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AAA+ ATPases are a diverse protein superfamily which power a vast number of cellular processes, from protein degradation to genome replication and ribosome biogenesis. The latest advances in cryo-EM have resulted in a spectacular increase in the number and quality of AAA+ ATPase structures. This abundance of new information enables closer examination of different types of structural insertions into the conserved core, revealing discrepancies in the current classification of AAA+ modules into clades. Additionally, combined with biochemical data, it has allowed rapid progress in our understanding of structure-functional relationships and provided arguments both in favour and against the existence of a unifying molecular mechanism for the ATPase activity and action on substrates, stimulating further intensive research.

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Introduction: the AAA+ domain architecture

AAA+ ATPases are widely used by cells as motors to power mechanical work or to act as molecular switches or scaffolds, often as parts of macromolecular machines [1–4]. The universal AAA+ ATPase module is composed of two subdomains [4–6]. The N-terminal 'large' αβα subdomain belongs to the ASCE group of P-loop NTPases, and is built around a central 5-strand β-sheet carrying the highly conserved Walker A and Walker B motifs, as well as sensor 1 (S1) and arginine finger (R-finger) residues (Figure 1a,b). The Walker A motif (i.e. the P-loop) stabilises ATP binding while Walker B coordinates an ATP-bound magnesium ion and provides the catalytic glutamate that, together with the polar S1 residue, primes a water molecule for ATP hydrolysis. Oligomerisation of AAA+ ATPases completes the ATP binding pocket, with most AAA+ proteins forming ring-shaped hexamers (Figure 1c). Oligomerisation allows the R-finger to act *in trans*, contacting the γ-phosphate of the ATP molecule bound to the anticlockwise neighbouring subunit during hydrolysis (Figure 1d). In most AAA+ ATPases, the large subdomain is fused to a 'small' C-terminal α-helical lid subdomain that closes over the nucleotide binding site and mediates oligomeric assembly. This subdomain often contributes a second arginine residue called sensor 2 (S2) to the ATP binding site, which acts either *in cis* or *in trans* depending on the AAA+ ATPase family (Figure 1d). ATPase activity results in relative movements between the large and the small subdomains, which are propagated within the oligomeric AAA+ assembly and transduced to the functional target.

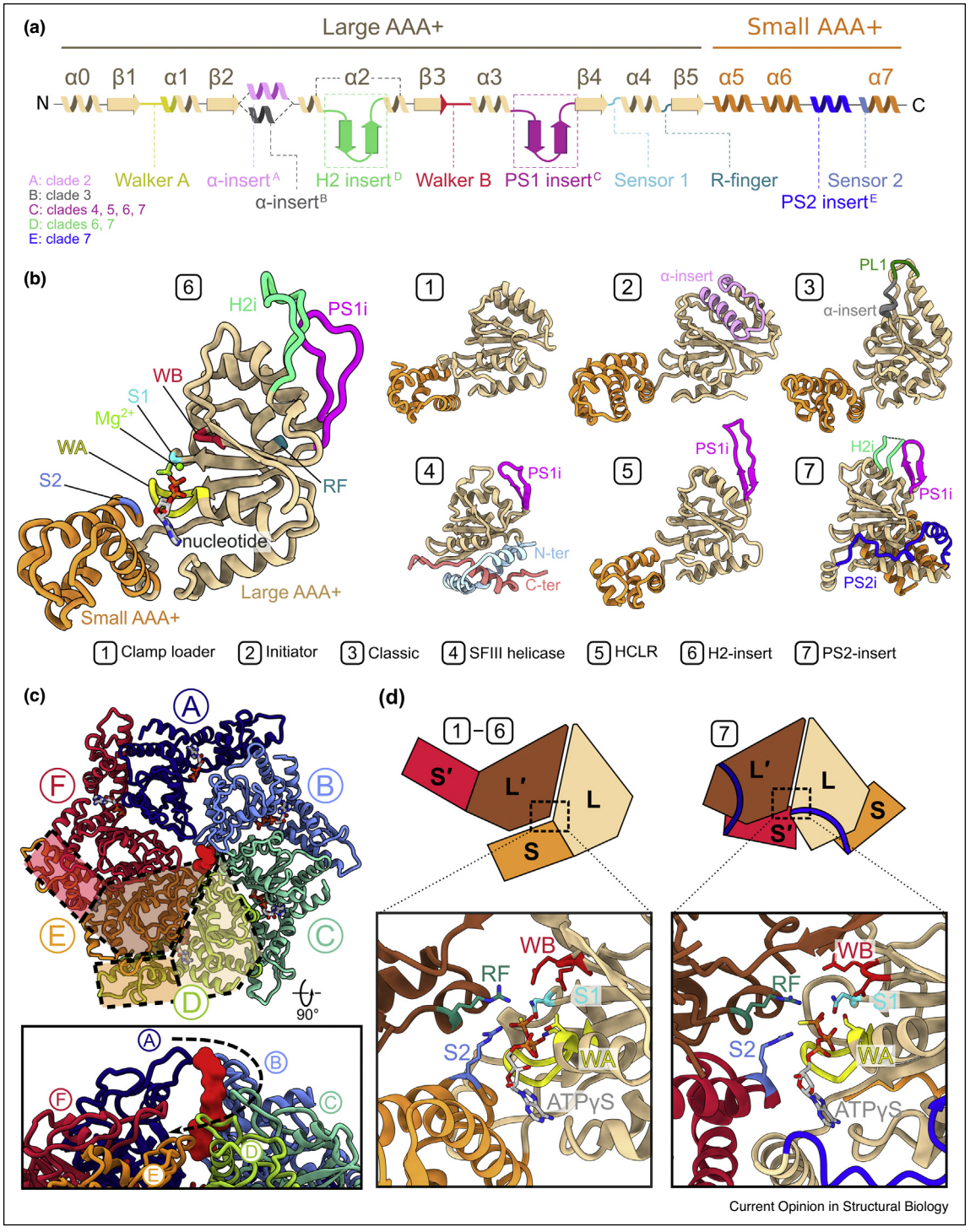
The name 'AAA+ (ATPases associated with various cellular activities)' highlights the remarkable diversity of this protein superfamily, which is due to the fact that the conserved AAA+ ATPase module can be appended to a plethora of different accessory domains conferring a vast number of functions [7••]. The most common function of AAA+ proteins is the ATP hydrolysis-driven translocation of protein or DNA substrates through the central hexameric channel. Often assisted by additional domains, cofactors, and binding partners, protein translocation by AAA+ ATPases results in target unfolding, disassembly and remodelling, whereas DNA translocation leads to unwinding during replication and transcription or packaging of viral genomes.

