# A long look at short prokaryotic Argonautes

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## Abstract

Argonaute proteins (Agos) use small 15-30 nucleotide-long guides to bind and/or cleave complementary target nucleic acids. Eukaryotic Agos mediate RNA-guided RNA silencing, while 'long' prokaryotic Agos (pAgos) use RNA or DNA guides to interfere with invading plasmid and viral DNA. Here, we review the function and mechanisms of truncated and highly divergent 'short' pAgos, which, until recently, remained functionally uncharacterized. Short pAgos retained the MID and PIWI domains important for guide-mediated target binding, but lack the ability to cleave their targets. Instead, emerging insights reveal that various short pAgos interact with distinct accessory 'effector' enzymes. Upon guide-mediated detection of invading DNA by short pAgos, their associated effector enzymes kill the host cell and consequentially prevent spread of the invader.

## Keywords

Prokaryotic Argonaute (pAgo), host defense, short pAgo, prokaryotic immunity, host defense, abortive infection

## Argonaute proteins are found in all domains of life

In all domains of life, Argonaute proteins (Agos) use small (15-30 nucleotides) oligonucleotides as guides to bind complementary nucleic acid targets. Eukaryotic Argonautes (eAgos) are the key effector enzymes in RNA silencing pathways and can be subdivided in two main clades: eAGO and ePIWI (lineage-specific eAgos are not discussed here). eAGOs generally bind small interfering RNA (siRNA) or microRNA (miRNA) guides generated by Dicer and/or Drosha nucleases [1]. Together with accessory proteins, eAGO forms an RNA-induced silencing complex (RISC) that silences mRNA targets to regulate gene expression [1]. ePIWIs bind PIWI-interacting RNA (piRNA) guides generated from longer genomic transcripts [2]. ePIWIs mainly silence transposons by cleaving their transcripts or by recruiting accessory proteins that induce heterochromatin formation [2–4].

Compared to eAgos, prokaryotic Argonaute proteins (pAgos) are highly diversified in sequence and domain composition [5–7]. They show a patchy distribution over the bacterial and archaeal phyla [5] and are often associated with other host defense genes in so called "defense islands": clusters of genes related to prokaryotic defense [7,8]. Based on their genetic association with other defense genes, their (predicted) nuclease activity, and because they are frequently subjected to horizontal gene transfer, it was hypothesized that pAgos play a role in host defense against invading DNA such as plasmids and viruses [7]. Based on their phylogeny, pAgos can be subdivided in long-A, long-B, and short pAgos (Figure 1A). Long-A and long-B pAgos have a canonical bilobed N-PAZ-MID-PIWI domain composition like eAgos [9–12] (Figures 1 and 2A-C; Box 1). While this suggests that Agos in all domains of life rely on similar mechanisms, proteins that are typically required in eukaryotic RNA silencing pathways (e.g. Dicer), have not been identified in prokaryotes [13]. Therefore, it is likely that pAgos execute different functions. In contrast, short pAgos lack the N- and PAZ domains, and are thus comprised of the MID and PIWI domains only (Figures 1 and 2D-I).

79% of the long-A pAgos have an intact catalytic DEDX tetrad (Box 1) in the PIWI domain, and most characterized long-A pAgos are capable of DNA guide-mediated target DNA cleavage [12,14–18][12,14–18]. However, at least *in vitro*, one

long-A pAgo uses RNA guides to target DNA [19], others use DNA guides to target RNA [20,21], and some do not have a clear preference for a specific type of guide and target [22,23]. In accordance with earlier hypotheses [7], catalytically active long-A pAgos have been implicated in host defense against invading nucleic acids such as plasmids and viruses [14,15,24,25]. Besides, long-A pAgos function beyond host defense by stimulating homologous recombination [26,27] and aiding in DNA replication [28]. No catalytically inactive long-A pAgos have been characterized to date. Much less is known about long-B pAgos. All long-B pAgos lack the catalytic DEDX tetrad, and often the PAZ domain is partially truncated (PAZ*) [5] (Box 1). The only canonical long-B pAgo that has been characterized to date is that of *Rhodobacter sphaeroides* (RsAgo), which uses RNA guides to bind DNA targets [29]. Despite its lack of nuclease activity, RsAgo lowers expression of plasmid-encoded genes and stimulates plasmid degradation in *E. coli* [29]. While the underlying mechanism is unclear, it was hypothesized that binding of RsAgo to target plasmids may cause their transcriptional silencing, and/or recruitment of nucleases that degrade the plasmids [29]. Another (non-canonical) long-B pAgo from *Archaeoglobus fulgidus* (AfAgo) has been used as structural model for Ago and Ago-nucleic acid interactions [30,31]. However, AfAgo is truncated: it is comprised of the MID and PIWI domains only (Figure 2I). As it can be considered to be a short pAgo, it will be discussed in more details below.

While pAgo research has predominantly focused on the eAgo-like long-A pAgos, the majority (59%) of pAgos are 'short pAgos' (Figure 1A) [5]. Like long-B pAgos, all short pAgos lack the DEDX tetrad required for target cleavage. Instead, they genetically associate in operons with putative enzymes previously predicted to be nucleases (Figures 1 and 2D-G) [5–7]. As the MID-PIWI domains of eAgos were shown to be sufficient for guide-mediated target binding [32,33], it has been hypothesized that short pAgo function in a modular host defense system, in which short pAgos act as guide-mediated target binders, while relying on the associated enzymatic domains for target degradation [6]. In this review we will discuss recent studies that uncovered that the functional mechanisms of short pAgos and their associated effector enzymes are fundamentally distinct from long pAgos and eAgos [34–37].

# Phylogeny of short pAgos

The majority of short pAgos form a distinct phylogenetic clade and strictly associate with proteins containing an "analog of PAZ" (APAZ) domain [5,6,36,38] (Figure 1A and Box 1). However, we also find truncated pAgos scattered over different branches of the long-A and long-B clades (Figure 1A) [5]. This implies that loss of the N- and PAZ domains occurred multiple times in the evolution of pAgos [5]. Consequentially, not all short pAgos are phylogenetically related: The short pAgo from *Sulfolobus islandicus* (SiAgo) and homologs thereof form a clade of pAgos that does not cluster with either long or short pAgos (the SiAgo-like clade; Figure 1A), and short pAgo from *Archaeoglobus fulgidus* (AfAgo) clusters with long-B clade pAgos (Figure 1A). SiAgo and AfAgo are not associated with APAZ domains, and rely on distinct functional mechanisms (described below). Therefore, from here onward we refer to the phylogenetically clustered short pAgos associated with APAZ domains as "short pAgos", and to other truncated pAgos as "pseudo-short pAgos".

