

Live and Let Die: insights into pseudoenzyme mechanisms from structure

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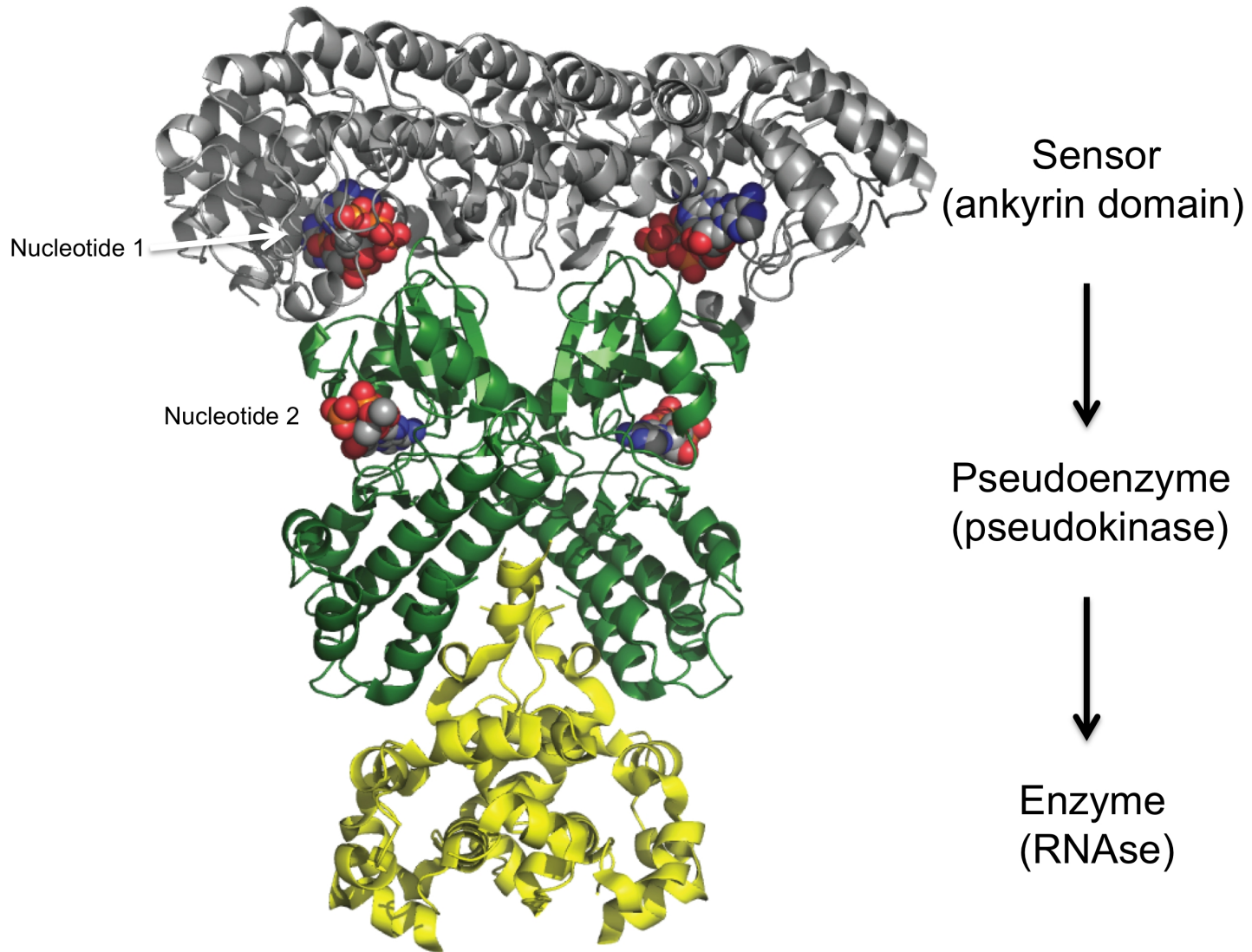
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Abstract

Pseudoenzymes were first described more than 50 years ago, when it was recognised that a subset of proteins that are structurally homologous to active enzymes lack amino acids necessary for catalytic activity. Recently, interest in pseudoenzymes has surged as it has become apparent that they constitute ~10% of proteomes and perform essential metabolic and signalling functions that can be experimentally distinguished from catalytic outputs of enzymes. Here, we highlight recent structural studies of pseudoenzymes, which have revealed the molecular basis for roles as allosteric regulators of conventional enzymes, as molecular switches and integrators, as hubs for assembling protein complexes, and as competitors of substrate availability and holoenzyme assembly. As structural studies continue to illuminate pseudoenzyme molecular mechanisms, we anticipate that our knowledge of the breadth of their biological functions will expand in parallel.

