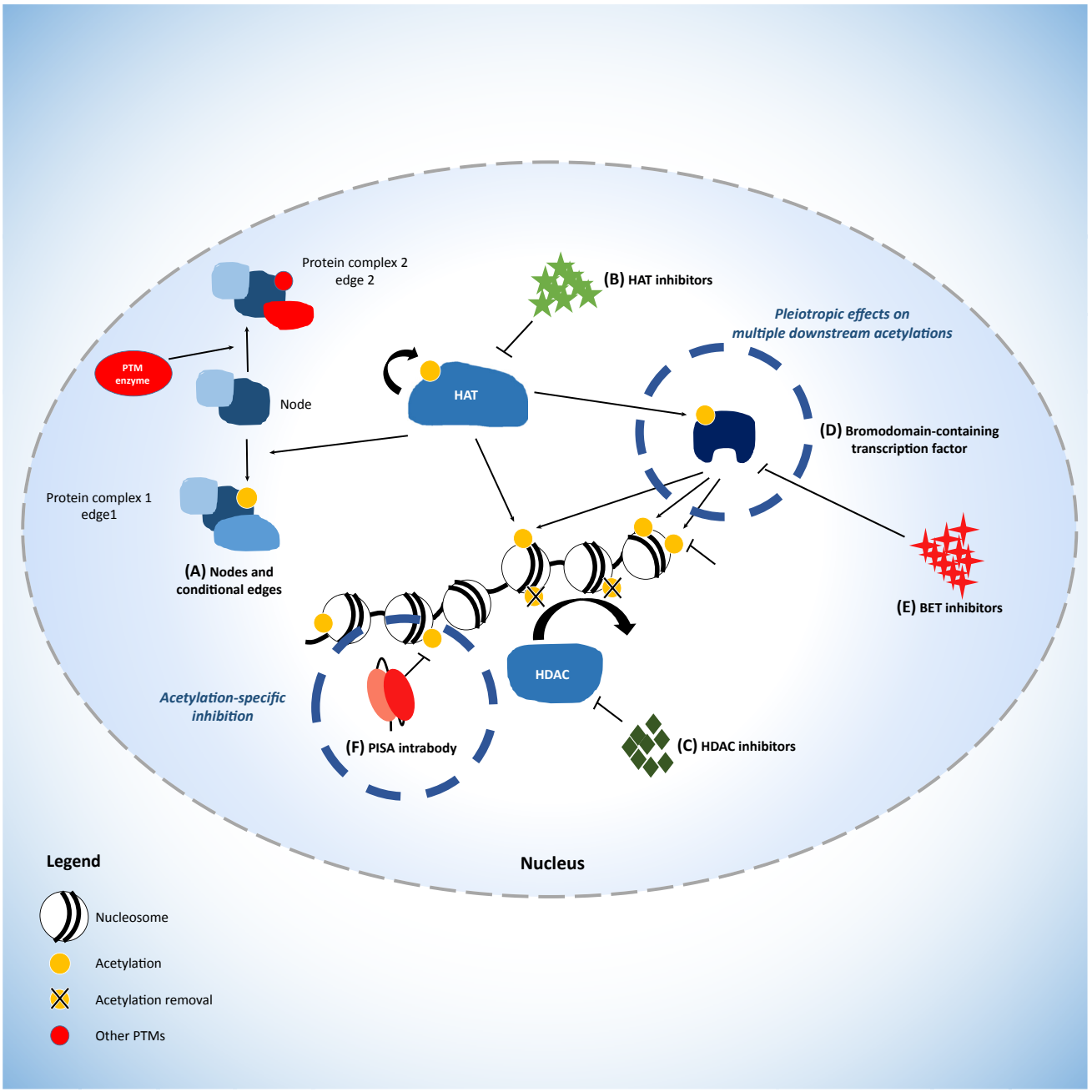
Cellular pathways can be pictured as an intricate **network** of **nodes** and **edges**. A network is the ensemble of protein–protein interactions (the edges) that mediate and allow a precise cellular function. Each protein represents therefore a node of the network. Such interactions are mediated by distinct parts of a protein, including PTMs. In the context of PTM-mediated interactions, edges are created by different PTM versions of the same protein node. Edges can thus shape a particular node (i.e., the nonmodified protein itself) by connecting it with different molecular partners, and by branching the node in different pathways (Figure 1A). PTMs are therefore conditional edges of the cellular protein network (interactome). Ablation of a protein node will result in the disruption of all the edges, while edge-selective interference will not affect all the other PTM edges.

Mechanistic understanding of human disease requires detailed knowledge of how disease-causing mutations, or even sporadic diseases, affect specific interactome properties, without necessarily affecting the nodes themselves. In fact, node removal can have very different consequences than edge removal (Figure 2). The study of '**edgetics**' uncovers specific loss or gain of interactions (edges) to interpret genotype-to-phenotype relationships [25–28]. What is hidden behind the intricacies of the PTM-mediated interactions remains unclear. Indeed, the lack of suitable methods to achieve experimentally edgetic perturbations makes the post-translational proteome the 'dark matter' of biology.

One recently demonstrated method for *de novo* selection of intracellular antibodies targeting PTM proteins in living cells is the PISA technology (post-translational intracellular silencing antibody) [29], which overcomes several difficulties associated with anti-PTM antibody

Glossary

Edge: in this context, a PTM-mediated interaction mediated by one of the possible PTM variants of a node. Ablation of a node results in the ablation of all the edges, but selective ablation of a certain edge does not affect interactions of other edges with the same node.
Edgetics: in general, genetic mutations that affect specific protein–protein interactions or edges, in this context interfering with edges, including PTM proteins and conditional edges (edgetic perturbations).
Epigenetic word: a PTM-containing epitope on a native protein involved in epigenetic processes, installed by a 'writer', removed by an 'eraser', and recognized by a 'reader'.
Intrabody: an intracellularly stable antibody domain targeting an intracellular protein.
Macrodrug: in this context, an antibody domain used as protein (e.g., internalized by cells), or expressed by cells from a recombinant DNA source.
Node: in this context, a nonmodified protein that is a connection point in a given protein network. Nodes are the macromolecules that can interact together forming a network.
Post-translational proteome: the ensemble of all proteins that display a chemical post-translational modification or a conformational change.
ScFv: single chain fragment variable; an antibody domain composed by the fusion of the variable light (VL) and variable heavy (VH) domain of an immunoglobulin.
Single domain antibody: an antibody domain made either of the VL or the VH domain alone. Also called nanobodies.



Trends in Biotechnology

Figure 1. Different Methods to Interfere with the Function of Acetylated Proteins. (A) Protein acetylation (as well as other PTMs) promotes specific protein–protein interactions, from which can branch specific pathways. Different PTM enzymes can modify therefore a protein node creating conditional edges, which can interact with distinct molecular partners in distinct pathways. (B and C) Commonly, epigenetics is studied through administration of chemical inhibitors of HATs and HDACs, which in turn target multiple acetylated sites, on different histones or transcription factors. (D) Many transcription factors are able to bind many different acetylated sites via their bromodomains (BETs), a general lysine acetylation-binding motif. (E) A successful class of chemical inhibitors is able to block the action of BETs, even though this effect extends to all downstream gene loci on which the transcription factor exerts its function. (F) PISA intrabodies are able to bind selectively a single acetylated residue on one histone type (a word), avoiding simultaneous inhibition of other PTM forms of the same histone or protein. Abbreviations: BET, bromodomain and extra terminal motif; HAT, histone acetyl transferase; HDAC, histone deacetylase; PISA, post-translational intracellular silencing antibody; PTM, post-translational modification.