94% of short pAgos are encoded by bacteria (proteobacteria (pseudomonadota): 54%, Bacteroidetes: 22%, Actinobacteria: 8%, other bacterial phyla combined: 10%) and only 6% by archaea (Euryarchaeota: 6%, TACK group archaea: 1%) (Figure 1B). Short pAgos can be divided in four phylogenetic clades: S1A, S1B, S2A, and S2B [36], and based on their phylogeny, clade S2B short pAgos are further subdivided in nine subclades (S2B-1 to S2B-9; Figure 1). The different (sub)clades of short pAgos are typically found in only a couple of prokaryotic phyla only (Figure 1B).

In all clades, short pAgos are encoded in operons that also encode an APAZ domain (Box 1). Initially, APAZ was predicted to functionally replace the PAZ domain in long pAgos [7], but later studies suggested that it is homologous to the N-domain of long Agos [39,40]. AlphaFold2-generated models [41] of short pAgo systems corroborate that the N domain and APAZ are homologous and assume the same position respective to the MID-PIWI lobe (Figure 2, see also [35]). The N-terminus

of APAZ is generally fused to a (putative) catalytic domain [5] (Figure 2D-G). In clade S1A and S1B APAZ is fused to a 'Silent Information Regulator 2' (SIR2, also known as Sirtuin) domain (Figure 2D-E). In clade S2A APAZ is fused to a Toll-interleukin-Receptor (TIR) domain (Figure 2F). In the different S2B subclades APAZ is fused to one of various domains (e.g. Mrr-like, DUF4365 or DHS-like) (Figure 1B and Figure 2G) [5,36,42]. In clade S1A, the APAZ domain-containing protein is fused to the N-terminus of short pAgo (Figure 2D). In other clades the APAZ-domain containing proteins are encoded by a separate gene upstream of short pAgo (Figure 2E-G), which suggests functional interdependence. We refer to short pAgo and its associated APAZ-domain containing protein as a 'short pAgo system'.

Like short pAgos, pseudo-short pAgos genetically co-localize with other proteins. For example, SiAgo is encoded in an operon with a predicted transcriptional regulator, while two other proteins are encoded on the opposite DNA strand [34] (Figure 2H). The presence of these three genes in close proximity of SiAgo is conserved for SiAgo homologs across different strains [34]. AfAgo has so far only been characterized as a standalone protein [43], despite it being encoded in an operon with two hypothetical proteins. Combined, this data suggests both short and pseudo-short pAgos function in conjunction with proteins encoded in their genomic context.

Indeed, recent studies confirm that (pseudo-)short pAgos form complexes with their associated proteins to protect their host against invading DNA such as plasmids and viruses [34–36]. Rather than triggering invader DNA degradation akin to long pAgos, these (psuedo-)short pAgo systems function as abortive infection systems [44] that kill their host to prevent replication and spread of the invader to others cells [34–37] (Figure 3). The diversified mechanisms on which these (pseudo-)short pAgo systems rely are detailed below.

# Functions and mechanisms of short pAgos

## SPARSA systems

Short prokaryotic Argonaute/SIR2-APAZ (SPARSA, also known as Sir2/Ago) systems found in clades S1A (fused SIR2-APAZ-pAgo) and S1B (co-encoded SIR2-APAZ and pAgo) are typified by the fusion of APAZ to a SIR2 domain (Figure 1 and Figure 2D-E) [5,36]. In eukaryotes, SIR2 proteins are involved in $NAD^+$-dependent protein or histone deacetylation or ADP-ribosylation, which can have implication for chromatin formation [45], DNA repair [46], and programmed cell death [47]. In prokaryotes, SIR2 domains are $NAD^+$-dependent deacetylases that play a role in stress resistance [48] and alter the immune response of their hosts through histone deacetylation [49]. Besides, it was shown that several prokaryotic abortive infection systems employ SIR2 to deplete $NAD^+$ upon detection of invading DNA [37,50]. This causes cell death of invaded cells, thereby preventing spread of the invader and providing population-based immunity (Figure 3).

Fused SPARSA systems from clade S1A (*Paraburkholderia graminis* (PgSPARSA) and *Joostella marina* (JomSPARSA) as well as co-encoded clade S1B SPARSA systems (*Geobacter sulfurreducens* (GsSPARSA), *Caballeronia cordobensis* (CcSPARSA) and *Xanthomonas vesicatoria* (XavSPARSA)) deplete $NAD^+$ upon detection of invader DNA [35–37]. PgSPARSA, GsSPARSA and CcSPARSA provide protection against double stranded (ds)DNA phage lambda-vir, while GsSPARSA and PgSPARSA also protect against dsDNA phage SECphi27. GsSPARSA and CcSPARSA also provide protection against transformation of plasmids containing a CloDF13 origin of replication (*ori*), but not against plasmids containing other *oris* (ColA, p15A and RSF1030)[35]. For GsSPARSA, it was shown that invader interference critically relies on both guide-binding by GsAgo and GsSIR2-APAZ NADase activity [35].

While GsSIR2-APAZ and GsAgo are encoded separately, the proteins form a heterodimeric complex [35]. When provided with an RNA guide and complementary single stranded (ss)DNA target *in vitro*, the complex is activated and degrades $NAD^+$ (Figure 4). *In vivo*, GsSPARSA associates with small 20 nt long guide RNAs with a 5'-AU sequence. While most guides are derived from genome-encoded genes, guides derived from plasmid-encoded transcripts are mostly obtained from their *oris*, suggesting guide acquisition relies on RNA-dependent priming plasmid replication. Replication of the phage lambda-vir relies on RNA-dependent priming as well [51], which might provide clues on how SPARSA obtains guides that

facilitate specific detection of plasmid- and viral invaders. Although other viruses and plasmids relying on the same replication mechanism are not affected by SPARSA [35], it should be noted that many prokaryotic immune systems only work against a subset of viruses and under specific conditions [50,52]. Combined, this shows that SPARSA provides population-based immunity by triggering cell death through $NAD^+$ depletion upon RNA-guided invader DNA detection (Figure 4, Key Figure).