Classification of AAA+ ATPases into clades

In an effort to infer evolutionary relationships and common functional and mechanistic principles between AAA+ ATPases, AAA+ modules were classified into seven distinct clades based on sequence and structural information available in 2004–2006 [3–5,8]. This analysis revealed that in addition to N-terminal and C-terminal accessory domains, the functions of AAA+ proteins are fine-tuned by insertions of specific structural elements in the conserved AAA+ core. Each clade is defined as an evolutionary lineage and is further subdivided into protein families. A summary of the structural features defining the AAA+ clades is presented in Figure 1a,b.

Clades 1 and 2 are mostly non-hexameric AAA+ proteins and contain DNA polymerase clamp loaders and

Figure 1



Overview of the AAA+ ATPase structure. **(a)** Overview of secondary structural features and key sequence motifs. The locations of clade-specific insertions in the AAA+ core are indicated (except for the N-terminal and C-terminal helices of Clade 4). **(b)** 3D structures of representative monomers from each of Clade 1–7. Clade 1 = RFC3 (PDB ID: 1SXJ), Clade 2 = Orc5 (PDB ID: 5V8F), Clade 3 = ClpA-NTD (PDB ID: 6UQE), Clade 4 = E1 helicase (PDB ID: 2V9P), Clade 5 = RuvB (PDB ID: 1HQC), Clade 6 = NtrC1 (PDB ID: 4LY6), Clade 7 = RavA (PDB 3NBX). The Clade 6 NtrC1 structure is enlarged to show the location of key motifs in (a). Key motifs and insertions are coloured as in (a), as well as the Clade 3 pore loop 1 (PL1) coloured dark green. **(c)** Hexameric structure of LonA (PDB ID: 6ON2) with monomers A–F coloured individually and bound substrate shown as a red surface in the centre of the hexameric ring. Dotted boxes are shown over two adjacent monomers, coloured as in (d). A side view (below) focussed on centre of the hexameric ring show a spiral staircase arrangement of pore loops around the substrate. **(d)** Overview of the ATP