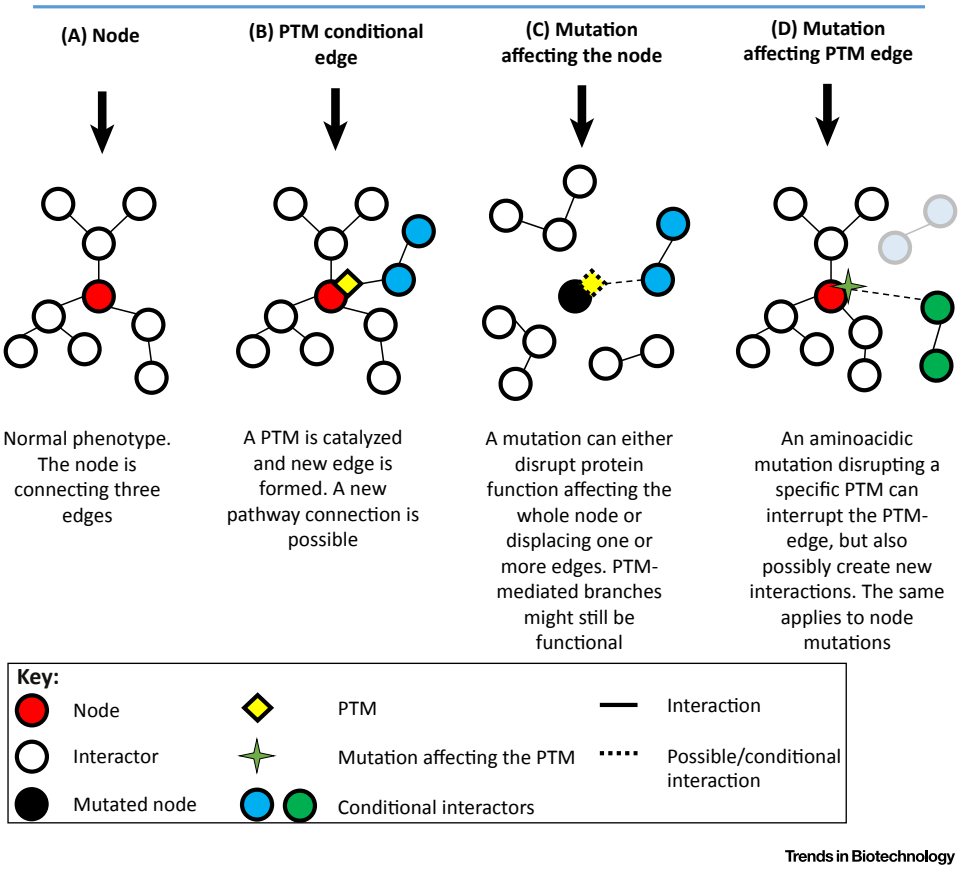


Figure 2. Intracellular Protein Network and PTM-Mediated Pathways. PTMs can branch a particular protein node (red circle) by connecting it with new molecular partners in a conditional way, acting as a pathway edge (A and B). Mutations occurring on the protein node (e.g., genetic diseases) might disrupt all or some of the possible interactions with the usual molecular partners (C). In the same way, the PTM-edge can be compromised by mutations in the PTM amino acid, or by mutations affecting modifying enzymes. Mutations causing substitutions could possibly generate new edges with deleterious effects (D). Adapted from [26]. Abbreviation: PTM, post-translational modification.

production (Figure 3). Indeed, it is generally not easy to produce a purified native protein harboring the desired PTM to elicit anti-PTM antibodies. Thus, current methods to select PTM-selective antibodies, such as animal immunization or phage display [22,23], use chemically modified linear peptides as antigens [30–33]. The ease of the panning selection with phage display is, however, offset by disadvantages with respect to the native PTM-oriented application. Pitfalls include using unstructured peptides with chemically synthesized PTMs instead of native PTM proteins, and generating antibody binders that are not adequate for their use in cells. Thus, the conformation of a linear unstructured PTM-containing peptide used as antigen will be different from that of the corresponding PTM-modified sequence in the context of the native protein (Figure 3). Therefore, these anti-PTM antibodies will not always recognize the native protein. Moreover, selecting antibody domains from phage display libraries that fold in the periplasm of *Escherichia coli* cells does not guarantee that the antibody will function as a neutralizing intracellular antibody within the protein network in the cell, because of the different redox conditions [34,35]. Thus, the antibody domains selected by monoclonal antibody or phage display technologies might be suitable for use as PTM detection probes, but not as intracellular function-neutralizing agents against native PTM proteins.

