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## Primer AAA+ proteins

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Many cellular processes occur using energy generated from nucleoside triphosphate (NTP) hydrolysis. Amongst the groups of proteins able to bind and hydrolyze NTP, the phosphate loop (P-loop)-containing proteins are the most abundant, possessing a characteristic  $\alpha/\beta$  Rossmann-fold that contains Walker A and Walker B motifs for NTP binding and hydrolysis. Belonging to this group are the AAA+ (ATPases associated with diverse cellular activities) proteins, which typically function as molecular machines that use chemical energy from ATP to generate mechanical work for a myriad of activities, such as chaperoning protein folding or unfolding, genome replication, and gene expression, among many others. In this Primer, we highlight the structural features defining the different clades of AAA+ proteins and describe the functional diversity of these proteins that allows them to perform such varied cellular tasks. We also discuss our current knowledge of the molecular mechanisms driving their activities.

### Overview of the AAA+ domain

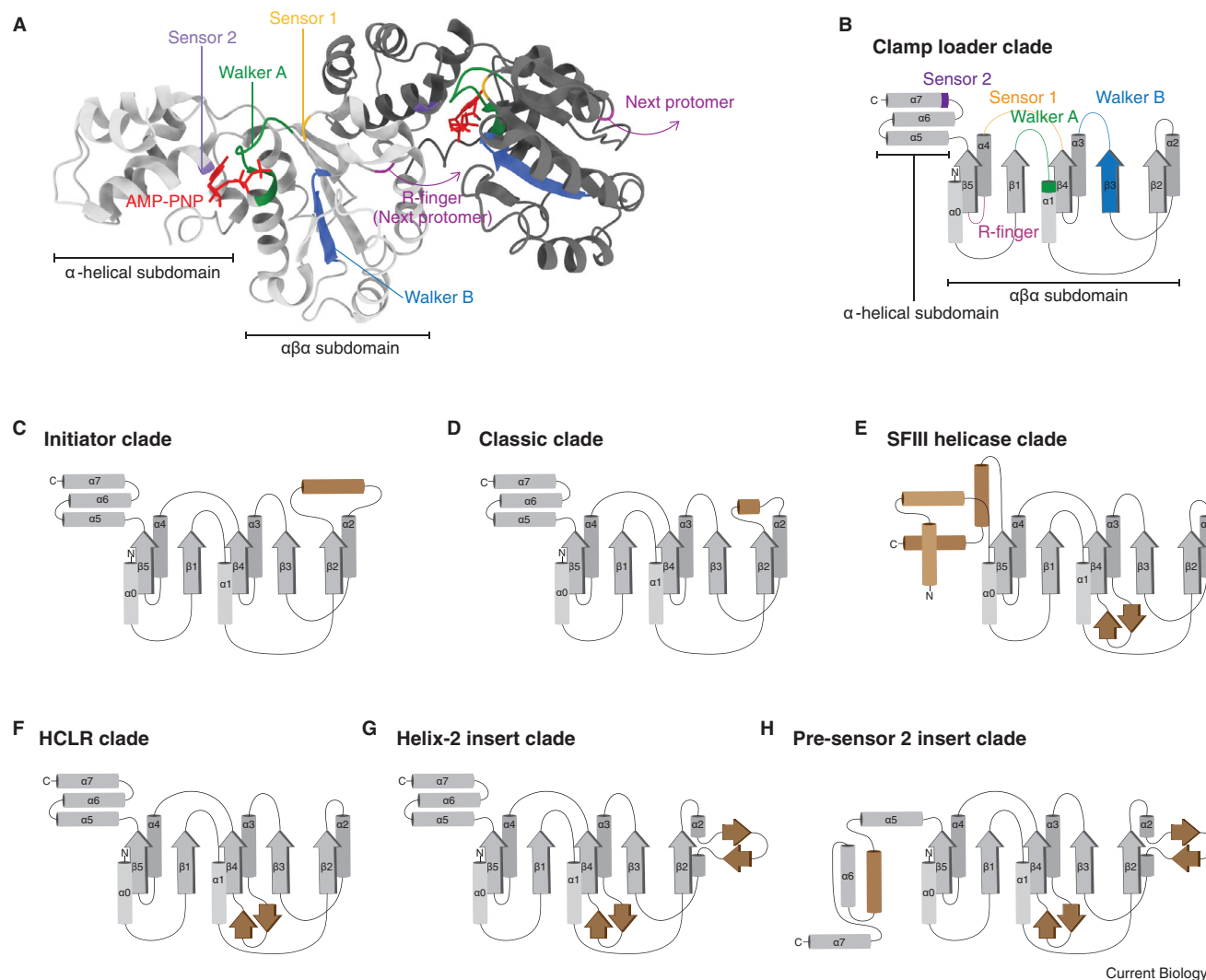
P-loop NTPases are divided into two groups: the kinase-GTPase (KG) group and the additional strand, catalytic E (ASCE) group. While proteins of the KG group have the Walker A (P-loop) and Walker B motifs adjacent to each other, members of the ASCE group have an additional  $\beta$ -strand inserted between these two motifs (Figure 1A,B; strand  $\beta 4$ ). AAA+ proteins belong to the ASCE group with the architecture of the ATP-binding pocket and their mechanism of ATP hydrolysis being conserved from bacteria to humans.

