Small RNAs and gene expression regulation

Roles for small RNAs in gene expression regulation

Three classes of small RNA can regulate genes by targeting transcripts in the cytoplasm. These are:

- **microRNAs (miRNAs)**, which are hairpin-derived RNAs with imperfect complementarity to targets and that cause translational repression;

- **small interfering RNAs (siRNAs)**, which have perfect complementarity to targets and cause transcript degradation;

- **PIWI-intereacting RNAs (piRNAs)**, which target transposon transcripts in animal germ lines.

Therapeutic use of RNAi

- Hematological Diseases:
  - Alteration due to reduction, mutation, or absence of gene function

- Oncology:
  - Inhibit oncogenes
  - Increase the efficacy of chemotherapy and radiotherapy

- Infectious Diseases - Targeting Viruses (HCV and HIV):
  - Inhibit cellular and viral factors
  - Target the Reverse Transcriptase RNA, inhibit viral replication
  - Induce resistance to viral infection in infected organisms

Approved RNAi therapeutics

Name	Target disease	Target protein	Development company	siRNA chemistry[b] and dosing frequency	Stage of clinical development and pivotal and/or latest trials (ClinicalTrials.gov registration and participant population)
Patisiran	Hereditary transthyretin-mediated amyloidosis with polyneuropathy	Transthyretin[37]	Alnylam	Partially modified siRNA (delivered via LNP); 3 weekly	Approved in the United States and Canada for the polyneuropathy of hATTR amyloidosis in adults, and in the European Union, Japan and other countries for treatment of hATTR amyloidosis in adults with stage 1 or stage 2 polyneuropathy: APOLLO phase 3 (ref. 38) (ClinicalTrials.gov: NCT01960348, *n*=225) Late-stage development for hATTR amyloidosis with cardiomyopathy: APOLLO-B phase 3 (ClinicalTrials.gov: NCT03997383, *n*=360)
Givosiran	Acute hepatic porphyria	Delta-aminolevulinic acid synthase 1 (ref. 131)	Alnylam	Fully modified ESC siRNA–GalNAc conjugate; monthly	Approved in the United States, Brazil and Canada for the treatment of adults with acute hepatic porphyria (AHP), and in the European Union and Japan for the treatment of AHP in adults and adolescents 12 years and older: ENVISION[132] (ClinicalTrials.gov: NCT03338816, *n*=94)
Lumasiran	Primary hyperoxaluria type 1	Glycolate oxidase	Alnylam	Fully modified ESC siRNA–GalNAc conjugate; monthly	Approved in the United States, European Union and Brazil for the treatment of primary hyperoxaluria type 1 in all age groups: ILLUMINATE-A[133] (ClinicalTrials.gov: NCT03681184, *n*=39) Late-stage development for primary hyperoxaluria type 1 in infants and young children: ILLUMINATE-B phase 3 (ClinicalTrials.gov: NCT03905694, *n*=18) Late-stage development for advanced primary hyperoxaluria type 1 in all age groups: ILLUMINATE-C phase 3 (ClinicalTrials.gov: NCT04152200, *n*=21)
Inclisiran	Hypercholesterolemia	Pro-protein convertase subtilisin/kexin type 9 (ref. 129)	Alnylam, Novartis, The Medicines Company	Fully modified ESC siRNA–GalNAc conjugate; 6 monthly	Approved in the United States as an adjunct to diet and maximally tolerated statin therapy for the treatment of adults with clinical atherosclerotic cardiovascular disease (ASCVD) or heterozygous familial hypercholesterolemia who require additional lowering of LDL-C, and in the European Union for use in adults with primary hypercholesterolemia (heterozygous familial and non-familial) or mixed dyslipidemia, as an adjunct to diet: ORION program[61,86,134] (also a poster at the International Society on Thrombosis and Haemostasis by K. J. Pasi in 2017) (ClinicalTrials.gov: NCT03397121, *n*=482; NCT03399370, *n*=1,561; NCT03400800, *n*=1,617)
Vutrisiran	Transthyretin-mediated amyloidosis with polyneuropathy and cardiomyopathy	Transthyretin[60]	Alnylam	Fully modified ESC siRNA–GalNAc conjugate; 3 monthly	Approved in the United States for the polyneuropathy of hATTR amyloidosis in adults: HELIOS-A phase 3 (ref. 135) (ClinicalTrials.gov: NCT03759379, *n*=164) Late-stage development for hATTR amyloidosis with cardiomyopathy: HELIOS-B phase 3 (ClinicalTrials.gov: NCT04153149, *n*=655)
Nedosiran	Primary hyperoxaluria	Lactate dehydrogenase subunit A[97,102]	Novo Nordisk (formerly Dicerna)	Fully modified Dicer-substrate siRNA–GalNAc conjugate; monthly	Approved in the United States to lower urinary oxalate levels in children 9 years of age and older and adults with primary hyperoxaluria type 1 (PH1) and relatively preserved kidney function (eGFR ≥30 ml min^{-1} 1.73 m^{-2})