DNA-replicative helicase loaders respectively; the first is the 'archetypal' AAA+ domain, whereas the second has an α-helical insertion between β2 and α2. Clade 3 also has an extra α-helix inserted between β2 and α2, but this helix is shorter and is followed by a substrate-binding loop (pore loop 1 or PL1) (Figure 1b). Members of this 'classic' protein-remodelling clade possess a second R-finger and lack an S2 residue. Clade 3 is the most widely studied and extensively reviewed clade [7••], and includes prominent members such as Vps4, katanin, and the N-terminal AAA+ domains of double-ringed unfoldases such as ClpB and Hsp104. Clades 4–7 all share a β-hairpin insertion between α3 and β4, before the S1 motif, and are therefore grouped into the pre-sensor 1 insert (PS1i) superclade. Clade 4 is composed exclusively of viral helicases, and possesses a unique domain formed by α-helices N-terminal and C-terminal to the αβα core instead of the canonical AAA+ lid subdomain. Clade 5 is termed the HCLR Clade, reflecting the main families that it is composed of — HslU/ClpX, ClpABC-CTD, Lon, and RuvB. Members of Clade 5 possess no additional features to the PS1i, and similarly to Clade 3, are involved in protein unfolding and remodelling. Clade 6 is characterised by a β-hairpin insertion in α2 termed the helix-2 insert (H2i), and contains bacterial enhancer binding proteins (bEBPs) such as NtrC1 and PspF, and the unusual 'AAA+ GTPase' McrB. Clade 7 members also possess the H2i, but have an additional α-helical insertion between α5 and α6 called the pre-sensor 2 insert (PS2i). Several functionally divergent families such as the MCM helicase, MoxR, and dynein families are attributed to this clade.

Cryo-EM insights into the AAA+ ATPase mechanism

As the classification of AAA+ ATPases was performed 15 years ago, only about a dozen high resolution structures of different representatives of this superfamily were available, mostly from X-ray crystallographic studies. At this time, the prevailing consensus was that AAA+ ATPases formed symmetric closed rings, with a few isolated exceptions [3,4,9]. Since the cryo-EM revolution in 2015 a vast number of new structures of AAA+ ATPases have been published, and there have been more than 50 publications presenting cryo-EM structures of AAA+ ATPases since the start of 2019 (Supplementary Table 1). It is now apparent that most AAA+ ATPases show an asymmetric spiral arrangement of six monomers around the central pore (Figure 1c). Many of the published structures were obtained in the presence of a substrate, which may play a role in the rearrangement of planar hexameric rings into spirals to facilitate threading of the substrate through the pore [10–12]. As the binding of ATP is known to be required for substrate engagement, most of the structures were obtained with ATP binding-efficient but hydrolysis-defective Walker B mutants, or in the presence of non-hydrolysable ATP analogues. Substrate-binding pore loops form a spiral staircase engaging the substrate, with pore loop positions correlating with the nucleotide state (ATP, ADP, or an apo 'seam'). These structures have allowed the inference of a common mechanism for ATP hydrolysis [7••,12] which is based on the sequential hydrolysis of ATP around the hexameric ring, with corresponding 'hand-over-hand' movements of pore loops driving unidirectional translocation of substrate through the pore. However, the universality of this mechanism is still under debate [13••]. In addition, the majority of structures used to define this mechanism are of proteins from the Classic Clade [7••,12]. In contrast, the PS1i superclade is much less studied despite its huge functional diversity. In this review, we present an overview of recent structures of AAA+ ATPases, with a particular focus on structural insertions in the AAA+ core of PS1i superclade members. We discuss the functional roles of these insertions, and revisit the classification of AAA+ ATPases in light of recently solved structures. Finally, we address the controversy surrounding the mechanism of AAA+ ATPase activity, underscoring the need for further investigation to clarify the general applicability of proposed ATP hydrolysis mechanisms to the entire AAA+ superfamily.

Structure and function of insertions in the AAA + core

Clade 3 is by far the best-characterised clade, with many recent high resolution cryo-EM structures of Clade 3 members, including those of the proteasome [14••,15–17], Vps4 [18], katanin [19], spastin [20,21], Bcs1 [22], Cdc48/p97/VCP [23–25], and ClpABC-NTD [26–29] yielding valuable insight into the action of protein-translocating AAA+ ATPases. As stated above, PL1, a short loop before α2, is responsible for binding to substrates during translocation. These loops usually contain an aromatic residue that non-specifically intercalates between protein substrate residues, forming a spiral staircase in the central pore (Figure 1c) [7••,12,30]. A secondary loop termed pore loop 2 (PL2), less conserved than PL1 [7••], is located between β3 and α3 forms another spiral staircase below PL1. The roles of PL1 and PL2 in

(Figure 1 Legend Continued) binding site of (left) Clades 1–6 (ClpA-CTD, PDB ID: 6UQE) and (right) Clade 7 (MCM, PDB ID 6XTX). Schematics of two neighbouring monomers are shown above; large and small subdomains of one AAA+ monomer are labelled L and S, while corresponding subdomains from a neighbouring monomer are labelled L′ and S′ respectively. As shown in zooms of the ATP binding site (below), the domain-swapped architecture of Clade 7 members means that the S2 arginine acts *in trans* and contacts the γ-phosphate of the neighbouring monomer, unlike the canonical S2 of Clades 1–6 which act *in cis*. Both binding sites are occupied by ATPγS, a commonly used non-hydrolysable ATP analogue. Key motifs are coloured as in (a), publication references for all PDB IDs are presented in Supplementary Table 2.

substrate translocation are extensively reviewed in Refs. [7••] and [12].