RNase L dimer



Introduction

Genomic sequencing of many organisms confirms that nearly all enzyme families include pseudoenzyme homologues, which are predicted to be enzymatically inactive due to the loss of key catalytic amino acid residues. Pseudoenzymes are already known to be conserved in ~20 different protein families [1], including well-studied examples of pseudokinases, pseudophosphatases and pseudoproteases [2-6]. In these families, incremental changes in catalytic and substrate-binding sites created new evolutionary trajectories that led to the evolution of pseudoenzymes from enzyme templates sharing a similar fold [7,8]. Although an absence of conserved catalytic residues is not proof of catalytic deficiency, very high sequence and/or structural conservation suggests that pseudoenzymes have been functionally selected across all branches of life, and preserved to regulate cell biology in a catalytically-independent manner. Although pseudoenzymes comprise a significant percentage of proteomes, we understand little about individual classes relative to their enzyme counterparts. However, since much of what we know about pseudoenzymes arose from structural studies, and the fold of proteins provides clues to functions, this review will focus on key examples that help illustrate the general pseudoenzyme principles underlying specialised non-catalytic functions.

Four classes of biological pseudoenzyme mechanism

In terms of predicted and known mechanisms, pseudoenzymes fall into four major classes (Figure 1). The first of these, for which a number of examples are known in the protein kinase, phosphatase and ubiquitination fields, still retain enzyme-like architecture, but have evolved an ability to regulate a catalytically active partner that generates a biological output in signalling or metabolism (Figure 1a). The second class acts as “switches” that integrate signals in the form of post-translational modifications or binding to metabolic ligands, which trigger interconversion between inactive and active conformations (Figure 1b). The third category appears to have gained new functions as protein interaction modules through structural specialisation, where they can act as cellular scaffolds to nucleate the assembly of protein complexes or regulate the localization or trafficking of a binding partner (Figure 1c). The fourth category has repurposed canonical features of a protein fold that is shared with active enzyme relatives to act as competitors for either substrate binding (as catalytic ‘traps’) or higher order complex assembly (Figure 1d).

How has structure illuminated the molecular mechanisms of pseudoenzymes?

Building upon a flood of genomic data, structural studies have provided key insights into the mechanisms by which pseudoenzymes operate as protein (and ligand) interaction domains to elicit biological responses. Additionally, pseudoenzyme structures have been instrumental in clarifying the molecular basis for deficient catalytic activity, whether it be: loss of canonical catalytic residues; loss of cofactor binding; loss of allosteric regulatory potential; occlusion of the active site by sequences divergent from those in an active enzyme counterpart; or active site blockade by non-canonical appendages. Importantly, while conventional enzymes are best understood for their catalytic functions, structural studies of pseudoenzymes are also providing an avenue to help understand non-catalytic functions of catalytically active enzymes, and in so-doing, are uncovering new strategies for therapeutic intervention.

1. Allosteric Activators

A conceptually simple mechanism that helps explain the prevalence of pseudoenzymes in biology is the finding that, upon binding, a pseudoenzyme can often impact a conventional enzyme's activity. The best characterised examples of such allostery involve pseudoenzymes regulating a structurally-related enzyme counterpart, as exemplified by several pseudokinases (Figure 2a) [9-13], and the regulation of the protease Caspase-8 by the pseudoprotease FLIP (Figure 2b) [14]. Multiple cases of domain duplication have led to tandem pseudoenzyme-enzyme domain architectures within the same polypeptide—for example, kinases like JAK1-3 and TYK2 (Figure 2a), the ATPase EccC (addressed in scaffolds section below) and the GTPase p190RhoGAP—in which a pseudoenzyme fold is sandwiched between neighbouring catalytic GTPase and GAP domains.

Further examples of allosteric regulation between inactive and active enzyme homologues lie in the ubiquitin system, particularly the Really Interesting New Gene (RING) proteins that comprise the largest class of ubiquitin ligase. There are numerous examples of RING heterodimers where one component has a functional binding site for a ubiquitin-conjugating (E2) enzyme, and one inactive RING cannot bind E2. Perhaps the best known is MDM2-MDMX, where the active RING MDM2

can form a functional complex with MDMX to promote ubiquitination of p53. MDMX plays this role because it retains a conserved C-terminal hydrophobic residue present in active RINGs, which is essential for stabilisation of the catalytic complex [15]. Additional examples include the BRCA1–BARD1 RING complex [16]; and Polycomb Repressive Complexes (Figure 2c), where an active RING1a/b component is able to partner with one of 6 different PCGF inactive RINGs (PCGF1–6). In addition to stabilising the catalytic complex, varying pseudoenzyme PCGF RINGs participate in substrate recognition and can modulate the intrinsic catalytic rate of their respective complexes [17,18]. On the flip-side to ubiquitin conjugation by RINGs, ubiquitin removal by the BRCC36 deubiquitinase is controlled by pseudoDUBs of the KIAA0157 family. KIAA0157 is essential for assembly of a heterotetrameric BRC366–KIAA0157 complex, in which BRC366 becomes catalytically active and the catalytic sites of BRC366 are ideally arranged to act upon their preferred Lys₆₃-linked ubiquitin substrate (Figure 2d; [19]).