RNAi for the treatment of neurodegenerative diseases

nature medicine

Silencing mutant SOD1 using RNAi protects against neurodegeneration and extends survival in an ALS model

G Scott Ralph[1], Pippa A Radcliffe[1], Denise M Day[1], Janine M Carthy[1], Marie A Leroux[1], Debbie C P Lee[1], Liang-Fong Wong[1], Lynsey G Bilsland[2], Linda Greensmith[2], Susan M Kingsman[1], Kyriacos A Mitrophanous[1], Nicholas D Mazarakis[1] & Mimoun Azzouz[1]

A synthetic, single-stranded DNA molecule designed to target the SOD1 gene.
An antisense oligonucleotide (ASO) that binds to the messenger RNA of the mutated SOD1 gene.

Tofersen is a targeted therapy that specifically addresses the root cause of SOD1-ALS. By reducing the production of the toxic SOD1 protein, tofersen may help slow disease progression. Tofersen is administered through intrathecal injection directly into the cerebrospinal fluid, ensuring delivery to the central nervous system.

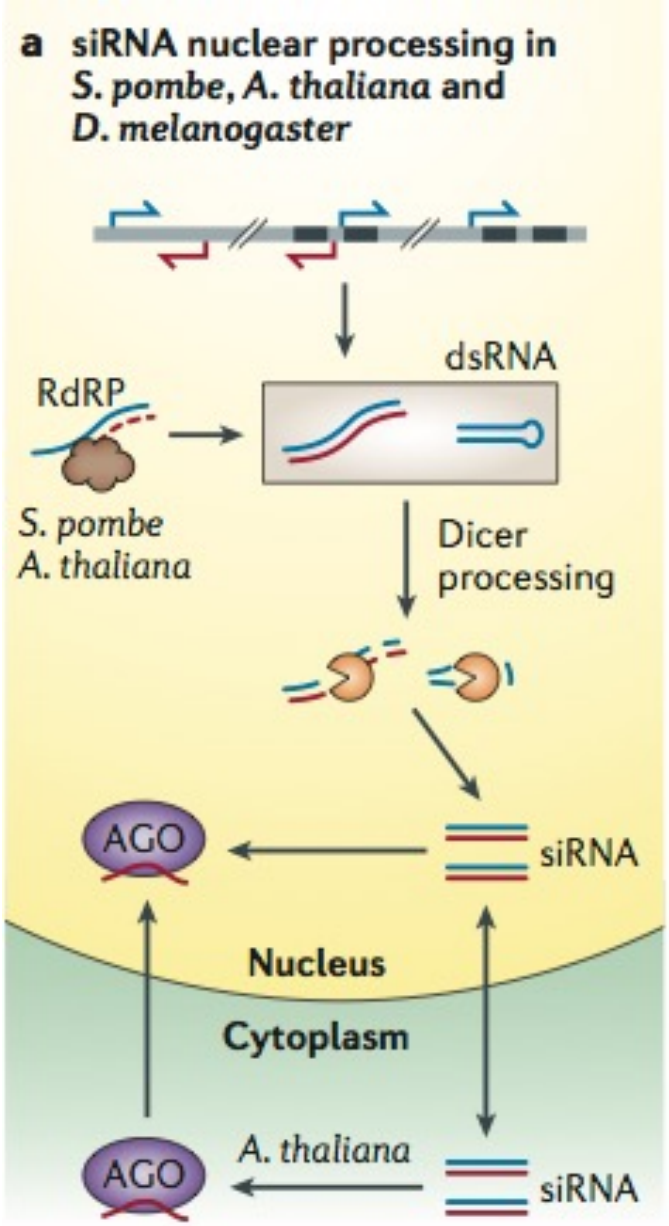
While tofersen shows promise, it's important to note that it is not a cure for ALS.