The Classic Clade is functionally similar to the HCLR Clade 5, with members of both being involved protein unfolding, remodelling, and proteolysis, as reviewed in Ref. [30]. For some, such as the Lon protease, the AAA+ module is fused to a dedicated protease domain. For others, protease binding partners such as ClpP and HslV degrade substrates. While Clade 5 lacks the α-helical insertion typical to Clade 3, several Clade 5 members also interact with substrate via a short loop between β2 and α2. For other members of Clade 5 as well as both Clade 6 and 7, other insertions in the AAA+ core, namely the PS1i and H2i, instead play roles in substrate recognition and translocation. Despite differences in the location of these insertions, the spiral staircase conformation seen for the Clade 3 PL1 is conserved across clades, as discussed below.

Pre-sensor 1 insert

The PS1i β-hairpin, often positioned in the centre of the hexameric ring, is crucial to the function of many AAA+ proteins in the PS1i superclade. For some, it fulfils a similar role to the PL1 in Clade 3. In the viral helicases of Clade 4, the PS1i protrudes directly into the central pore and interacts with double-stranded DNA during origin recognition and with single-stranded DNA during helicase unwinding, forming a spiral staircase around substrate [31,32]. Similarly, in the Clade 7 DNA helicase MCM, recent structures revealed that the PS1i is involved in coordinating the phosphate backbone of single-stranded DNA during translocation [33,34•, 35–37]. For others, however, the PS1i plays more diverse roles. Early studies on RuvB, an evolutionarily distinct member of Clade 5, showed that the PS1i is not involved in substrate translocation but rather in mediating an interaction with its binding partner RuvA [38]. Two recent cryo-EM structures of the Clade 5 unfoldase ClpX bound to substrate demonstrate that the PS1i is involved in substrate recognition rather than translocation, with the majority of substrate-interacting residues coming instead from PL1 [39,40]. The structure of the *Escherichia coli* ClpX reveals that the PS1i reaches into and above the central pore to engage substrate, which is later unfolded and translocated through the hexameric pore [40]. In contrast, high-resolution cryo-EM structures of substrate-engaged ClpA [26], ClpB [27,28] and Hsp104 [41•] have all been published recently showing that the PS1i in the C-terminal AAA+ domain is displaced away from the centre of the pore (Figure 2), with inserted loops in helix α2 instead binding the substrate during translocation. Although these insertions are close to the location of the Clade 3 PL1, they appear to be much longer and are in the middle of α2, bearing a closer resemblance to the H2i of Clade 6 and 7 members. The relative arrangements of PS1i loops and these substrate-coordinating pore loops for the ClpABC-CTD family are similar to those seen in recent cryo-EM structures of LonA [42,43], another Clade 5 protein, and several members of Clades 6 and 7 (Figure 2).