A typical AAA+ protein contains a central  $\beta$ -sheet organized in a  $\beta 5$ – $\beta 1$ – $\beta 4$ – $\beta 3$ – $\beta 2$  order flanked by  $\alpha$ -helices to form an  $\alpha\beta\alpha$  sandwich (Figure 1A,B). Within this  $\alpha\beta\alpha$  subdomain lies the Walker A and Walker B motifs, and the sensor 1 and arginine finger (R-finger) elements. The Walker A motif is found in the loop between strand  $\beta 1$  and the

beginning of helix  $\alpha 1$  and is generally composed of the amino acid residues GXXXXGK[T/S], where X represents any amino acid. This motif is involved in ATP binding and proper positioning of the triphosphate group of the NTP. The Walker B motif is formed by  $\Phi\Phi\Phi\Phi$ [D/E] residues ( $\Phi$  represents a hydrophobic amino acid) found in strand  $\beta 3$ . Walker B is essential for the water activation event preceding the nucleophilic attack of the  $\gamma$ -phosphate of the bound ATP.

AAA+ proteins also possess a region named the second region of homology (SRH), which contains the sensor 1 and R-finger elements. The SRH is a characteristic feature of AAA+ proteins and is formed by strand  $\beta 4$ , loop, helix  $\alpha 4$  and the loop between  $\alpha 4$  and strand  $\beta 5$ . Sensor 1, typically formed by the polar residues asparagine or threonine in the loop between  $\beta 4$  and  $\alpha 4$ , works in coordination with the Walker B motif to promote the nucleophilic attack on the ATP  $\gamma$ -phosphate group by a water molecule. The R-finger is a *trans*-acting arginine residue located at the end of the SRH, in the loop between  $\alpha 4$  and  $\beta 5$ , pointing towards the ATP-binding pocket of a neighboring protomer in AAA+ oligomers (Figure 1A), helping to stabilize the ATP during hydrolysis. Therefore, oligomerization of AAA+ proteins, typically into hexamers, is important for the formation of the NTP-binding pocket.

With a few exceptions, most AAA+ proteins have an additional  $\alpha$ -helical carboxy-terminal subdomain that functions as a lid for the ATP-binding pocket and mediates interactions between AAA+ protomers. In this  $\alpha$ -helical bundle, more specifically in helix  $\alpha 7$  (Figure 1A,B), the sensor 2 arginine residue interacts with the  $\gamma$ -phosphate of the bound ATP. Although not present in all AAA+ proteins, sensor 2 is proposed to mediate the movement of the  $\alpha$ -helical bundle relative to the  $\alpha\beta\alpha$  region and SRH. Therefore, NTP binding, hydrolysis and exchange provide the chemical energy promoting conformational changes necessary for AAA+ proteins to perform their functional activities. In addition, AAA+ proteins often have insertions of additional elements into their AAA+ domains and are classified into seven distinct clades according to their similarities by evolutionary analysis.



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### Figure 1. Features of the different clades of AAA+ proteins.

(A) The 3D structure of the simplest AAA+ domain present in replication factor C (RFC) is shown (PDB 2CHQ). Only two protomers are shown and are colored in different shades of gray. Walker A (green) and Walker B (blue) motifs, and sensor 1 (orange), sensor 2 (purple) and R-finger (magenta) elements are also indicated. (B–H) Structure diagrams of representative AAA+ proteins belonging to each of the seven clades. Arrows and cylinders represent  $\beta$ -strands and  $\alpha$ -helices, respectively. Structural elements colored in brown represent insertions characteristic of each AAA+ clade. See text for further details.

#### Clade 1: Clamp loaders

The first and minimal AAA+ clade, the clamp loader, is conserved in bacterial, archaeal and eukaryotic species and its functions include DNA replication and repair. Proteins belonging to this clade do not have any additional structural elements inserted in their AAA+ domain (Figure 1B). Instead, they have specialized regions outside of the AAA+ domain. Clamp loaders form heteropentameric complexes organized in a spiral manner that are responsible for the opening and loading of the DNA polymerase processivity clamp (sliding clamp) onto DNA. Examples of clamp loaders are proteins belonging to the

archaeal/eukaryotic replication factor C (RFC), bacterial *HolB*/DnaX and bacterial/eukaryotic WHIP families.