Ongoing research is exploring the potential of ASOs for other neurodegenerative diseases.

RNA interference in the nucleus

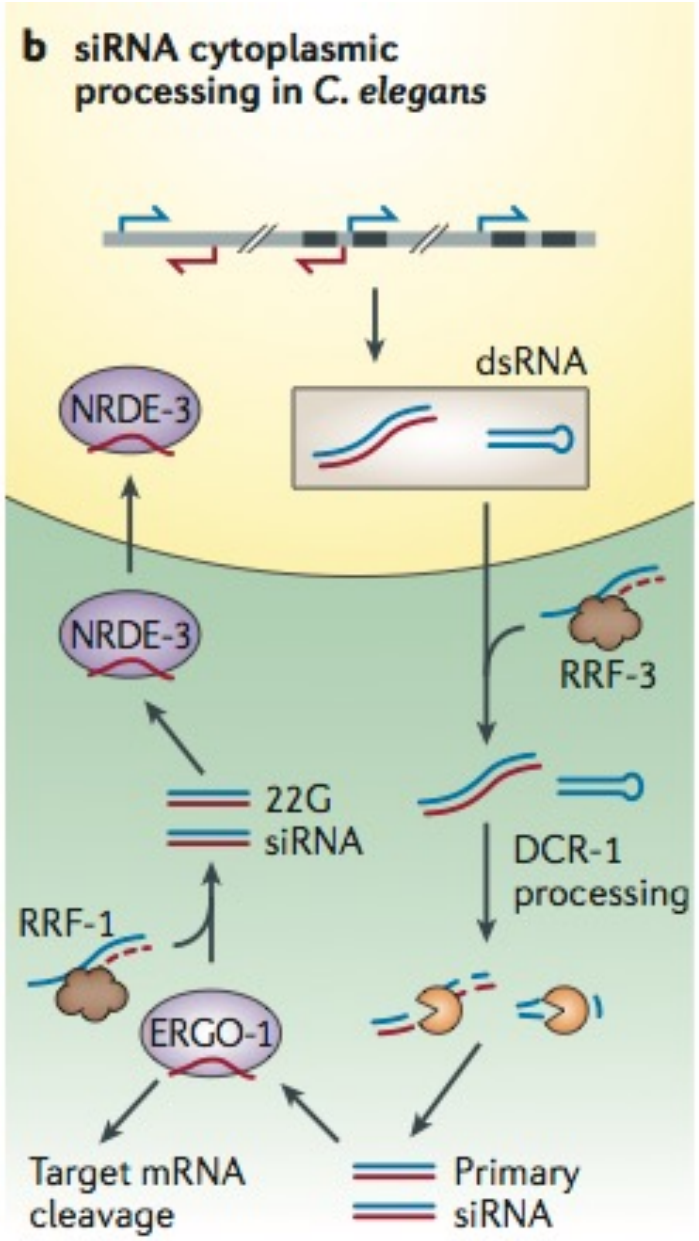
Biogenesis of nuclear small RNAs

siRNA processing takes place in the nucleus in *Schizosaccharomyces pombe* and *Drosophila melanogaster* and in the nucleolus in *Arabidopsis thaliana*. DsRNA can be produced by convergent transcription, complementary transcripts, structured loci or RNA-directed RNA polymerase (RdRP) activity in *A. thaliana* and *S. pombe*. Dicer proteins generate siRNAs that are loaded into an Argonaute protein (AGO). In *A. thaliana,* siRNAs are transported to the cytoplasm, where Argonaute is loaded and then imported into the nucleus.



