A surveillance mechanism for Argonaute-bound small RNAs in *Schizosaccharomyces pombe*
Erklärung


Eidesstattliche Versicherung

Diese Dissertation wurde eigenständig und ohne unerlaubte Hilfe erarbeitet.

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A mio nonno Amedeo,
perché avrebbe voluto studiare e non ha potuto farlo.

E a me stessa,
perché penso sempre di non farcela e alla fine ce la faccio.
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SUMMARY

RNA-interference (RNAi) is a mechanism conserved from fission yeast to humans through which small RNAs direct gene silencing in a transcriptional and post-transcriptional manner. Several classes of regulatory small RNAs with similar and different functions have evolved along with diverse biogenesis pathways. Despite the differences in their origin and maturation process, small RNAs have a common mediator to accomplish their regulatory role: the Argonaute proteins. How the Argonaute-associated small RNAs are targeted for degradation in order to promote their turnover or how the cell avoids wrong classes of small RNAs to be loaded into Argonaute are two open questions. In the last decade, several studies have shown that tailing of small RNAs can lead to their destabilization and degradation, where tailing represents the addition of untemplated nucleotides at the small RNAs 3’-end by nucleotidyltransferases.

This work shows that in Schizosaccharomyces pombe, also known as fission yeast, the adenylyltransferase Cid14, a member of the TRAMP complex, and the uridylyltransferase Cid16 add untemplated nucleotides to 3’-end of the Argonaute-bound small RNAs. This tailing recruits the 3’-to-5’ exonuclease Rrp6 which degrades the Argonaute-bound small RNAs. In absence of Cid14, the cells undergo an uncontrolled RNAi which silences ectopic targets, like euchromatin loci. This is caused by a defect in the Argonaute-bound small RNAs surveillance mechanism which results in the accumulation of “noise” small RNAs on Argonaute and in the targeting of diverse euchromatic genes by the RNA-induced transcriptional silencing (RITS) complex. To protect themselves, Cid14 deletion cells exploit the uncontrolled RNAi to silence genes that are essential for the RNAi machinery itself, like the RNA-dependent RNA polymerase Rdpg1 which is responsible for the secondary small RNAs generation. Overall, the results discussed here describe a surveillance mechanism of the Argonaute-bound small RNAs based on the nucleotidyltransferases Cid14/Cid16 and the nuclear exosome. Moreover, the data show how fission yeast can rely on a rapid RNAi-based, heterochromatin-independent response to adapt and survive to stress conditions.
1. INTRODUCTION

1.2 RNA interference: how an unexpected result can turn into a great discovery

In 1990, Jorgensen and colleagues introduced a pigment-producing gene under the control of a strong promoter with the aim of intensifying the purple pigmentation in petunias. Instead of an increased color intensity, they observed loss of pigmentation associated with suppression of both endogenous gene and transgene: hence the name of “co-suppression” for this phenomenon (Napoli et al., 1990). Called with the name of “quelling”, the same transgene-mediated silencing was reported in the fungus Neurospora crassa and was described as sequence-homology dependent and promoter independent (Romano and Macino, 1992; Cogoni et al., 1996). Studies in Caenorhabditis elegans finally described this phenomenon as a double stranded RNA (dsRNA) based mechanism and referred to it as RNA interference (RNAi) (Guo and Kemphues, 1995; Fire et al., 1998). Later on, RNAi was shown to be associated with the presence of 21-23 nucleotide (nt) long RNA molecules, hence called small interfering RNAs (siRNAs), generated by Dicer, an enzyme belonging to the RNase III family, and bound to Argonaute, a member of the piwi/sting/argonaute/zwille/eIF2C gene family, both effectors being conserved from fungi to vertebrates (Hamilton and Baulcombe, 1999; Tabara et al., 1999; Hammond et al., 2000; Zamore et al., 2000; Elbashir et al., 2001; Bernstein et al., 2001; Hammond et al., 2001; Martinez et al., 2002). At the present, several classes of regulatory small RNAs have been reported and their role in transcriptional gene silencing (TGS) and post-transcriptional gene silencing (PTGS) has been described (Ghildiyal and Zamore, 2009; Carthew and Sontheimer, 2009).

1.2.1 siRNAs

Small interfering RNAs (siRNAs) are 21-23 nt long small RNAs deriving from cleavage of dsRNA, typically but not exclusively exogenous, by Dicer proteins (Fig. 1). Dicer contains a double RNase III domain, a dsRNA binding domain, a Helicase-like domain and a PIWI-ARGONAUTE-ZWILLE (PAZ) domain (Bernstein et al., 2001) and it is associated with another dsRNA binding protein, called R2D2 in fly (Liu et al., 2003). The siRNAs duplexes generated by Dicer are then loaded on Argonaute proteins to form the RNA-induced silencing complex (RISC) in which Argonaute performs transcript slicing through base pairing between the single stranded small RNA, called the “guide strand”, and the target RNA (Fig.1) (Tabara et al., 1999; Hammond et al., 2000; Hammond et al., 2001; Martinez et al., 2002). Although the number of Argonaute proteins varies among different species, in
eukaryotes they are characterized by an N-terminal domain, a PAZ domain, a MID (middle) domain and a PIWI domain (Tolia and Joshua-Tor, 2007). The PAZ domain contains the small RNA binding pocket which accommodates the 3'-end of the guide strand (Lingel et al., 2003; Song et al., 2003; Yan et al., 2003; Lingel et al., 2004; Ma et al., 2004). The MID domain recognizes the 5'-end through the interaction between the basic binding pocket present in the MID-PIWI interface and the 5'-end phosphate of the small RNA, influencing the 5'-end nucleotide preference that different Argonaute proteins have (Parker et al., 2005; Frank et al., 2010; Frank et al., 2012). The PIWI domain is an RNase H-like domain which is responsible for the slicer activity of those Argonaute proteins that are able to cleave the target RNAs, most likely through the catalytic site Asp-Asp-His/Asp (DDH/D) (Song et al., 2004; Liu et al., 2004; Rivas et al., 2005). Argonaute proteins with no slicer activity are unable to cleave the target RNA (Meister et al., 2004; Liu et al., 2004).

The RISC assembly process comprehends the siRNA duplex loading, which leads to the formation of the pre-RISC, and the complex maturation, which consists in the removal of the strand complementary to the guide strand, called “passenger strand” (Kobayashi and Tomari, 2016). In fly (Iwasaki et al., 2010; Miyoshi et al., 2010c), mammals (Iwasaki et al., 2010; Pare et al., 2013) and plants (Iki et al., 2010), the loading step is ATP-hydrolysis dependent and mediated by the heat shock chaperone machinery. In *Drosophila melanogaster*, the loading step involves also the RISC-loading complex (RLC), which consists in Dcr-2 (Dicer protein) and R2D2 (Tomari et al., 2004; Pham and Sontheimer, 2005) which reflects the separation of siRNAs and miRNAs pathways in fly and the difference in the Dicer and Argonaute proteins involved (Förstemann et al., 2007; Tomari et al., 2007; Czech et al., 2009). The RLC also defines the polarity of the siRNA duplex in terms of thermodynamic stability of the siRNA ends, which is the guide strand selection criterion: the strand with the least 5'-end base pairing stability is selected as guide strand (Schwarz et al., 2003; Khororova et al., 2003; Tomari et al., 2004). Mammalian siRNA duplex loading into RISC does not require Dicer, and the four Argonaute proteins (Ago1-4) do not distinguish between siRNAs and miRNAs (Liu et al., 2004; Meister et al., 2004; Azuma-Mukai et al., 2008; Murchison et al., 2005; Yoda et al., 2010; Betancur and Tomari, 2012).

The passenger strand release was suggested to start with a process of wedging, during which the N-terminal domain of Argonaute opens up the duplex from the 3’-end of the guide strand (Kwak and Tomari, 2012), and to terminate with a slicer-dependent or independent ejection: in presence of a perfect complementary duplex and a slicer competent Argonaute, the passenger strand is cleaved and hence destabilized (Matranga et al., 2005; Rand et al., 2005; Miyoshi et al., 2005), otherwise the
PAZ domain, anchoring the 3'-end, might help the duplex unzipping process (Gu et al., 2012).

Figure 1. From Carthew and Sontheimer, 2009: siRNAs biogenesis pathway.

1.2.3 miRNAs

Micro-RNAs (miRNAs) are 21-22 nt long single stranded RNAs generated from hairpin-shaped primary-transcripts (pri-miRNAs) (Lee et al., 2002; Winter et al., 2009). The pri-miRNAs are transcribed mostly by RNA Pol II (Fig. 2) (Lee et al., 2004; Cai et al., 2004), although some miRNAs deriving from Alu repeats were reported to be transcribed by the RNA Pol III (Borchert et al., 2006). Therefore, the first difference with the siRNAs is that miRNAs arise from endogenously transcribed precursors and their biogenesis starts in the nucleus. In fact, pri-miRNAs are processed by the nuclear RNase III-type protein Drosha (Lee et al., 2003) that, together with the co-factor DGCR8/Pasha, forms the microprocessor complex which releases the precursor miRNA (pre-miRNA) (Fig. 2) (Han et al., 2004; Denli et al., 2004; Gregory et al., 2004; Landthaler et al., 2004; Zeng et al., 2005; Zeng and Cullen, 2005; Han et al., 2006). This is then recognized by the Exportin-5 and traslocated to the cytoplasm (Yi et al., 2003; Bohnsack et al., 2004; Lund et al., 2004; Okada et al., 2009) where it is processed by Dicer (Grishok et al., 2001; Hutvágner et al., 2001; Ketting et al., 2001;
Knight and Bass, 2001) into mature 22 nt miRNA duplex (Fig. 2). As mentioned before, in *D. melanogaster* the siRNAs and miRNAs pathways are clearly separated and involve different effectors, starting from Dicer and its association with dsRNA-binding proteins: Dcr-1, together with the dsRNA-binding protein Loquacious, is required for miRNAs biogenesis while Dcr-2, associated with R2D2, processes siRNAs (Förstemann et al., 2005; Jiang et al., 2005; Saito et al., 2005). After Dicer cleavage step, according to the “sorting” model, miRNA duplexes with mismatches are loaded on Ago1 whereas perfectly complementary siRNA duplexes are loaded on Ago2 (Förstemann et al., 2007; Tomari et al., 2007). Similarly, in *C. elegans* miRNAs are sorted into ALG-1 and siRNAs are bound by RDE-1 (Steiner et al., 2007). Although it was initially observed that one strand of the miRNA duplex is preferentially selected as the mature miRNA associated with RISC (Khvorova et al., 2003; Schwarz et al., 2003) recent evidences coming from next-generation sequencing data suggest that both strands of the miRNA duplex can be loaded on Argonaute, generating the 5p- and 3p- mature miRNA depending on whether it derives from the 5’-arm or the 3’-arm of the hairpin precursor, respectively (Yang et al., 2011; Li et al., 2012; Zhou et al., 2012; Kuo et al., 2015).

The mechanism by which miRNAs control post-transcriptional gene expression depends on the degree of complementarity between the miRNA and the target sequences: nearly fully complementary miRNAs in animals trigger endonucleolytic cleavage of the target-RNA (Yekta et al., 2004; Davis et al., 2005) whereas miRNAs with a complementarity limited to the first 8 nucleotide of the miRNA 5’-end, called “seed region” (Lewis et al., 2003; Brennecke et al., 2005; Lewis et al., 2005), repress the translation of the mRNA, usually interacting with the 3’-untranslated region (UTR) (Valencia-Sanchez et al., 2006; Baek et al., 2008; Selbach et al., 2008). Some studies support the idea that the miRNA-mediated translation repression happens at the initiation step of the protein synthesis (Pillai et al., 2005; Humphreys et al., 2005; Mathonnet et al., 2007). However, other studies showed this repression to accour after the initiation step (Olsen and Ambros, 1999; Seggerson et al., 2002; Petersen et al., 2006) or at even later stages (Kim et al., 2004; Nelson et al., 2004; Maroney et al., 2006; Nottrott et al., 2006), suggesting that there might be more than one mechanism dictating the miRNA-mediated translation repression (Kong et al., 2008). Besides translation repression, miRNAs were reported to promote RNA destabilization (Wu et al., 2006; Wakiyama et al., 2007) and to address the target-RNA to dedicated cytoplasmic foci, called P-bodies, which contain most enzymes required for mRNA degradation (Bashkirov et al., 1997; Sheth and Parker, 2003; Liu et al., 2005; Bhattacharyya et al., 2006; Pauley et al., 2006; Eulalio et al., 2007).
piRNAs

Piwi-interacting RNAs (piRNAs) are 24-30 nt long single stranded small RNAs that, as suggested by the name, interact with the germline-specific subclass of Argonaute proteins named Piwi and conserved from nematode to mammals (not present in plants), playing an important role in germ cell development (Cox et al., 1998; Luteijn and Ketting, 2013; Iwasaki et al., 2015). They were discovered first in *D. melanogaster* as rasiRNAs, small RNAs essential for transposons silencing (Aravin et al., 2001; Aravin et al., 2003; Saito et al., 2006; Vagin et al., 2006; Brennecke et al., 2007; Gunawardane et al., 2007; Brennecke et al., 2008) and subsequently in mouse (Aravin et al., 2006; Girard et al., 2006; Grivna et al., 2006; Carmell et al., 2007; Aravin et al., 2007; Aravin et al., 2008; Kuramochi-Miyagawa et al., 2008), rat (Lau et al., 2006) and *Danio rerio* (Zebrafish) (Houwing et al., 2007). In contrast to miRNAs, piRNAs are generated from a single-stranded precursor RNA deriving
from specific loci named piRNA clusters (Fig. 3). The precursor is then processed in the mature piRNAs in a Dicer-independent manner (for Dicer-independent small RNAs see paragraph 1.2.6) (Vagin et al., 2006; Houwing et al., 2007; Brennecke et al., 2007; Li et al., 2013b). However, piRNAs have been reported to be generated from non-transposon-related transcripts/mRNAs as well (Robine et al., 2009; Rouget et al., 2010).

In C. elegans, piRNAs are known as 21U-RNAs, whose precursor is transcribed from separated genes harboring a Forkhead transcription factor consensus motif, and they are associated to the Piwi protein PRG-1 (Ruby et al., 2006; Das et al., 2008; Batista et al., 2008; Cecere et al., 2012; Billi et al., 2013).

According to the current model, fly piRNAs biogenesis begins with their 5'-end definition through an endonucleolytic cleavage mediated by Zucchini (Fig. 3), generating primary piRNAs (Nishimasu et al., 2012; Ipsaro et al., 2012). It can also be directed by the Piwi proteins Aubergine (Aub) and Ago3 (Brennecke et al., 2007; Gunawardane et al., 2007), liberating in this case secondary piRNAs (Fig. 3). The secondary pathway is also known as ping-pong loop (Fig. 3) since the Aub and Ago3 Piwi proteins are associated with antisense and sense piRNAs, respectively, and direct the cleavage of the sense and antisense transposons, respectively, generating new piRNAs for each other (Brennecke et al., 2007; Gunawardane et al., 2007; Houwing et al., 2007; Aravin et al., 2007).

The 3'-end formation depends on either Zucchini or Aub/Ago3. Zucchini-mediated cleavage directly defines a mature piRNA 3'-ends (Han et al., 2015; Mohn et al., 2015) while Aub and Ago3 generate pre-piRNAs that require further processing by the 3’-to-5’ exoribonuclease Nibbler (Feltzin et al., 2015; Wang et al., 2016a; Hayashi et al., 2016), known as PNLDC1 in Bombyx mori (silkworm) (Izumi et al., 2016) and as PARN-1 in C. elegans (Tang et al., 2016).