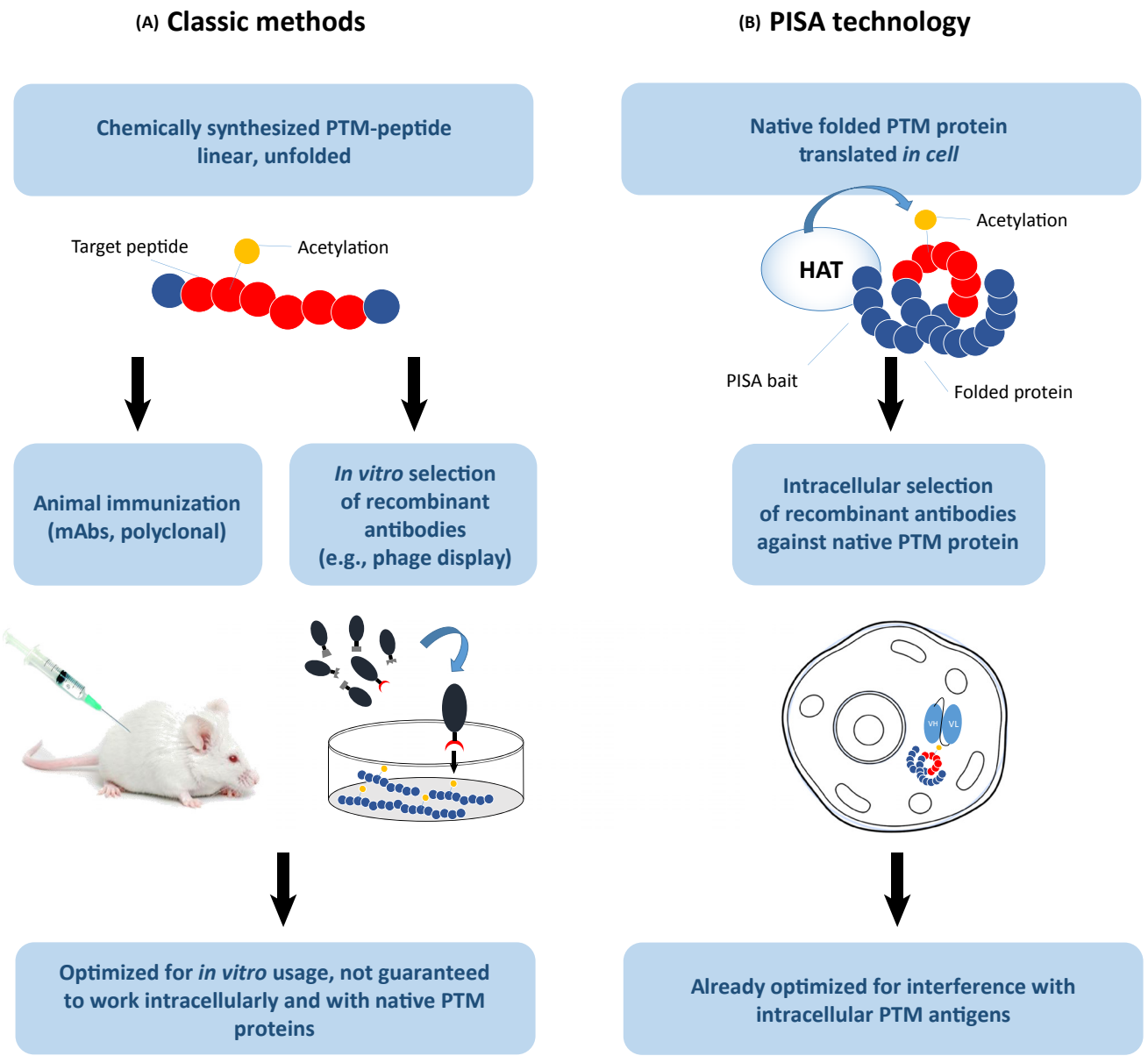


Figure 3. Current Methods to Isolate Antibodies Targeting PTMs versus PISA Technology. (A) Currently, it is not generally feasible to have the quantities of a purified native protein harboring the desired PTM required to elicit anti-PTM antibodies, which are obtained using synthetic peptides containing the target PTM, such as acetylation. These short epitopes are unfolded, linear, and can fail to mimic the PTM containing epitope in the structural context of the native protein in the intracellular environment. Such peptides are used for animal immunization, to yield polyclonal or monoclonal antibodies or as panning antigens in phage display selections, to yield recombinant antibodies (e.g., Fabs, ScFvs, and nanobodies). In both cases, the antibody is selected outside of the intracellular context or *in vitro*, against linear peptides, limiting their immediate usage as intrabodies. (B) With PISA technology, tethered catalysis is used to induce the desired PTM in the target protein. In the example shown, a native-folded target protein is intracellularly acetylated by a HAT enzyme. The protein bait is therefore challenged directly in the intracellular environment with a library of recombinant antibodies. The resulting positive antibody clones are ready-to-use anti-PTM intrabodies recognizing the PTM in its native structural context and in the cell. PISA technology promises to be a 'one-stop shop', from the systematic derivation of orthogonal anti-PTM antibodies to the intracellular silencing of PTMs in mammalian and other cells. Abbreviations: HAT, histone acetyl transferase; PISA, post-translational intracellular silencing antibody; PTM, post-translational modification.

PISA Technology, a New Method to Target Protein PTMs *in vivo*

PISA technology selects antibody domains (in either **ScFv** or **single domain** format) directly in the reducing environment of a cell cytoplasm, against a cell-installed PTM protein target (Figure 4).

Past work has shown that the natural repertoire of antibodies contains a subset of more stable antibody variable domains, which can be selected to tolerate the absence of intrachain disulfide bonds when expressed under the reducing conditions of the cytoplasm (intracellular antibody capture technology; IACT) and are therefore validated as functional intrabodies [36–38]. The PISA platform is based on the IACT yeast two-hybrid system, in which naïve or immune (human, mouse, or camelid) antibody domain libraries are challenged with a target antigen bait [29,39]. The novelty of the PISA platform resides in the new set of PTM protein baits, prepared by genetically fusing to the desired protein target the catalytic domain of the PTM modifier enzyme, such as acetylases or kinases. The protein target therefore receives the PTM by a tethered catalysis process [40] performed in the cell, requiring no protein manipulation. The selection occurs in auxotroph yeast cells: the interaction between the PTM-bait and the selected antibody allows yeast growth on histidine-lacking plates (Figure 4A). A counter-screening is performed against a mutated version of the bait unable to install the desired PTM (Figure 4B). The antibody genes are finally recovered from the selected colonies, expressed in bacteria, and tested for *in vitro* interactions (Figure 4C). The genes coding for the selected antibody domains are naturally suitable for use as intrabodies *in vivo* against native PTM proteins and have the advantage of targeting a specific edge of the protein network directly. Indeed, the PISA selection system is designed in a way that the epitope recognized by the intrabody is physically the PTM itself.

PISA is designed to tackle a specific PTM-mediated interaction edge of a cellular pathway. Since the PISA selection platform exploits native protein targets that are modified by their natural acetylases or kinases, the selected antibody binders are intrinsically able to recognize the PTM in the native structural context, with the additional property of being orthogonal to the cell protein network.

Site-Specific Acetylation-Selective Interference: Targeting Individual Epigenetic Words

Histone acetylation is one of the main modulators of chromatin. Acetylated histones correlate with relaxed chromatin structure and enhanced gene expression [41]. Moreover, acetylated proteins function as a hub for the so-called bromodomains, which are general acetyl-lysine binding modules found in different classes of proteins, such as transcription factors and HATs [42]. Recently, a novel class of drugs, BET inhibitors [15], have shown promising applications in cancer therapy [43]. Indeed, many cancers rely on chromatin modulation and epigenetic enhancement of proto-oncogenes transcription [44]. Alongside BET inhibitors, small chemicals targeting HDACs and HATs are currently in clinical trials for oncogenic and neurological pathologies [9,45,46], but despite their potential, questions can be raised about their specificity, their mechanisms, and their use for epigenetic research [47]. Indeed, being antagonists of general protein motifs, their action cannot be restricted to a defined protein, simultaneously affecting several classes of enzymes, and causing unknown side effects. Much of the current understanding of epigenetics was obtained using inhibitors of HDACs (e.g., valproic acid and trichostatin A) [48,49] or HATs (e.g., anachardic acid) [50]. However, the target specificity of HDAC, HATs, and bromodomains is broad [51–53]. In this way, it is not possible to study mechanistic aspects of epigenetic phenomena by investigating the role of single PTMs, such as single epigenetic words, represented by a specific acetylation of a particular protein. The study of epigenetic words would in fact require specific anti-PTM inhibitors, which is precisely what the PISA platform allows for.