The frequency of pseudoenzymes regulating structurally-similar enzymes has been proposed to arise from gene duplications that liberate the second gene from selective pressures that normally ensure it catalyzes a chemical reaction, therefore encouraging regulatory specialisation [7,8]. Interestingly, many enzymes are now appreciated to possess ‘pseudoenzyme’ modes of allostery, where the catalytic potential of the enzyme is overridden in favour of a pseudoenzyme-like conformational output for a biological function. Examples include a non-enzyme scaffolding function of Caspase-8 in the immune system [20], and a non-catalytic function for the conformationally-flexible canonical kinase Aurora A as an N-Myc regulator in neuroblastoma [21].

2. Signal integrators/molecular switches

Modification of, or ligand binding to, pseudoenzymes can enable them to act as “receivers” of information from upstream regulators to control a downstream output. Structural and biochemical studies of the nucleotide-binding pseudokinase, Mixed lineage kinase domain-like (MLKL; Figure 3a), are illustrative of how post-translational modification, in this case phosphorylation of the pseudokinase domain activation loop by the upstream activator kinase, RIPK3, can toggle a molecular switch to induce a downstream effector function [22,23]. MLKL phosphorylation is proposed to induce a conformational change in the pseudokinase domain that

relieves a suppressive interaction between the pseudokinase domain and the N-terminal four-helix bundle domain (Figure 3a). Release of the latter permits MLKL oligomerisation, membrane translocation and death of a cell by the regulated death pathway, necroptosis [24].

In addition to covalent modifications, molecular switch functions could analogously be imparted upon ligand binding to pseudoenzymes. Structural studies of ADCK3 (COQ8A), considered a pseudokinase because of a contorted active site that confers a preference for ADP over ATP [25], have revealed the importance of nucleotide binding to its function. While precise details are still emerging, nucleotide binding to ADCK3 promotes assembly of a functional Coenzyme Q biosynthetic holoenzyme and, furthermore, induces conformational changes that expose putative lipid binding pockets adjacent to the substrate-occluding N-terminal helices (Figure 3b; [26]).

Ligand binding has also been shown to exert subtle switching effects on pseudoenzymes that are not always clear from crystal structures. In the case of RNaseL, binding of nucleotide to the pseudokinase domain and/or the cyclic nucleotide second messenger, 2',5'-oligoadenylate (2-5A), to a channel between the N-terminal ankyrin repeat and pseudokinase domains promotes activity of the C-terminal nuclease domains within the RNaseL homodimer (Figure 3c; [27]). Because *apo* and ligand-bound crystal structures are not obviously different, based on solution scattering data it was proposed that the role of nucleotide binding was to lock the pseudokinase domain into a closed conformation to facilitate nuclease activity [27]. These data suggest that ATP binding by the related Ire1, which contains a conventional protein kinase domain rather than a pseudokinase domain [28], may similarly serve a conformational role to augment nuclease activity. While these examples are illustrative of the propensity of some pseudoenzyme functions to be tuned by modification or ligand binding, not all pseudoenzymes are expected to undergo conformational switching; biochemical studies suggest that fewer than half of all pseudokinases retain binding to conventional nucleotides [29,30].

3. Scaffolds for assembly of protein complexes

Several multimeric complex structures have provided insights into how pseudoenzymes assemble more than one partner simultaneously to create higher order scaffolds or “hubs”. The pseudokinase PAN3 provides an interesting case in

point. PAN3 forms an asymmetric dimer [31], which scaffolds the assembly of a higher order complex with the mRNA deadenylation enzyme, PAN2, which itself contains a pseudo-ubiquitin C-terminal hydrolase (UCH) domain (Figure 3d). Beyond simply scaffolding complex formation, PAN3 abuts the PAN2 RNase domain within the complex and promotes RNase activity, whilst the pseudokinase ATP-binding site recruits poly(A) substrates to the holoenzyme [32].