## SPARTA systems

Short prokaryotic Argonaute/TIR-APAZ (SPARTA) systems make up clade S2A and are typified by the fusion of APAZ to a TIR domain (Figure 1 and Figure 2F)[5,36]. TIR domains were originally identified as scaffolding proteins associated with eukaryotic receptor proteins [53], but later they were found to possess NADase activity [54] which is important for their immune function both in eukaryotes [55–60] and prokaryotes [50,61–63]. While some TIR domains deplete cellular $NAD^+$ and function as abortive infection systems (Figure 3), others generate signaling molecules (e.g. cyclic ADPR and v-ADPR) to trigger downstream effects [50,54,55,64].

Akin to SPARSA, SPARTA systems from *Crenotalea thermophila* (CrtSPARTA) and *Maribacter polysiphoniae* (MapSPARTA) degrade $NAD^+$ (and $NADP^+$) in the presence of plasmid DNA [36]. In contrast to SPARSA, however, no direct interference with plasmid transformation was observed; instead, SPARTA lowers cell viability of plasmid-invaded cells through $NAD(P)^+$ depletion, removing invaded cells from bacterial cultures. Also, no SPARTA-mediated immunity against bacteriophages was observed. While both SPARSA and SPARTA are short pAgo systems that degrade $NAD^+$, these differences suggest that SPARSA and SPARTA might rely on different mechanisms to detect invaders. However, the different experimental conditions at which SPARSA and SPARTA have been functionality characterized could also have played a role in the observed differences. Further research is required to establish differences and similarities between SPARSA and SPARTA mechanisms.

As expected, the NAD(P)ase activity of SPARTA is attributed to the TIR domain of TIR-APAZ, which is catalytically active in absence of the short pAgo partner [36]. In contrast to TIR domains that generate signaling molecules c-ADPR or v-ADPR [50,54,55,64], SPARTA converts $NAD(P)^+$ to non-cyclic ADPR(P) and NAM. In heterodimeric short pAgo/TIR-APAZ complexes this activity is quenched, implying short pAgo controls the activity of TIR-APAZ. Through *in vitro* experiments it was determined that guide RNA-mediated target ssDNA binding by SPARTA induces tetramerization of four guide/target-bound SPARTA heterodimers, which reinstates the NAD(P)ase activity of the TIR domain (Figure 4). Oligomerization-dependent activation of TIR domains is a general mechanism conserved from prokaryotic to eukaryotic immune systems [61,62,65,66].

*In vivo*, SPARTA associates with 15-25 nt long guide RNAs with a 5'-A that are mostly derived from highly transcribed genes encoded on multicopy plasmids [36]. In line with this observation, plasmids with a high copy number and/or encoding highly transcribed genes activate SPARTA, whereas low copy plasmids lacking highly transcribed genes do not. Invading DNA exploiting their host cell for propagation are often present in high copy numbers and highly transcribed. Combined, this implies SPARTA senses invader activity through the high abundance of both their DNA and their RNA transcripts which triggers its NAD(P)ase activity and consequentially cell death, thereby removing invaded cells from the bacterial population (Figure 4).

## S2B-clade short pAgo system from *Kordia jejudonensis*

S2B clade short pAgo systems are the most diverse in terms of distinct protein domains that are fused to APAZ, but they are also the least explored: only the S2B system of *Kordia jejudonensis* has been studied *in vitro* [42] (Figure 1 and Figure 2G). The KjAgo-associated APAZ protein is fused to an Mrr-like domain [36] which is homologous to *E. coli* Mrr that acts as a methylation-dependent DNA nuclease [67] (Figure 2). Akin to short pAgos from other clades [35,36], KjAgo forms a heterodimeric complex with Mrr-APAZ [42]. *In vitro*, this complex catalyzes RNA/DNA guided cleavage of ssDNA targets, but also non-specific cleavage of ssDNA and dsDNA [42]. No functional role of the KjAgo/Mrr-APAZ system was determined, but it is conceivable that the KjAgo system mediates prokaryotic immunity akin to other short pAgo systems.

Yet, the results suggest that the KjAgo system relies on mechanisms that are clearly different from SPARSA and SPARTA: upon its activation the KjAgo/Mrr-APAZ system might indiscriminately degrade nucleic acids to shut down the cell, an abortive infection strategy that has been described for other prokaryotic immune systems (e.g. CBASS, CRISPR-Cas13, type III CRISPR systems) [68–70]. Alternatively, the Mrr-like domain might compensate for loss of the catalytic activity of the PIWI domain and specifically cleave guide-bound (invader) nucleic acids analogous to long-A pAgos and other CRISPR-Cas systems [14,24,71–73].

Taken together, these studies show that short pAgos and their associated APAZ-domain containing proteins are fused or form heterodimeric complexes (Figure 2D-G, Figure 4). The fact that fusion of short pAgos with their associated APAZ-effectors occurred multiple times in evolution underscores the importance of their complexation. In short pAgo systems, the short pAgo acts as a 'sensor' that facilitates guide-mediated recognition and binding of nucleic acid invaders. Upon target binding, the APAZ-fused domain is catalytically activated and acts as an 'effector'. In SPARSA and SPARTA systems, target detection results in unleashed SIR2- or TIR-mediated NAD(P)ase activity that consequentially kills the host of the invaded cells (Figure 3, Figure 4). For clade S2B short pAgos the exact catalytic mechanism activated and the consequences thereof remain to be determined.