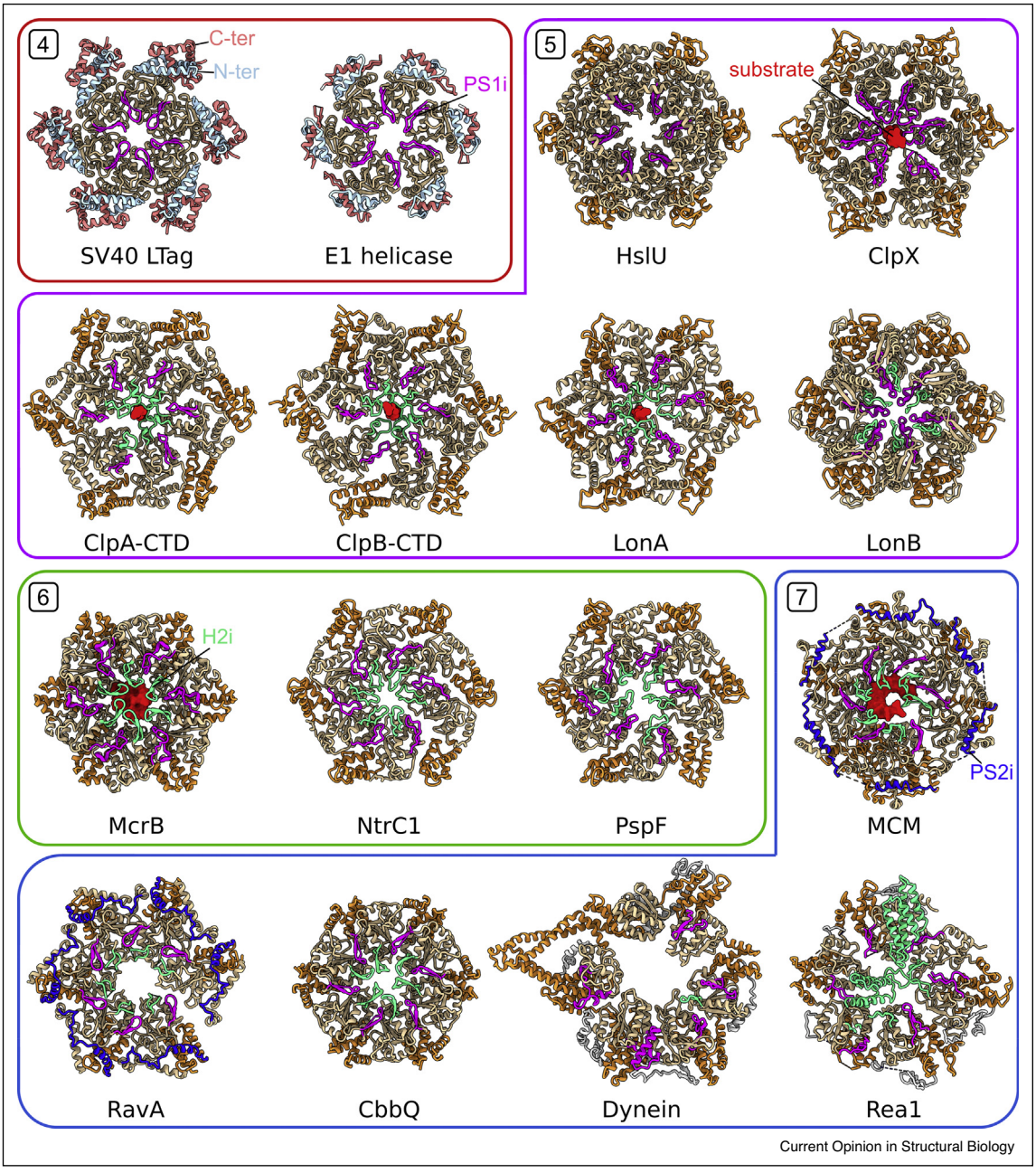
The PS1i is termed loop 2 (L2) in the Clade 6 members McrB and bEBPs such as NtrC1 and PspF. In bEBPs, the PS1 inserts lack the typical β-hairpin secondary structure and are more disordered compared to other members of the PS1i superclade, and interact with substrates along with the H2i (see below) [44]. In some cases, such as for the MoxR family CbbQ, classified into Clade 7, the PS1i interacts with the H2i which would allow the transmission of conformational changes from the H2i to the nucleotide binding site [45].

H2 insert

Similarly to the PS1i, the H2i β-hairpin plays a crucial role in interactions with substrate or partner proteins. In Clade 6 bEBPs (extensively reviewed in Ref. [44]), a conserved motif in the H2i (termed loop 1, L1 or PL1 due to its similarity to the Clade 3 PL1) facilitates the interaction between the AAA+ module and σ^{54}-bound RNA polymerase. bEBPs use the energy from ATP hydrolysis to remodel RNA polymerase from a closed to an open conformation, thereby activating transcription. The cryo-EM structure of PspF showed that the H2 inserts of PspF sit in the centre of the hexameric ring and facilitate an interaction with promoter DNA [46]. In NtrC1, another bEBP, the H2 inserts are arranged in a spiral staircase [47]. However, these proteins do not act as motors but rather as molecular switches, and as such are unlikely to translocate substrate through the central channel [44]. The McrB H2i facilitates protein-protein interactions with the endonuclease McrC, which sits in the central hexameric pore, but it is unclear whether the GTPase activity of McrB is used for threading DNA substrate through the pore [48•].