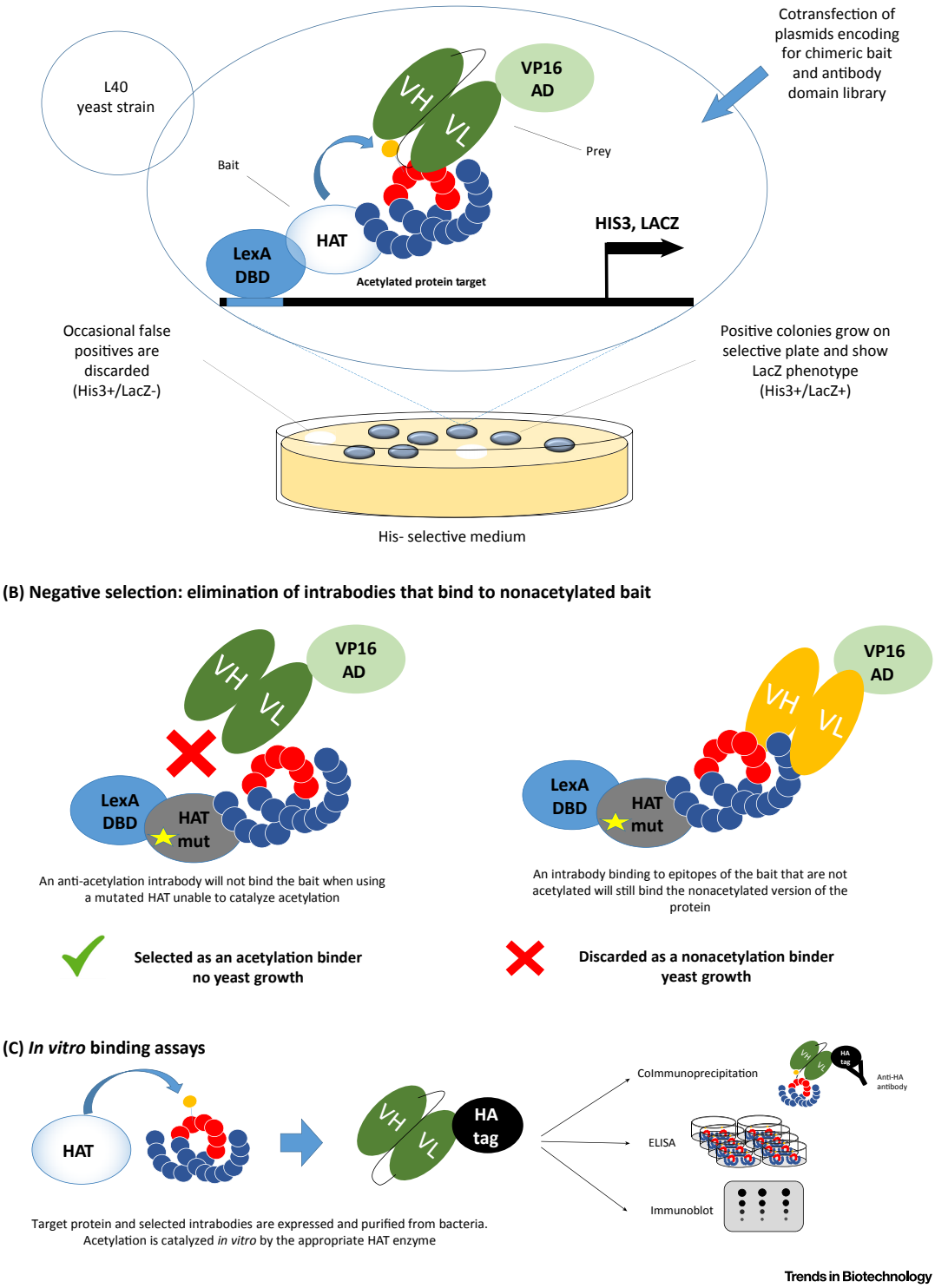


Figure 4. PISA Selection Scheme. (A) Yeast L40 strain is a two-hybrid system strain auxotroph for histidine. The yeast is cotransfected with two plasmids: the bait plasmid is encoding the chimeric acetylated target protein (HATwt-Target) fused to the DNA binding domain of LexA (LexA DBD). The prey plasmid is instead part of an

(Figure legend continued on the bottom of the next page.)

PISA has isolated an intrabody (ScFv-58F) that can target acetylated histone H3 on Lys9 (AcK9H3) specifically [29]. With intrabodies targeting histone PTMs, it is possible to intervene directly on a desired histone modification, downstream to the modifying HAT writer enzymes or of the BET readers. This inhibition can be extended to all the modified histones (e.g., all AcK9H3), by a general expression of the anti-AcK9H3 in the nucleoplasm, or it could be restricted to a particular genetic locus, exploiting CRISPR/Cas9 technologies [18]. The latter example would represent the finest and the most specific way to orthogonally deplete the function of a single epigenetic word. The systematic use of intrabodies for single-PTM epigenetic editing could open new opportunities for the study of basic mechanisms and pathways of the cells, which for technical reasons might have been hidden until now. Finally, a more precise study of the mechanistic aspect of chromatin regulation would also open new therapeutic opportunities by validation of pathogenic PTM targets.

Conformational-Selective Interference

Selective protein conformation changes are a class of PTMs playing a fundamental role for a number of physiological and pathological processes, characterizing the so-called protein misfolding diseases. It has become clear that the protein misfolding in the cell is more general than previously imagined, and that its study can provide unique insights into the nature of the functional forms of peptides and proteins, as well as in designing a new generation of drugs by which protein homeostasis can be restored and protein metastasis avoided [54]. Indeed, oligomeric states of proteins represent another example of edge in protein networks, which introduce new molecular interactions upon allosteric or conformational changes.

Intrabodies have proven to be an elective approach to target selected protein conformers in the cell (conformational selective interference; CSI) [55] and to study protein conformational changes in the cellular context [37,55–58]. Their importance is particularly evident in the field of neurodegenerative diseases, where the impact of protein misfolding on the proteostatic equilibrium is under intense investigation, and where conformation-selective molecular tools would allow a finer cell biology of protein misfolding and aggregation to be performed. Recently, the availability of conformation-sensitive anti-Aβ oligomers (AβOs) intrabodies [37] has been exploited to demonstrate that the critical pathogenic oligomerization of the Aβ peptide begins in the endoplasmic reticulum, validating precise amyloid conformations of the protein, emerging in this subcellular compartment, as a target for potential treatments [55]. This has provided a proof of principle of the use of CSI to study the cell biology of protein misfolding in neurodegenerative diseases. CSI and PISA can now be extended to other misfolded proteins, involved in different neurodegenerative diseases, such as α-synuclein, microtubule-associated protein τ, huntingtin, TDP-43, and others.

In addition to protein misfolding diseases, alterations in conformational changes of proteins involved in signaling also play a significant role in cancer and other pathologies, which can be caused – or sustained – by aberrant stabilization of protein conformations that normally cycle between different allosteric forms in a signal-regulated way. A particularly relevant conformational target in oncology is the p21-ras protein, whose aberrant signal-independent activation is the most common cause of human cancers [59]. Intrabodies specific for the activated

antibody domain library (in either single domain or ScFv format) fused to VP16 activation domain. The interaction between the intrabody and the target protein allows the yeast cell to survive by inducing transcription of His3, an enzyme involved in histidine production. Positive bait/prey interaction generates also transcription of LacZ. Double-positive clones are selected for next step. (B) To select clones binding specifically to acetylation, a counter screening is performed against a bait in which HAT enzyme carries a point mutation (LexA-HATmut-Target) and against other truncated baits. In this case, yeast growth indicates that the epitope targeted by the intrabody is not acetylation and therefore the clone is discarded. (C) Anti-acetylation intrabodies and target proteins are purified and their interaction is confirmed through one or more *in vitro* binding assays. As discussed, an intrabody is not necessarily working *in vitro* despite its functional intracellular activity. Abbreviations: HAT, histone acetyl transferase; PISA, post-translational intracellular silencing antibody.

conformation of p21-ras have provided a pioneering benchmark approach [60] in the quest for antitumor drugs able to selectively inhibit the constitutively active form of p21-ras but not its signal-activated counterpart. Following this line, a conformation-dependent anti-RAS single domain intrabody was demonstrated to prevent tumorigenic transformation in cells and mice models [56] and is currently being developed as a gene therapy approach for cancer [61,62]. Other notable examples of conformational targeting with intrabodies include recombinant antibody domains to the Small GTPase Rab6 [57] and scFvs targeting and stabilizing the oxidized forms of PTP1 and inhibiting its phosphatase activity, an established target for diabetes and obesity [58].