# Functions and mechanisms of pseudo-short pAgos

## *Sulfolobus islandicus* pAgo

The archaeal SiAgo and homologs thereof do not cluster with either long or short pAgos, but form a separate branch in the phylogenetic pAgo tree (Figure 1). SiAgo functionality relies on two genetically associated proteins (Figure 1 and Figure 2H): Ago associated protein 1 (SiAga1) and Ago associated protein 2 (SiAga2) [34]. These three proteins together function as a prokaryotic immune system that protect its host against infection of the dsDNA virus SMV1 [34]. Akin to short pAgos, SiAgo and SiAga1 form heterodimeric complexes upon their co-expression [34]. However, SiAgo and SiAga1 alone do not confer defense against bacteriophages: they additionally rely on the effector Aga2. Aga2 is a membrane protein that forms large oligomeric complexes and binds phospholipids. While SiAgo-Aga1 complexes reside in the cytoplasm, Aga2 mostly localizes in the membrane. Upon viral infection, the SiAgo-Aga1 complex is directed to the membrane, triggering activation of Aga2. This results in loss of membrane polarity and consequentially triggers cell death in invaded cells [34].

While *in vitro* SiAgo appears to bind RNA guides, the SiAgo-SiAga1 complex associates with all guide/target type (DNA/RNA) combinations, [34]. However, it is unknown what combination of guides/target type activates the system [34]. Nevertheless, a model is proposed wherein the SiAgo-SiAga1 complex recognizes invading nucleic acids through guide-target binding, consequentially activating Aga2 to initiate abortive infection [34]. Contrary to short pAgo systems, the SiAgo system does not seem to target plasmids in the reported experimental setup: overexpression of genes from plasmids does not cause substantial Aga2 activation. In conclusion, although it relies on different accessory proteins than short pAgos, also the SiAgo system mediates population-based immunity by abortive infection upon detection of invading DNA (Figure 4).

## *Archaeoglobus fulgidus* pAgo

AfAgo is a truncated long-B pAgo that is comprised of the MID and PIWI domains only [74] (Figure 1, Figure 2I). AfAgo was one of the first Ago proteins to be crystalized and served as a model to study Ago-nucleic acid interactions [75]. In these structures AfAgo crystallizes as a homodimer, but the functional relevance of AfAgo dimerization remained unknown. A recent study showed that AfAgo also forms dimers in solution, and that dimerization is stabilized upon binding of dsDNA ends [43]. As each AfAgo component in the dimer can accommodate a separate dsDNA end, AfAgo dimers can stimulate formation of DNA loops. This suggests that AfAgo might be involved in DNA repair or integration of mobile genetic elements. Also long-A pAgos have been implicated in homologous recombination [26,27], but there are no indications that the dimerization is required in this process. Although its natural preferences for DNA/RNA have not been studied, AfAgo interacts with both RNA and DNA *in vitro* [75]. It is also unknown if AfAgo, like other pAgos, acts as host defense system.

Akin to other (pseudo-) short pAgos, AfAgo is encoded in an operon that also encodes hypothetical proteins. Further experimentation is required to determine the biological function of AfAgo.

# Concluding remarks

Short pAgos and pseudo-short pAgos are prokaryotic Argonaute proteins comprised of the MID and PIWI domains only, lack catalytic activity, and can have different phylogenetic origins. As they retain their ability to use guides to bind complementary targets, they serve as 'sensors' that detect invading DNA. All (pseudo-)short pAgos functionally characterized thus far mediate abortive infection (Figure 3): They form a complex with 'effector' proteins that are activated upon guide-mediated invader detection, thereby killing their host in order to protect neighbouring cells (Figure 4).

Despite the major advances in understanding of (pseudo-)short pAgo functionality, many aspects of their functionality remain mysterious (see Outstanding questions). It is unknown how (pseudo-)short pAgos interact with their effector partners. As the APAZ domain associated with short pAgos is homologous to the N domain found in long pAgos and eAgos, it might facilitate interactions with short pAgo and/or play a role in guide loading and/or target release. However, it is also conceivable that APAZ serves to activate the effector domain upon guide-mediated target binding by Ago, presumably through a conformational change. Likewise, in the SiAgo system, SiAga1 may connect SiAgo to SiAga2 in response to guide-mediated target binding, which induces membrane depolarization by Aga2. Besides, the SiAgo/SiAga1 complex showed increased affinity for nucleic acids compared to the SiAgo monomer, which suggests that SiAga1 either aids in binding guide and/or target nucleic acids or induces a conformation of SiAgo to favor nucleic acid binding. Structure-derived insights into protein-protein and protein-nucleic acid interactions are required to reveal the role of APAZ in short pAgo systems and Aga1 in the SiAgo system, and could illuminate the mechanism by which (pseudo-)short pAgos control the activity of their associated effectors.

Short pAgos characterized to date utilize small RNA guides to DNA, but it remains largely unknown how (pseudo-)short pAgos distinguish self (genomic DNA) from non-self (invader DNA). Potentially, guide RNA biogenesis specifically yields invader-targeting guide RNAs. In contrast with that hypothesis, both short and long-B pAgos acquire guide RNAs from the entire transcriptome [29,35,36], which results in the loading of self-targeting guide RNAs. Yet, these observations were made while overexpressing the pAgos in heterologous hosts, and it cannot be ruled out guide RNA biogenesis and loading is more specific in the natural host under native expression conditions. Eukaryotes have dedicated pathways for guide RNA biogenesis: In general eAGOs are loaded with microRNA (miRNA) or small interfering RNA (siRNA) guides that are generated from dsRNA substrates such as RNA hairpins or bidirectional transcripts by the RNase III family proteins Dicer and/or Drosha [76–78]PIWIs bind PIWI-interacting RNAs (piRNA) guides that are derived from transcripts of piRNA clusters to target transposon transcripts, or secondary piRNAs that are generated from targeted transposon transcripts in an ePIWI-dependent manner [79]. While prokaryotes lack Dicer and Drosha homologs, bacteria encode simpler RNase III family proteins [13] as well as an array of other housekeeping RNases that could play a role in guide RNA biogenesis. Analogous to the ePIWI pathway, CRISPR-Cas systems rely CRISPR RNA guides (crRNAs) derived from transcripts of CRISPR loci to target nucleic acid invaders [80]. Although some pAgos are encoded in the context of Cas genes [5,6,38], so far, no association has been found between crRNA guides and pAgos. While (pseudo-)short pAgos do not strictly rely on genome-encoded small RNAs [35,36], it can also not be ruled out that certain pAgo hosts encode precursors of invader-targeting guide RNAs on the genome.