The Tribbles (TRIB) family of proteins (and the homolog SgK495/STK40) also employ an atypical pseudokinase domain to bring a catalytic enzyme into proximity of its substrate. In this case, a ternary complex is formed between TRIB1, COP1 ubiquitin ligase and the C/EBP substrate, which is appropriately positioned for ubiquitination [29,33,34]. The structure of TRIB1 has demonstrated that its C-terminal tail, which binds COP1, can also self-associate with the pseudokinase domain *in cis* [29], likely through a mutually exclusive mechanism (Figure 3e; [35]). While the structural mechanism is still not clear, it appears that formation of a Substrate–TRIB1–COP1 holocomplex must involve an evolutionary imprinted conformational change in the TRIB1 pseudokinase, which is likely conserved in all eukaryotic Tribbles proteins [32]. Another pseudokinase, KSR2, serves as a hub to orchestrate Raf communication with the effector kinase, MEK1. KSR2 is able to activate the RAF kinases via a “back-to-back” dimer interface (Figure 2a), but also bind to MEK1 through a “face-to-face” interaction to promote phosphorylation of MEK1 by RAF [36].

In some cases, including the EccC family of hexameric ATPases (see below) and the RBR ubiquitin ligases, pseudoenzyme domains are embedded in the context of tandem arrays with conventional enzyme counterparts, where they have evolved dual functions as both hubs and as allosteric regulators. In RBR ubiquitin ligases, a benign-catalytic region that lacks a catalytic cysteine residue (BRcat; also known as IBR for in-between-RING), lies adjacent to a catalytic RBR RING domain, [37]. Structures of autoinhibited and active RBR ligases (Parkin and HOIP, respectively) have demonstrated important roles that BRcat plays in each state. In inactive Parkin, the BRcat stabilises an autoinhibited conformation that blocks the active site, whereas in active HOIP the BRcat contacts the E2-Ub conjugate and binds an additional ubiquitin that stabilises the catalytically competent complex [38-40].

The EccC protein, a type VII secretion ATPase of bacterial pathogens, is composed of an array of three linked ATPase domains, two of which are pseudoenzymes lacking Walker A Glu residues, permitting them to bind, but not

hydrolyse ATP (Figure 3f; [41]). EsxB, a secreted substrate of EccC, is instrumental in the ordered assembly and activation of the active complex. EsxB binding by the terminal pseudoATPase domain simultaneously promotes release of autoinhibition between the ATPase domain and central pseudoATPase, and drives multimerization and activation of the hexameric holoenzyme (Figure 3f; [41]).

PseudoGTPases are particularly abundant throughout nature [1], and structural insights into their scaffolding and allosteric functions are eagerly awaited. Such structures are already proving important to enhance our understanding of how pseudoGTPases such as the kinetochore-regulating scaffold CENP-M, which is unable to bind GTP, functions to assemble and regulate a multi-protein complex that recruits CENP-T/W proteins to the kinetochores of metazoan chromosomes [42].

4. Competition for substrate or enzyme binding

Lastly, examples of pseudoenzymes that act as competitors in various guises have been identified (Figure 4). The structural capacity of some pseudoenzymes to sequester substrates is vividly illustrated by the pseudo-chitinase YKL-39 (Figure 4a; [43,44]). YKL-39 lacks the essential Glu within the DxxDxDxE catalytic motif, meaning the protein can bind chito-oligosaccharides with a nanomolar affinity, but not hydrolyse them, and thus sequester them away from catalytically-active counterparts (or immunological receptors) to prevent processing. Although structures are yet to be reported, another competitive mode of action is illustrated by pseudophosphatases (Figure 4b), which bind substrates with high affinity to antagonize conventional enzymes. In the case of the pseudophosphatases, EGG-4 and EGG-5, interaction with the phosphorylated activation loop of the protein kinase MBK-2 prevents regulatory dephosphorylation [45,46]. A related mode of action is exemplified by the pseudophosphatase STYX, which has been shown to compete with the dual-specificity phosphatase DUSP4 for binding to the canonical protein kinases, ERK1 and ERK2 [47]. As a result, STYX binding precludes DUSP4 engagement and ERK1/2 dephosphorylation and inactivation, which is likely to be important in cells, where compartmentalization of the ERK module regulates transforming potential [48]. Because these substrate “traps” could evolve from simple loss of catalytic functions, we anticipate this group is abundant in nature. This idea is supported by the impressive array of pseudophosphatase domains within the myotubularin and receptor tyrosine phosphatase families in higher eukaryotes [5].

In addition to substrate competition, enzyme sequestration represents another mode of pseudoenzyme action. This is exemplified by a naturally-occurring, catalytically-defective variant of aldehyde dehydrogenase-2 (ALDH2), termed ALDH2*2, which can compete with the catalytically-active counterpart, ALDH2*1, to 'poison' complex assembly into an active homo-tetramer, thus inhibiting activity (Figure 4c; [49]).