Beside the slicing activity on the target RNA reported in D. melanogaster, (Brennecke et al., 2007; Gunawardane et al., 2007) evidences of translational and transcriptional silencing mediated by mammalian Piwi proteins have been shown as well (Grivna et al., 2006; Carmell et al., 2007; Aravin et al., 2007; Aravin et al., 2008).
1.2.5 Drosha-independent miRNAs

Besides the canonical miRNA biogenesis pathway, several alternative mechanisms lead to mature miRNAs formation without the microprocessor cleavage step (Miyoshi et al., 2010a). The first class to be characterized was the one of the mirtrons in fly and worm firstly (Ruby et al., 2007a; Okamura et al., 2007; Chung et al., 2011) and in vertebrates secondly (Berezikov et al., 2007; Babiarz et al., 2008; Glazov et al., 2008; Chiang et al., 2010; Sibley et al., 2012; Ladewig et al., 2012). miRNAs whose processing does not require Drosha cleavage but depends on Dicer were reported to derive also from small nucleolar RNAs (snoRNAs) (Ender et al., 2008; Saraiya and Wang, 2008), which are 70-100 nt long small RNAs found in small nucleolar ribonucleoprotein complexes involved in ribosomal
RNA (rRNA) editing (Matera et al., 2007). A study performed in mouse embryonic stem cells identified a class of miRNAs generated from precursor microRNA-like hairpins termed endogenous short-hairpin RNAs (endo-shRNAs) and from the isoleucine transfer RNA (tRNA-Ile) precursor (Babiarz et al., 2008). Moreover, several groups identified human tRNA-derived small RNAs (Kawaji et al., 2008; Lee et al., 2009b; Cole et al., 2009; Haussecker et al., 2010), of which the first type (type I tsRNA) was described as Dicer substrate, after processing by Rnase P and Rnase Z and nuclear export (Haussecker et al., 2010).

More recently, a microprocessor-independent mechanism was shown for the pre-miR-320, an RNA pol II transcript that harbors a 7-methylguanosine (m7G)-cap at the 5′-end and is exported to the cytoplasm through the PHAX (phosphorylated adaptor for RNA export)-dependent Exportin-1 pathway. Because of the 5′-(m7G)-cap, only the 3p-miRNA is efficiently loaded on Argonaute after Dicer cleavage (Xie et al., 2013).

1.2.5.1 mirtrons

As mentioned above, mirtrons form a microprocessor-independent class of pri-miRNAs that gives rise to mature and functional miRNAs through a biogenesis pathway involving splicing machinery, nuclear export and dicing step in the cytoplasm (Westholm and Lai, 2011). In this case, the mature miRNA resides inside a protein-coding gene intron which, once excised by the splicing machinery as a lariat, can be debranched by the lariat debranching enzyme (Ldbr) and transferred to the cytoplasm as a canonical pre-miRNA by Exportin-5 (Fig. 4). Once in the cytoplasm, it is cleaved by Dicer and loaded on Argonaute (Ruby et al., 2007a; Okamura et al., 2007; Ladewig et al., 2012). Although the conventional mirtron feature is that the ends of the hairpin precisely map to the 5′ and 3′ splice sites of the intron, mirtron-like loci characterized by a single strand extension at either 5′- or 3′-ends or at both ends of the hairpin have been found and, thus, named “tailed” mirtrons (Ruby et al., 2007a; Babiarz et al., 2008; Glazov et al., 2008; Valen et al., 2011; Ladewig et al., 2012; Wen et al., 2015) and “two-tailed” mirtrons, respectively. (Castellano and Stebbing, 2013). The biogenesis of this type of mirtrons requires a trimming step of the 5′/3′ extension between the lariat resolution and the nuclear export steps. The 3′-tail of the hairpin was reported to be trimmed by the RNA exosome (Flynt et al., 2010) the major 3′-to-5′ exoribonuclease in eukaryotes. Regarding the 5′-tailed mirtron, there is still no biochemical evidence of the potential 5′-to-3′ exonuclease.
It is important to mention that also canonical human miRNA loci can be located within the introns of transcription units (Rodriguez, 2004) and cleaved by Drosha before the splicing event (Kim and Kim, 2007), providing an example of Drosha-dependent co-transcriptional processing of canonical pri-miRNAs (Morlando et al., 2008).

1.2.5.2 endo-siRNAs or esiRNAs

Endogenous short interfering RNAs (endo-siRNAs) represent a class of regulatory small RNAs generated from perfectly complementary genome encoded double stranded transcripts that are processed by Dicer proteins and loaded on Argonaute proteins (Piatek and Werner, 2014). 

*C. elegans* owns three types of endo-siRNAs: 21U-, 26G and 22G-RNAs (Ambros et al., 2003; Ruby et al., 2006). The 21U-RNAs present a 5’-uridine bias (Ruby et al., 2006) and, as mentioned before,
were shown to be the worm piRNAs required for transposon suppression and for the maintenance of temperature-dependent fertility (Das et al., 2008; Batista et al., 2008).

The 26G-RNAs starts with 5'-guanine and derives from RRF-3 (RNA-dependent RNA Polymerase, RdRP)-dependent transcripts which are processed by DCR-1 and ERI-1 (Dicer and exonuclease, respectively) (Vasale et al., 2010). A group of 26G-RNAs is expressed in oocytes, embryos and somatic tissues and is found in complex with Argonaute ERGO-1 (Vasale et al., 2010). Another group interacts with Argonaute ALG-3 and ALG-4 and regulates spermatogenic gene expression (Han et al., 2009; Conine et al., 2010).

The 22G-RNAs, also characterized by a 5'-guanine and synthesized by RdRPs (Gu et al., 2009; Claycomb et al., 2009; Lee et al., 2012), were shown to have different functions depending on the Argonaute proteins they are associated with: 1) when bound to CSR-1, they are required for targeting of germline-expressed genes and correct chromosome segregation (Claycomb et al., 2009; van Wolfswinkel et al., 2009; Conine et al., 2013; Seth et al., 2013; Wedeles et al., 2013). 2) when associated with WAGOs (Worm-specific AGO)S, they silence protein-coding genes, transposons, pseudogenes, and cryptic loci through both transcriptional and post-transcriptional mechanisms (Gu et al., 2009; Ashe et al., 2012; Luteijn et al., 2012; Buckley et al., 2012; Mao et al., 2015).

In *D. melanogaster* endo-siRNAs are generated upon a Dcr-2 and Loqs-PD (a specific isoform of Loquacious) mediated processing (Hartig et al., 2009; Zhou et al., 2009; Marques et al., 2010; Miyoshi et al., 2010b) of transposable elements (TEs), *cis*-natural antisense transcripts (*cis*-NATs), which comprise convergently transcribed 3' UTRs, and long hairpin RNAs containing a long duplex structure (Chung et al., 2008; Czech et al., 2008; Kawamura et al., 2008; Ghildiyal et al., 2008; Okamura et al., 2008a; Okamura et al., 2008b). First reported to be involved in transposons control and viral defence (Chung et al., 2008; Czech et al., 2008; Ghildiyal et al., 2008; Kawamura et al., 2008; Mirkovic-Hösle and Förstemann, 2014), several studies suggested a role of fly endo-siRNAs in protein coding gene regulation (Lucchetta et al., 2009; Lim et al., 2011; Lim et al., 2013). In addition to fly endo-siRNAs, which are found in both somatic and germline cells, endo-siRNAs arise from dsRNAs composed of gene:pseudogene transcripts in mouse oocytes (Tam et al., 2008; Watanabe et al., 2008). Moreover, in mESCs, some miRNAs are actually endo-siRNAs deriving from one convergent Alu/B1 SINE element located on chromosome 7 and on chromosome 4 (Babiarz et al., 2008; Castellano and Stebbing, 2013).
1.2.6 Dicer-independent miRNAs and small RNAs

Besides the Drosha-independent miRNAs, also Dicer-independent miRNAs have been discovered (Miyoshi et al., 2010a). The biogenesis of predicted mirtrons, miR-1225 and miR-1228, was shown to be dependent on Drosha but not on splicing, DGCR8, Dicer or Ago2 and, therefore, it was named simtron (splicing-independent mirtron-like miRNA) biogenesis pathway (Havens et al., 2012). In Zebrafish, mouse and human pre-mir-451 is directly cleaved and trimmed by Ago2, bypassing Dicer cleavage after Drosha processing (Cheloufi et al., 2010; Cifuentes et al., 2010; Yang et al., 2010). A novel class comprehends miRNAs deriving from processing at the 5'- or 3'-end of mature or precursor tRNAs (Lee et al., 2009b; Haussecker et al., 2010).

In this frame, primal small RNAs (priRNAs) discovered in Schizosaccharomyces pombe fit as Dicer-independent small RNAs (Halic and Moazed, 2010). They are generated from transcripts processed by a mechanism involving Argo1aute (Ago1) and the PARN family nuclease Triman (Tri1) (Marasovic et al., 2013). Longer small RNA precursors are loaded on Ago1 and trimmed by Tri1 to the functional length of 22 nt. In this way, the priRNAs direct Ago1 to nascent heterochromatic RNAs where, in turn, it induces co-transcriptional and transcriptional gene silencing (CTGS and TGC).

1.2.7 Small RNAs in plants

In plants, small RNAs are generated from hairpin-shaped or perfect long double stranded RNAs that are processed by DICER-LIKE (DCL) proteins (Fang and Qi, 2016). The classes discovered so far are: miRNAs produced by DCL1 (Reinhart et al., 2002; Kurihara and Watanabe, 2004; Qi et al., 2005), trans-acting small interfering RNAs (ta-siRNAs) generated by DCL4 (Peragine et al., 2004; Vazquez et al., 2004; Gasciolli et al., 2005; Xie et al., 2005), heterochromatic siRNAs (hc-siRNAs) processed by DCL3 (Xie et al., 2004; Qi et al., 2005; Henderson et al., 2006), natural antisense siRNAs (NAT-siRNAs) (Borsani et al., 2005), long siRNAs (Pontes et al., 2006), long miRNAs (ImiRNAs) (Wu et al., 2010), double-strand-break (DSB)-induced sRNAs (diRNAs) (Wei et al., 2012), and DCL-independent siRNA (sidRNAs) (Ye et al., 2016). miRNAs, ta-siRNAs, and NAT-siRNAs direct PTGS, while hc-siRNAs, ImiRNAs, and sidRNAs guide TGS through DNA methylation (Fang and Qi, 2016).

Differently with what mentioned before about fly small RNAs, in plants small RNAs sorting depends on the 5'-end nucleotide of the small RNA: AGO1, the main effector of miRNAs, binds to 5'-uridine, AGO4 is loaded mainly with small RNAs starting with adenine and AGO5 has a bias for 5'-cytosine (Mi et al., 2008; Montgomery et al., 2008; Takeda et al., 2008). Differently from animals, plant miRNAs are characterized by a high degree of sequence complementarity to their target RNAs and they were found to induce silencing by endonucleolytic cleavage activity of AGO1, the main miRNAs
and ta-siRNAs Argonaute protein (Baumberger and Baulcombe, 2005; Llave et al., 2002; Tang et al., 2003). However, beside the slicing-mediated silencing, many miRNAs were shown to repress translation as well (Aukerman and Sakai, 2003; Chen, 2004; Gandikota et al., 2007; Brodersen et al., 2008; Yang et al., 2012; Li et al., 2013a).

1.3 RNAi in *Schizosaccharomyces pombe*

*Schizosaccharomyces pombe*, also known as fission yeast, is an ascomycete yeast that, unlike the budding yeast *Saccharomyces cerevisiae*, has retained the genes encoding for the RNAi machinery effectors (Hoffman et al., 2015). RNAi in fission yeast is essential for the establishment and the maintenance of silenced chromatin, known as heterochromatin, at the pericentromeric regions (Volpe et al., 2002; Volpe et al., 2003) and for the heterochromatin establishment at the mating-type locus (mat2/3), where the maintenance is instead mediated by another pathway involving the DNA-binding proteins Atf1 and Pcr1, the histone deacetylases (HDAC) Clr3, Clr6 and the HP1 (Heterochromatin Protein 1) protein Swi6 (Hall et al., 2002; Jia et al., 2004; Yamada et al., 2005). RNAi can establish heterochromatin at subtelomeric regions as well, acting redundantly with the Shelterin complex, which binds to telomeres and recruits chromatin modifiers (Kanoh et al., 2005; Hansen, 2006; Sugiyama et al., 2007; Wang et al., 2016b). A recent work showed that at subtelomeric regions heterochromatic RNAs are retained on chromatin, forming DNA:RNA hybrids, with the need of being degraded by the Ccr4-Not complex, containing the deadenylase essential for downstream mRNA decapping and decay, or RNAi in order for heterochromatin to be maintained (Brönner et al., 2017).

The *S. pombe* centromeric regions consist in outer repeats (*otr*) subdivided into two elements called *dg* and *dh*, flanking a central domain. The central domain includes the innermost repeats (*imr*), and a central core. The I, II and III chromosomes harbor cen1, cen2, and cen3 which occupy 40, 60, and 120 kilobases, respectively (Steiner et al., 1993; Pidoux and Allshire, 2004). The noncoding DNA organized in repetitive sequences at the pericentromeric region in fission yeast, similar to the more complex centromeres structure of metazoans, is transcribed at low levels by RNA polymerase II and subsequently targeted by the RNAi machinery which in turn directs CTGS and TGS (Fig. 5), inducing methylation of the Histone 3 Lysine 9 (H3K9me) (Volpe et al., 2002; Verdel et al., 2004; Noma et al., 2004; Kato et al., 2005; Bühler et al., 2006; Djupedal et al., 2005; Shimada et al., 2016).

The heterochromatin establishment at centromeres occurs when RNAs transcribed from the *dg* and *dh* DNA repeats are processed into Dicer (Dcr1)-independent 21-23 nt long primal RNAs (priRNAs) (Halic and Moazed, 2010) by Argonaute (Ago1) and Triman (Tri1), mediating then the initial silencing
Ago1 is part of two distinct complexes: the RNA-induced transcriptional silencing (RITS) complex (Verdel et al., 2004) and the Argonaute chaperone (ARC) complex (Buker et al., 2007) (Fig. 5). In the latter, Ago1 is coupled with Arb1 and Arb2 that act as chaperones in the loading process of Ago1 with the siRNA duplex generated by Dcr1 from the dg/dh transcripts (Provost et al., 2002; Reinhart and Bartel, 2002; Colmenares et al., 2007; Buker et al., 2007; Holoch and Moazed, 2015a). The subsequent cleavage and release of the passenger strand leads to maturation of an active RITS complex (Buker et al., 2007; Jain et al., 2016). In this complex, Ago1 is associated with the GW (Glycine Tryptophan) domain protein Tas3 and the chromodomain protein Chp1 (Verdel et al., 2004).

Besides the base pairing between Ago1-bound small RNAs and the pericentromeric transcripts, the RITS complex is bound to the chromatin through the interaction between the H3K9me and the chromodomain of Chp1 as well (Partridge et al., 2002; Petrie et al., 2005; Zocco et al., 2016). However, strong evidences indicate that siRNAs can be generated independently of H3K9me, meaning that heterochromatin is not a prerequisite for siRNAs generation (Gerace et al., 2010; Halic and Moazed, 2010).

Targeting of the RITS complex to the pericentromeric region recruits other two complexes essential for the heterochromatin formation: the RNA-dependent RNA polymerase complex (RDRC) and the Clr4-Rik1-Cul4 (CLRC) complex (Fig. 5). The latter contains the histone methyl-transferase Clr4 which is responsible for di- and tri-methylation of H3K9 (Nakayama et al., 2001; Zhang et al., 2008; Al-Sady et al., 2013). The RDRC complex, consisting of the RNA-dependent RNA polymerase Rdp1, the RNA helicase Hrr1, and the non-canonical poly-(A) polymerase Cid12, synthesizes the RNA strand complementary to the nascent pericentromeric transcripts targeted by RITS (Motamedi et al., 2004; Sugiyama et al., 2005). This RNA polymerase activity leads to the formation of dsRNA which is then processed by Dcr1 into 21-25 nt siRNA duplexes (Reinhart and Bartel, 2002; Motamedi et al., 2004; Colmenares et al., 2007) and, by consequence, to the amplification of the RNAi by a positive feedback loop. Moreover, a newly characterized protein named Dsh1, was suggested to localize Dcr1 and the RDRC to the nuclear periphery and form the “siRNA amplification compartment” (Kawakami et al., 2012).