Besides preferential guide generation, the availability of target DNA might play a role in differentiating self and non-self DNA. All (pseudo-)short pAgos reported to date target ssDNA *in vitro*, but act on dsDNA invaders *in vivo* [34–36]. Being unable to unwind dsDNA targets, pAgos might rely on other enzymes or processes (e.g. replication or transcription) to make DNA susceptible for detection. If such process or enzymes are different for genomic and invading DNA, they could contribute to invader-specific targeting by pAgos. Resolving the mechanisms facilitating pAgo-mediated dsDNA recognition can therefore contribute to understanding how pAgos distinguish self from non-self.

The majority of the vast (pseudo-)short pAgo-diversity await exploration. For example, certain S2A clade TIR-APAZ domains are additionally fused to an Mrr domain (Figure 2), and many S2B-clade short pAgos associate with domains of which the functions are not clear. For most pseudo-short pAgos the genetic context is yet to be investigated. Besides, more distant pAgos homologs have been identified (e.g. PIWI-RE [6,81] and DdmE [82]). With the rapidly expanding metagenomics data available it is not unlikely that additional clades containing distinct (pseudo-)short or long pAgos or more distantly related homologs thereof will be discovered in the near future. Characterization of pAgo variants will determine what functional roles they can fulfil, which mechanism they convey, and what evolutionary turns pAgos have taken.

Finally, short pAgo systems can be isolated and programmed with short (synthetic) RNA guides with sequences of choice, akin to CRISPR-Cas systems [83–86] and long pAgos [87]. As target binding triggers a specific catalytic activity, they could be repurposed for a range of sequence-specific applications. For example, SPARTA has been repurposed for the detection of ssDNA and dsDNA sequences [36]. Possibly, other pAgo systems with a distinct catalytic activity could be repurposed for targeted nucleic acid modification or genome editing. As such, the characterization (pseudo-)short pAgos not only uncovered a novel, highly diverse class of immune systems, but it could also inspire a new generation of programmable molecular tools.

# BOX 1

The characteristics of pAgo domains and other structural features of Agos are described in this box.

**MID**

The MID (Middle) domain forms a pocket that in most pAgos contains four conserved residues (Y/R, K, Q and K [5]) and coordinates a divalent cation [75] which together anchor the 5'-phosphate (5'-P) group of the guide. Certain pAgos preferentially bind 5'-hydroxylated (5'-OH) guides instead, which is attributed to a more hydrophobic binding pocket and absence of the divalent cation [5,11], while other pAgos show no clear preference for 5'-P or 5'-OH guides [21,22]. The 5'-nucleotide of the guide is also bound in the MID domain pocket (sometimes in a sequence-specific manner [88]), and therefore it is unavailable for base-pairing with the target.

**PIWI(*)**

The PIWI (P-element-Induced Wimpy Testis) domain is homologous to RNAse H, which cleaves the RNA strand of RNA/DNA substrates [89]. In Agos, the PIWI domain coordinates and pre-orders the 'seed' nucleotides of the guide (nucleotide 2-7 or 2-8) in a helical conformation with bases exposed to the solvent [90]. This promotes guide/target base pairing by lowering the entropic costs of duplex formation. PIWI is also the domain responsible for target cleavage (slicing): Slicing Agos contain a DEDX motif (see DEDX) in the PIWI domain, while this motif is mutated in Agos that rely on target binding for their function. PIWI domains lacking the DEDX tetrad are referred to as PIWI*.

**DEDX catalytic tetrad**

The DEDX motif (where X denotes D, H or, K) found in the PIWI domain of slicing Agos facilitates target strand cleavage. The glutamic acid residue is located on a structural feature termed the glutamate finger [91] that completes the catalytic tetrad upon target binding. The DEDX tetrad coordinates two divalent cations that catalyse hydrolysis of the target strand phosphate backbone between nucleotides 10 and 11 [92]. Most eAgos from the AGO clade, some long-A pAgos, and long-B and (pseudo-)short pAgos have mutations in the DEDX motif that abolish cleaving activity [5]. However, even in slicing Agos target cleavage can be affected by mismatches between the guide and the target [93,94].

**MID-PIWI (PIWI lobe)**

The MID and PIWI domains together form the PIWI lobe, which is sufficient for guide-mediated target binding and cleavage [32,33]. In line with their functional importance, these domains show the highest conservation over Agos from all domains of life, and it are the only domains found in all Argonaute family proteins [6,95]. Short pAgos are generally comprised of the MID-PIWI domains only.

**N domain**

The N domain is the least conserved domain in Agos and functions in guide loading and target cleavage: It acts as a wedge to facilitate removal of the passenger strand during guide loading and removal of cleaved target strands in slicing Agos [96,97].

**PAZ(*)**

The PAZ (PIWI-Argonaute-Zwille) domain interacts with the 3' end of the guide protecting it from degradation [33]. The PAZ domain usually displays no sequence preference, although PAZ domains of ePIWIs specifically recognize methylated 3' ends [98]. Upon guide-mediated target binding, the 3'-end of the guide is released from the PAZ domain [99,100]. The associated conformational changes result in target cleavage. All long-B pAgos and some long-A pAgos have a truncated PAZ domain which is referred to as PAZ* [5]. In PAZ* the guide 3' end binding pocket is at least partially lost [101,102]. Possibly, this allows for more extensive duplex formation between the guide and the target [101,102].

**N-PAZ (N lobe)**

The N and PAZ domains together form the N lobe, which is not required for guide-mediated target binding but enhances on-target cleavage specificity [32,33]. All (pseudo-)short pAgos lack the N lobe [5].

**APAZ**

Short pAgos lack both the N and PAZ domains, but are generally co-encoded in an operon or fused to an Analogue of PAZ (APAZ) domain-containing protein. The APAZ domain is usually fused to a variable catalytic domain (Figure 2) [5–7]. The APAZ domain was originally thought to be a functional analogue of the PAZ domain [7]. However, other predictions [39,40] as well as Alphafold2 modeling (Figure 2) show that at least part of the APAZ domain is homologous to the N domain instead. Furthermore, the Alphafold2 models suggest that APAZ assumes the same position respective to the MID-PIWI domains as the N-PAZ domains in long pAgos. The function of APAZ is currently unknown: it could have a 'wedging' function like the N domain, control short pAgo-mediated activation of the effector domain, or it could have another unknown function.