Conclusions

In this review, we have sought to illustrate the diversity of mechanisms underlying pseudoenzyme functions as revealed from recent structural studies. An underlying theme is that pseudoenzymes behave as protein interaction modules, whether in the guise of allosteric regulators, signal integrators, nucleators of protein complex assembly or as substrate competitors with conventional enzymes. It is also becoming clear that pseudoenzymes can often perform several of these functions simultaneously. We expect that future multidisciplinary studies will reveal new and unexpected modes of action of pseudoenzymes, which will in turn expand knowledge of the repertoire of non-catalytic functions that may be performed by conventional enzymes.

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Figure Legends

Figure 1. Modes of pseudoenzyme function

Pseudoenzymes (green) exert their effects on signal transduction or metabolism through interactions with other proteins, including client enzymes (grey), or substrates (yellow).

(a) Allosteric binding of a pseudoenzyme can positively or negatively regulate the catalytic activity of a conventional enzyme client.

(b) Pseudoenzyme domains can act as receivers for post-translational modifications, such as (de)ubiquitylation, (de)phosphorylation or proteolytic cleavage, and can promote conformational switching and effector functions.

(c) As protein interaction domains, pseudoenzymes can nucleate the assembly of protein complexes to bring enzyme and substrate pairs into proximity (top), or regulate protein localization, stability or quality control in a particular trafficking pathway or organelle (bottom).

(d) Pseudoenzymes can compete with conventional enzymes to prevent assembly of higher order protein complexes (top), or sequester substrates to protect them from enzymatic processing (bottom).

Figure 2. Allosteric regulation of active enzymes

(a) *Stabilisation of active kinases by pseudokinase partners.* Shown are complexes between KSR–BRAF (based on superposition of KSR2 onto the BRAF homodimer; PDBs 5kk7 and 3og7; [50] and [51] respectively), the crystal structure of HER3 pseudokinase bound to the EGFR kinase domain (PDB 4riw; [9]), and the TYK2 pseudokinase–kinase complex (PDB 4oli; [10]). PsK=pseudokinase, K=kinase.

(b) *Regulation of Caspase-8 activity by a pseudocaspase partner.* Structure of the complex between proCaspase-8 (grey) and FLIP (green) (PDB 3h11; [14]). The intersubunit linker of Caspase-8, which undergoes proteolysis upon activation, is not visible in the crystal structure but is represented by a dotted line.

(c) *Active–inactive RING heterocomplexes regulate RING activity.* Model of the putative RING1b–Bmi1–Histone–E2–Ub substrate complex, based on the structure from PDB 4r8p; [17]. Ubiquitin is modelled by superposition of primed E2–Ub

conjugate from PDB 4ap4; [52]. The non-E2 binding Bmi1 RING domain stabilises the E2-Ub conjugate in the closed conformation primed for attack by a substrate lysine from the histone substrate.

(d) *DUB regulation within a DUB-pseudoDUB complex.* Structure of the complex between BRCC36 (grey surface) and the KIAA0157 pseudoDUB (green cartoon) (PDB 5cw3; [19]). A putative Lys₆₃-linked Ub substrate is positioned by superposition into the active sites of BRCC36 (based on PDB 2zmv; [53]), which demonstrates the compatibility of the tetrameric BRCC36–KIAA0157 complex with its preferred substrate.

Figure 3. Assembly of signalling complexes

(a) *The MLKL pseudokinase.* MLKL is thought to exist in a basal state (left) where the N-terminal four-helix bundle domain is sequestered by the C-terminal pseudokinase domain (green). Upon phosphorylation of the pseudokinase domain activation loop, MLKL is proposed to undergo a conformational change (middle; PDB 4btf; [22]), leading to exposure of the four-helix bundle, oligomerisation, membrane translocation, permeabilisation of plasma membranes and cell death (right).

(b) *ADCK3 pseudokinase.* The human mitochondrial UbiB pseudoenzyme ADCK3 is the first ancient UbiB protein kinase-like (PKL) family member to be characterised structurally [25,26], revealing UbiB-specific features that prevent canonical protein kinase activity (PDB 5i35). These include distinct N-terminal helical extensions that occlude the substrate-binding pocket (grey cartoon).

(c) *RNase L pseudokinase.* RNase L is a homodimeric assembly containing ankyrin repeat domains linked to dual pseudokinase-ribonuclease domains. The pseudoenzyme domain (green) contains non-canonical adaptations in both N and C-lobes, which prevent catalytic activity [54]. RNase L drives the IFN-induced antiviral response in humans, and is activated by the 2',5'-oligoadenylate (2-5A) second messenger, which binds in a cleft between the ankyrin repeats and pseudokinase domain. Crystal structures of RNase L (PDBs 4o1o and 4o1p) demonstrate how 2-5A and the pseudokinase region to drive the RNase domain (yellow) into an enzyme conformation compatible with catalysis [27].