The recruitment of both the CLRC and the RDRC complex was recently shown to be independent on Ago1-slicer activity (Jain et al., 2016), in contrast with the previously reported idea that Ago1 slicer activity is required for siRNA amplification and silencing (Irvine et al., 2006), although the observed silencing was directed by a priRNA in an Ago1 over-expressing background (Jain et al., 2016).
slicing activity of Ago1 is indeed critical for the generation of an active RITS complex, upon ejection of the passenger strand, and for the release of RITS from chromatin, which could be a key step for RITS recycling (Jain et al., 2016).

Another protein complex that was reported to be involved in the RNAi machinery of fission yeast is the TRAMP-like complex, which consists of the non-canonical poly-(A) polymerase Cid14, associated with the zinc knuckle subunit Air1 and the ATP-dependent RNA helicase Mtr4 (Bühler et al., 2007), all homologous proteins of the subunits of the budding yeast Trf4p/Air2p/Mtr4p (TRAMP) complex (Wyers et al., 2005; Vanácová et al., 2005; LaCava et al., 2005). While a role of the budding yeast TRAMP complex in targeting aberrant RNAs for exosome-mediated degradation was described (LaCava et al., 2005), a similar role for the fission yeast TRAMP complex has not been shown directly, besides the observation that Cid14 polyadenylates ribosomal RNA (rRNA) (Win et al., 2006) and that cells lacking Cid14 accumulate antisense ribosomal siRNAs (Bühler et al., 2008).

Figure 5. From Martienssen and Moazed, 2015: RNAi-mediated co-transcriptional, transcriptional and post-transcriptional gene silencing.
A role of RNAi in directing epigenetic silencing was reported also in nematode, plants, fly and mammals (reviewed in Wassenegger, 2005; Castel and Martienssen, 2013; Chen and Aravin, 2015; Martienssen and Moazed, 2015).

### 1.4 3'-end tailing of regulatory small RNAs from plants to humans

In the last decade, numerous studies have shown that small RNAs are modified at their 3'-end with few nucleotides that do not match to the genome. For this reason, they are described as untemplated nucleotides and the phenomenon as “tailing”. The first evidence of 3'-end post-transcriptional modification of small RNAs was observed in *Arabidopsis thaliana* (Li et al., 2005) followed by *C. elegans* (Ruby et al., 2006), *D. melanogaster* (Ruby et al., 2007b) and mammalian cells (Landgraf et al., 2007). In plants, upon deletion of the methyltransferase *hen1*, small RNAs were reduced and uridylated at the 3'-end (Yu, 2005; Li et al., 2005). It was then proposed that 2'-O-methylation is required to protect small RNAs from uridylation and it might prevent then the small RNAs decay. The nucleotidyltransferases responsible for the uridylation of unmethylated small RNAs in plants are HESO1 and URT1 (Zhao et al., 2012; Ren et al., 2012; Tu et al., 2015; Wang et al., 2015b). They showed to have different substrates preference and different processivity, with URT1 mainly mono-uridylating the miRNAs, and same ability to uridylate AGO1-bound miRNAs. In addition to triggering their decay, uridylation of miRNAs was shown to impair the effectiveness of AGO1-bound miRNAs reducing the slicing activity (Tu et al., 2015).

The nucleotidyltransferase MUT68 was discovered to uridylate the 3'-end of mature miRNAs and siRNAs in *Chlamydomonas reinhardtii*, promoting their degradation by RRP6 exosome subunit *in vitro* (Ibrahim et al., 2010). Deletion of MUT68, in fact, resulted in increased abundance of miRNAs and siRNAs, supporting the role of MUT68 and RRP6 in the mature small RNAs turnover.

Consistently with the findings in plants, a study in Zebrafish showed that in testis lacking of *hen1* the piRNAs are adenylated and uridyalted, with the uridylation being more frequent on retrotransposon-derived piRNAs (Kamminga et al., 2010). This was associated with a reduction of piRNA levels and a mild derepression of transposon transcripts.

The same group observed that in *C. elegans henn-1* mutants the majority of 26G RNAs shows reduced stability and increased 3'-end uridylation frequency (Kamminga et al., 2012). Moreover, another study showed that the CSR-1 (Argonaute protein)-bound siRNAs are uridylated by the nucleotidyltransferase CDE-1 (van Wolswinkel et al., 2009). Loss of CDE-1 was associated with siRNAs accumulation and defects in meiotic and mitotic chromosome segregation.

Another uridylintransferase in worm is PUP-2 which was shown to uridylates pre-let-7 miRNA and
regulates its processing (Lehrbach et al., 2009).

In 2010, Ameres et al. showed how high level of complementarity between D. melanogaster Ago1-bound miRNAs and target-RNAs induces tailing and trimming of the miRNAs, but not of the Ago2-bound siRNAs, which are protected by the 2'-O-methylation (Ameres et al., 2010). The untemplated nucleotides added to the 3'-end of the targeted miRNAs were uridines and adenines mostly.

In agreement with this, it was shown that miRNAs undergo 3'-end uridylation upon target-RNA regulation in mammals (Baccarini et al., 2011). In fact, in cells overexpressing the target-RNA the relative abundance of uridylated miR-223 was higher and the decay rate was faster.

Analysis of high-throughput sequencing data revealed that the tailing frequency was higher for 3p species with the difference that in D. melanogaster both uridines and adenines showed a bias for the 3'-arm derived miRNAs (Berezikov et al., 2011) while in mammalian cells the tailing rate of 3p-miRNAs was higher for the uridines only (Chiang et al., 2010; Burroughs et al., 2010) suggesting that uridylation might happen mainly on pre-miRNAs. However, a following work showed that there is no substantial difference in the modification rates of 5p and 3p canonical miRNA reads, indicating that there is no clear preference for uridylation of canonical pre-miRNAs (Westholm et al., 2012). The authors explained the discrepancy with the study from Berezikov et al. with the presence of mirtron-derived reads in the overall pool of miRNA species. In fact, they observed a high 3'-end uridylation frequency in fly, worm, mouse and human mirtron-3p miRNAs (Westholm et al., 2012; Wen et al., 2015). Three years later, two studies in D. melanogaster identified Tailor as the enzyme that preferentially uridylates mirtron hairpins, inhibiting their biogenesis (Bortolamiol-Becet et al., 2015; Reimão-Pinto et al., 2015).

In mammalian embryonic stem cells, as in C. elegans, pre-let7 miRNA is uridylated at the 3'-end by the uridylyltransferase TUT4 (ZCCHC11), in a Lin28-dependent manner, and this uridylation impairs the correct processing by Dicer and targets pre-let7 miRNA for degradation mediated by the 3'-to-5' exonuclease Dis3L2 (Viswanathan et al., 2008; Newman et al., 2008; Rybak et al., 2008; Heo et al., 2008; Heo et al., 2009; Hagan et al., 2009; Chang et al., 2013; Ustianenko et al., 2013; Faehnle et al., 2014). The related nucleotidyltransferase TUT7 (ZCCHC6, previously found to uridylate miRNAs in human cells (Wyman et al., 2011)) acts redundantly with TUT4 and knockdown of both nucleotidyltransferases leads to increased let-7 levels in embryonic stem cells (Thornton et al., 2012). Moreover, an RNA-specific co-factor, the E3 ligase Trim25, was shown to activate TUT4 by binding the conserved terminal loop of pre-let-7 and make the Lin28-mediated uridylation more efficient (Choudhury et al., 2014).
On the other hand, in differentiated cells with no Lin28 expression a Lin28-independent pre-let-7 monouridylation was observed as well (Newman et al., 2011). In human somatic cells the nucleotidyltransferases TUT7, TUT4, and TUT2 mono-uridylate pre-let-7, which is efficiently processed by Dicer since the 2 nt 3'-overhang is a better substrate than a 1 nt 3'-overhang (Heo et al., 2012). Another study in mammalian cells showed that TUT7 and TUT4 together with the exosome provide a quality control mechanism that prevents defective pre-miRNAs to be loaded onto Argonaute: TUT7/4 not only can mono-uridylate 3'-end of pre-miRNAs to restore the optimal overhang of 2 nt, but they can also oligo-uridylate pre-miRNAs and target them for decay mediated by DIS3 which, contrary to RRP6, prefers pre-miRNAs with a 3'-overhang longer than 2 nt (Liu et al., 2014). TUT7/4 have also been shown to oligo-uridylate truncated pre-miRNAs with a 5'-overhang, with the hypothesis that this oligo-U tail might promote their degradation (Kim et al., 2015).

If 3'-end uridylation has been associated to small RNA decay, the first findings about 3'-end adenylation supported a stabilization role upon addition of one or few adenosines (Lu et al., 2009; Katoh et al., 2009; Burns et al., 2011; D’Ambrogio et al., 2012). In Populus trichocarpa (black cottonwood) adenylated miRNAs were degraded at a slower rate, indicating that 3'-end adenylation plays a negative role in miRNA decay (Lu et al., 2009). In human hepatocytes and mouse liver, miR-122 is adenylated by the non-canonical poly-(A) polymerase GLD-2 (PAPD4) after Dicer mediated processing (Katoh et al., 2009). In liver of GLD-2 knocked out mice, the miR-122 levels were reduced, suggesting a stabilization role for miRNAs by 3'-end adenylation. However, despite its liver-specificity, miR-122 is also present in human fibroblasts where it was observed to be mono-adenylated by GLD-2 and subsequently targeted for degradation, disproving a miRNA stabilization by 3'-end adenylation (Burns et al., 2011; D’Ambrogio et al., 2012). A study in human THP-1 cells showed that 3'-end adenylation of the oncomir miR-21 by the non-canonical poly-(A) polymerase PAPD5, rather than PAPD4 (GLD-2), leads to miR-21 destabilization (Boele et al., 2014). In fact, knocking down either PAPD5 or the exonuclease PARN resulted in increased miR-21 levels, suggesting that PAPD5-mediated adenylation of miR-21 triggers its 3'-to-5' digestion by PARN exonuclease. Moreover, this degradation pathway of miR-21 was deregulated in several proliferative diseases, supporting the role of PAPD5 in preventing the miR-21 mediated repression of tumor suppressor targets (Boele et al., 2014). A previous study in the same cell line observed that PAPD4 (and PAPD5 as well, although to a lesser extent) adenylates about the 20% of the miRNAs deriving from a given miRNA locus and showed that Ago2 and Ago3 proteins were associated with a reduced number of 3'-end adenylated miRNAs (Burroughs et al., 2010). This is consistent with (i) the reduced
expression of the target mRNAs upon reduction of adenylation in PAPD4 knocked down cells, without affecting the miRNAs levels, and (ii) the derepression of target mRNAs upon miRNAs adenylation. Overall, Burroughs et al. suggested that 3′-end adenylation is likely to happen between Dicer processing and Argonaute loading and it might modulate the uptake of miRNAs by Ago2 and Ago3 containing RISC complex. In agreement with this, a meta-analysis of small RNAs sequencing data in plants showed that the combination of 5′-end deletion and 3′-end uridine addition can determine the preference of the miRNA for a specific Argonaute protein (Ebhardt et al., 2009).

Interestingly, several analyses of regulatory small RNAs isolated from virus-infected mammalian cells revealed that 3′-end tailing can be a consequence of the host-pathogen interaction, representing either a viral strategy to regulate the accumulation of specific viral miRNAs or a host defense response against pathogen invasion (Dolken et al., 2007). Small RNAs from poxvirus-treated mammalian and insect cells were discovered to be polyadenylated by the virally encoded poly-(A) polymerase and subsequently degraded (Backes et al., 2012). This activity was not observed on endogenous siRNAs (esiRNAs) containing 2′-O-methylation, suggesting that virus-mediated small RNA degradation might have contributed to 2′-O-methylation as a protection mechanism against viral infections. The lytic murine cytomegalovirus (MCMV) transcript m169 targets the miR-27a and the miR-27b via a single binding site at the 3′ UTR, thereby triggering 3′-end tailing and trimming of miR-27 and promoting the host infection (Marcinowski et al., 2012).

In 2014, the Kim group proposed that adenylation of mature miRNAs in fly contribute to the elimination of the maternally deposited miRNAs during the maternal to zygotic transition (Lee et al., 2014). They, in fact, discovered that maternally inherited miRNAs in D. melanogaster are highly adenylated at their 3′-end (feature conserved in sea urchin, Strongylocentrotus purpuratus, and mouse) by the fly GLD-2 homologue named Wispy. While the knockout resulted in miRNAs accumulation in eggs, over-expression of Wispy was associated with reduced level of miRNAs and de-repression of the target mRNA-reporter.

Supporting this role of Wispy, a previous study in D. melanogaster showed how 3′-end untemplated nucleotides addition is biologically regulated and associated with a specific stage of development: the authors observed that adenylation was predominant at early stages of development while uridylation was increased in adult tissues (Fernandez-Valverde et al., 2010).

Later on, two studies observed that the frequency of 3′-end tailing on specific miRNAs changes with differentiation of human embryonic stem cells and that in human cell B adenylated miRNAs were enriched inside the cells whereas their uridylated isoforms were overrepresented in exosome
vesicles (Wyman et al., 2011; Koppers-Lalic et al., 2014).

Altogether, these works have shown that (i) 3'-end adenylation and uridylation can dictate not only the temporal specificity of regulatory small RNAs, but they can also be tissue-specific and spatially separated among the cell compartments and that (ii) different nucleotidyltransferases can target specific pre-miRNAs/miRNAs and trigger their degradation, promote their biogenesis/stability and/or modulate their effectiveness in target RNA regulation.
2. AIM OF THE STUDY

Previously published high-throughput sequencing data showed that in fission yeast about 20% of the Argonaute-bound small RNAs contain 1-2 untemplated nucleotide(s) at their 3’-end, majority of which being adenine and uridines (Halic and Moazed, 2010). The two non-canonical poly-(A) polymerase Cid12, subunit of the RDRC complex, and Cid14, part of the TRAMP complex, were suggested to be the nucleotidyltransferases responsible for the 3’-end modifications of the Argonaute-bound small RNAs in *S. pombe*. The questions arising from these data were: are the Argonaute-bound small RNAs stabilized or targeted for degradation upon adenylation and uridylation? Which one(s) of the six nucleotidyltransferase is indeed responsible for the Argonaute-bound small RNAs tailing? Given the role of RNAi in heterochromatin formation in fission yeast, does the 3’-end addition of untemplated nucleotides affect the silencing at heterochromatic regions? How small RNAs are removed from Argonaute proteins in order for new small RNAs to be loaded is still not entirely clear. A defect in the Argonaute-bound small RNAs decay machinery might result in a decreased specificity of RNAi and might subsequently affect the expression of genome. The aim of this study was then to investigate the role of 3’-end tailing in the Argonaute-bound small RNAs turnover and, hence, in the gene regulation of fission yeast.
3. RESULTS

3.1 Argonaute-bound small RNAs are modified at their 3'-end

To investigate whether in *S. pombe* the small RNAs are tailed at their 3'-end as well as in other organisms, total and Argonaute-bound (Ago1-bound) small RNAs were purified from wild type cells and sequenced on a HiSeq Illumina system (Halic and Moazed, 2010). When classified, the majority of reads corresponding to Ago1-bound small RNAs fraction mapped to centromeric repeats, with the 8% and 1.5% mapping to ribosomal RNA (rRNA) and to messenger/non-coding RNA (mRNA/ncRNA), respectively (Fig. 1a). In the total small RNA fraction, which comprehends both Ago1-bound and unbound small RNAs, reads mapping to centromeric repeats were about 27%, with many reads representing degradation products of rRNA, tRNAs and mRNA/ncRNA (Fig. 1a).