# Figures

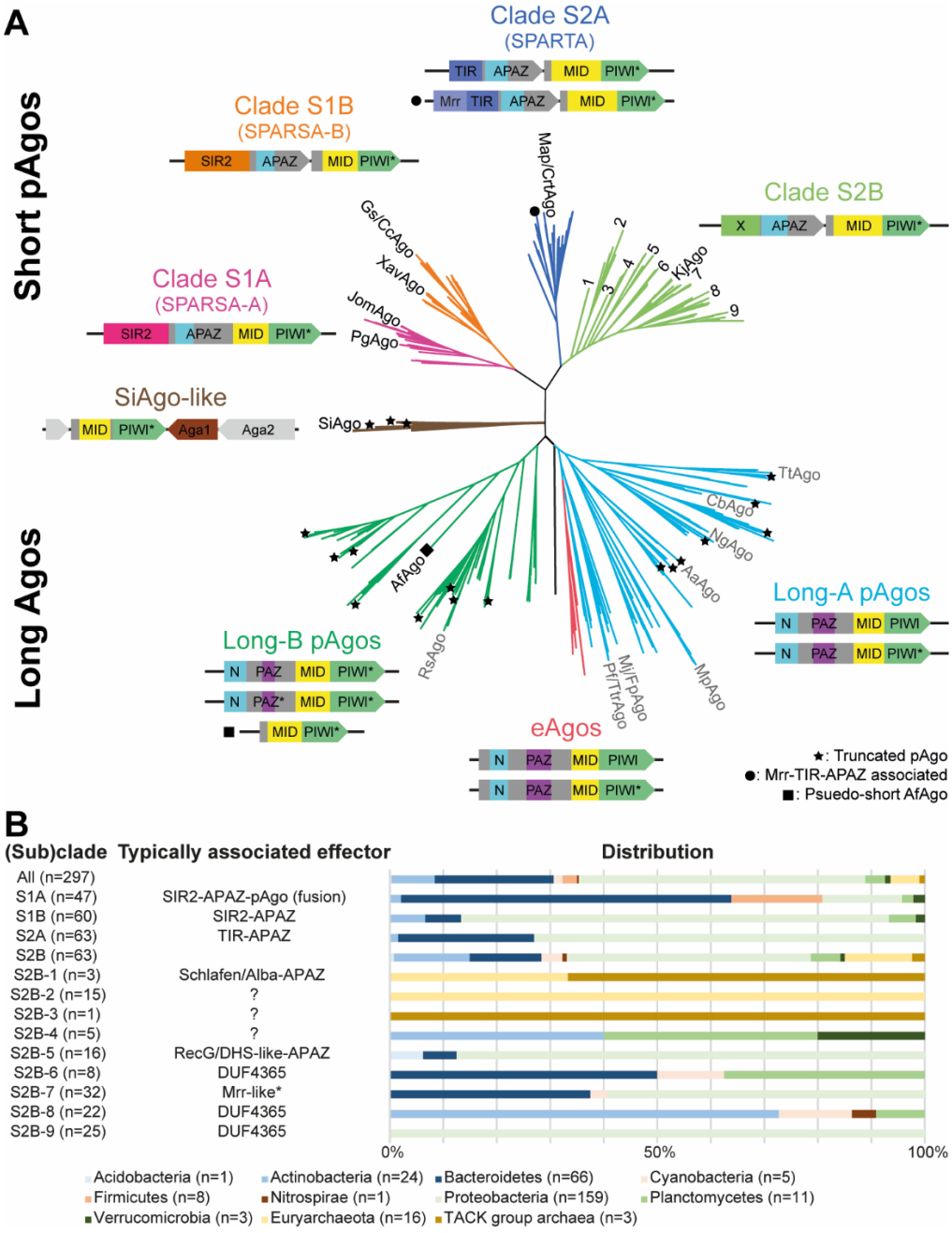


**Figure 1 Phylogeny of prokaryotic Argonaute proteins.**

(A) Maximum-likelihood-based unrooted phylogenetic tree containing all bacterial and archaeal pAgo homologs identified in the RefSeq database containing at least scaffold-level assemblies, as well as selected experimentally characterized pAgos, eAgos, and SiAgo homologs. Analysis performed as previously [36]. Clade S1A: SIR2-APAZ-pAgo fusion (SPARSA-A). Clade S1B: operon with pAgo and SIR2-APAZ (SPARSA-B). Clade S2A: operon with pAgo and TIR-APAZ (SPARTA). Clade S2B: operon with pAgo and APAZ fused to one of various domains (denoted 'X'), including Mrr, DUF4365, and DHS-like domains (see also panel B). ★: truncated pAgos in Long-A, Long-B, and SiAgo-like clades. ●: Short pAgos associated with Mrr-TIR-APAZ. ▪: Psuedo-short AfAgo. The fully annotated tree can be downloaded from Mendeley Data (https://dx.doi.org/10.17632/mpgn9by7z2.1).

(B) Typically associated effector domains and distribution of 297 short pAgos from panel A. Typically putative associated effectors domains as identified by InterPro (identified in >50% of short pAgo systems in this specific clade). Schlafen/Alba: IPR038461. RecG: IPR038475. DHS-like: IPR029035. DUF4365: IPR025375. ?: Effector domain not identified by InterPro. *: The Mrr-like domain in clade S2B-7 effector proteins is not identified by InterPro but is based on earlier studies [42] and AlphaFold2 predictions (Figure 2) [41,103].