#### Clade 2: Initiators

The second clade, known as initiators (Figure 1C), is present in bacteria, archaea and eukaryotes, and includes proteins that recognize the DNA origin of replication, promoting the assembly of the DNA replisome. The feature defining this clade is the insertion of an  $\alpha$ -helix between strand  $\beta$ 2 and helix  $\alpha$ 2 (Figure 1C). Mutations in this insertion impair the binding of the initiator protein to the origin of replication. Bacterial and archaeal members of the DnaA/

DnaC family of initiators are typically monomeric and assemble into spiral oligomers when bound to nucleotide and DNA. Similarly, members of the eukaryotic ORC/Cdc6 family are found in complexes formed by three to five protomers containing AAA+ domains, and play a role in recognizing the origin of replication and loading the replicative helicase MCM.

#### Clade 3: Classic

Defined by the insertion of a short  $\alpha$ -helix between strand  $\beta$ 2 and helix  $\alpha$ 2 is the third and most functionally diverse AAA+ clade known as the classic clade. This short helical insertion

is localized close to the central pore of these hexameric proteins, which plays a role in substrate binding. Members of the classic clade share a common role in protein remodeling, despite being involved in distinct cellular processes. Classic AAA+ proteins have additional domains located amino- or carboxy-terminal to the AAA+ domain, conferring specialization and functional diversity.

Proteasomal ATPases, CDC48/p97, NSF, FtsH, katanin, Pex1/6 and Bcs1 are examples of classic AAA+ families. Proteasomal ATPases and p97 function in the clearance of ubiquitinated polypeptides, an interesting example of a collaboration between two AAA+ proteins. p97 contains two AAA+ domains and uses ATP-driven mechanical force to extract ubiquitinated proteins from complexes and membranes, subsequently transferring them to proteasomal ATPases for degradation. FtsH is present in bacteria and eukaryotes and is involved in membrane protein quality control and degradation. Katanin, Pex1/6 and Bcs1 are eukaryotic protein families involved in microtubule severing, peroxisome biogenesis and the assembly of mitochondrial membrane protein complexes, respectively.

The ClpAB family contains two AAA+ domains, ClpAB-N and ClpAB-C. The amino-terminal AAA+ domain, ClpAB-N, is found in bacteria, archaea and eukaryotes and is part of the classic clade. The carboxy-terminal AAA+ domain, ClpAB-C, is part of the HCLR clade and is discussed below. ClpAB-N family members are proteins with a role in, for example, protein degradation, disaggregation, and thermotolerance. The bacterial and eukaryotic AFG1 family of AAA+ proteins function as novel mitochondrial quality control factors. Lastly, the TIP49 family is found in archaea and eukaryotes, having two members known as RUVBL1 and RUVBL2. The most distinctive and unique feature of TIP49 proteins is a large ~170 amino acid insertion between helix  $\alpha$ 2 and strand  $\beta$ 3 that is proposed to bind to DNA and other proteins. RUVBL1 and RUVBL2 play essential roles in chromatin remodeling and the assembly of macromolecular complexes as part of the R2TP chaperone complex.

### Pre-sensor 1 $\beta$ -hairpin superclade

The remaining four clades of AAA+ proteins are grouped together into a superclade termed pre-sensor 1  $\beta$ -hairpin. All proteins within this superclade present an insertion of a  $\beta$ -hairpin between helix  $\alpha$ 3 and strand  $\beta$ 4, before the sensor 1 motif (Figure 1E–H).

### Clade 4: SFIII helicase

The fourth clade includes the SFIII helicases encoded by small viruses, such as simian virus 40, adeno-associated virus type 2 and papillomavirus. These helicases play a critical role in the maintenance of the viral genome, promoting the unwinding of the viral DNA prior to its replication by the host cell machinery. In addition to the  $\beta$ -hairpin insertion, SFIII helicases have an  $\alpha$ -helical subdomain formed by elements present both amino- and carboxy-terminal to the  $\alpha\beta\alpha$  subdomain.

### Clade 5: HslU/Clp/Lon/RuvB (HCLR)

The fifth clade, dubbed HCLR, has the  $\beta$ -hairpin insertion as its only additional feature within its AAA+ core (Figure 1F). This clade is formed by the hexameric HslU/ClpX, ClpAB-C, Lon and RuvB families. Bacterial HslU and bacterial/eukaryotic ClpX are two protease-associated machines that unfold and feed substrate polypeptides into HslV and ClpP proteases, respectively. The ClpAB-C family encompasses the carboxy-terminal AAA+ domain of ClpA, ClpB, Hsp104 and their orthologs. This family also includes torsin ATPases, a set of eukaryotic proteins found in the endoplasmic reticulum and critical for nuclear envelope integrity. The Lon family is formed by bacterial, archaeal and eukaryotic proteases, controlling protein turnover and degradation of misfolded proteins. In eukaryotes, Lon also controls mitochondrial morphology and dynamics. Lastly, the bacterial RuvB family is the most functionally distinct member of the HCLR clade, acting on Holliday junctions during homologous recombination and DNA repair in concert with the DNA-binding protein RuvA and the endonuclease RuvC as part of the resolvase complex.