Figure 1. Ago1-bound small RNAs are enriched of centromeric siRNAs and show the common 5’-uridine preference.

a. Ago1-bound and total small RNAs (Halic and Moazed, 2010) analyzed by high-throughput sequencing from wild type cells and classified as indicated below the pie charts. Pie charts illustrate percentages for the individual small RNA classes relative to the total number of reads for each strain.

b. 5’-nucleotide preference of Ago1-bound and size selected total small RNAs in wild type cells.
The total small RNA fraction did not show any preference for the 5'-end nucleotide, while in the Ago1-bound fraction small RNAs showed a strong bias for uridine, as already shown in fission yeast and other organisms (Bühler et al., 2008; Djupedal et al., 2009; Halic and Moazed, 2010; Brennecke et al., 2007; Mi et al., 2008; Wu et al., 2009; Reuter et al., 2011; Cora et al., 2014) (Fig. 1b).

This feature indicates that these small RNAs are indeed bound to Ago1. Although small RNAs target transposons in many organisms (Obbard et al., 2009; Koonin, 2017), only few Dicer-independent priRNAs and no siRNAs mapped to the transposable element Tf2 and LTR elements (Fig. 2a). Moreover, neither Tf2 nor LTR were silenced by RNAi (Fig. 2b).

The sequences of Ago1-bound and total small RNAs were analysed for the presence of mismatches at the 3’-end and more than 20% of the Ago1-bound small RNAs harbored 1-2 untemplated nucleotide(s), against the ca. 5% of the total fraction small RNAs (Fig. 3a). More than 70% of the untemplated nucleotides was represented by adenines, the 25% by uridines. When classified, about 25% of Ago1-bound siRNAs and priRNAs generated from centromeric transcripts, mRNAs and ncRNAs was adenylated or uridylylated at the 3’-end (Fig. 3b). In contrast, only around 5% of small RNAs generated from tRNAs and sense rRNA had untemplated nucleotides at the 3’-end (Fig. 3b).
Figure 3. Ago1-bound small RNAs have untemplated nucleotides at the 3’-end.

a. Quantification of all small RNAs (left panel) and centromeric small RNAs (right panel) that have untemplated nucleotides at the 3’-end in Ago1-bound and total small RNA sample. Error bars indicate s.e.m. of two independent small RNA sequencing experiments. One set is from Halic and Moazed, 2010.

b. Quantification of small RNAs that have untemplated nucleotides at the 3’-end in different classes of Ago1-bound small RNAs.

c. Quantification of small RNAs that have untemplated nucleotides at the 3’-end in different classes of size selected fraction.

d. Ago1-bound and total small RNA reads from wild type cells were plotted over centromeric region. The location of genes is indicated below the small RNA peaks. Reads from + and - strands are depicted in green and blue, respectively. Scale bars on the right denote small RNA read numbers normalized per one million reads.
Results

Interestingly, small RNAs generated from antisense rRNA were modified more frequently than the ones generated from sense rRNA (Fig. 3b). This suggests that Ago1-bound small RNAs deriving from centromeric region, mRNAs/ncRNAs and antisense rRNA might be processed in the same way, while priRNAs generated from sense rRNA and tRNAs form a distinct class of small RNAs. Alternatively, priRNAs deriving from tRNAs and sense rRNA might be loaded on Argonaute during cell disruption without being bona fide in vivo Argonaute-bound small RNAs.

Unlike the Ago1-bound small RNAs, the total small RNAs were adenylated or uridylated less frequently (Fig. 3a). Centromeric siRNAs were modified at the similar rate in both the total and the Ago1-bound small RNAs samples (Fig. 3a, b and c), suggesting that the majority of the siRNAs deriving from centromeric repeats are indeed loaded on Ago1 (Fig. 3d). On the other hand, small RNA degradation products originating from mRNAs, tRNAs and rRNA were rarely adenylated or uridylated in the total small RNAs fraction (Fig. 3c). Altogether, these results suggest that (i) only Ago1-bound small RNAs are adenylated or uridylated at their 3'-end and that (ii) the untemplated adenine(s) and uridine(s) might be added to the 3'-end of small RNAs after they are loaded on Ago1 (Fig. 3b and c).

The sequence reads corresponding to the small RNAs that are adenylated or uridylated at the 3'-end showed that the modified small RNAs are distributed over the entire genome with reduction for rRNA and tRNAs (Fig. 4a and b). Interestingly, the Ago1-bound priRNAs generated from noncoding antisense RNAs resulted to be more frequently adenylated, whereas Ago1-bound priRNAs deriving from sense mRNA transcripts were found to be more frequently uridylated (Fig. 4c). Despite of some variation in addition of untemplated nucleotides at the 3'-end of different classes of small RNAs, these data show that almost all classes of Ago1-bound small RNAs are adenylated and uridylated.
Figure 4. Untemplated adenines and uridines are enriched at small RNAs originating from noncoding RNAs and mRNAs, respectively.

a. Ago1-bound small RNAs were analyzed by high-throughput sequencing from indicated cells and classified as indicated below the pie charts. Pie charts illustrate percentages for the individual small RNA classes relative to the total number of reads for each strain.

b. Ago1-bound small RNA reads from wild type cells were plotted over euchromatic genes. Reads having untemplated adenine(s) or uridine(s) at the 3'-end are shown in separated tracks. The location of genes is indicated below the small RNA peaks. Reads from + and - strands are depicted in green and blue, respectively. Scale bars on the right denote small RNA reads numbers normalized per one million reads.

c. Quantification of Ago1-bound small RNAs that have untemplated adenine(s) or uridine(s) at the 3'-end.
3.2 Cid14 adenylates and Cid16 uridylates Ago1-bound small RNAs

Each of the six non-canonical poly-(A) polymerases of fission yeast (Stevenson and Norbury, 2006) was deleted in order to identify the nucleotidyltransferases responsible for the tailing of the Ago1-bound small RNAs. When compared to the wild type, the percentage of adenylation and uridylation resulted to be decreased in *cid14Δ* and *cid16Δ* cells, respectively (Fig. 5a). To search for a possible redundancy, Ago1-bound small RNAs were isolated and sequenced from double deletion mutants as well. All the *cid14Δ* and all the *cid16Δ* double mutants showed the same reduction of adenylation and uridylation, respectively (Fig. 5b). In *cid14Δcid16Δ* cells the percentage of 3'-end untemplated nucleotides was greatly reduced due to the lack of both adenine(s) and uridine(s) (Fig. 5b).

Also, the *cid14Δcid12Δ* mutant showed a decrease of untemplated uridine(s) besides adenine(s), but this might be due to the severe loss of siRNAs that characterizes *cid12Δ* and other RNAi mutants rather than a redundancy (data not shown; Halic and Moazed, 2010) (Fig. 5b).

Once the nucleotidyltransferases were identified, a second round of sequencing of Ago1-bound small RNAs from *cid14Δ*, *cid16Δ* and *cid14Δcid16Δ* mutants was performed to confirm the results observed in the previous screening (Fig. 6a). The strong 5'-end preference for a uridine indicated that the purified small RNAs were indeed Ago1-bound small RNAs (Fig. 6b).

The small RNAs deriving from centromeric repeats and mRNAs/ncRNAs showed a comparable loss of adenylation in *cid14Δ* cells and uridylation in *cid16Δ* cells (Fig. 6c). When the length of the sequences was calculated, the Ago1-bound small RNAs from *cid14Δcid16Δ* cells were observed to be shorter than the wild type, consistently with the deficiency of the 3'-end untemplated nucleotides (Fig. 6d).

These data indicate that the Ago1-bound small RNAs are adenylated and uridylated by Cid14 and Cid16, respectively.
Figure 5. Cid14 adenylates and Cid16 uridylates the 3’-end of Ago1-bound small RNAs.

a. Quantification of Ago1-bound small RNAs that have untemplated nucleotides at the 3’-end in indicated single mutants.
b. Quantification of Ago1-bound small RNAs that have untemplated nucleotides at the 3’-end in indicated double mutants.
Results

Figure 6. Cid14 adenylates and Cid16 uridylates all classes of Ago1-bound small RNAs.

a. Quantification of Ago1-bound small RNAs that have untemplated nucleotides at the 3’-end in indicated strains. Error bars indicate s.e.m. of two independent small RNA sequencing experiments.

b. 5’-nucleotide preference of Ago1-bound small RNAs in indicated cells.

c. Quantification of Ago1-bound small RNAs from centromeric region (left panel) and from mRNAs and ncRNAs (right panel) that have untemplated nucleotides at the 3’-end in indicated cells. Error bars indicate s.e.m. of two independent small RNA sequencing experiments.
3.4 Adenylation and uridylation of Ago1-bound small RNAs is not essential for silencing at centromeric repeats

Because centromeric siRNAs represent the most abundant class of Ago1-bound small RNAs, since RNAi is essential for heterochromatin formation at centromers, the first question was whether the tailing of the Ago1-bound small RNAs might have a role in silencing of centromeric transcripts. The percentage of all classes of Ago1-bound small RNAs was calculated for cid14Δ, cid16Δ and cid14Δcid16Δ cells and compared to the wild type. A small reduction of the centromeric siRNAs was observed, especially for the cid14Δcid16Δ mutant (28% less), although it was not comparable to the severe loss shown in dcr1Δ (Halic and Moazed, 2010; Marasovic et al., 2013) (Fig. 7a and b).

Centromeric siRNAs were analyzed by northern blotting of the total small RNAs fraction isolated from the wild type, dcr1Δ, cid14Δ, cid16Δ and cid14Δcid16Δ. A mild decrease of the dg/dh was detected in the double mutant, if compared to the complete loss of dg/dh siRNAs in dcr1Δ (Fig. 7c), consistently with the sequencing results (Fig 7a).

Centromeric dg transcripts were 2 and 3-fold up-regulated in cid14Δcid16Δ and cid14Δ, respectively, when analyzed by Reverse Transcription-quantitative PCR (RT-qPCR) (Fig. 8a) and di-methylation of Histone 3 Lysine 9 (H3K9me2) at centromeric loci was reduced 2 and 3-fold in cid14Δcid16Δ and cid14Δ, respectively (Fig. 8b). Nevertheless, these variations were not as pronounced as the up-regulation of centromeric transcripts in dcr1Δ (45 fold) and the loss of H3K9me2 in clr4Δ (Fig. 8a and b).

These results show that in cid14Δ, cid16Δ and cid14Δcid16Δ cells heterochromatic silencing at centromeric loci is only moderately reduced when compared to RNAi mutants, indicating that tailing of Ago1-bound small RNAs by Cid14 and Cid16 is not essential for silencing of centromeric repeats.
**Figure 7.** Deletion of Cid14 and Cid16 is not associated with a complete loss of centromeric small RNAs.

a. Ago1-bound small RNAs were analyzed by high-throughput sequencing from indicated cells and classified as indicated below the pie charts. Pie charts illustrate percentages for the individual small RNA classes relative to the total number of reads for each strain. Data set of dcr1Δ is from Marasovic et al., 2013.

b. Ago1-bound small RNA reads from indicated cells were plotted over centromeric region. The location of genes is indicated below the small RNA peaks. Reads from + and - strands are depicted in green and blue, respectively. Scale bars on the right denote small RNA reads numbers normalized per one million reads.

c. Northern blotting showing dg and dh transcripts from total RNA isolated from the indicated strains. In cid14Δcid16Δ cells centromeric small RNAs are reduced. Two independent cid14Δ strains show near wild type levels of centromeric siRNAs. Strain cid14Δ_2 is from Bühler et al., 2007.
Figure 8. Deletion of Cid14 and Cid16 is not associated with a complete loss of centromeric silencing.

a. Quantification of centromeric dg transcripts in indicated strains by RT-qPCR. In cid14Δ and cid14Δcid16Δ cells dg RNA is accumulating. Error bars indicate s.e.m. of > six independent experiments. “/” indicates fold change.

b. ChIP experiment showing H3K9me2 levels at centromeric dg repeats in cid16Δ, cid14Δ and cid14Δcid16Δ cells. Error bars indicate s.e.m. of three independent experiments.
3.5 Cid14 and Cid16 protect the genome from uncontrolled RNAi

**Figure 9.** *cid14Δ* and *cid14Δcid16Δ* cells show accumulation of small RNAs into Ago1.

a. Autoradiograph of denaturing polyacrylamide gel showing Ago1-bound RNAs purified from wild type, *cid16Δ*, *cid14Δ* and *cid14cid16Δ* cells. Quantification is based on two independent biological replicates. Each band was normalized to higher unspecific bands of its lane and compared to wild type. Lower panels show western blotting detection of Flag-Ago1 protein in immunoprecipitation assays.

b. Quantification of euchromatic Ago1-bound siRNAs and priRNAs mapping to mRNAs and ncRNAs in indicated strains. Error bars indicate s.e.m. of two independent small RNA sequencing experiments.

c. Quantification of intronic Ago1-bound siRNAs and priRNAs in indicated strains. Error bars indicate s.e.m. of two independent small RNA sequencing experiments.
In *cid14Δ* and *cid14Δcid16Δ* cells Ago1 was associated with a higher amount of small RNAs than in wild type cells (Fig. 9a) and the sequencing data showed accumulation of some classes of Ago1-bound small RNAs in *cid14Δ* and *cid14Δcid16Δ* cells (Fig. 9b and c). For example, Ago1-bound priRNAs originating from mRNAs and ncRNAs were 2 to 3-fold more abundant in *cid14Δ* and *cid14Δcid16Δ* cells, and to a lesser extent in *cid16Δ* cells (Fig. 9b). Another example was represented by Ago1-bound priRNAs generated from intronic regions, which increased of 2, 4.5 and 7.5-fold in *cid16Δ*, *cid14Δ* and *cid14Δcid16Δ* cells, respectively (Fig. 9b). This suggested that Cid14 and Cid16 are required for elimination of these classes of small RNAs.

When the Ago1-bound small RNAs sequencing data were plotted against the *S. pombe* genome and visualized with Integrative Genomics Viewer (IGV), generation of siRNAs was observed for many euchromatic genes in *cid14Δ*, *cid14Δcid16Δ* and, to at lower levels, in *cid16Δ* mutant, but not in the wild type cells (Fig. 10).

**Figure 10. In *cid14Δ* and *cid14Δcid16Δ* cells RNAi targets euchromatic genes.**

Ago1-bound small RNA reads from indicated strains were plotted over euchromatic genes. The location of genes is indicated below the small RNA peaks. Reads from + and - strands are depicted in green and blue, respectively. Scale bars on the right denote small RNA reads numbers normalized per one million reads.
Results

Initiation of siRNA generation is consistent with the increase of Ago1-bound priRNAs in cid14Δ and cid14Δcid16Δ cells, since accumulation of priRNAs will trigger RNAi and generation of secondary siRNAs at euchromatic loci. Total RNA sequencing data showed that the transcript level of the genes characterized by siRNAs accumulation was reduced in cid14Δ and cid14Δcid16Δ cells, indicating that the ectopic siRNAs are functional and they lead to uncontrolled RNAi (Fig. 11).