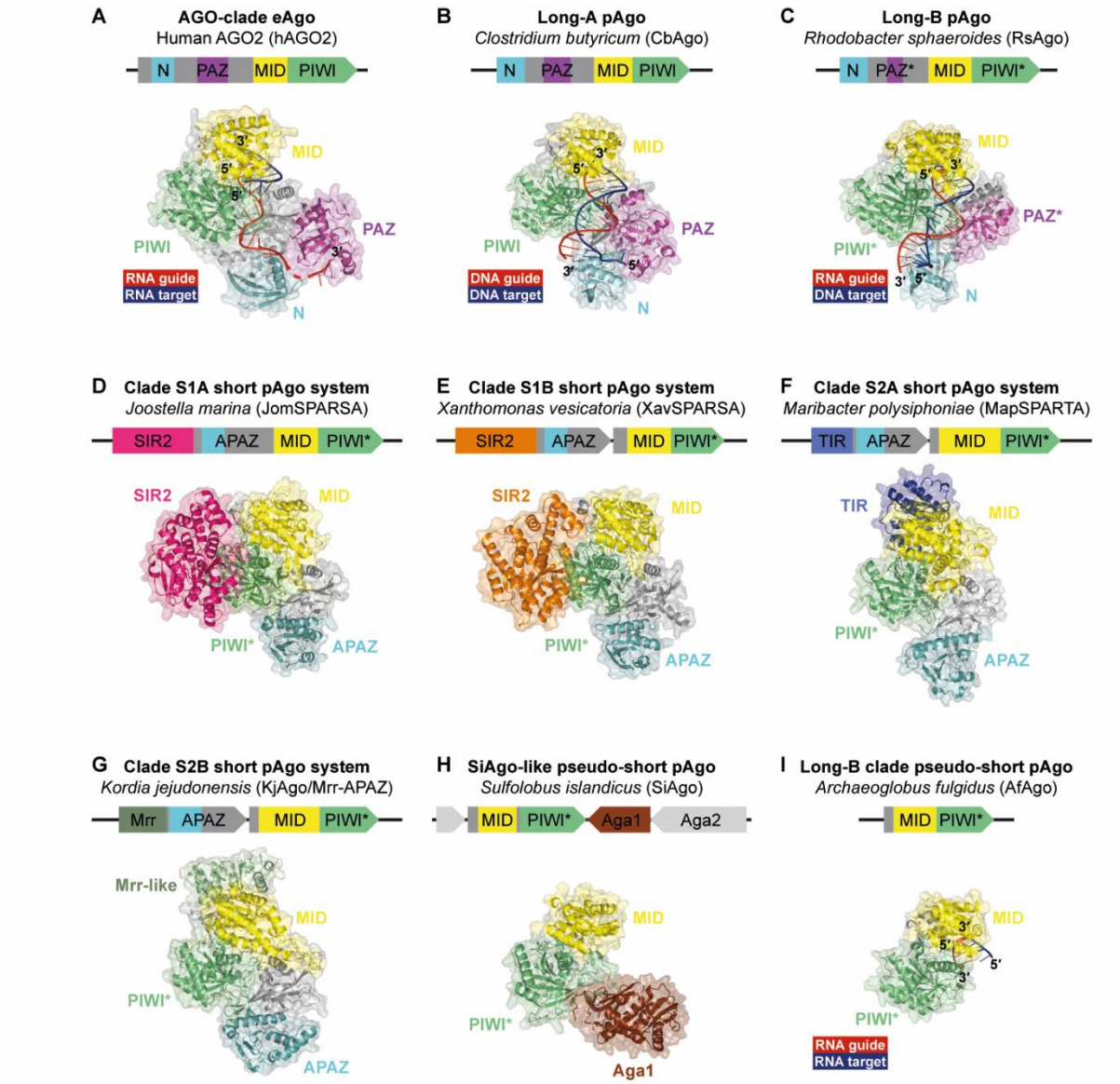


**Figure 2 Operon structure, domain composition, and (predicted) structural architecture of prokaryotic Argonaute proteins.**

Crystal structures of (A) human AGO2 (PDB: 4W5O), (B) *Clostridium butyricum* long-A pAgo (CbAgo; PDB: 6QZK), (C) *Rhodobacter sphaeroides* long-B pAgo (PDB: 5AWH), and (I) *Archaeoglobus fulgidus* truncated long-B pAgo (PDB: 1YTU) in complex with guide and target strands, and Alphafold2-predicted models [41] for (pseudo-)short pAgo systems from distinct phylogenetic clades including (D) clade S1A *Joostella marina* SPARSA (JomSPARSA), (E) clade S2B *Xanthomonas vesicatoria* SPARSA (XavSPARSA), (F) clade S2A *Maribacter polysiphoniae* SPARTA (MapSPARTA), (G) the clade S2B *Kordia jejudonensis* (KjAgo/Mrr-APAZ) system, and (H) the pseudo-short *Sulfolobus islandicus* (SiAgo-SiAga1-SiAga2) system. The related (predicted) domain composition (with corresponding colors) and operon structure are given above each structural model. Explanation of domain functions are given in Box 1. Alphafold2 predictions can be downloaded from Mendeley Data (https://dx.doi.org/10.17632/mpgn9by7z2.1).