Proteolytic cleavage from precursors, to yield a mature protein, is another form of post-translational processing. A relevant case is represented by the neurotrophin nerve growth factor (NGF), which is cleaved from its precursor proNGF. NGF and proNGF coexist in the brain, where they display opposing actions, neurotrophic and death inducing, respectively. A conformational recombinant anti-NGF antibody selectively recognizing mature NGF versus uncleaved proNGF, was expressed in the brain of transgenic mice, yielding comprehensive Alzheimer's neurodegeneration [63–65]. This neurodegenerative phenotype deriving from a proNGF/NGF imbalance, is not reproduced by the ablation of *NGF* gene by homologous recombination [66]; a further demonstration that antibody interference against post-translationally modified epitopes can provide a complementary approach to gene based approaches.

In all such cases, selective interference with protein conformers or allosteric states can only be achieved with tools such as conformational selective antibodies.

Equipping Anti-PTM Intrabodies with Effector Functions

The binding domains of conformation- and PTM-selective antibody domains can be equipped with a number of different effector functions for imaging, degradation, and cell death, to provide a diverse range of applications (Figure 5A–D).

Intrabodies can be fused to fluorescent proteins to image and sense intracellular epitopes. For instance, anti-acetylated histone intrabodies in fusion with GFP can track histone modifications *in vivo* (mintbodies) [67]. An appealing extension of this technology would be equipping PISA intrabodies with FRET pairs of fluorescent proteins, to build an 'AND gate' sensor for the diagnostic detection of a particular couple of PTMs on target proteins, or for the appearance of a pathological PTM (Figure 5A). In the same context of intrabody-based sensors, PISA antibodies could also be fused to transcriptional actuators [68] to link PTM detection to programmed transcriptional cellular responses.

An alternative to protein inhibition or neutralization by binding competition is protein degradation. Indeed, there are cases in which it would be desirable to deplete the targeted PTM protein from the total protein pool, for instance, when the modified protein has a pathological role. Indeed, protein inhibition needs a known active site, requires a protracted target engagement, and typically requires drug exposure for extended periods. On the contrary, with protein degradation, proteins without a known active site can be targeted and even transient interactions between drug and target can result in durable loss of protein activity.

A pioneering approach exploited E3 ubiquitin ligases for targeted degradation, by fusing intrabodies to ubiquitin–proteasome pathway substrates to achieve ligand-inducible fast degradation of the target protein (Figure 5B) (suicide intrabody technology; SIT) [69]. This method was used to conditionally and reversibly target the microtubule-associated τ protein for

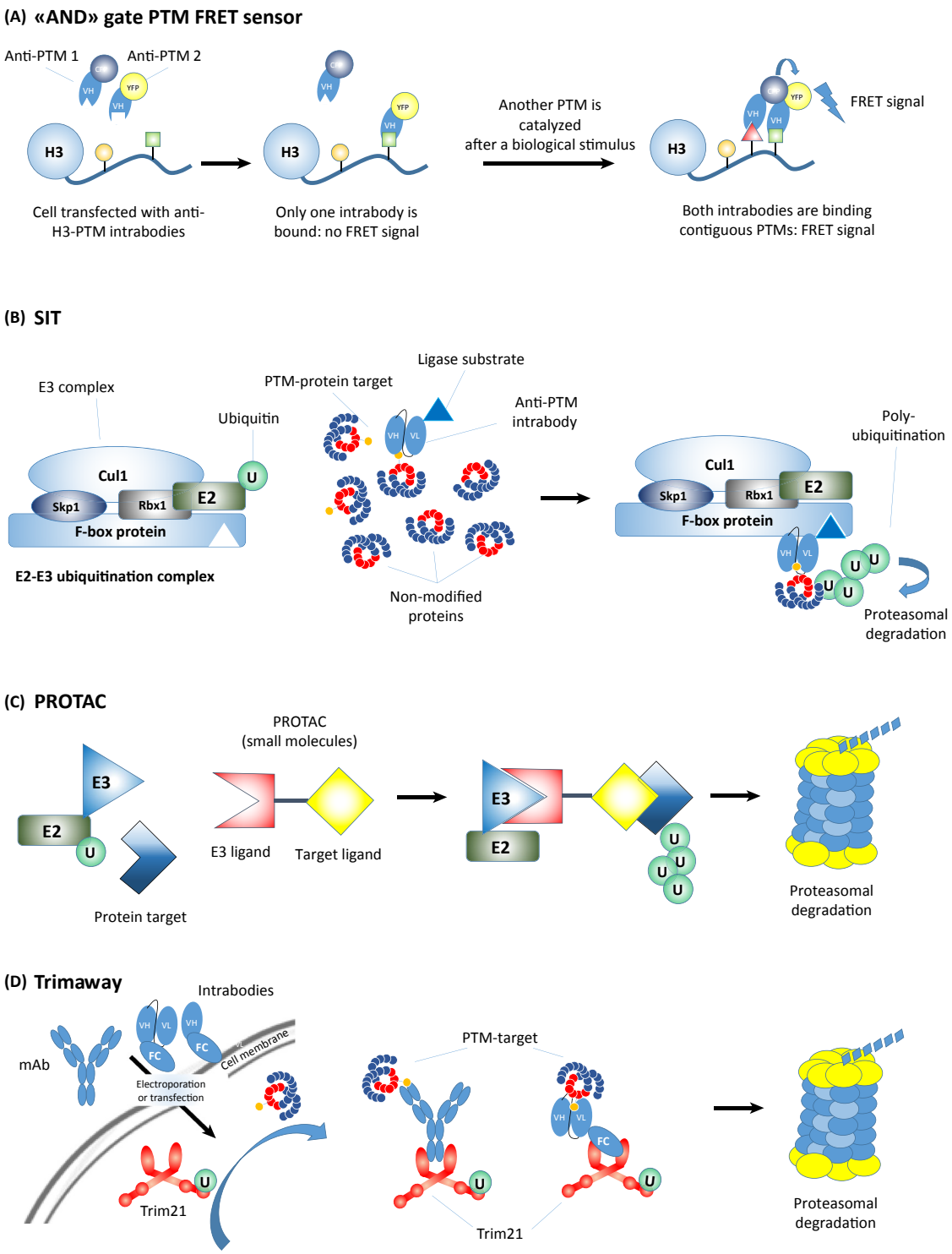


Figure 5. Equipping Anti-PTM Intrabodies with Effector Functions. (A) Anti-PTM intrabodies can be used as sensors in a Boolean 'AND gate'. A FRET signal is observed only when the desired two PTMs are present. This system could be used for monitoring dynamics of two PTMs at the time, following certain stimuli, or to

(Figure legend continued on the bottom of the next page.)

proteasome degradation. In light of the pathogenic role of phosphorylated and acetylated τ in Alzheimer's disease and tauopathies, an extension of SIT with new anti-PTM-τ intrabodies could help elucidating τ pathogenesis. SIT was recently used for the reversible modulation of synaptic activity, achieved by fusing an E3 ligase to an intrabody targeting gephyrin; a component of the postsynaptic protein network of inhibitory synapses [70]. SIT would allow one to selectively deplete a cell of the PTM protein, without affecting the levels of the unmodified protein. In some ways, SIT is reminiscent of the PROTAC (proteolysis targeting chimera) strategy for targeted protein degradation [71,72]. A PROTAC is a heterobifunctional small molecule compound that contains two ligands connected by a linker unit (Figure 5C). One ligand binds an E3 ubiquitin ligase protein, while the other ligand binds to the target protein of interest, thereby bringing the ligase and the target in close proximity. Unlike PROTAC, for PTM targets, SIT does not rely on the case-by-case design, synthesis, and validation of cell permeating heterobifunctional small molecule compounds able to specifically bind the target PTM. The combination of SIT with PISA antibodies allows achievement of conditional, selective knock down of PTM proteins.