As already shown by Bühler et al. (Bühler et al., 2008), an increased number of small RNAs was detected at the ribosomal RNA (rRNA) locus in cid14Δ cells (Fig. 12a). Moreover, an increase in H3K9me2 levels at rDNA in cid14Δ cells and cid14Δcid16Δ was observed (Fig. 12b). However, no level of H3K9me2 could be detected at other euchromatic genes targeted by RNAi in cid14Δ and cid14Δcid16Δ cells, indicating that RNAi does not establish heterochromatin at these loci (Fig. 12c). The results described here suggest that adenylation of small RNAs by Cid14 protects the genome from uncontrolled RNAi implicating that adenylation of Ago1-bound small RNAs promotes their degradation. A defect in surveillance of Ago1-bound small RNAs results in accumulation of priRNAs on Ago1 that will lead to silencing of genomic loci that are not targets of RNAi in normal conditions.
Figure 11. Uncontrolled RNAi in \textit{cid14\Delta} and \textit{cid14\Delta cid16\Delta} cells silences ectopic targets.

a. RNA sequencing reads in indicated cells are plotted over the several euchromatic genes. In \textit{cid14\Delta} cells genes that are targeted by RNAi are silenced. Scale bars on the right denote RNA reads numbers normalized per one million reads.

b. Read counts of RNA sequencing from indicated strains for genes shown in Fig. 11a.

c. Box plot of differential expression of RNA in \textit{cid14\Delta} cells compared to wild type cells for all genes and genes that generate siRNAs in \textit{cid14\Delta} cells. Genes that generate siRNAs show reduced level of mRNAs in \textit{cid14\Delta} cells indicating silencing by RNAi. Two sided t-test for two independent datasets with high variance was used to calculate the p-value. *** P < 1E-10. Analysis performed by Mario Halic.
Figure 12. Uncontrolled RNAi silences euchromatic gene without establishing heterochromatin.

a. Ago1-bound small RNA reads from indicated strains were plotted over rDNA. The location of the locus is indicated below the small RNA peaks. Reads from + and - strands are depicted in green and blue, respectively. Scale bars on the right denote small RNA reads numbers normalized per one million reads.

b. ChIP experiment showing the H3K9me2 level in cid14Δ and cid14cid16Δ cells at rDNA. Error bars indicate s.e.m. of three independent experiments.

c. ChIP experiment showing that in cid16Δ, cid14Δ and cid14cid16Δ cells H3K9me2 is not established at targeted genes. Error bars indicate s.e.m. of three independent experiments.
3.6 Rapid adaptation to uncontrolled RNAi

Among the euchromatic genes showing accumulation of siRNAs there were *ago1* and *rdp1* (Fig. 13). The latter encodes for the RNA-dependent RNA polymerase part of the RDRC complex. The synthesis of dsRNA by Rdp1 is essential for the generation of centromeric siRNAs by Dicer and for enforcing the positive feedback loop for the heterochromatin formation at centromeric repeats (Motamedi et al., 2004; Sugiyama et al., 2005).

The total RNA sequencing data showed that *rdp1* transcript was reduced by the 25% in *cid14Δ* cells (Fig. 14a). The result was confirmed by RT-qPCR which showed a 2-fold decrease in *cid14Δ* cells and a 3-fold decrease in *cid14Δcid16Δ* cells (Fig. 14b). No level of H3K9me2 could be detected at this locus, indicating that the *rdp1* silencing might occur post-transcriptionally and/or independently of heterochromatin (Fig. 14c). Taken together, these data suggest that in *cid14Δ* cells RNAi targets *rdp1*, which is essential for the RNAi machinery itself, to avoid propagation of uncontrolled RNAi and protect the genome.

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**Figure 13. Uncontrolled RNAi targets essential genes for the RNAi machinery itself.**

Argonaute-bound small RNA reads from indicated strains were plotted over *rdp1* gene and *ago1* gene. The location of genes is indicated below the small RNA peaks. Reads from + and - strands are depicted in green and blue, respectively. Scale bars on the right denote small RNA reads numbers normalized per one million reads.
Figure 14. Uncontrolled RNAi silences *rdp1* gene independently of heterochromatin.

a. RNA sequencing reads in indicated cells are plotted over the several euchromatic genes. In *cid14Δ* cells genes that are targeted by RNAi are silenced. Scale bars on the right denote RNA reads numbers normalized per one million reads.

b. Quantification of *rdp1* transcripts in indicated strains by RT-qPCR. Error bars indicate s.e.m. of three independent experiments.

c. ChIP experiment showing that in *cid16Δ, cid14Δ* and *cid14cid16Δ* cells H3K9me2 is not established at *rdp1* locus. Error bars indicate s.e.m. of three independent experiments.
Results

*rdp1* was cloned under the strong *nmt1* promoter and over-expressed in *cid14Δ* cells, whose viability was tested by growth assay. The over-expression of *Rdp1* severely impaired the growth of *cid14Δ* cells, supporting the idea that reducing the level of uncontrolled RNAi, by silencing a gene encoding for an RNAi effector, is essential for the cells to survive under stress conditions (Fig. 15a and b).

**Figure 15.** Over-expression of *Rdp1* causes a severe growth defect of *cid14Δ* cells.

a. Growth assay showing strong reduction in viability of *cid14Δ* cells that over-express *rdp1* gene. Cells were growing for two days before imaging.

b. Same growth assay. Cells were growing for three days before imaging.

**Figure 16.** Over-expression of *Rdp1* induces propagation of uncontrolled RNAi in *cid14Δ* cells.

Ago1-bound small RNA reads from indicated strains were plotted over rDNA and euchromatic loci. Reads from + and - strands are depicted in green and blue, respectively. Scale bars on the right denote small RNA read numbers normalized per one million reads.
Sequencing of Ago1-bound small RNAs isolated from *cid14Δ* cells over-expressing Rdpl showed appearance of new siRNAs at some euchromatic loci and an even greater accumulation of siRNAs at the rRNA locus: while in *cid14Δ* cells siRNAs appeared at the 3'-end of the 25S and at the 3' External Transcribed Spacer (ETS), *cid14Δ* cells over-expressing Rdpl showed accumulation of siRNAs along the entire rRNA locus (Fig. 16).

Moreover, rRNA transcript levels were 2-fold reduced *cid14Δ* cells over-expressing Rdpl (Fig. 17a). The H3K9me2 was enriched along the rRNA locus in *cid14Δ* cells, comparing with the wild type, and 5-fold increased at the Non-Transcribed Spacer (NTS) in *cid14Δ* cells over-expressing Rdpl (Fig. 17b).

The results described here show that fission yeast cells exploit RNAi to down-regulate the RNAi machinery itself in response to an improper small RNAs decay mechanism. Interestingly, *rdp1* mRNA is targeted by RNAi, in at least two independent strains, and silenced. This rapid adaptation to a misregulated RNAi enable *S. pombe* cells to reprogram their gene expression and survive to the stress condition.

Figure 17. Over-expression of Rdpl causes silencing of the rRNA locus in *cid14Δ* cells.

a. Quantification of 25S rRNA transcript in indicated strains by RT-qPCR. Error bars indicate s.e.m. of three independent experiments.

b. ChIP-seq reads from indicated strains were plotted over rDNA. Scale bars on the right denote small RNA read numbers normalized per one million reads.
3.7 Cid14 and Ago1 interact in vivo

The small RNA sequencing data showed that Cid14 and Cid16 add untemplated nucleotides to Ago1-bound small RNAs. Therefore, co-immunoprecipitation experiments were performed to investigate a potential interaction between the two nucleotidyltransferases and Ago1. Cid16, which was endogenously tagged as Cid14 and Ago1, happened to be very low expressed and not detectable neither in the input nor in the immunoprecipitated fraction by Western blotting analysis. On the other hand, Cid14 and Ago1 did interact in vivo (Fig. 18).

![Figure 18. Cid14 and Ago1 interact in vivo.](image)

Western blotting analysis of a co-immunoprecipitation assay. Experiment performed with Henry Fabian Thomas.

3.8 Cid14 and Cid16 recruit the 3'-to-5' exonuclease Rrp6 to degrade Ago1-bound small RNAs in vitro

The accumulation of Ago1-bound small RNAs at euchromatic loci and rRNA locus observed in *cid14Δ* and *cid14Δcid16Δ* cells suggested that (i) Cid14, and to a lesser extent Cid16, might be required for Ago1-bound small RNAs removal and that (ii) the loss of such a surveillance mechanism results in ectopic RNAi. For this reason, Cid14 and Cid16 were over-expressed and purified, along with their activity mutants, to study their activity and molecular function in vitro (Fig. 19).

While the activity mutants failed in addition of untemplated adenines and uridines, Cid14 added 10-20 adenines and Cid16 added 1-3 uridines to free 22 nt small RNA (Fig. 20a). Both Cid14 and Cid16 were specific in the addition of adenines and uridines, respectively (Fig. 20b).
The nucleotidyltransferase activity was tested on double strand RNA (dsRNA) as well: Cid14 was able to adenylate dsRNA too, while Cid16 showed a weak activity on dsRNA similarly to TUT4 in mammalian cells (Jones et al., 2009) (Fig. 21).

Therefore, the same experiment was performed with 22 nt small RNAs previously loaded on Ago1 (see 5.2 Methods section). Cid14 added either 1-2 or 15-20 untemplated adenines to the 3'-end of Ago1-bound small RNAs, indicating that the Cid14 activity is modulated by Ago1 (Fig. 22). In a previous work, it has been shown that 10-20% of 22 nt small RNAs dissociate from Ago1 in course of the assay, suggesting that 15-20 untemplated adenines might be added to free small RNAs. Moreover, one has to consider that the assay was performed with α-32P-ATP, meaning that the addition of 10-20 adenines should result in a 10-fold higher signal which is not the case shown here. This is consistent with the sequencing of Ago1-bound small RNAs showing 1-2 untemplated nucleotides at the 3'-end of small RNAs. Similarly, to Cid14, Cid16 added 1-2 nucleotides to Ago1-bound small RNAs in the in vitro assay (Fig. 22).
Results

Previous Ago1-bound small RNAs sequencing data from mutants of Tri1, Rrp6, Dis3 and Dis3l2 were analyzed for the presence of untemplated nucleotides at the 3′-end. Adenylated Ago1-bound small RNAs accumulated in tri1Δ cells, but not in rrp6Δ, dis3l2Δ and dis3-54 cells (Fig. 23).

Figure 20. Cid14 and Cid16 specifically adenylates and uridylylates small RNAs in vitro, respectively.

a. Left panel: autoradiograph of denaturing polyacrylamide gel showing Cid14 activity on free small RNA. 22 nucleotides long small RNA was incubated with wt and catalitically inactive Cid14 and [α-32P] ATP. Right panel: autoradiograph of denaturing polyacrylamide gel showing Cid16 activity on free small RNA. 22 nucleotides long small RNA was incubated with wt and catalitically inactive Cid16 and [α-32P] UTP.

b. Autoradiograph of denaturing polyacrylamide gel showing Cid14 and Cid16 activity on free small RNA and specificity for ATP and UTP, respectively. 22 nucleotide long small RNA was incubated with Cid14/Cid16 and [α-32P] ATP/UTP.
Results

Ago1 recruits Tri1 to process small RNAs to the final length of 22 nucleotides (Marasovic et al., 2013). Thus, it is very likely that Tri1 actively removes untemplated adenines from the 3′-end of small RNAs, consistently with Tri1 belonging to the PARN family of deadenylases. Cid14 and Tri1 might act together to control the length and stability of small RNAs. Small RNAs length is essential for Ago1 slicing activity and addition/removal of untemplated nucleotides can change small RNA efficiency. On the contrary, uridylation did not accumulate on Argonaute-bound small RNAs in tri1Δ cells, indicating that uridines are not removed by Tri1 (Fig. 23).

Uridylated Ago1-bound small RNAs resulted to be longer than average Ago1-bound small RNAs in wild type cells, suggesting that uridine(s) at the 3′-end actually protect(s) small RNAs from trimming (Fig. 24).

Figure 21. Cid14 can adenylate ds small RNA while Cid16 preferentially uridylates ss small RNA in vitro.

Autoradiograph of polyacrylamide gel showing Cid14 and Cid16 activity on free dsRNA and ssRNA. 22 nucleotides single and double stranded small RNAs were incubated with Cid14 and Cid16. Small RNAs were analyzed on a polyacrylamide gel.
**Results**

**Figure 22. Cid16 uridylates and Cid14 adenylates Ago1-bound small RNAs in vitro.**

Autoradiograph of denaturing polyacrylamide gel showing Cid16 and Cid14 activity on Ago1-bound small RNAs. 22 nucleotides long small RNA was loaded on empty Ago1 purified from dcr1Δtri1Δ cells and incubated with Cid14/Cid16 and [α-32P] ATP/UTP. Small RNAs were analyzed on denaturing polyacrylamide gel.

**Figure 23. 3’-end adenylated Ago1-bound small RNAs accumulate in tri1Δ cells.**

Quantification of Ago1-bound small RNAs that have untemplated nucleotides at the 3’-end in indicated strains. Data from Halic and Moazed, 2010 and Marasovic et al., 2013.
As already mentioned before, Cid14 is a member of the TRAMP complex which was suggested to recruit the nuclear exosome to targeted RNAs and promotes their degradation. Therefore, Ago1 was loaded with radioactively labeled 22 nt small RNAs and incubated with Cid14 or Cid16 together with Tri1 or Rrp6. Neither Tri1 nor Rrp6 were able to degrade the Ago1-bound small RNAs by themselves, although Rrp6 did degrade short contaminating unbound RNAs. In presence of Cid14 and Cid16, however, Rrp6 removed and degraded the Ago1-bound small RNAs completely (Fig. 25).

![Graph](image)

**Figure 24. Uridylated Ago1-bound small RNAs are longer than average Ago1-bound small RNAs.**

Length of uridylated Ago1-bound small RNAs in wild type cells calculated from Ago1-bound small RNA high-throughput sequencing data.

When incubated with the activity mutants of Cid14 and Cid16, Rrp6 was not able to degrade the Ago1-bound small RNAs, indicating that adenylation and uridylation are required for Ago1-bound small RNAs removal and degradation by Rrp6 *in vitro* (Fig. 26).
Results

Figure 25. Cid14 and Cid16 recruit Rrp6 to degrade Ago1-bound small RNAs in vitro.

Autoradiograph of denaturing polyacrylamide gel showing degradation of Ago1-bound small RNA by Cid14 (left panel), Cid16 (right panel) and Rrp6. 5’-32P labeled 22 nucleotides long small RNA was loaded on empty Ago1 purified from dcr1Δtri1Δ cells and incubated with Cid14, Cid16 and Tri1 or Rrp6.

Figure 26. Rrp6-mediated Ago1-bound small RNAs degradation in vitro depends on Cid14 and Cid16 nucleotidyltransferase activity.

Autoradiograph of denaturing polyacrylamide gels showing degradation of Ago1-bound small RNA by Cid14 and Rrp6. 5’-32P labeled 22 nucleotides long small RNA was loaded on empty Ago1 purified from dcr1Δtri1Δ cells and incubated with Rrp6, Cid14 and Cid14DADA (left panel) or Rrp6, Cid16 and Cid16DADA (right panel). 30 nucleotides long DNA was used as a loading control.
Results

Time course experiments showed the appearance of intermediate degradation products (Fig. 27). These results observed in vitro suggest that adenylation by Cid14 might recruit either Tri1 or Rrp6 in vivo as well. While Rrp6 degrades Ago1-bound small RNAs, Tri1 shortens them to 22 nucleotides to make them functional again.

![Figure 27](image)

**Figure 27. Time course experiments show intermediate degradation products.**

Autoradiograph of denaturing polyacrylamide gels showing time course of Ago1-bound small RNAs degradation by Cid14/Cid16 and Rrp6. 5'−32P labeled 22 nucleotides long small RNA was loaded on empty Ago1 purified from dcr1Δtri1Δ cells and incubated with Rrp6, Cid14 (h) or Cid16 (i). Time at which the reaction was stopped is indicated above the image.

### 3.9 Ago1-bound small RNAs are more stable in cid14Δ cells

If Cid14 recruits Rrp6 to remove and degrade the Ago1-bound small RNAs, the following question was whether the loss of Cid14 could result in a longer half-life of the Ago1-bound small RNAs. To answer this question, a construct expressing ura4 hairpin (Iida et al., 2008) under the control of the repressible nmt1 promoter was inserted into wild type and cid14Δ cells. In absence of thiamine, the ura4 hairpin is transcribed at high level and processed by Dicer into ura4 siRNAs (Fig. 28a).
Results

Figure 28. The *ura4* hairpin is equally repressed in wild type and in *cid14Δ* cells upon thiamine addition.

  a. Schematic diagram of the *ura4* hairpin construct under the *nmt1* promoter.
  
  b. Quantification of *ura4* hairpin RNA in indicated strains by RT-qPCR. Error bars indicate standard error s.e.m. of three independent experiments.

Figure 29. The total *ura4* siRNAs are Dicer products.

Northern blotting analysis showing *ura4* small RNAs isolated from total small RNA fraction from the indicated strains. Lower panel shows the loading control *snoR69*.
In presence of thiamine, the *nmot1* promoter is repressed and the *ura4* hairpin levels rapidly decrease in both wild type and *cid14Δ* cells equally (Fig. 28b).