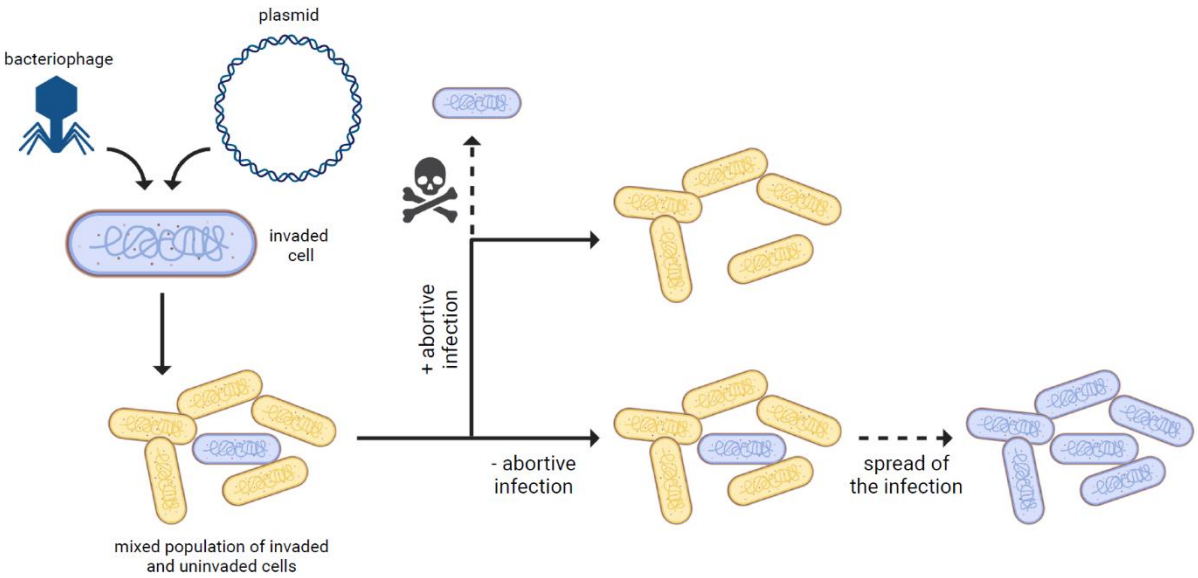


**Figure 3 General mechanism of Abortive infection systems**

Invading nucleic acid such as phages or plasmids can enter prokaryotic cells and, in absence of abortive infection systems, may persist in the population, providing a metabolic burden, or they can spread to neighboring cells through lysis and infection of neighboring cells (phages), or through conjugation (conjugative plasmids). Abortive infection systems prevent spread of invading nucleic acids by sensing the invader and subsequently killing their host cell. This will remove invaded cells from the population. This figure was created using BioRender (https://biorender.com/).

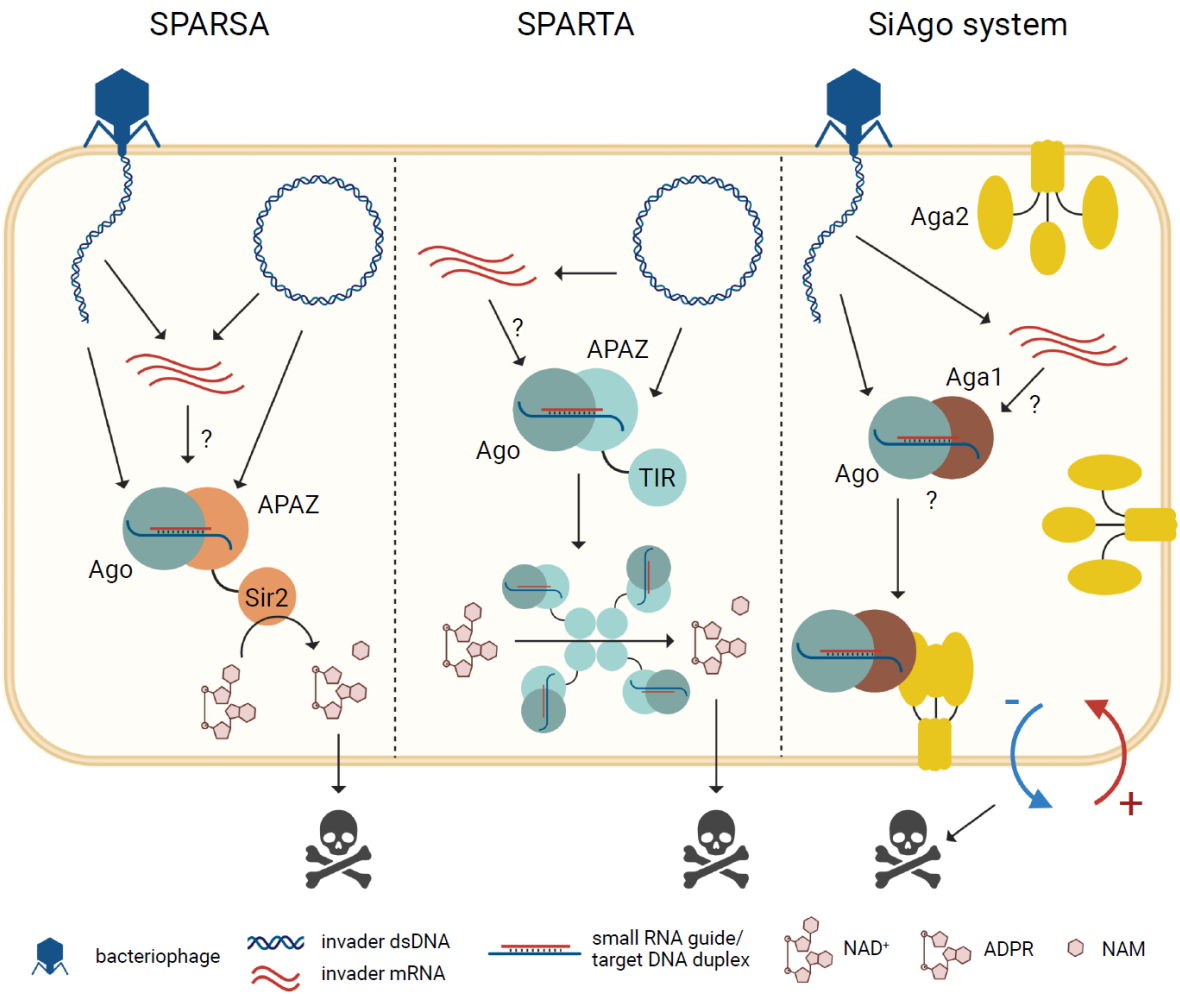


**Figure 4 Schematic representation of host defense against invading DNA by (pseudo-) short pAgo systems.** Short pAgos from SPARSA (left), SPARTA (middle), and pseudo-short SiAgo (right) systems form heterodimeric complexes with their accessory effector proteins. Invading viral and plasmid dsDNA (blue) enter the cell, after which the (pseudo-)short pAgo system acquires small guide RNAs from invader RNA transcripts (red) by an unknown mechanism. The guide RNA facilitates sequence-specific recognition of invading DNA strands (blue) which results in catalytic activation of the effector domains: SIR2 (SPARSA), TIR (SPARTA), or Aga2 (SiAgo system). In SPARSA and SPARTA, SIR2/TIR activation leads to the conversion of $NAD(P)^+$ to NAM and ADPR(P). In SPARTA, catalytic activation requires tetramerization of four guide/target-bound heterodimeric SPARTA complexes. Upon invader detection, the SiAgo-SiAga1 complex is recruited to the membrane protein SiAga2 which induces membrane depolarization. Both $NAD(P)^+$ depletion and membrane depolarization induce cell death, which protects neighboring cells and cures metabolically costly plasmids from the population (see also Figure 3). This figure was created using BioRender (https://biorender.com/).


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## Declaration of interests

D.C.S. and B.K. (together with Ana Potocnik) submitted a patent application regarding the utilization of short pAgo systems for nucleic acid detection.

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