Another approach to attain antibody-mediated degradation exploits the cellular Trim21 protein, an intracellular Fc receptor that promotes proteasomal protein degradation of the antibody–target complex (TrimAway) [73]. In TrimAway [73], antibodies are injected or electroporated as proteins in cells, but in principle this method could also be implemented with gene encoded intrabodies, by equipping them with a recombinant Fc receptor (Figure 5D).

Intrabodies were also used to trigger cell death upon antigen binding [74]. The AIDA strategy is based on the dual targeting of two adjacent epitopes on the target protein, by two antibody fragments linked to procaspase, which results in antigen-dependent intrabody-mediated dimerization of procaspase and its self-activation through internal proteolysis and induction of apoptosis. In this context, anti-PTM antibodies targeting pathogenic PTM proteins, or sensing particular combinations of adjacent phosphorylations or PTMs, could be adopted to trigger cell death, in a variation of the scheme in Figure 5A.

Concluding Remarks and Future Perspectives

Exploring in detail the epigenetic regulation of gene expression, as well as the functions of other cellular networks, requires an accurate and selective targeting of PTMs. The refined tools that we described could provide a much needed new approach in the quest to develop a new generation of drugs targeting PTMs. Technologies providing tools for the inhibition of specifically modified residues on proteins (such as the epigenetic words or kinase words) pave the way to the discovery of new molecular interactions and new pathways. This could be fundamental to increase our understanding of cellular networks, in order to validate new therapeutic targets, especially in epigenetic-related diseases like several cancers, neurodegenerative diseases, or intractable forms of chronic pain. PISA antibodies will provide new therapeutic tools to interfere selectively with PTMs, the dark matter of biology. Still, some challenges remain to fully exploit the potential of anti-PTM intrabodies (see Outstanding Questions).

Outstanding Questions

The tethered catalysis approach to generate anti-PTM intrabodies, despite its successes and its potential, has limitations. These include: (i) the modifying enzyme of the target protein bait must be known; (ii) the fusion protein is large; and (iii) the site of the PTM cannot be predicted *a priori* and can only be verified *a posteriori*. Can we surpass limitations of tethered catalysis by producing specific PTM baits also for those proteins for which the modifying enzyme is not known?

How feasible is it to use expanded genetic code approaches for the site-specific genetic installment of the desired PTM in the target protein?

Protein delivery of the anti-PTM intrabodies is still a challenge. How feasible is it to exploit biological Trojan horse mechanisms that deliver proteins in different compartments of cells?

Can we exploit anti-PTM intrabodies to unravel new epigenetic pathways and mechanisms regulated by single PTMs?

Can we demonstrate the prediction that inhibiting specific PTMs on proteins is a more specific and more selective approach than that of inhibiting the writer/eraser enzymes?

Can we show that inhibiting a phosphorylated protein leads to more specific effects than inhibiting the corresponding kinase?

monitor the appearance of a single PTM adjacent to a structural unmodified epitope. Only after the PTM is installed would a FRET signal be detected. (B) With SIT, an intrabody can carry a target to degradation via the ubiquitin–proteasome pathway, in response to a ligand. The intrabody is fused to a E3 ligase substrate, which is bound and polyubiquitinated. The whole complex is finally degraded. Adapted from [69]. SIT allows one to selectively deplete a cell of the PTM protein, without affecting the levels of the unmodified protein. (C) PROTAC is a small molecule with two binding sites: one for the E2/E3 ligase and the other for the target to be degraded. The chimera is processed by the proteasome as well. Adapted from [72]. (D) With TrimAway, cells are transfected/electroporated with antibodies containing the FC fragment. The intracellular FC receptor Trim21 is able to bind the antibody and degrade the antibody-target complex via proteasome. One can also fuse the FC fragment to nanobodies or ScFvs to elicit the same degradation effect. Adapted from [73]. Abbreviations: PROTAC, proteolysis targeting chimera; PTM, post-translational modification; SIT, suicide intrabody technology.

Permeating Cell Membrane with Intrabodies

Using anti-PTM intrabodies as **macrodrugs** will require overcoming challenges represented by their delivery to cells. Indeed, the therapeutic applications of intrabodies are currently limited to those systems in which gene therapy is established or possible.

Many are the internalization strategies attempted in the past. Studies showed that anthrax toxin protective antigen in conjugation with N-terminal domain of the anthrax lethal factor enzyme, and *Pseudomonas* exotoxin A are able to transport antibody mimics in mammalian cells [75,76]. Other approaches exploit instead natural sequences of antibody variable domains (e.g., CDR stretches) that have penetrating properties [77].

State of the art for internalization of antibodies is, however, substantially restricted to two main technological approaches. The first one involves the use of cell-penetrating peptides (CPPs) such as the cyclic, arginine-rich peptide cR_{10}. These CPPs were fused to nanobodies (camelid single domain antibodies) to cross mammalian cell plasma membranes and to carry therapeutically relevant proteins such a Mecp2. Nanobodies carried with this system are able to be intracellularly localized with signal peptides, and show functional interaction with endogenous proteins [78].

The other approach uses so-called endolytic peptides, which are incorporated into endosomes, to internalize full immunoglobulins. Futaki and coworkers demonstrated that internalization of antibodies is possible by exploiting simple chemical features of biological membranes and without antibody modifications. Indeed, progressive acidification of the endosome leads to protonation of the endolytic peptide, which causes, in turn, disruption of the endosomal membrane, releasing the antibody [79]. Finally, an intriguing way to promote antibody internalization would be that of exploiting mechanisms of the intracellular immunization, by which antibodies attached to viral particles Trojan horses are naturally able to enter the cells [80].

Quantifying Protein Internalization

Introducing cargos into the cell requires quantitative evaluation of the internalized protein; ideally being able to distinguish delivery to different compartments such as the cytosol and endosomal system, and evaluating the efficiency of endosomal escape. Recently, Verdurmen and colleagues quantitatively compared different protein uptake systems in different cell lines via a ligase ubiquitin assay [81]. This prokaryotic ligase was expressed as a reporter for the biotinylation of an 'avi' tag fused to a non-selected DARPin (designed ankyrin repeat protein). Authors demonstrated how endosomal uptake does not necessarily correlate with cytosol delivery. Indeed, endosomal uptake was best achieved with CPPs, receptor-targeting moieties, or supercharged proteins such as scGFP. Conversely, endosomal escape was efficiently achieved with bacterial toxin systems, but only poorly by fusing proteins to CPPs or scGFP [81].

From Macrodrugs to Drugs: Intrabody-based Drugs

Alternatively to their usage as macrodrugs [82], intrabodies can be used to design small molecules from cocrystal structures of antibody domains with their targets, or used as competitors to screen target inhibitors, as demonstrated with an intrabody targeting the mutated form of RAS [60,61,83]. Following early work on p21-ras intrabodies [60], Quevedo and coworkers generated a high-affinity inhibitor able to block RAS protein–protein interactions in human cancer cells, affecting their vitality [83]. Similarly to the anti-RAS intrabody case, which targets the oncogenic form specifically, PTM-selective PISA-derived intrabodies could provide templates to select or design the first generation of chemicals targeting PTMs specifically.