Therefore, the degradation of Ago1-bound and total *ura4* small RNAs in wild type and *cid14Δ* cells was followed over three time-points. Total *ura4* small RNAs were not detectable in *dcr1Δ* or *dcr1Δcid14Δ* cells, proving that they are *bona fide* siRNAs (Fig. 29).

Wild type cells showed a rapid degradation of *ura4* siRNAs in total small RNA fraction with the majority of *ura4* siRNAs being degraded in less than 5 hours (Fig. 30a and b).

In *cid14Δ* cells, after initial rapid degradation, a reduction in the degradation rate of *ura4* siRNAs was detected (Fig. 30a and b).

![Figure 30. Total *ura4* siRNAs show a slower degradation rate after 3 h of repression in *cid14Δ* cells.](image)

- a. Northern blot showing *ura4* small RNAs isolated from total small RNA fraction from the indicated strains. *ura4* small RNAs were normalized to *snoR69* shown in the lower panel. Quantification is relative to time point 0 when thiamine was added and is shown below the image.

- b. Quantification of *ura4* small RNAs from total small RNA fraction in wild type and *cid14Δ* cells. Quantification is relative to time point 0 when thiamine was added. *ura4* small RNAs were normalized to *snoR69*. Error bars indicate s.e.m. of three independent experiments.
Since the total fraction comprehends both Ago1-bound and unbound small RNAs, the result suggested a possible scenario where the unbound ura4 siRNAs are rapidly degraded, independently of Cid14, whereas Ago1-bound small RNAs, which are expected to be less prone to degradation than the unbound small RNAs, might be stabilized in cid14Δ cells as shown in the latest time-points (Fig. 30a and b). To investigate this hypothesis, the degradation of the Ago1-bound small RNAs was followed.

**Figure 31. The stability of Ago1-bound ura4 siRNAs is increased in cid14Δ cells.**

a. Northern blotting analysis showing Ago1-bound ura4 small RNAs isolated from wild type and cid14Δ cells. The ura4 small RNAs were normalized to centromeric small RNAs shown in the lower panel. Quantification is relative to time point 0 when thiamine was added and is shown below the image.

b. Quantification of Ago1-bound ura4 small RNAs in wild type and cid14Δ cells. The ura4 small RNAs were normalized to centromeric siRNAs. Quantification is relative to time point 0 when thiamine was added. Error bars indicate s.e.m. of three independent experiments.

In cid14Δ cells Ago1-bound ura4 siRNAs displayed indeed more stability than in wild type cells (Fig. 31a and b).

This experiment showed that Ago1-bound small RNAs are more stable in cid14Δ cells when compared to the wild type cells. In wild type cells Ago1-bound small RNAs were degraded at a slower rate than total small RNAs, indicating that binding to Ago1 increases the stability of the small RNAs (Fig. 32a). Nevertheless, the stability of Ago1-bound ura4 siRNAs over the total ura4 siRNAs was more pronounced in cid14Δ than in wild type cells, taking into account an equal doubling time (Fig. 32b).
Figure 32. Ago1-bound *ura4* siRNAs are more stable than the total *ura4* siRNA and in *cid14Δ* this stability is increased.

a. Quantification of total and Ago1-bound *ura4* small RNAs half-life in wt. Quantification is relative to time point 0 after addition of thiamine. Error bars indicate standard error s.e.m. of three independent experiments.

b. Quantification of total and Ago1-bound *ura4* small RNAs half-life in *cid14Δ*. Quantification is relative to time point 0 after addition of thiamine. Error bars indicate s.e.m. of three independent experiments.
4. DISCUSSION

Regulatory small RNAs have been shown to provide protection of the genome against external DNA and to control gene expression through transcriptional and post-transcriptional regulation pathways (Ghildiyal and Zamore, 2009; Kim et al., 2009; Moazed, 2009; Holoch and Moazed, 2015b). In particular, miRNAs in metazoans regulate crucial biological processes like cell differentiation, development, tumorogenesis and apoptosis (Bartel and Chen, 2004; Yekta et al., 2008; Stefani and Slack, 2008; Wang and Lee, 2008). Therefore, the regulation of the small RNAs themselves is of fundamental importance. Many miRNAs, in fact, are controlled by developmental and/or tissue-specific signals (Landgraf et al., 2007) and a deregulation in their expression pathway is often associated with human diseases (Jiang et al., 2009). The first step of regulation is transcription (Xie et al., 2010) and several miRNAs were reported to be under the control of tumor-suppressors or oncogenic transcription factors (He et al., 2007).

Post-transcriptional regulation mechanisms can involve (i) RNA-binding protein, like the case of Lin28 suppressing pre-let7 biogenesis (see the 1.4 paragraph), (ii) exoribonucleases, described in plants (Ramachandran and Chen, 2008) and nematode (Kennedy et al., 2004), (iii) RNA editing machinery, based on adenine deaminases (ADARs) which change adenines to inosines possibly modulating the precursor biogenesis or the mature miRNA-transcript specificity (Yang et al., 2006; Kawahara et al., 2007; Kawahara et al., 2008; Warnefors et al., 2014; Tomaselli et al., 2015; Nishikura, 2015), and (iv) RNA modification, such as the 2'-O-methylation involved in stability and the methylation of adenine and cytosine, playing a role in several physiological processes like stress response, metabolism and immunity (Zhang et al., 2016).

An important class of small RNA modifications is represented by the 3’-end addition of untemplated nucleotides, subsequently described as “tailing” and reported in plants and animals. As discussed before, tailing can address the small RNAs to different destinies, like promoting their stability, inducing their exonuclease-mediated degradation or modulating their target recognition (Song et al., 2015).

This study investigated the 3’-end untemplated nucleotides addition to Argonaute-bound small RNAs and its role inside the RNAi machinery of Schizosaccharomyces pombe. The results show that:

- Ago1-bound small RNAs are adenylated by Cid14 and uridylated by Cid16 in vitro and in vivo.
- Cid14 and Cid16 recruits the nuclear exosome subunit Rrp6 which, in turn, degrades the Ago1-bound small RNAs in vitro. Moreover, the Rrp6-mediated degradation depends on the
Cid14 and Cid16 activity.

- The loss of Cid14 is associated with uncontrolled RNAi induced by the accumulation of siRNAs at euchromatic loci which, in wild type condition, are not targets of the RNAi machinery. Therefore, taken together, the data presented in this work shed light on a surveillance mechanism for Argonaute-bound small RNAs that protects fission yeast genome from uncontrolled and, consequently, harmful RNAi (Pisacane and Halic, 2017).

### 4.1 A surveillance mechanism mediated by Cid14 and Rrp6

Like Wispy in *D. melanogaster* was shown to interact *in vivo* with Argonaute and to adenylate Argonaute-bound miRNAs (Lee et al., 2014), the data described in this study show that Cid14 interacts with Ago1 *in vivo* and adenylates Ago1-bound small RNAs *in vitro* and *in vivo*. As the loss of Wispy was associated with accumulation of miRNAs (Lee et al., 2014), the deletion of Cid14 resulted in 1) generation of siRNAs at euchromatic loci that are not targeted by RNAi in normal conditions, and 2) stabilization of the *ura4* siRNAs generated from the exogenously introduced hairpin (Fig. 31a and b). It would be interesting then to investigate the effect of the Cid14 over-expression on the Ago1-bound small RNAs turnover, since over-expression of Wispy caused a reduction of miRNAs level (Lee et al., 2014).

It was previously shown that loss of Cid14 results in accumulation of antisense ribosomal siRNAs (Bühler et al., 2008), suggesting a role for the TRAMP complex in RNA quality control as it was reported in *S. cerevisiae* (LaCava et al., 2005). However, the low quality of the Ago1-bound small RNA sequencing data did not allow the authors to detect siRNAs generated and accumulated at euchromatic loci, as it is described in this work. Moreover, the MTREC complex, more than the TRAMP complex, was suggested to target aberrant RNAs for nuclear exosome-mediated degradation in fission yeast (Zhou et al., 2015). The *cid14Δ* high-throughput sequencing data indicates a Cid14 dependent adenylation of Ago1-bound small RNAs *in vivo* and suggest a role of Cid14 in recruiting Rrp6 to degrade “noise” Ago1-bound small RNAs, since *rrp6Δ* cells undergo the same uncontrolled RNAi observed in *cid14Δ* (Marasovic et al., 2013; Yamanaka et al., 2013). However, a further proof would be to observe the ectopic siRNAs accumulation in cells with the endogenous catalitically inactive Cid14. Nevertheless, the results observed *in vitro* strongly support a role of Cid14 and Rrp6 in maintaining the Ago1-bound small RNAs background level under the threshold required for the RNAi machinery to generate siRNAs and induce unspecific PTGS *in vivo* as well.

This surveillance mechanism will control, however, the least abundant siRNAs in order to keep them below the threshold and prevent them from being loaded on Argonaute.
This might explain why the loss of Cid14 does not affect the very abundant and constantly generated centromeric siRNAs, as it was previously stated (Bühler et al., 2007), and, therefore, does not cause a severe loss of silencing at pericentromeric repeats, as it was reported for other RNAi mutants (Provost et al., 2002; Volpe et al., 2002; Volpe et al., 2003). An effector that fits in this frame is Eri1, a ribonuclease that was shown to negatively regulate heterochromatin formation in fission yeast, most likely degrading the siRNA duplexes processed by Dcr1 (Iida et al., 2006). Eri1 might have a role in controlling the amounts of cellular siRNA duplexes and, thereby, in suppressing a potential uncontrolled RNAi, similarly to the role of Cid14 and Rrp6 on Ago1-bound small RNAs.

Moreover, a recent study from Motamedi group reported the accumulation of Ago1-associated small RNAs mapping to euchromatic genes during quiescence transition. They observed a G0-specific down-regulation of the TRAMP and the exosome components and an upregulation of the RNAi effectors, leading to euchromatic genes targeting and transcriptional silencing (Joh et al., 2016). Even though based on epigenetic regulation, these findings are in line with the data discussed here.

4.2 An internal sabotage to rapidly adapt and survive

Interestingly, one of the locus showing siRNAs accumulation in cid14Δ is rdp1 gene, encoding for the RNA-dependent RNA polymerase, part of the RDRC complex, that is responsible for the dsRNA formation and, hence, for the siRNAs amplification (Motamedi et al., 2004; Sugiyama et al., 2005). Moreover, rdp1 transcript levels were reduced in cid14Δ cells indicating an effective silencing mediated by the siRNAs observed (Fig. 14a and b). In other words, the uncontrolled RNAi, driven by a stress condition such as the lack of a surveillance mechanism for the Ago1-bound small RNAs, targeted a gene which is essential for the RNAi machinery itself, counteracting the already harmful dysfunction of this pathway in the cell. In line with this, overexpression of Rdp1 in cid14Δ cells severely impaired their growth (Fig. 15).

The rdp1 silencing, however, along with the other loci reported in this work, was independent from H3K9 methylation (Fig. 14c and 12c), suggesting that it is a case of post-transcriptional gene silencing more than transcriptional gene silencing. A PTGS of euchromatic loci, besides heterochromatic transcript, has not been confirmed yet in fission yeast, although Ekwall group did show a possible PTGS mechanism for repression of gene expression (Smialowska et al., 2014). They observed, however, a reduction at the protein level instead of the mRNA level. Further investigation needs to be carried out to surely affirm a role of the RNAi in PTGS of euchromatic genes in S. pombe as it was described in other organisms.
The data remain anyway an indication that the RNAi in fission yeast can be uncoupled from heterochromatin and that H3K9 methylation is not a prerequisite for siRNAs generation and silencing.

Why specifically *rdp1*? Is this a random or a specific choice? It is likely that *rdp1* was targeted randomly by the uncontrolled RNAi and that the cells with silenced *rdp1* were isolated because of natural selection. More isolates should be analyzed to search for other possible genes essential for the RNAi that might have been silenced by the same mechanism.

A recent study in fission yeast cells deleted of the putative histone demethylase *epe1* and the histone acetyltransferase *mst2*, described a heterochromatin-mediated adaptation mechanism: *epe1Δmst2Δ* cells accumulate H3K9 methylation at the locus encoding for the H3K9 methyltransferase Clr4 to prevent uncontrolled spreading of heterochromatin (Wang et al., 2015a), as *cid14Δ* and *cid14Δcid16Δ* cells accumulate siRNAs at the *rdp1* locus to counteract uncontrolled RNAi. Compared to genetic mutations, epigenetic and/or RNAi-based adaptations represent a faster response and a reversible solution.

In conclusion, these data show that *S. pombe* cells can exploit RNAi to reprogram their genome expression and adapt to external or internal stress stimuli.

### 4.3 Ribosomal small RNAs respond to external and internal stimuli

Among the genomic loci displaying accumulation of siRNAs upon loss of Cid14 and, even more, with the Rdp1 over-expression, there is the ribosomal RNA (rRNA) locus. In this case, siRNA generation is associated not only with the rRNA transcript repression, but also with an increased of H3K9 methylation at the rDNA repeats in *cid14Δ* cells over-expressing Rdp1 (Fig. 17a and b), indicating a TGS mechanism. In agreement with this, over-expression of Rdp1 in *cid14Δ* caused a severe growth defect of the cells (Fig.15).

Similarly, stress stimuli and loss of *susi-1*, the *C. elegans* homolog of the human 3'-to-5' exonuclease DIS3L2, result in the accumulation of ERI(Dicer)-independent RdRP-synthesized antisense ribosomal siRNAs (risiRNAs) that enter the nuclear RNAi pathway and silence rRNA (Zhou et al., 2017). Other examples come from *N. crassa* where DNA damage induces qiRNAs (QDE-2 interacting small RNAs, where QDE-2 is an Argonaute protein) deriving from the rRNA locus (Lee et al., 2009a), and from *A. thaliana* where the antiviral response triggers the ribosomal siRNAs (risiRNAs) formation (Cao et al., 2014).
4.4 Cid16: a nuclear or cytoplasmic quality controller?

Although the results reported here clearly describe Cid16 as the nucleotidyltransferase responsible for the Ago1-bound small RNAs uridylation, the role of this uncharacterized protein in the surveillance mechanism remains to be elucidated. The single deletion of Cid16, in fact, does not cause the same accumulation of siRNAs observed in cid14Δ cells, not even at the rdp1 locus, with consequent lack of silencing.

On the other hand, the in vitro assays support the idea that, like Cid14, Cid16 recruits Rrp6 to degrade the Ago1-bound small RNAs, suggesting that Cid16 is a functional homologue of the MUT68 uridylyltransferase in the unicellular green alga (Ibrahim et al., 2010) and of the TUT4/7 uridylyltransferases in mammalian cells (Liu et al., 2014).

Another factor to consider is that unlike Cid14, that was found to be nuclear, Cid16 was detected as cytoplasmic protein in fission yeast (Matsuyama et al., 2006), consistent with the observation that small RNAs generated from mRNAs are more frequently uridylated and the small RNAs deriving from ncRNAs are highly adenylated (Fig. 4c). Therefore, how does Cid16 act on the Ago1-bound small RNAs and promote their degradation by Rrp6, which is part of the nuclear exosome?

It is not exactly clear how Argonaute is loaded with small RNAs. Buker et al. have reported that Arb1 and Arb2, part of the ARC complex, localize throughout the nucleoplasm as well as in cytoplasmic foci, especially Arb2 (Buker et al., 2007). Holoch and Moazed, have shown that double strand small RNA loading on Argonaute is Arb1-dependent, with Arb2 being possibly important for localization of Arb1-Ago1 complex (Holoch and Moazed, 2015a). Argonaute might shuttle from the nucleus to the cytoplasm in a process of small RNA loading/maturation.

Therefore, the possible scenarios are:

1. uridylated small RNAs are imported into the nucleus and degraded by Rrp6.
2. Cid16 is present inside the nucleus as well.
3. mRNA degradation products are loaded on Argonaute during the import to the nucleus, after being synthesized, and are removed by the exosome in the nucleus, where Argonaute is predominantly localized.