The H2 inserts of Clade 7 members play a variety of roles. The H2i of MCM acts together with the PS1i to coordinate substrate DNA, meaning that each MCM protomer contributes two loops to a continuous spiral staircase (Figure 2) [33,34•,35–37]. Key residues in the H2i are necessary for the MoxR protein CbbQ to function as a RuBisCO activase, and in the CbbQ hexamer the H2is protrude into the central hexameric pore [45]. The H2i also plays important functional roles in the motor protein dynein and the ribosome biogenesis protein Midasin/Rea1. The H2i in the second AAA+ domain of dynein (AAA2) is critical for dynein's motor activity, with AAA2 H2i mutants still able to hydrolyse ATP but not to perform the power stroke associated with motor function [49]. Cryo-EM structures of Midasin/Rea1 showed that AAA2 H2i is instead extended by an α-helical bundle, which sits in the centre of the hexameric ring as a plug

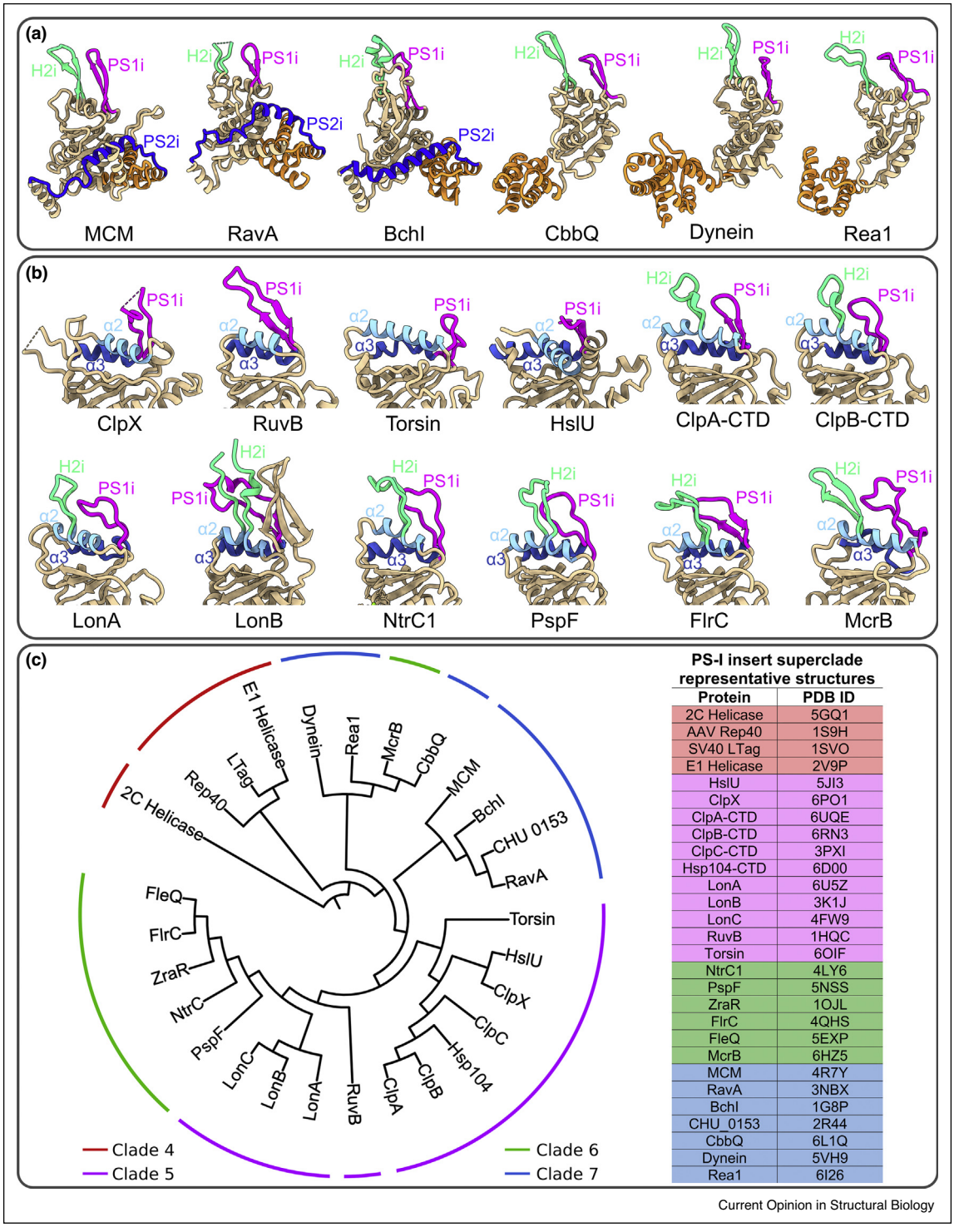
Figure 2



Hexamer structures of selected PSI-insert superclade members showing PS1i, H2i and PS2i locations. Coloured boxes indicate clade classification: red = Clade 4, magenta = Clade 5, green = Clade 6, blue = Clade 7. Insertions are coloured as for Figure 1 (large AAA+ subdomain = light brown, small AAA+ subdomain = orange, PS1i = magenta, H2i = green, PS2i = navy blue, Clade 4-specific N-terminal and C-terminal helices = light blue and coral respectively, grey = inter-protomer linkers). Bound substrates, when present, are coloured red. All PDB IDs are listed in Figure 3d, except for LonA (6ON2), MCM (6XTX) and RavA (6SZB), and publication references for all PDB IDs are presented in Supplementary Table 2.