(d) *PseudoDUB and pseudokinase modules orient activity of the PAN2/3 complex.* The PAN2/3 complex is shown with the dimer of PAN3 in cartoon representation, and

PAN2 as a surface (PDB 4q8j; [32]). The pseudokinase domains of PAN3 are shown in green, with the nucleotide-binding site (proposed to bind to polyA tails) indicated with spheres. The PseudoDUB domain of PAN2 (green) makes extensive contacts with the RNase domain that contains the deadenylase active site (indicated with a black sphere).

(e) *Pseudokinase-based assembly of an E3-ligase complex.* The C-terminal COP1-binding motif of TRIB1 binds to the N-terminal lobe of the pseudokinase domain (left-most structure, based on PDB 5cem [29]), but upon substrate binding must be dislodged from the pseudokinase domain to be recruited into a complex with the ubiquitin E3-ligase COP1 (right; TRIB1 tail–COP1 complex based on PDB 4igo [35]). Bound substrate is represented with a yellow line.

(f) *Oligomerisation of an active hexameric ATPase via pseudo-ATPase modules.* EccC is comprised of an N-terminal active ATPase (grey) with two N-terminal pseudo-ATPase domains (green). The C-terminal pseudo-ATPase domain of EccC binds to peptides, which themselves emanate from dimeric substrates, and hence promote association of the active EccC ATPase hexamer [41].

Figure 4. Competition for substrate or enzyme binding

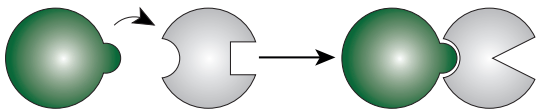
(a) *Sequestration of chito-oligosaccharides by the pseudochitinase, YKL-39.* The crystal structure of YKL-39 has been solved bound to chito-oligosaccharides of varying residue length [43,44], the longest being polymeric GlcNAC₆ (yellow) (PDB 4p8x).

(b) *Pseudophosphatase binding to phosphorylated canonical kinases occludes conventional phosphatases.* Left, binding of the pseudophosphatase, EGG-4 or EGG-5, shields the activation loop of the kinase MBK-2 from dephosphorylation [45,46]. Right, pseudophosphatase STYX regulates ERK activation by competing with the conventional phosphatase, DUSP4, for substrate binding [47].

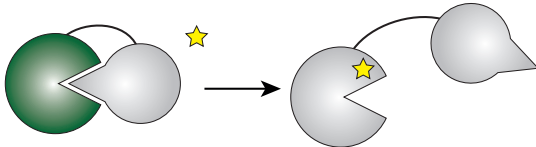
(c) *Pseudodehydrogenase can block assembly higher order enzyme or signalling complexes.* Left, schematic of how the catalytically-defective variant, ALDH2*2, might compete with the active paralog, ALDH2*1, to prevent assembly of, and allosteric activation within, an ALDH2*1 homo-tetramer [49]. Right, homo-tetrameric structures of ALDH2*2 (green; PDB 1zum) and ALDH2*1 (grey; PDB 1nzz).

Figure 1

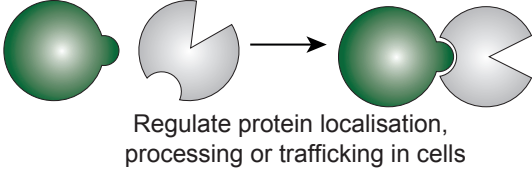
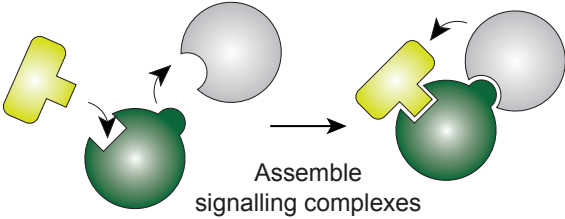
(a) Allosteric regulation of active enzyme