It is worthy to mention that Eri1, described as an RNAi negative regulator able to degrade double stranded siRNAs, was found to localize in the cytoplasm, despite of its role in limiting the siRNAs availability for the RITS complex (Iida et al., 2006).

To the present, cytoplasmic RNAi has not been reported in fission yeast and the current data describe a sole nuclear RNAi pathway. Thus, a potential cytoplasmic RNAi pathway, cytoplasmic small RNA
loading and tailing as well as the previously mentioned potential RNAi-mediated post-transcriptional repression of euchromatic genes in *S. pombe* need to be profoundly investigated.

4.5 Conclusions and future perspectives

The aim of this study was to analyze the Ago1-bound small RNAs 3’-end tailing and investigate its role in the Ago1-bound small RNAs turnover inside the RNAi pathway of fission yeast. The discussed results indicate that:

➢ Cid14 adenylates and Cid16 uridylates Ago1-bound small RNAs in fission yeast (Fig. 33a).

➢ Both Cid14 and Cid16 recruit the 3’-to-5’ exonuclease Rrp6 to actively remove small RNAs from Ago1 and degrade them *in vitro* (Fig. 33a).

➢ Cid14 can recruit either Tri1 or Rrp6. Rrp6 will degrade Ago1-bound small RNAs whereas Tri1 will trim them to the 22 nucleotides optimal length and make them functional again (Fig. 33a).

➢ Degradation of Ago1-bound small RNAs is necessary to reduce the accumulation of “noise” small RNAs into Ago1 and, therefore, to protect the genome from uncontrolled RNAi.

➢ In *cid14Δ* and *cid14Δcid16Δ* cells uncontrolled RNAi targets and silences *rdp1*, a gene which is essential for the RNAi machinery itself, to avoid its propagation (Fig. 33b).

➢ Over-expression of Rdp1 in *cid14Δ* cells silences rRNA and many euchromatic genes, strongly impairing the cellular growth. This indicates that the reprogramming of *rdp1* expression is essential for the viability of *cid14Δ* cells (Fig. 33b).
Figure 33. Cid14/Cid16 and Rrp6 degrade Ago1-bound small RNAs to protect the genome from uncontrolled RNAi.

a. Cid14 adenylates and Cid16 uridylates Ago1-bound small RNAs in fission yeast. Both Cid14 and Cid16 recruit the Rrp6 nuclease to actively degrade small RNAs from Ago1 \textit{in vitro}. Adenylation by Cid14 can recruit either Tri1 or Rrp6. Rrp6 will degrade Ago1-bound small RNAs, whereas Tri1 will trim Ago1-bound small RNAs to 22 nucleotides and make them functional again.

b. Degradation of Ago1-bound small RNAs is necessary to reduce accumulation of “noise” small RNAs into Ago1 and to protect the genome from uncontrolled RNAi. In \textit{cid14Δ} and \textit{cid14Δcid16Δ} cells RNAi targets the \textit{rdp1} gene and suppresses itself. Over-expression of Rdp1 in \textit{cid14Δ} cells silences rRNA and many euchromatic genes, impairing the normal growth. This indicates that the reprogramming of \textit{rdp1} expression is essential for the viability of \textit{cid14Δ} and \textit{cid14Δcid16Δ} cells.
Further investigation needs to be carried out to definitely state a role of the 3'-end tailing in the exosome-mediated Ago1-bound small RNAs degradation in vivo. Another aspect to clarify is how exactly the uncontrolled RNAi post-transcriptionally silences the euchromatic loci at which siRNAs accumulate upon loss of Cid14.

Moreover, since Cid16 remains a poorly characterized protein, proteomic analysis would help to assign other biological functions, besides the uridyltransferase activity reported in this work, and to better understand the role of Cid16 in recruiting Rrp6 to degrade uridylated Ago1-bound small RNAs, given its cytoplasmic localization (Matsuyama et al., 2006).

Degradation of Argonaute-bound miRNAs may be necessary to exchange miRNAs from Argonaute during cellular differentiation and development in mammalian cells, and a defect in their turnover might lead to uncontrolled silencing and, potentially, to deregulation of cellular proliferation. Therefore, it would be of great interest to investigate tailing and degradation of Argonaute-bound small RNAs in various mammalian cell types and during several biological processes such as cellular differentiation, cellular development and tumorigenesis.
5. MATERIALS AND METHODS

5. 1 MATERIALS

Table 5.1.1: Strains used in this study

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### Table 5.1.2: Strains + plasmid

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### Table 5.1.3: Plasmids used in this study

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Table 5.1.4: Oligonucleotides used in this study

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**Materials and Methods**

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<th>ura4-hp5</th>
<th>CTTACGTTTTGGAGATCCCG</th>
<th>RT</th>
</tr>
</thead>
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Primers used for ChIP and qRT-PCR if no specification, RT: reverse transcription, NB: Northern Blot, ET: endogenous tagging, iPCR: inverse PCR, underlined sequences: restriction sites.

### Table 5.1.5: Sequencing data

The sequencing data that support the findings of this study have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (GEO) and are accessible through the GEO Series accession number GSE95821

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### Materials and Methods

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**Table 5.1.6: Media and solutions**

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<tbody>
<tr>
<td>YES medium (S. pombe)</td>
<td>5g/l Yeast Extract, 30g/l glucose, 0.225 g/l amino acids (leucine, histidine, lysine), 0.225 g/l adenine (+ 20g/l agar for solid plates)</td>
</tr>
<tr>
<td>EMMc-leu medium (S. pombe)</td>
<td>12.4 g/l EMM without dextrose (Formedium), 0.225 g/l adenine, 0.225 g/l leucine, 0.056 g/l uracil (+ 20g/l agar for solid plates)</td>
</tr>
<tr>
<td>LB medium (E. coli)</td>
<td>10g/l NaCl, 5g/l Yeast Extract, 10g/l Tryptone</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>100mg/ml 1000X stock</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>50 mg/ml 1000X stock</td>
</tr>
<tr>
<td>IPTG for bacterial induction</td>
<td>1M IPTG stock, used at 0.2 mM final concentration</td>
</tr>
<tr>
<td>Lysis buffer Ago1-bound small RNA purification</td>
<td>50 mM HEPES pH 7.5, 1.5 M NaOAc, 5 mM MgCl2, 2 mM EDTA pH 8, 2 mM EGTA pH 8, 0.1% Nonidet P-40, 20% Glycerol, 1 mM PMSF, 0.8 mM DTT and Complete EDTA free Protease Inhibitor Cocktail (Roche)</td>
</tr>
<tr>
<td>Lysis buffer ChIP</td>
<td>50 mM HEPES pH 7.5, 1.5 M NaOAc, 5 mM MgCl2, 2 mM EDTA pH 8, 2 mM EGTA pH 8, 0.1% Nonidet P-40, 20% Glycerol, 1 mM PMSF and Complete EDTA free Protease Inhibitor Cocktail (Roche)</td>
</tr>
<tr>
<td>Elution buffer ChIP</td>
<td>50 mM Tris-Cl pH 8, 10 mM EDTA, 1% SDS</td>
</tr>
<tr>
<td>Lysis buffer Ni-NTA purification</td>
<td>50 mM NaH$_2$PO$_4$/Na$_2$HPO$_4$ pH 8, 1 M NaCl, 20 mM imidazole, 3 mM b-mercaptoethanol and 0.5 mM PMSF</td>
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<td>Material/Buffer Type</td>
<td>Description</td>
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<td><strong>Materials and Methods</strong></td>
<td></td>
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<tr>
<td><strong>Materials and Methods</strong></td>
<td></td>
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<tr>
<td><strong>Wash buffer Ni-NTA purification</strong></td>
<td>50 mM NaH₂PO₄/Na₂HPO₄ pH 8, 1 M NaCl, 40 mM imidazole, 3 mM b-mercaptoethanol and 0.5 mM PMSF</td>
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<td><strong>Elution buffer Ni-NTA purification</strong></td>
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<td><strong>Dialysis buffer Ni-NTA purification</strong></td>
<td>50 mM Tris-Cl pH 7.5, 150 mM NaCl, 0.1 mM DTT, 0.1 mM EDTA</td>
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<td><strong>Elution buffer Glutathione purification</strong></td>
<td>50 mM Tris-Cl pH 8, 500 mM NaCl, 10 mM reduced glutathione</td>
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<td><strong>Dialysis buffer Glutathione purification</strong></td>
<td>50 mM Tris-Cl pH 7.5, 200 mM NaCl, 1 mM DTT, 0.1 mM EDTA, 10% glycerol</td>
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<td><strong>Elution buffer FLAG purification</strong></td>
<td>25 mM HEPES pH 7.5, 200 mM KCl, 2 mM MgCl₂, 0.1% Nonidet P-40, 20% Glycerol with 0.2 mg/ml 3xFLAG peptide</td>
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<tr>
<td><strong>1X Tris-glycine running buffer</strong></td>
<td>25 mM Tris-Cl, 250 mM glycine, 0.1% SDS</td>
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<tr>
<td><strong>Blotting buffer</strong></td>
<td>20% Ethanol, 39 mM Glycine, 48 mM Tris base, 0.037% SDS</td>
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<tr>
<td><strong>1X TBS-T</strong></td>
<td>50 mM Tris-Cl pH 7.5, 150 mM NaCl, 0.05% Tween-20</td>
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<tr>
<td><strong>Blocking solution</strong></td>
<td>50 mM Tris-Cl pH 7.5, 150 mM NaCl, 0.05% Tween-20 + 2.5% (m/v) Skimmed Milk powder</td>
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<tr>
<td><strong>1X TAE</strong></td>
<td>40 mM Tris-Cl pH 7.6, 20 mM acetic acid, 1mM EDTA</td>
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<td><strong>1X TBE</strong></td>
<td>89 mM Tris-Cl pH 7.6, 89 mM boric acid, 2 mM EDTA</td>
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<td><strong>Church buffer</strong></td>
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<td><strong>2X SSC buffer</strong></td>
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### Table 5.1.7: Antibodies

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<tr>
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5.2 METHODS

5.2.1 Strain construction, plasmid generation and genomic integration
All S. pombe strains used in this study are listed in Table 5.1.1 and Table 5.1.2. The strains were constructed by electroporation (Bio-Rad MicroPulser program ShS) with plasmid or a PCR-based gene targeting product leading to deletion or epitope-tagging of specific genes. Plasmids, listed in Table 5.1.3, were generated via cloning based on restriction enzymes or inverse Polymerase Chain Reaction (iPCR). PCR was performed using lab purified Phusion enzyme or commercial Phusion Flash High-fidelity PCR Master Mix (Thermo Fischer Scientific) and primers listed in Table 5.1.4. Digestion of plasmids and inserts was performed at 37°C for 1 hour using 1 U of restriction enzyme (New England Biolabs and Thermo Fischer Scientific) for 1 ug of DNA in 50 µl of reaction. The digested product were purified with the NucleoSpin Gel and PCR Clean-Up kit (Macherey-Nagel) according to the manufacturer's instructions and ligated with the T4 DNA ligase (Thermo Fischer Scientific) at 22°C for 30 min in 20 µl of reaction. XL1 Blue E. coli competent cells were heat-shock transformed and plated on LB plates containing the appropriate antibiotic. Positive colonies were confirmed by PCR and sequencing. For transformation, S. pombe cells were grown in YES at OD600 0.3-0.4 at 32 °C, harvested and washed in 1.2 M Sorbitol (Roth) twice. Pellets were then re-suspended with 200 µl 1.2 M Sorbitol and mixed with 200 - 500 ng of DNA. Positive colonies were selected on YES plates containing 100 – 200 mg/ml antibiotics and were confirmed by PCR and sequencing. Strains containing plasmids, listed in Table 5.1.2, were grown on Complete Edinburgh Minimal Medium - Leu. For endogenous C-terminal tagging, plasmid p85 harboring 3xHA tag was used together with primers 523 and 524 for Cid14 and Cid16, respectively, to amplify the targeting cassette. cid14 was cloned into pRSF-Duet with a GST tag on the N-terminal using primers 461; consequently a six histidine tag on the C-terminal was inserted with primers 712 via inverse PCR. cid16 was cloned into pREP1 with a FLAG tag on the N-terminal using primers 460 F and 665 R; consequently a six histidine tag on the C-terminal was inserted with primers 713 via inverse PCR. The ura4 hairpin was excised from the original plasmid pnatMX ART sh ura4 – 5 and ligated into pREP1 after a double digestion with Xmal and Ndel.

5.2.2 Ago1-bound siRNA sequencing
Endogenous 3xFLAG-tagged Ago1 was purified from different mutants by protein affinity purification. The pellet of a 2.5 L log-phase culture was resuspended 1:1 in lysis buffer (50 mM HEPES pH 7.5, 1.5 M NaOAc, 5 mM MgCl2, 2 mM EDTA pH 8, 2 mM EGTA pH 8, 0.1% Nonidet P-40, 20%
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Glycerol) containing 1 mM PMSF, 0.8 mM DTT and Complete EDTA free Protease Inhibitor Cocktail (Roche). Lysis was performed with glass beads (Roth) and the BioSpec FastPrep-24 bead beater (MP-Biomedicals), 8 cycles at 6.5 m/s for 30s and 3 min on ice. Cell debris was removed by centrifugation at 13000 rpm for 15 min. The supernatant was incubated with 30 µl FLAG-M2 agarose beads (Sigma-Aldrich, A2220) at 4°C for 1.5 hour. The resin was washed 5 times with lysis buffer. Ago1 was eluted with 1% SDS, 300 mM NaOAc. The protein-bound RNA was recovered by phenol-chloroform-isoamylalcohol (25:24:1, Roth) extraction and ethanol precipitation. Small RNAs with the length 20-30 nt were excised from an 18% polyacrylamide urea gel. 2 pmol of a preadenylated 3' adaptor oligonucleotide (miRNA Cloning Linker-1 from IDT, 5'-App CTG TAG GCA CCA TCA AT/ddC/-3') were ligated in a 10 µl reaction with 5 U T4 RNA ligase (TaKaRa), ligation buffer without ATP and 5 U RNasin (Promega) at 20°C for 2 hours. The 3' ligated products were purified on an 18% acrylamide urea gel with subsequent phenol-chloroform purification and ethanol purification. The 5' adaptor ligation was performed in a 10 µl reaction with 2 pmol 5' adaptor oligonucleotide (5'-GUU CAG AGU UCU ACA GUC CGA CGA UC-3'), 5 U RNasin (Promega), 0.06 µg BSA, 5 U T4 RNA ligase (Thermo Fischer Scientific) and 1x ligation buffer with ATP (Thermo Fischer Scientific) at 20°C for 2 hours. The ligated products were gel purified and reverse transcribed with 10 pmol primer (RT primer: 5'-GTG ACT GGA GTT CAG AGT GTG TA CTT TCC CTA CAC GAC G-3') and the SuperScript III First Strand Synthesis System (Thermo Fisher Scientific). The cDNA was PCR-amplified with Q5 High-Fidelity 2x Master Mix (NEB) for 14-20 cycles using the Illumina P5 5' primer (5' -AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT CTT TCC CTA CAC GAC G-3') and the Illumina P7 3' primer with inserted barcode (5'-CAA GCA GAA GAC GGC ATA CTA GAT XXXXX GTG ACT GGA GTT CAG ACG TG -3'). Single end sequencing was performed on an Illumina GAIIX sequencer at the LAFUGA core facility of the Gene Center, Munich. The Galaxy platform was used to demultiplex the obtained reads.