and inhibits ATPase action of the hexamer when not bound to its substrate [50,51]. Additionally, the recent cryo-EM structure of Rea1 in complex with the Rix1/pre-60S ribosome particle showed that the H2i of AAA2 promotes an interaction with Rix1 while the H2i of AAA5 instead contacts the Rea1 MIDAS domain [52]. However, it is unclear whether these pore loop-facilitated interactions are involved in threading substrate, or rather in mediating protein-protein interactions alone.

Figure 3



Discrepancies in AAA+ ATPase classification. **(a)** Comparison of monomer structures for Clade 7 members. While MCM, RavA, BchI and CHU_0153 (not shown) possess the clade-defining PS2i (coloured navy blue) and display repositioned small AAA+ subdomains, CbbQ, Dynein (AAA2 monomer shown) and Rea1 (AAA3 monomer shown) instead display a canonical arrangement of AAA+ subdomains. **(b)** Comparison of selected Clade 5 and 6 monomers, focussed on the region surrounding α2 (light blue) and α3 (medium blue). Insertions in α2 are coloured light green and the PS1i is coloured magenta. While the Clade 5 members ClpX, RuvB, Torsin and HslU possess a continuous α2, ClpA-CTD, ClpB-CTD, LonA and LonB possess an insertion very similar to those seen for Clade 6 members NtrC1, PspF, FlrC, McrB, as well as FleQ and ZraR (not shown but almost identical to NtrC1). ClpC-CTD and Hsp104-CTD structures (3PXI and 6D00 respectively) lack built-in residues at this location, however the number of missing residues at the break in α2 are consistent with an inserted loop. **(c)** Structural similarity dendrogram of

Pre-sensor 2 insert

The PS2i, the defining feature of Clade 7 AAA+ ATPases, forms a long α-helix that drastically repositions the small subdomain relative to the large subdomain [4]. Despite this repositioning, the overall arrangement of large and small subdomains in the hexamer and the architecture of the ATP binding site is preserved (Figure 1d). In other AAA+ clades, the linker between large and small subdomains operates as a hinge, with ATP hydrolysis linked to conformational changes within a monomer. The recent cryo-EM structure of the Clade 7 ATPase RavA from the MoxR family showed that these hinge-like motions are conserved, but occur instead between the large and small subdomains of neighbouring monomers [53•]. In addition, similarities between twofold symmetric closed ring states of ClpX [54] and RavA [53•], and in particular the presence of a nucleotide-free 'double seam', support the idea of a conserved ATPase mechanism between Clades 5 and 7, despite the huge differences in domain architecture. Although there are several recent cryo-EM structures of Clade 7 ATPases with the PS2i, in particular MCM and RavA [34•,35–37,53•], the effects of AAA+ subdomain repositioning are not well-explored. Clade 7 is much less characterised than other clades, and further investigation may uncover the role of the PS2i in modulating the ATPase activity. Finally, several AAA+ proteins classified into Clade 7 clearly lack the PS2i, as discussed below, raising questions about the current classification scheme.

Discrepancies in AAA+ classification

Just as the vast number of recent AAA+ ATPase structures has facilitated the increased understanding of their molecular mechanism, it also offers us a chance to make extensive structural comparisons and reveals discrepancies in the current classification scheme, particularly in the PS1i superclade. Because the grouping into clades is often used to extend hypotheses from one protein to members of the same clade and to contrast observations between members of different clades [4,42], working within the framework of an accurate and up-to-date classification system seems important, in particular for the investigation of the general applicability of currently proposed AAA+ ATPase mechanisms, as discussed below.