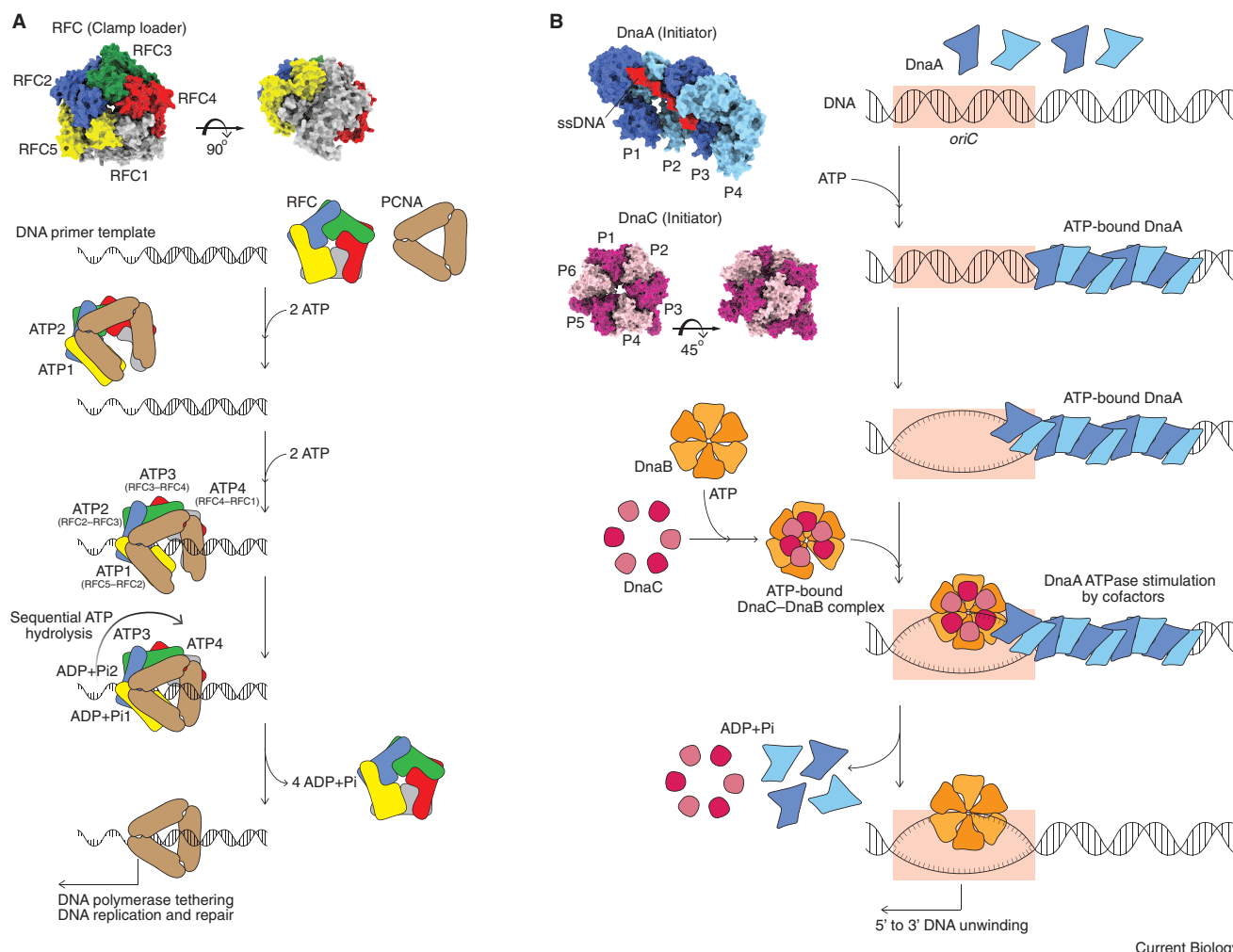
### Clade 6: Helix-2 insert

The insertion of an additional  $\beta$ -hairpin that breaks helix  $\alpha$ 2 is the feature that

distinguishes the sixth clade, named helix-2 insert clade (Figure 1G). This extra  $\beta$ -hairpin is located at the central pore of these proteins and plays a role in substrate binding. The first example of this clade is the bacterial NtrC family, functioning as transcriptional activators in concert with the RNA polymerase  $\sigma^{54}$  holoenzyme. RNA polymerase  $\sigma^{54}$  is involved in the expression of nitrogen-related genes, but the polymerase needs to be activated by NtrC members. The second example of helix-2 insert proteins is the McrB family, found in bacteria, archaea and in the Animalia kingdom. Interestingly, McrB members bind and hydrolyze GTP rather than ATP. In bacteria and archaea, the McrB family plays a role as part of a restriction endonuclease system, but the functions of McrB-related proteins in animals are still poorly understood.