Acknowledgments

We gratefully acknowledge the discussions with members of the Intrabody laboratory at Bio@SNS, and specially Simonetta Lisi, Marco Fantini, Martina Goracci, Raffaele Sarnataro, Matteo Trovato, Ottavia Vitaloni, as well as Giovanni Meli (European Brain Research Institute). Work in the laboratory of AC has been funded by the EU Flagship Human Brain Project (N 604602).

References

1. Beltrao, P. *et al.* (2013) Evolution and functional cross-talk of protein post-translational modifications. *Mol. Syst. Biol.* 9, 714
2. Silmon de Monerri, N.C. *et al.* (2015) The ubiquitin proteome of *Toxoplasma gondii* reveals roles for protein ubiquitination in cell-cycle transitions. *Cell Host Microbe* 18, 621–633
3. Cohen, P. (2000) The regulation of protein function by multisite phosphorylation – a 25 year update. *Trends Biochem. Sci.* 25, 596–601
4. Hunter, T. (1995) Protein kinases and phosphatases: the Yin and Yang of protein phosphorylation and signaling. *Cell* 80, 225–236
5. Jenuwein, T. and Allis, C.D. (2001) Translating the histone code. *Science* 293, 1074–1080
6. Stevens, F.J. *et al.* (2000) Protein conformation and disease: pathological consequences of analogous mutations in homologous proteins. *Biochemistry* 39, 15291–15296
7. Nussinov, R. *et al.* (2012) Allosteric post-translational modification codes. *Trends Biochem. Sci.* 37, 447–455
8. Krueger, K.E. and Srivastava, S. (2006) Posttranslational protein modifications: current implications for cancer detection, prevention, and therapeutics. *Mol. Cell. Proteomics* 5, 1799–1810
9. Ahuja, N. *et al.* (2016) Epigenetic therapeutics: a new weapon in the war against cancer. *Annu. Rev. Med.* 67, 73–89
10. Ali, I. *et al.* (2018) Lysine acetylation goes global: from epigenetics to metabolism and therapeutics. *Chem. Rev.* 118, 1216–1252
11. Di Martile, M. *et al.* (2016) Histone acetyltransferase inhibitor CPTH6 preferentially targets lung cancer stem-like cells. *Oncotarget* 7, 11332–11348
12. Merschbaecher, K. *et al.* (2016) Inhibition of different histone acetyltransferases (HATs) uncovers transcription-dependent and -independent acetylation-mediated mechanisms in memory formation. *Learn. Mem.* 23, 83–89
13. Marks, P.A. (2010) The clinical development of histone deacetylase inhibitors as targeted anticancer drugs. *Expert Opin. Investig. Drugs* 19, 1049–1066
14. Falkenberg, K.J. and Johnstone, R.W. (2014) Histone deacetylases and their inhibitors in cancer, neurological diseases and immune disorders. *Nat. Rev. Drug Discov.* 13, 673–691
15. Filippakopoulos, P. *et al.* (2010) Selective inhibition of BET bromodomains. *Nature* 468, 1067–1073
16. Dhalluin, C. *et al.* (1999) Structure and ligand of a histone acetyltransferase bromodomain. *Nature* 399, 491–496
17. Filippakopoulos, P. and Knapp, S. (2014) Targeting bromodomains: epigenetic readers of lysine acetylation. *Nat. Rev. Drug Discov.* 13, 337–356
18. Kwon, D.Y. *et al.* (2017) Locus-specific histone deacetylation using a synthetic CRISPR-Cas9-based HDAC. *Nat. Commun.* 8, 15315
19. Andreoli, F. and Del Rio, A. (2015) Computer-aided molecular design of compounds targeting histone modifying enzymes. *Comput. Struct. Biotechnol. J.* 13, 358–365
20. Hsu, K.-C. *et al.* (2017) Novel class IIa-selective histone deacetylase inhibitors discovered using an *in silico* virtual screening approach. *Sci. Rep.* 7, 3228
21. Rudolf, A.F. *et al.* (2014) A comparison of protein kinases inhibitor screening methods using both enzymatic activity and binding affinity determination. *PLoS One* 9, e98800
22. Bradbury, A.R.M. *et al.* (2011) Beyond natural antibodies: the power of *in vitro* display technologies. *Nat. Biotechnol.* 29, 245–254
23. Gargano, N. *et al.* (1997) From phage libraries to intracellular immunization. In *Intracellular Antibodies*, pp. 173–186, Springer
24. Cattaneo, A. and Biocca, S. (1997) *Intracellular Antibodies: Development and Applications,* Springer
25. Zhong, Q. *et al.* (2009) Edgetic perturbation models of human inherited disorders. *Mol. Syst. Biol.* 5, 321
26. Sahni, N. *et al.* (2013) Edgotype: a fundamental link between genotype and phenotype. *Curr. Opin. Genet. Dev.* 23, 649–657
27. Charloteaux, B. *et al.* (2011) Protein–protein interactions and networks: forward and reverse edgetics. *Methods Mol. Biol.* 759, 197–213
28. Wang, Y. *et al.* (2015) Global edgetic rewiring in cancer networks. *Cell Syst.* 1, 251–253
29. Chirichella, M. *et al.* (2017) Post-translational selective intracellular silencing of acetylated proteins with *de novo* selected intrabodies. *Nat. Methods* 14, 279–282
30. Hattori, T. *et al.* (2013) Recombinant antibodies to histone post-translational modifications. *Nat. Methods* 10, 992–995
31. Koerber, J.T. *et al.* (2013) Nature-inspired design of motif-specific antibody scaffolds. *Nat. Biotechnol.* 31, 916–921
32. Guan, K.-L. *et al.* (2010) Generation of acetyllysine antibodies and affinity enrichment of acetylated peptides. *Nat. Protoc.* 5, 1583–1595
33. Kehoe, J.W. *et al.* (2006) Using phage display to select antibodies recognizing post-translational modifications independently of sequence context. *Mol. Cell. Proteomics* 5, 2350–2363
34. Biocca, S. *et al.* (1995) Redox state of single chain Fv fragments targeted to the endoplasmic reticulum, cytosol and mitochondria. *Biotechnology (N. Y.)* 13, 1110–1115
35. Cattaneo, A. and Biocca, S. (1999) The selection of intracellular antibodies. *Trends Biotechnol.* 17, 115–121
36. Visintin, M. *et al.* (1999) Selection of antibodies for intracellular function using a two-hybrid *in vivo* system. *Proc. Natl. Acad. Sci. U. S. A.* 96, 11723–11728
37. Meli, G. *et al.* (2009) Direct *in vivo* intracellular selection of conformation-sensitive antibody domains targeting Alzheimer's Amyloid-β oligomers. *J. Mol. Biol.* 387, 584–606
38. Visintin, M. *et al.* (2004) Intracellular antibodies for proteomics. *J. Immunol. Methods* 290, 135–153
39. Fantini, M. *et al.* (2017) Assessment of antibody library diversity through next generation sequencing and technical error compensation. *PLoS One* 12, e0177574
40. Guo, D. *et al.* (2004) A tethered catalysis, two-hybrid system to identify protein-protein interactions requiring post-translational modifications. *Nat. Biotechnol.* 22, 888–892
41. Sterner, D.E. and Berger, S.L. (2000) Acetylation of histones and transcription-related factors. *Microbiol. Mol. Biol. Rev.* 64, 435–459
42. Zeng, L. and Zhou, M.M. (2002) Bromodomain: an acetyl-lysine binding domain. *FEBS Lett.* 513, 124–128
43. Pérez-Salvia, M. and Esteller, M. (2017) Bromodomain inhibitors and cancer therapy: From structures to applications. *Epigenetics* 12, 323–339
44. Sharma, S. *et al.* (2010) Epigenetics in cancer. *Carcinogenesis* 31, 27–36
45. Caponigro, F. *et al.* (2016) Phase II clinical study of valproic acid plus cisplatin and cetuximab in recurrent and/or metastatic squamous cell carcinoma of Head and Neck-V-CHANCE trial. *BMC Cancer* 16, 918