Members of Clade 7 offer the most apparent example of discrepancies in classification. Indeed, structural alignment of Clade 7 AAA+ ATPases shows that while several members possess the characteristic PS2i, dynein, Midasin/Rea1 and CbbQ lack it and retain the canonical arrangement of large and small AAA+ subdomains (Figure 3a) [53•,55]. In addition, although dynein is classified an H2i AAA+ ATPase, the sequence stretch originally identified [3] as being an H2i in AAA3, the third tandem AAA+ domain, in fact appears as a loop between α2 and β3 while the only insertion in the middle of α2 appears to be in AAA2 [55]. This suggests that either the other five AAA+ domains in dynein have subsequently lost their H2i, or probably more likely, there was a later independent insertion into α2 of AAA2 after branching from other PS1i proteins [55]. In contrast, all six tandem AAA+ domains in Midasin/Rea1 possess an H2i [50].

As introduced above, several members of Clade 5, namely ClpA-CTD, ClpB-CTD, ClpC-CTD, Hsp104-CTD and Lon also have insertions in α2 that interact with substrates in a similar way to the H2is of Clade 6 and 7. On the face of it, this feature would place these proteins in the H2i-containing Clade 6. Comparison of the monomer structures of these proteins with those of Clade 6 and 7 shows a high degree of structural conservation (Figure 3b). However, as is possibly the case for the H2i in the AAA2 domain of dynein, it is conceivable that these insertions arose independently multiple times. Indeed, a structural similarity dendrogram based on structural alignment of monomers across the PS1i superclade, generated using the distance matrix-based structural alignment algorithm on the DALI server [56], does not maintain a single grouping for proteins containing H2is but rather splits them into several different groups (Figure 3c). Therefore, the original classification of AAA+ ATPases based on structural PS1i, H2i and PS2i insertions and assuming their emergence only once in the course of the superfamily evolution may be outdated. The evolutionary history of the AAA+ superfamily may be more complex, suggesting that a fresh look at the classification system based on large-scale analysis is needed in light of the ever-growing structural information, particularly for the comparatively understudied Clades 6 and 7. In addition, some proteins such as Pch2/TRIP13 do not fit in the current classification scheme, but possess features of multiple clades [57]. Finally, it may be worth asking whether is it still useful to refer to clades at all, or whether it is better to group AAA+ ATPases according to their functional similarities.

Perspectives — a universal AAA+ ATPase mechanism?

Although the recent wealth of structural information has yielded substantial insight into the mechanism of

(Figure 3 Legend Continued) PSI-insert superclade AAA+ ATPases generated from a structural alignment using DALI [56] with outside lines coloured according to the clade classification. The 'all-against-all' option was used — this carries out sequential pairwise comparisons between all PDB files, generating distance matrices that describe distances between equivalent Cα atoms, and hierarchically clusters these matrices into a Newick format dendrogram [61]. Where necessary, missing loop residues were modelled using the Phyre2 server [62] before structural alignment. Dendrogram visualisation was carried out using iTOL [63]. A table of PDB IDs used as input for the creating the dendrogram is shown on the right, with proteins coloured by clade. Publication references for all PDB IDs used are presented in Supplementary Table 2.

AAA+ ATPase action and the orthodox 'hand-over-hand' model of sequential ATP hydrolysis has become widely accepted, several recent publications call it into question [30,40,53•,58••]. For example, high-speed atomic force microscopy of the histone chaperone Abo1 has provided direct evidence for ATP hydrolysis at non-adjacent sites in the hexameric ring [58••]. In parallel, two recent cryo-EM papers on the Clade 5 unfoldase ClpXP have reignited debate over whether a sequential or stochastic ATP hydrolysis mechanism is correct by interpreting very similar structures in terms of two very different but plausible mechanisms [13••,39,40]. Despite the attractiveness of the idea of a unifying ATPase mechanism for all AAA+ proteins, the reality may be more nuanced. It is conceivable that depending on cellular conditions and interactions with binding partners or cofactors, AAA+ ATPases may be able to switch between strictly sequential and stochastic modes of action [7••,30]. In addition, while most AAA+ ATPases act as motors with continual ATP turnover, others such as the Clade 1 clamp loaders, Clade 2 helicase loaders and Clade 6 bEBPs instead act as 'switches', with a single ATPase cycle linked to a single event. Whether these switch-like ATPases all function with the same mechanism as the more extensively characterised AAA+ ATPases 'motors' is still uncertain [44], although the recent cryo-EM structure of the Clade 1 RFC suggests that this is unlikely [59]. Besides, other non-conventional AAA+ proteins exert force on their protein substrate laterally such as dynein, or act on membranes via unexplored mechanisms such as the unusual Clade 5 ATPase Torsin, which forms long helical polymers [60]. Therefore, in parallel to allowing investigation of mechanistic commonalities, the current flurry of cryo-EM structures may also inspire the disentangling of clade or even protein-specific functional differences and their structural underpinning.

Conflict of interest statement

Nothing declared.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.sbi.2020.10.027.

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