(b) Signal integrator/molecular switch



(c) Nucleate assembly of protein complexes



(d) Competition for holoenzyme assembly or substrates

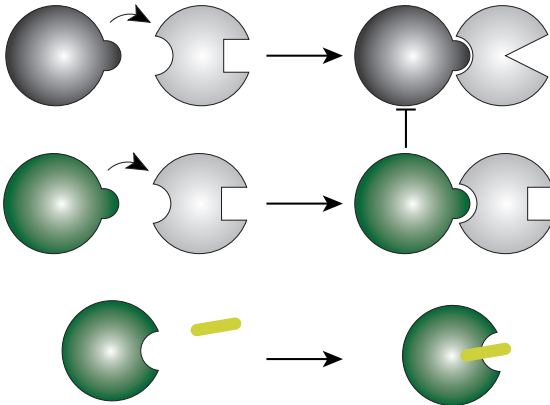
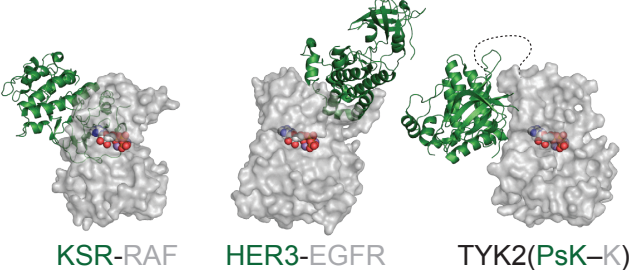
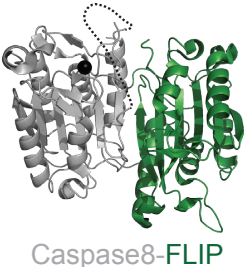


Figure 2

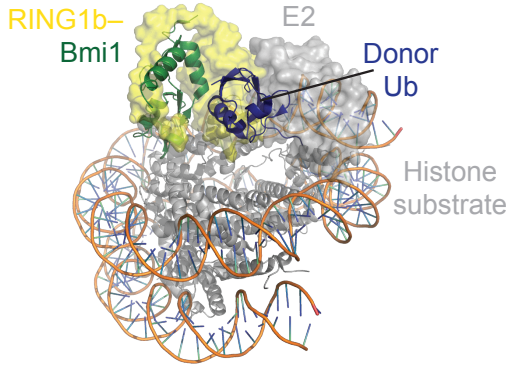
(a) Kinase allosteric stabilisation of active site



(b) Caspase stabilisation of active site across dimer



(c) RING E3 ligase stabilisation of primed E2-Ub conjugate



(d) DUB Allosteric stabilisation of active site via oligomerisation

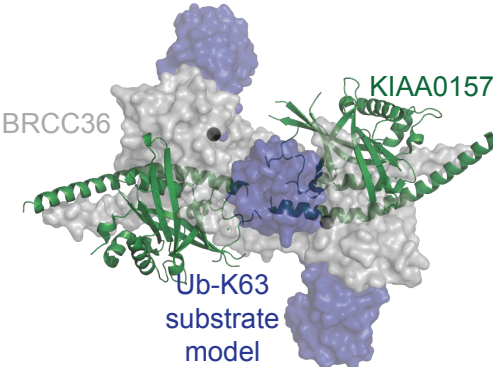
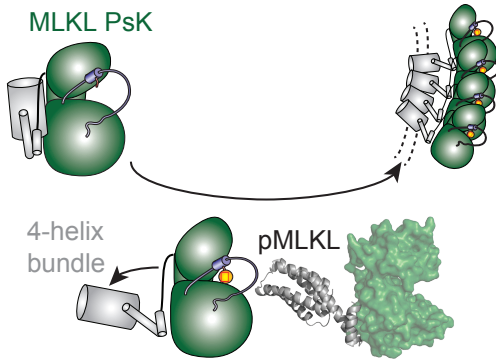
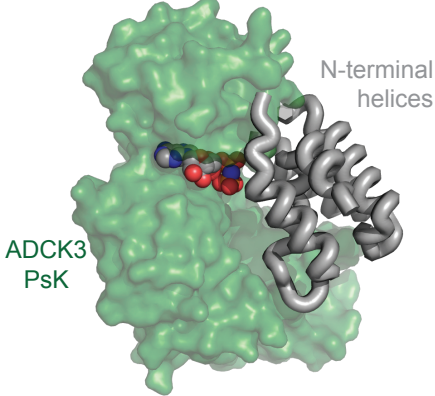