46. Gajer, J.M. *et al.* (2015) Histone acetyltransferase inhibitors block neuroblastoma cell growth *in vivo*. *Oncogenesis* 4, e137
47. Andrieu, G. *et al.* (2016) Clinical trials for BET inhibitors run ahead of the science. *Drug Discov. Today Technol.* 19, 45–50
48. Lennartsson, A. *et al.* (2015) Remodeling of retrotransposon elements during epigenetic induction of adult visual cortical plasticity by HDAC inhibitors. *Epigenet. Chrom.* 8, 55
49. Contreras-Leal, E. *et al.* (2016) Histone deacetylase inhibitors promote the expression of ATp2A3 gene in breast cancer cell lines. *Mol. Carcinog.* 55, 1477–1485
50. Eliseeva, E.D. *et al.* (2007) Characterization of novel inhibitors of histone acetyltransferases. *Mol. Cancer Ther.* 6, 2391–2398
51. Witt, O. *et al.* (2009) HDAC family: What are the cancer relevant targets? *Cancer Lett.* 277, 8–21
52. Anamika, K. *et al.* (2010) Lessons from genome-wide studies: an integrated definition of the coactivator function of histone acetyl transferases. *Epigenet. Chrom.* 3, 18
53. Muller, S. *et al.* (2011) Bromodomains as therapeutic targets. *Expert Rev. Mol. Med.* 13, e29
54. Knowles, T.P.J. *et al.* (2014) The amyloid state and its association with protein misfolding diseases. *Nat. Rev. Mol. Cell Biol.* 15, 384–396
55. Meli, G. *et al.* (2014) Conformational targeting of intracellular Aβ oligomers demonstrates their pathological oligomerization inside the endoplasmic reticulum. *Nat. Commun.* 5, 3867
56. Tanaka, T. *et al.* (2007) Tumour prevention by a single antibody domain targeting the interaction of signal transduction proteins with RAS. *EMBO J.* 26, 3250–3259
57. Nizak, C. *et al.* (2003) Recombinant antibodies to the small GTPase Rab6 as conformation sensors. *Science* 300, 984–987
58. Haque, A. *et al.* (2011) Conformation-sensing antibodies stabilize the oxidized form of PTP1B and inhibit its phosphatase activity. *Cell* 147, 185–198
59. Fernández-Medarde, A. and Santos, E. (2011) Ras in cancer and developmental diseases. *Genes Cancer* 2, 344–358
60. Biocca, S. *et al.* (1994) Intracellular immunization with cytosolic recombinant antibodies. *Biotechnology* 12, 396–399
61. Pérez-Martínez, D. *et al.* (2010) Intracellular antibodies and cancer: new technologies offer therapeutic opportunities. *Bioessays* 32, 589–598
62. Tanaka, T. and Rabbitts, T.H. (2010) Interfering with RAS–effector protein interactions prevent RAS-dependent tumour initiation and causes stop–start control of cancer growth. *Oncogene* 29, 6064–6070
63. Capsoni, S. *et al.* (2000) Alzheimer-like neurodegeneration in aged antinerve growth factor transgenic mice. *Proc. Natl. Acad. Sci. U. S. A.* 97, 6826–6831
64. Capsoni, S. *et al.* (2012) Intranasal "painless" human nerve growth factors slows amyloid neurodegeneration and prevents memory deficits in App X PS1 mice. *PLoS One* 7, e37555
65. Capsoni, S. *et al.* (2011) A dual mechanism linking NGF/proNGF imbalance and early inflammation to Alzheimer's disease neurodegeneration in the AD11 anti-NGF mouse model. *CNS Neurol. Disord. Drug Targets* 10, 635–647
66. Müller, M. *et al.* (2012) Loss of NGF-TrkA signaling from the CNS is not sufficient to induce cognitive impairments in young adult or intermediate-aged mice. *J. Neurosci.* 32, 14885–14898
67. Sato, Y. *et al.* (2013) Genetically encoded system to track histone modification *in vivo*. *Sci. Rep.* 3, 1–7
68. Siciliano, V. *et al.* (2018) Engineering modular intracellular protein sensor-actuator devices. *Nat. Commun.* 9, 1881
69. Melchionna, T. and Cattaneo, A. (2007) A protein silencing switch by ligand-induced proteasome-targeting intrabodies. *J. Mol. Biol.* 374, 641–654
70. Gross, G.G. *et al.* (2016) An E3-ligase-based method for ablating inhibitory synapses. *Nat. Methods* 13, 673–678
71. Lu, J. *et al.* (2015) Hijacking the E3 ubiquitin ligase cereblon to efficiently target BRD4. *Chem. Biol.* 22, 755–763
72. Carmony, K.C. and Kim, K.-B. (2012) PROTAC-induced proteolytic targeting. *Methods Mol. Biol.* 832, 627–638
73. Clift, D. *et al.* (2017) A method for the acute and rapid degradation of endogenous proteins. *Cell* 171, 1692–1706
74. Tse, E. and Rabbitts, T.H. (2000) Intracellular antibody-caspase-mediated cell killing: an approach for application in cancer therapy. *Proc. Natl. Acad. Sci. U. S. A.* 97, 12266–12271
75. Liao, X. *et al.* (2014) Delivery of antibody mimics into mammalian cells via anthrax toxin protective antigen. *ChemBioChem* 15, 2458–2466
76. Verdurmen, W.P.R. (2015) Efficient cell-specific uptake of binding proteins into the cytoplasm through engineered modular transport systems. *J. Control. Release* 200, 13–22
77. Choi, D.-K. *et al.* (2014) A general strategy for generating intact, full-length IgG antibodies that penetrate into the cytosol of living cells. *MAbs* 6, 1402–1414
78. Herce, H.D. *et al.* (2017) Cell-permeable nanobodies for targeted immunolabelling and antigen manipulation in living cells. *Nat. Chem.* 9, 762–771
79. Akishiba, M. *et al.* (2017) Cytosolic antibody delivery by lipid-sensitive endosomolytic peptide. *Nat. Chem.* 9, 751–761
80. Mallery, D.L. *et al.* (2010) Antibodies mediate intracellular immunity through tripartite motif-containing 21 (TRIM21). *Proc. Natl. Acad. Sci. U. S. A.* 107, 19985–19990
81. Verdurmen, W.P.R. *et al.* (2017) A quantitative comparison of cytosolic delivery via different protein uptake systems. *Sci. Rep.* 7, 13194
82. Tanaka, T. and Rabbitts, T.H. (2008) Interfering with protein–protein interactions: potential for cancer therapy. *Cell Cycle* 7, 1569–1574
83. Quevedo, C.E. *et al.* (2018) Small molecule inhibitors of RAS-effector protein interactions derived using an intracellular antibody fragment. *Nat. Commun.* 9, 3169