### Clade 7: Pre-sensor 2 insert

The seventh and last AAA+ clade is the pre-sensor 2 insert (Figure 1H). This clade differs from the sixth clade due to an insertion of an  $\alpha$ -helix between helices  $\alpha$ 5 and  $\alpha$ 6, which causes a remodeling in the  $\alpha$ -helical subdomain, repositioning the sensor 2 motif and turning it into a *trans*-acting residue (i.e. acting on the neighboring protomer) rather than a *cis*-acting one. More importantly, the pre-sensor 2 helical insertion specific to this clade and the  $\beta$ -hairpin (which is also present in the helix-2 insert clade) are projected toward the central pore of these hexameric machines for substrate interaction. MCM helicases, chelatases, YifB, MoxR and dynein/midasin are all members of this clade. MCM proteins are present in archaea and eukaryotes and form homohexamers and heterohexamers, respectively. They play a role in DNA unwinding at the origin of replication and DNA replication fork progression by translocating DNA through their central pore. The loading of MCM proteins onto the origin of replication is performed by the initiator AAA+ proteins ORC/Cdc6. Another family in the seventh clade is the chelatases, proteins that catalyze the insertion of metal ions into cofactors during their biosynthesis, such as  $Mg^{2+}$  in the porphyrin ring of chlorophyll and  $Co^{2+}$  into the tetrapyrrole of cobalamin. Chelatases are present in photosynthetic and autotrophic



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**Figure 2. Activity of AAA+ proteins belonging to clamp loader and initiator clades.**

(A) Structure of the yeast RFC complex (PDB 1SJX) and its mechanism of clamp opening and loading onto DNA during DNA replication. The RFC complex interacts with the sliding clamp PCNA and DNA upon ATP binding. Occupancy of the first two ATP-binding sites induces a lock washer conformation of RFC, consequently opening the bound PCNA. The remaining two ATP-binding sites of RFC are occupied by nucleotides after interaction with DNA. A sequential mechanism of ATP hydrolysis, starting at RFC2, takes place to promote PCNA closure and PCNA-DNA complex release from the RFC complex. (B) Structure of bacterial DnaA (PDB 3R8F) and DnaC (PDB 6QEM) oligomers and mechanism of DNA unwinding and DnaB-DnaC recruitment. The binding of ATP to DnaA triggers its oligomerization on the origin of replication (*oriC*) of the target DNA, which causes DNA unwinding and the exposure of single-stranded DNA. In the presence of ATP, DnaB helicase and DNA, DnaC undergoes oligomerization to form a spiral-like hexamer that aids loading of DnaB onto single-stranded DNA. Upon ATP hydrolysis, DnaA and DnaC oligomers fall apart, releasing and activating DnaB helicase to proceed with its DNA unwinding activity.

prokaryotes and in the Plantae kingdom. Chelataes are often found associated with proteins containing von Willebrand factor type A (VWA) domains that are involved in protein-protein interaction.

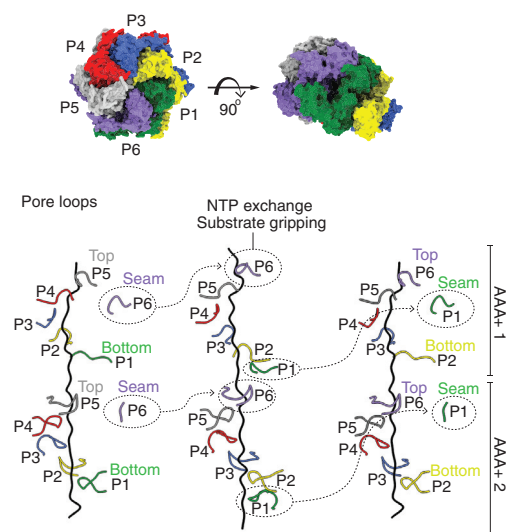
Proposed to have chaperone-like functions in the assembly of enzymatic complexes and insertion of cofactors into proteins, the MoxR family is another bacterial and archaeal member of the seventh clade of AAA+ proteins. For instance, RavA is a MoxR protein interacting with the fumarate

reductase electron transport complex, and therefore involved in anaerobic respiration in *Escherichia coli*. Like chelataes, RavA associates with a VWA domain-containing protein, in this instance ViaA. MoxR family members are also involved in the biogenesis of methanol dehydrogenase, nitric oxide reductases and Rubisco complexes. The bacterial YifB family is of unknown function as yet, but has Lon protease-like and chelatae homology domains in addition to the AAA+ domain. Lastly, eukaryotic dynein/midasin make up

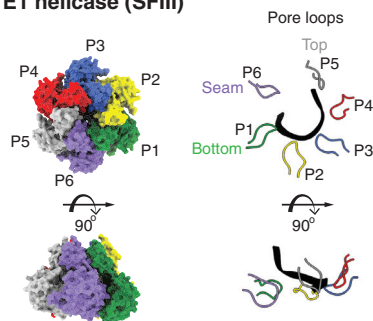
an interesting family of proteins that is composed of large proteins containing six fused AAA+ domains displaying a hexamer-like topology. Different dynein complexes are involved in flagella and cilia beating, as well as in cargo transport along the cytoskeleton in the cytoplasm. Midasin complexes provide mechanical force to remove assembly factors associated with the initial stages of large ribosomal subunit biogenesis in the nucleus, allowing for translocation of this subunit to the cytoplasm to form the mature ribosome. Unlike chelataes