Figure 3

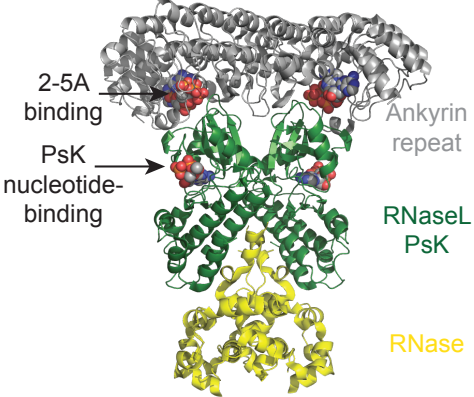
(a) MLKL phosphorylation-based switch



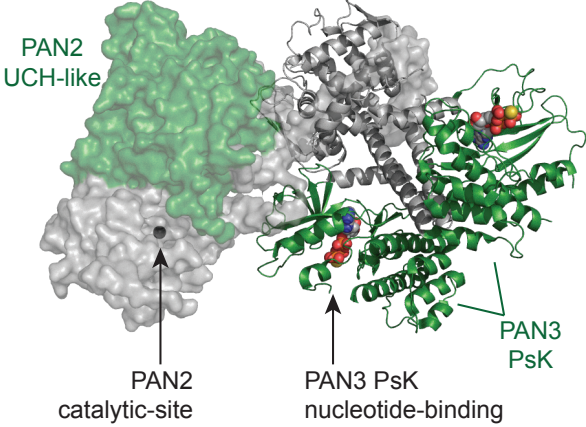
(b) Nucleotide-sensitive binding switch



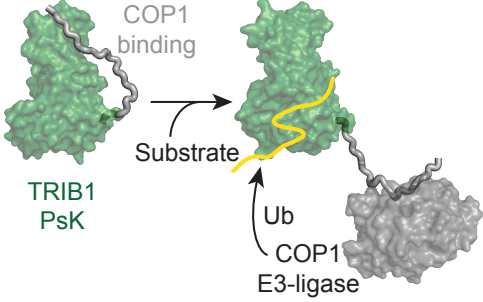
(c) Nucleotide-based RNase activation



(d) PAN2/3 deadenylase complex assembly



(e) TRIB1-COP1 E3-ligase assembly



(f) EccC ATPase assembly

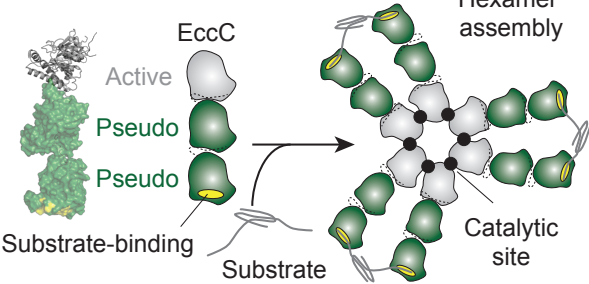
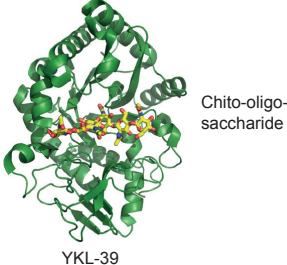
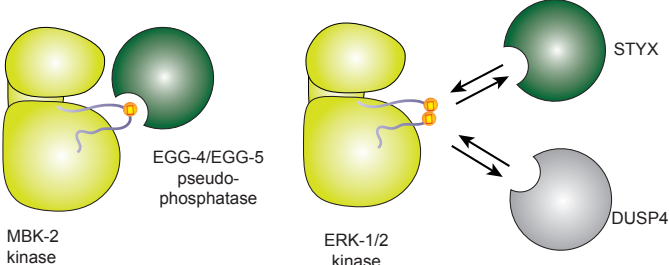


Figure 4

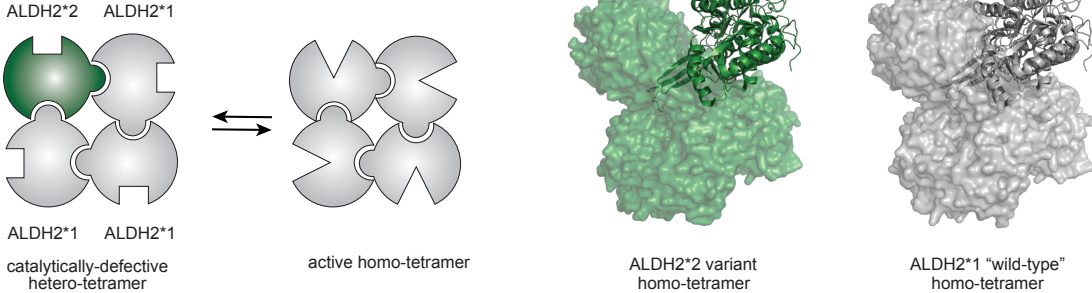
(a) Substrate sequestration



(b) Substrate occlusion



(c) Block holoenzyme assembly



Highlights:

Pseudoenzymes are structural, but catalytically-defective, homologs of enzymes

Examples of pseudoenzymes have been identified in all kingdoms of life

Pseudoenzymes regulate metabolic and signalling enzymes and processes

They function allosterically, as signal integrators, scaffolds, and competitors

Structural biology is helping to drive a 'pseudoenzyme revolution'