5.2.3 total RNA isolation and total small RNA enrichment

Total RNA was isolated from mid-log phase yeast culture with the TRI Reagent Solution (Ambion) according to the manufacturer's instructions. DNase I (Thermo Fisher Scientific) treatment was performed at 37°C for 1-2 hours. DNase was removed by a second phenol-chloroform-isoamylalcohol extraction or heat inactivated according to the manufacturer's instructions. Total RNA was then precipitated with 100% ethanol, washed with 75% ethanol and resuspended in nuclease-free water. To enrich for the small RNA fraction, the volume of the dissolved total RNA was brought to 50 ul, mixed with 175 ul of Lysis buffer of RNeasy Midi kit (Qiagen) and 125 ul of 100% ethanol and transferred on a miRNeasy column (Qiagen). After centrifuging for 15 seconds at 9500
rpm, the flow-through containing the <200 nucleotides total RNA fraction was collected, mixed with 227.5 ul of 100% ethanol and transferred to a new miRNeasy column. After centrifuging at 9500 rpm for 15 seconds the flow-through was discarded and the column was washed once with 500 ul of RPE buffer supplemented with ethanol. After drying the column at 9500 rpm for 1 min, the <200 nucleotides total RNA fraction was eluted from the column in 15-20 ul of nuclease-free water.

5.2.4 Reverse Transcription (RT)
250 ng of total RNA was reverse transcribed with SuperScript III First Strand Synthesis System (Thermo Fischer Scientific) and 0.2 pmol of specific reverse primers (Table 5.1.4). Reactions of 20 ul were incubated at 50°C for 1 hour, followed by 10 min at 70°C to inactivate the reverse transcriptase.

5.2.5 Quantitative Real-Time PCR (qPCR)
qPCR was performed on ChIP samples or after reverse transcription (RT) using the 2X DyNAmo Flash SyBR Green Master Mix qPCR kit (BioZym) and the Toptical thermocycler (Biometra). 10 μl PCR reactions were assembled in a 96-well plate (4titude) with 3.5 ng of cDNA and specific primers 0.4 μM forward and reverse primers (listed in Table 4). For RT-qPCR, untranscribed RNA was used as template as negative control to detect possible contaminating DNA species. For ChIP assays, both INPUT and Immunoprecipitated DNA were analyzed by qPCR. Both RT and ChIP samples were normalized on the tdh house-keeping gene, which is not affected by H3K9 methylation or transcript levels variation in the mutants analyzed.

The qPCR cycle consisted in an initial step of denaturation at 95°C for 3 minutes, followed by 46X cycles (95°C denaturation for 10 seconds, 59°C annealing for 20 seconds, 72°C elongation for 15 seconds) and a final melting temperature calculation step ranging from 60°C to 95°C. Oligonucleotides used for qPCR in this study are listed in Table 5.1.4.

5.2.6 total RNA sequencing
rRNA of 1 μg total RNA was degraded with Terminator nuclease (Epicentre) in buffer A at 30°C for 2 hours. The RNA library was obtained using the NEBNext Ultra Directional RNA Library Prep Kit for Illumina (NEB). Single end sequencing was performed on an Illumina GAIIX sequencer at the LAFUGA core facility of the Gene Center, Munich. The Galaxy platform was used to demultiplex the obtained reads.

5.2.7 Growth assay
Tenfold serial dilutions of cultures with OD₆₀₀ between 0.4 and 0.7 were made so that the highest density spot contained 10⁵ cells. Cells were spotted on non-selective YES medium plates. The plates
were incubated at 32ºC for 2-3 days and imaged.

5.2.8 Chromatin immunoprecipitation (ChIP)

50 ml mid-log phase yeast cultures were cross-linked with 1% formaldehyde (Roth) for 15 min at room temperature. The reaction was quenched with 125 mM glycine for 5 min. The frozen pellet was resuspended in 500 µl lysis buffer (250 mM KCl, 1x Triton-X, 0.1% SDS, 0.1% Na-Desoxycholate, 50 mM HEPES pH 7.5, 2 mM EDTA, 2 mM EGTA, 5 mM MgCl2, 0.1% Nonidet P-40, 20% Glycerol) with 1 mM PMSF and Complete EDTA free Protease Inhibitor Cocktail (Roche). Lysis was performed with glass beads (Roth) and the BioSpec FastPrep-24 bead beater (MP-Biomedicals), 8 cycles at 6.5 m/s for 30s and 3 min on ice. DNA was sheared by sonication (Bioruptor, Diagenode) 35 times for 30 s with a 30 s break. Cell debris were removed by centrifugation at 13000 x g for 15 min. The crude lysate was normalized based on the RNA and Protein concentration (Nanodrop, Thermo Fischer Scientific) and incubated with 1.2 µg immobilized (Dynabeads Protein A, Thermo Scientific) antibody against dimethylated H3K9 (H3K9me2, Abcam AB1220) for 2 hr or overnight at 4°C. The resin with immunoprecipitates was washed five times with each 1 ml of lysis buffer and eluted with 150 µl of elution buffer (50 mM Tris-Cl pH 8.0, 10 mM EDTA, 1% SDS) at 65°C for 15 min. RNase A (Thermo Fischer Scientific) treatment was performed for 20 min at 65°C and subsequent Proteinase K (Roche) treatment was performed for at least 5 hr or ON at 65°C. DNA was recovered by phenol-chloroform-isoamylalcohol (25:24:1, Roth) extraction with subsequent ethanol precipitation. DNA levels were quantified by qRT-PCR and normalized to *tdh1* background levels. Oligonucleotides used for quantification are listed in Supplementary Table 5.1.4. For sequencing, a ChIP-seq library was made using the NEBNext Ultra II DNA Library Prep Kit for Illumina kit (NEB).

5.2.9 Analysis of sequencing data

Demultiplexed illumina reads were mapped to the *S. pombe* genome, allowing 2 nucleotides mismatch to the genome using Novoalign (http://www.novocraft.com). Small RNA reads mapping to multiple locations were randomly assigned. The datasets were normalized to the number of reads per million sequences for small RNA-seq and ChIP-seq or reads mapping to coding sequences for total RNA-seq. Additionally, the datasets were normalized to total amounts of reads (for small RNAs) that were associated with Ago1 in different mutant strains as determined by Ago1 pulldowns and quantification of bound small RNAs. The genome sequence and annotation that were available from the *S. pombe* Genome Project were used (Wood et al., 2002). The data are displayed using the Integrative Genomics Viewer (IGV) (Thorvaldsdottir et al., 2013). Sequenced strains are listed in Supplementary Table 5.1.5.
5.2.10 siRNA Purification and Detection
siRNAs associated with Argonaute were purified as described in Marasovic et al., 2013. FLAG-Ago1 was immunoprecipitated with anti-FLAG resin as described above. siRNAs were recovered from FLAG-Ago1 by phenol-chloroform extraction and ethanol precipitation, dephosphorilated, $[\gamma^{32}\text{P}]$-ATP labeled and run on 18% denaturing polyacrylamide gel. The gel was wrapped in cling film and exposed to a storage phosphor screen (BAS MS 2025 – Fujifilm Corporation) overnight at -80°C. The screen was scanned with a Typhoon FLA 9500 (GE Healthcare).

5.2.11 Protein expression and purification
GST-Cid14-6xHis were expressed in *E. coli* with 0.2 mM IPTG (Roth) at 18°C overnight. Pelleted cells were resuspended in lysis buffer (50 mM NaH$_2$PO$_4$/Na$_2$HPO$_4$ pH 8, 1 M NaCl, 20 mM imidazole, 3 mM β-mercaptoethanol and 0.5 mM PMSF) and lysed with the French Press and the clear lysate was incubated with Ni Sepharose 6 Fast Flow (GE Healthcare, 17-5318-01) at 4°C for 30 min. The resin was washed three times with 50 ml of lysis buffer, once with 4 resin volume of 40 mM imidazole lysis buffer and the protein was eluted with 6 resin volume of elution buffer (50 mM NaH$_2$PO$_4$/Na$_2$HPO$_4$ pH 8, 500 mM NaCl, 300 mM imidazole, 3 mM β-mercaptoethanol and 1 mM PMSF). The elution fraction was dialysed in 50 mM Tris-Cl pH 7.5, 150 mM NaCl, 0.1 mM DTT, 0.1 mM EDTA and incubated with Glutathione Sepharose 4 Fast Flow (GE Healthcare, 17-5318-01) at 4°C for 30 min. The resin was washed twice with dialysis buffer and the protein was eluted with 6x column volume of elution buffer (50 mM Tris-Cl pH 8, 500 mM NaCl, 10 mM reduced glutathione). The elution fraction was dialysed in 50 mM Tris-Cl pH 7.5, 200 mM NaCl, 1 mM DTT, 0.1 mM EDTA, 10% glycerol). FLAG-Cid16-6xHis was expressed in *S. pombe*. Pelleted cells were resuspended in lysis buffer (50 mM HEPES pH 7.5, 1.5 M NaOAc, 5 mM MgCl$_2$, 2 mM EDTA pH 8, 2 mM EGTA pH 8, 0.1% Nonidet P-40, 20% Glycerol) containing 1 mM PMSF, 0.8 mM DTT and Complete EDTA free Protease Inhibitor Cocktail (Roche). Frozen cells were lysed with freezer mill and the protein was purified with Ni-NTA resin as described above. The dialyzed elution fraction was incubated with FLAG-M2 agarose beads (Sigma-Aldrich, A2220) at 4°C for 2 hours. The beads were washed with 20 column volumes of dialysis buffer. Protein was eluted with 2x column volume of elution buffer (25 mM Hepes pH 7.5, 200 mM KCl, 2 mM MgCl$_2$, 0.1% Nonidet P-40, 20% Glycerol) with 0.2 mg/ml 3xFLAG peptide. FLAG-Rrp6 (kindly provided by François Bachand) was purified with FLAG-M2 agarose beads (Sigma-Aldrich, A2220) as described above.

5.2.12 Co-immunoprecipitation (Co-IP)
FLAG immunoprecipitation was performed as described above but in low salt conditions (50 mM
HEPES pH 7.5, 100 mM NaOAc, 5 mM MgCl\(_2\), 2 mM EDTA pH 8, 2 mM EGTA pH 8, 0.1% Nonidet P-40, 20% Glycerol) containing 1 mM PMSF, 0.8 mM DTT and Complete EDTA free Protease Inhibitor Cocktail (Roche). Lysis was performed with glass beads (Roth) and the BioSpec FastPrep-24 bead beater (MP-Biomedicals), 8 cycles at 6.5 m/s for 30s and 3 min on ice. Cell debris was removed by centrifugation at 7000 rpm for 20 min. The crude lysate was normalized based on the Protein concentration (Nanodrop, Thermo Fischer Scientific) and incubated with FLAG-M2 agarose beads (Sigma-Aldrich, A2220). Immunoprecipitated samples and corresponding inputs were analyzed by immunoblot. Protein were separated on an 8% polyacrylamide SDS-page and transferred on a PVDF membrane (Roth Immobilon-P) for 1 hour at 15 voltage using a Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad). The membrane was blocked with 5% milk (w/v) in 1x TBS-T buffer for 1 hour, incubated with the Anti-HA antibody (Santa Cruz Biotechnology, sc-7392, 1:200 in 1x TBS-T) for 1 hour and washed three times with 1x TBS-T for 10 min. The membrane was then incubated with the secondary anti-mouse antibody coupled to peroxidase (Bio-Rad, #1721011, 1:3000 in 1x TBS-T) for 1 hour at room temperature and washed three times with 1x TBS-T for 10 min.

The membrane was developed using the Super Signal West Pico Chemiluminescence Substrate. Photos were taken with the Fujifilm LAS 3000 camera. The membrane was incubated with 20 ml of Restore Western Blot Stripping Buffer (Thermo Fisher Scientific) for 20 min, blocked with 5% milk (w/v) in 1x TBS-T for 1 hour and incubated with the peroxidase conjugated Anti-FLAG antibody (Sigma-Aldrich, #8592, 1:1000 in 1X TBS-T) for 1 hour. The membrane was developed and imaged as described above.

5.2.13 Degradation of Argonaute-associated small RNAs in vitro

Ago1 was purified from dcr1\(\Delta\)tri1\(\Delta\) and loaded with 22 nucleotides long small RNA (Table 5.1.4) as described in Marasovic et al., 2013. Argonaute was purified from dcr1\(\Delta\)tri1\(\Delta\) cells as described above, with the exception that Ago1 remained associated with the FLAG resin. Ago1 associated with the resin was incubated with 1-0.5 pmol of [\(\gamma\)-\(^{32}\)P]-ATP radiolabeled small RNAs for 2 hr 32°C with gentle shacking. Resin was washed with buffer containing 25 mM HEPES pH 7.5, 2 mM MgCl\(_2\), 2 mM DTT, 0.02% NP-40, and 100 mM NaOAc to remove unbound small RNAs. Ago1-associated small RNAs were incubated with 80 ng of Cid14, Cid16, Tri1 and Rrp6 in buffer containing 1 mM Hepes pH 7.5, 0.5 mM MgCl\(_2\), 0.5 mM MnCl\(_2\), 25 mM KCl, 0.2 mM DTT and 5 mM ATP/UTP (Roche) at 32°C for 1 hour, mixing the reactions every 5-10 min. For the time course experiment, reactions were up – scaled and aliquots were taken after the indicated time points. RNA was extracted from Ago1 and detected as described above.
5.2.14 Northern Blotting analysis

2-5 µg of total small RNAs were run on 18% denaturing polyacrylamide gel and transferred to a positively charged nylon membrane (GE Healthcare Amersham Hybond N+) on a Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad). The RNA was UV-cross-linked to the membrane with Spectrolinker XL-1500 (Spectroline, “optimal crosslink”). Prehybridization was performed with Church Buffer (500 mM NaH2PO4/Na2HPO4 pH 7.2, 1 mM EDTA, 7% SDS) at 37°C over night. 10 pmol of DNA probes (Table 5.14) were labeled with T4 PNK (NEB) and 10 pmol [γ-32P]-ATP (Hartmann Analytic) at 37°C for 1 hour. The labeled probes were purified with an Illustra MicroSpin G-25 column (GE Healthcare), mixed with 5 ml Church Buffer, and incubated with the membrane for 5 hours at 37°C. The membrane was rinsed once with 2x SSC buffer (300 mM NaCl, 30 mM sodium citrate, pH 7.0) and then washed three times with 2x SSC buffer for 15 min at 37°C. The membrane was wrapped in cling film and exposed to a storage phosphor screen (BAS MS 2025 - Fujifilm Corporation) overnight up to one week at -80°C. The screen was scanned with a Typhoon FLA 9500 (GE Healthcare). For a second hybridization, the membrane was stripped in boiling 0.1% SDS for 5 min and subsequently prehybridized.

5.2.15 Small RNAs tailing assay

500 fmol – 1 pmol of double-strand, single strand or Ago1-bound 22 nucleotides small RNAs (Table 4) were incubated with 80 ng of Cid14 and Cid16 in buffer containing 1 mM Hepes pH 7.5, 0.5 mM MgCl2, 0.5 mM MnCl2, 25 mM KCl, 0.2 mM DTT, 40U Ribolock (Thermo Fischer Scientific) and 150 nM [α-32P]-ATP/UTP (Hartmann Analytic) at 32°C for 1-2 hours. Double-strand RNA was obtained by incubating RNAs 71 and 72 (Table 5.14) at same molar concentration in annealing buffer (10 mM Tris-Cl pH 7.5, 50 mM KCl, 1 mM EDTA) at 95°C for 5 min followed by incubation at room temperature for 1 hour. RNA was extracted and detected as described above. The single strand 22 nucleotides small RNA was phosphorylated by PNK (Thermo Fischer Scientific) before being loaded into resin-bound Ago1.

5.2.16 Small RNAs half-lives detection

Wild type and cid14Δ cells with the ura4 hairpin expressed under the nmt1 promoter were grown until log-phase. At time zero the medium was supplemented with 15 µM thiamine to repress the nmt1 promoter. Aliquots were taken after 1, 3 and 5 hours to monitor the half-life of ura4 small RNAs. Total small RNAs and Ago1-bound small RNAs were purified and detected via Northern blotting analysis as described above. The quantity of the Ago1-bound small RNAs to be loaded was determined by loading the same volume of purified Ago1-small RNAs on a 18% denaturing
polyacrylamide gel, staining it with sybr gold and scanning it in order to quantify the samples. For quantification, *ura4* small RNAs from total fraction were normalized to *snoR69* and Ago1-bound *ura4* small RNAs were normalized to centromeric small RNAs.
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