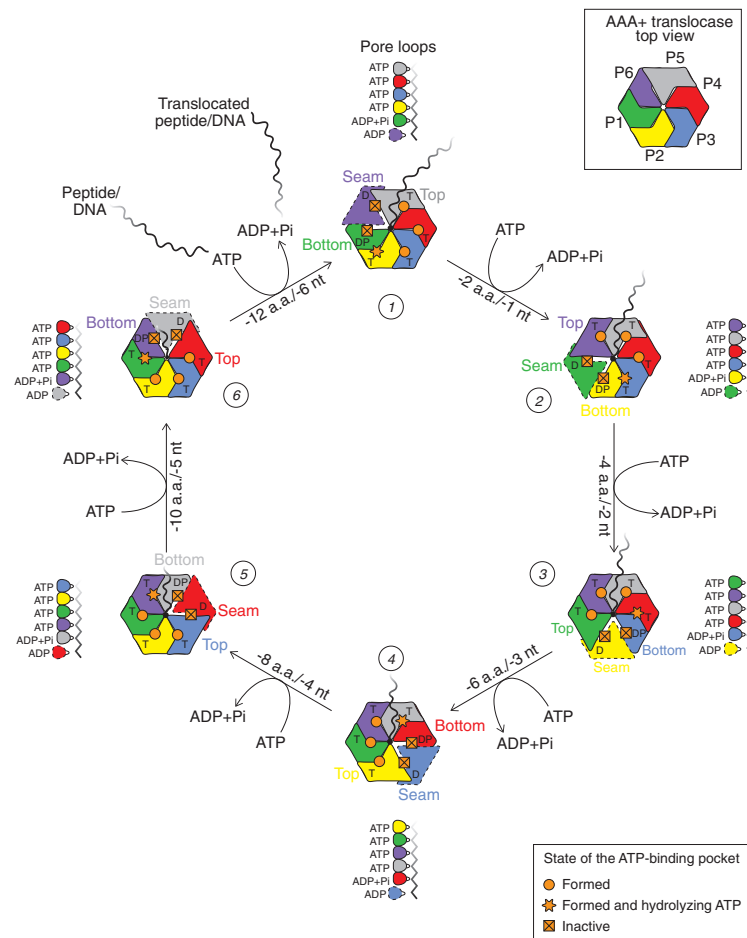
## A Hsp104 (Classic/HCLR)



## B E1 helicase (SFIII)



## C



**Figure 3. Spiral staircase architecture and hand-over-hand mechanism of substrate translocation by AAA+ proteins.**

(A) Structure and spiral staircase organization of the pore loops of yeast Hsp104 (PDB 5VJH), a protein-translocating complex. The protomer P6 moves from the so-called ‘seam’ position to the top of the spiral to grip the substrate after nucleotide exchange. Subsequently, the bottom protomer P1 moves to the seam position. (B) Structure and spiral staircase organization of the pore loops of bovine papillomavirus E1 helicase (PDB 2GXA), a DNA-translocating enzyme. (C) Mechanism of a generic AAA+ protein translocating a substrate. The different protomers (P1 to P6) of the hexameric AAA+ protein are represented by different colors and organized in a spiral. The positions of the respective pore loops are displayed next to the hexamers with the seam subunit outlined by dashed line. ATP, ADP+Pi and ADP-bound protomers are represented by T, DP and D, respectively. Nucleotide-binding pockets that are either formed, or formed and ATPase active, or inactive are shown as orange filled circles, orange filled bursting stars and orange filled crossed out squares, respectively. 1: The cycle starts when a substrate is recruited to the central channel of a AAA+ translocase, where pore loops are located and organized in a spiral manner. The nucleotide state of each protomer dictates its position in the spiral and pore-loop-substrate interactions. Protomer P5 (top), followed by P4, P3 and P2 are ATP bound while P1 (bottom) is ADP+Pi bound; P1 to P5 are substrate engaged. P6 (seam) is nucleotide free or ADP bound and does not bind the substrate. 2: ATP hydrolysis by P2, Pi release by P1 and nucleotide exchange by P6 induce conformational changes, so that ATP-bound P6 grips the substrate at an upper position, becoming the top subunit. P1 becomes the seam position and P2 the bottom protomer. 3–6: The anti-clockwise rotary ATP hydrolysis cycle proceeds in such a way by which each protomer experiences each position in the spiral staircase within one cycle. Two amino acid residues and one nucleotide are translocated per ATP molecule hydrolyzed.

and MoxR proteins, midasins have a VWA domain in their sequence.

## Mechanism of function of AAA+ proteins

The fact that AAA+ proteins participate in many cellular processes yet have similar overall folds is of great interest to the field. Therefore, a

detailed analysis of how ATP binding and hydrolysis is connected to conformational changes is necessary for an in-depth understanding of the molecular basis of their activity. A combination of structural and functional techniques has been applied over the years to study these proteins and has substantially contributed to our current

knowledge. More recently, propelled by the cryo-electron microscopy revolution, a growing number of AAA+ protein structures in different conformations has been published, allowing the observation of striking molecular details and providing insights into the mechanisms by which AAA+ proteins operate. Here, we discuss

three examples of mechanisms underlying AAA+ protein function.

### **Nucleotide-induced clamp opening by DNA clamp loaders**

The first example is that of the clamp loader replication factor C (RFC) complex (Figure 2A). RFC is a heteropentameric complex formed by RFC1, RFC4, RFC3, RFC2 and RFC5 that binds to the homotrimeric sliding clamp protein PCNA. This interaction catalyzes the opening of PCNA, allowing it to be loaded onto the target DNA. ATP binding to the clamp loader triggers conformational changes resulting in a spiral arrangement of RFC AAA+ domains resembling a lock washer and creating a gap between RFC5 and RFC1 (Figure 2A). Nucleotide binding to RFC also induces the interaction of PCNA with DNA and subsequent ATP hydrolysis causes RFC to dissociate from the PCNA–DNA complex.

Studies on the yeast RFC complex revealed that it has four functional ATP-binding sites with distinct ATP-binding properties (Figure 2A). The first two ATP molecules that bind RFC prime it to interact with PCNA, whereas the third and fourth ATP molecules bind RFC only after it interacts with PCNA and target DNA, respectively. Thereafter, the process of closure and release of the sliding clamp and DNA by the clamp loader starts with sequential and coordinated ATP hydrolysis. PCNA stimulates RFC2 ATP hydrolysis, inducing its disengagement from PCNA together with RFC5, leading to PCNA closure. Stimulated by DNA, ATP is hydrolyzed by the next protomer RFC3. Subsequently, RFC4 and RFC1 undergo ATP hydrolysis to complete the process of clamp loader closure and release of DNA-bound PCNA (Figure 2A).

### **Oligomerization of AAA+ proteins triggered by nucleotides**

A second interesting example comes from the prokaryotic initiators DnaA and DnaC (Figure 2B). The binding of ATP to monomeric DnaA causes conformational changes promoting its oligomerization on the origin of replication (*oriC*). Interestingly, self-association of ATP-bound DnaA positions the *trans*-acting R-finger of one protomer to face towards the ATP-binding pocket of the neighboring protomer. DnaA homo-oligomers are

arranged in a spiral manner with a single DNA strand occupying their inner side. DNA unwinding by DnaA is a necessary step prior to loading of the DnaC–DnaB complex. DnaB is a RecA-type hexameric helicase belonging to the DNA helicase superfamily 4. Although DnaB uses ATP to unwind DNA using a hand-over-hand mechanism, in which substrates are translocated via central pore loops in an ATP-dependent coordinated manner (see next section for details), to provide single-stranded DNA for the DNA polymerase III holoenzyme during replication, it is not a AAA+ protein. On the other hand, DnaC is a monomeric AAA+ protein that undergoes self-association into spiral hexamers in the presence of ATP, DNA and DnaB to promote DnaB ring opening, analogously to the effects of RFC on PCNA, hence loading DnaB onto the single DNA strand provided by DnaA. Once this step is over, ATP hydrolysis by DnaA is stimulated by DnaB and other co-factors, causing the monomerization of DnaA. DnaC also hydrolyzes ATP, dissociating from and activating DnaB, which now unwinds the DNA.

### **A hand-over-hand mechanism and rotary nucleotide hydrolysis for substrate translocation**

The third example comes from structures of AAA+ proteins that translocate DNA or peptides. For instance, proteins belonging to classic (CDC48, VAT, Hsp104, proteasomal ATPases, AFG3L2, YME1, katanin, spastin, Vps4, Rix7), SFIII helicase (E1 helicase), HCLR (ClpX, ClpA, Hsp104), helix-2 insert (NtrC1, McrB) and pre-sensor 2 insert (MCM) clades display a spiral staircase organization of their central pore loops and these proteins function using a hand-over-hand mechanism driven by rotary-type coordinated ATP hydrolysis for substrate translocation. Structures of two hexameric AAA+ proteins — Hsp104 and E1 helicase — are shown together with the organization of their central pore loops in Figure 3A,B. There is a spiral staircase where five of the pore loops are engaged with the substrate passing through the central channel (Figure 3A,B — P1 to P5, bottom to top) whereas one pore loop is disengaged (P6). More importantly, this organization and substrate engagement

correlate with the position and nucleotide state of each protomer within the oligomer. In the mechanochemical cycle of a generic AAA+ protein (shown in Figure 3C), the protomer at the top of the spiral and the next three clockwise protomers with descending spatial positions are ATP bound, substrate engaged and ATPase competent, with nucleotide-binding pockets and *trans*-acting R-fingers optimally arranged. The next protomer is located at the bottom part of the spiral and is found with a slightly more open nucleotide-binding pocket, in a post-hydrolysis state (ADP+Pi) yet bound to substrate. The P6 protomer, dubbed ‘seam’, is disconnected from the substrate and localizes between the bottom and top protomers in an intermediate position. Its nucleotide-binding pocket is open to allow for nucleotide exchange and is often free or ADP bound. Subsequently, nucleotide exchange in the seam protomer induces conformational changes allowing it to grip the substrate at an upper position. The seam now becomes the top protomer and the bottom protomer becomes the new seam (Figure 3A, pore loops). The cycle proceeds with sequential and coordinated ATP hydrolysis, where the substrate is passed from one loop to another hand-over-hand and, hence, undergoes translocation. Using this mechanism, peptides are typically translocated two amino acids at a time per ATP hydrolyzed, whereas for nucleic acids one nucleotide is translocated per ATP hydrolyzed. It should be noted that this hand-over-hand mechanism is not yet universally accepted.

### **Concluding remarks**

AAA+ proteins actively function as oligomers and this is one of the noteworthy features that they have in common, despite their diversity. The complementarity of nucleotide-binding sites between AAA+ interfaces, the mechanism of ATP hydrolysis and the conformational changes activating or deactivating their NTP-binding pocket ensure a functional cycle that creates mechanical force to promote remodeling of substrates. The adoption of a spiral staircase organization also seems to be a shared feature of AAA+ proteins regardless of their function. However, the mechanism of action of many AAA+ proteins is still

unknown, and questions remain as to the molecular details of the induced conformational changes that these proteins experience. Furthermore, the effects of cofactors and substrates during their functional cycle needs to be further investigated. In the coming years, one can expect that functional and structural studies will answer these and other questions arising along the way.

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### Serial dependence in perception requires conscious awareness

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Perception depends not only on the currently viewed stimulus, but also on expectations built from previous perceptual history, often termed perceptual *priors*. Perhaps the clearest example is ‘serial dependence’, where perceptual judgements — in orientation, numerosity, and so on — are robustly biased towards recent experience [1–3]. Here we ask whether stimuli need to be consciously perceived in order to influence perception, by measuring serial dependence for orientation following binocular rivalry between orthogonal gratings. We find that only the perceptually dominant rivalrous stimulus biased perceived orientation of subsequent stimuli, suggesting that the construction of perceptual priors requires conscious awareness of stimulus history.

Sixteen participants viewed trial sequences that alternated between a rivalrous display of orthogonal gratings presented separately to each eye, and a monocular test grating (Figure 1A). After each stimulus presentation, whether rivalrous or monocular, participants reproduced the orientation of the perceived stimulus by matching it with a rotatable line. On test trials, a grating was presented to the eye of the previous dominant or previous suppressed percept (randomly, with equal probability), rotated  $\pm 10^\circ$  from that orientation (see Supplemental Information for full details). The orientation of the other eye’s grating, whether suppressed or dominant, was always  $\pm 80^\circ$  away from the test stimulus and thus outside the range of serial dependence [2,3]. The results (Figure 1B) show that when the test orientation was near that of the dominant rivalrous grating, estimates were strongly biased towards the previous dominant orientation, with

a strong positive serial dependence of about 20%. But when the test orientation was near that of the suppressed rivalrous grating, there was no significant serial effect.

We measured significance in several ways. Figure 1C illustrates the permutation test where we shuffled the labels ( $\pm 10^\circ$ ) across the trials, and calculated the average serial dependence for 10,000 independent shuffles. On not one reiteration did the perturbation of the dominant condition exceed a bias of  $1^\circ$  (while the effect size was  $2^\circ$ ), implying that the probability of the observed effect arising by chance was far less than  $p < 10^{-4}$ . On the other hand, the probability that the negative effect for the suppressed condition arose by chance was  $p = 0.44$ , not significant ( $\alpha = 0.025$  on two-tailed sign test). The complementary approach was the standard bootstrap, retaining the labels and sampling (with replacement) the aggregated data 10,000 times, measuring the average effect on each reiteration. Again, for the dominant condition, all reiterations produced positive serial dependence, yielding  $p < 10^{-4}$  by bootstrap sign test. For the suppressed condition, however, 5215 reiterations were positive, giving  $p = 0.48$ , not significant. Finally, we considered the results separately for the individual participants and tested significance by paired two-tailed t-test and confirmed the same results: for the dominant condition,  $t_{15} = 5.6$ ,  $p < 0.001$ ; for the suppressed  $t_{15} = -0.12$ ,  $p = 0.91$ , failing significance in either direction.

The results show that, for stimuli to act as a perceptual *prior*, they need to be consciously perceived. This is interesting in light of conflict in the literature over the role of attention, with one report [1] suggesting serial dependence requires attention (which squares with our finding) while another [4] that it occurs automatically, so that one object feature will yield a positive serial effect even when a different feature was attended to on the previous trial. With attentional manipulations there is always a query over how effective the manipulation was and whether observers followed instructions. Our use of binocular rivalry has the benefit that conscious awareness of the stimulus can be manipulated with a greater certainty.