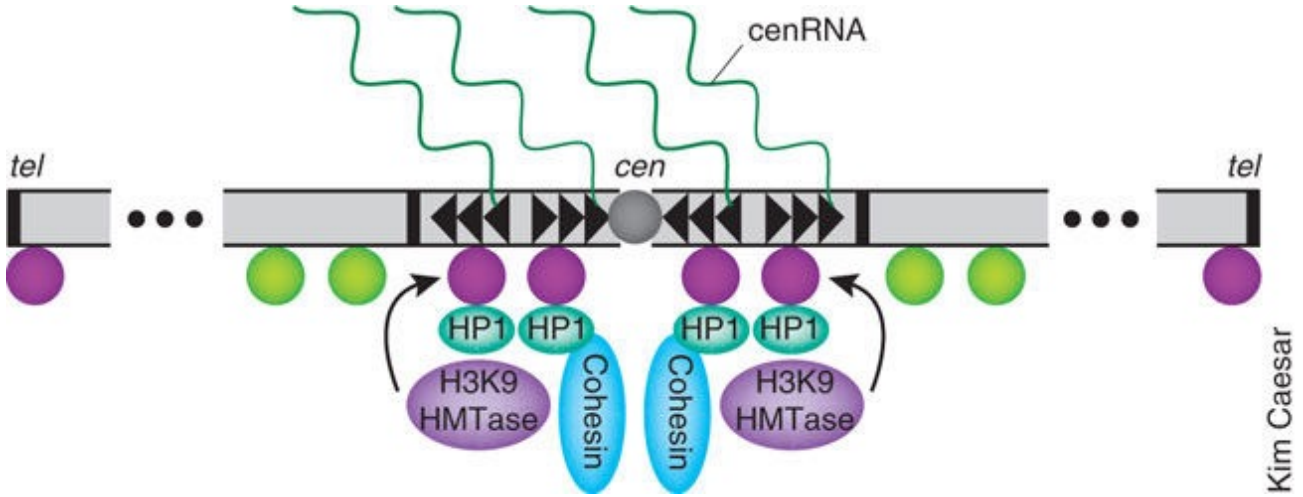
Biogenesis of nuclear small RNAs

In *Caenorhabditis elegans,* siRNA processing occurs in the cytoplasm in a two-step fashion. Primary trigger dsRNA arises from nuclear transcription or the RdRP activity of RRF-3, which acts on transcripts in the cytoplasm. Primary processing by DCR-1 produces primary 26-nucleotide siRNAs, which are loaded into the **Argonaute ERGO-1**. Loaded ERGO-1 can both facilitate post-transcriptional gene silencing (PTGS) in the cytoplasm and with RRF-1 can generate secondary 22G siRNAs. In the cytoplasm, secondary 22G siRNAs are loaded into the **nuclear Argonaute NRDE-3**, which is then transported into the nucleus.



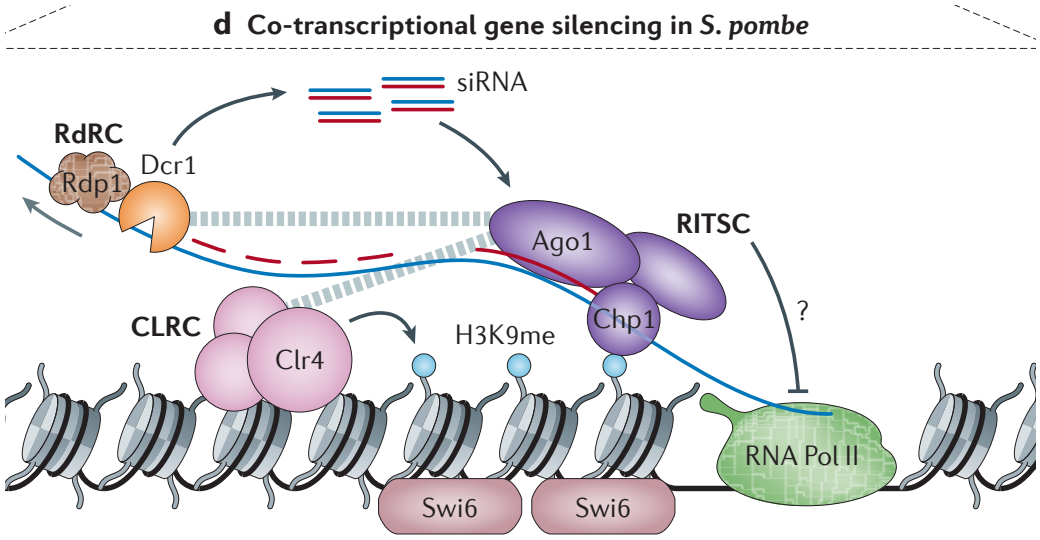
RNAi-mediated heterochromatin assembly in fission yeast

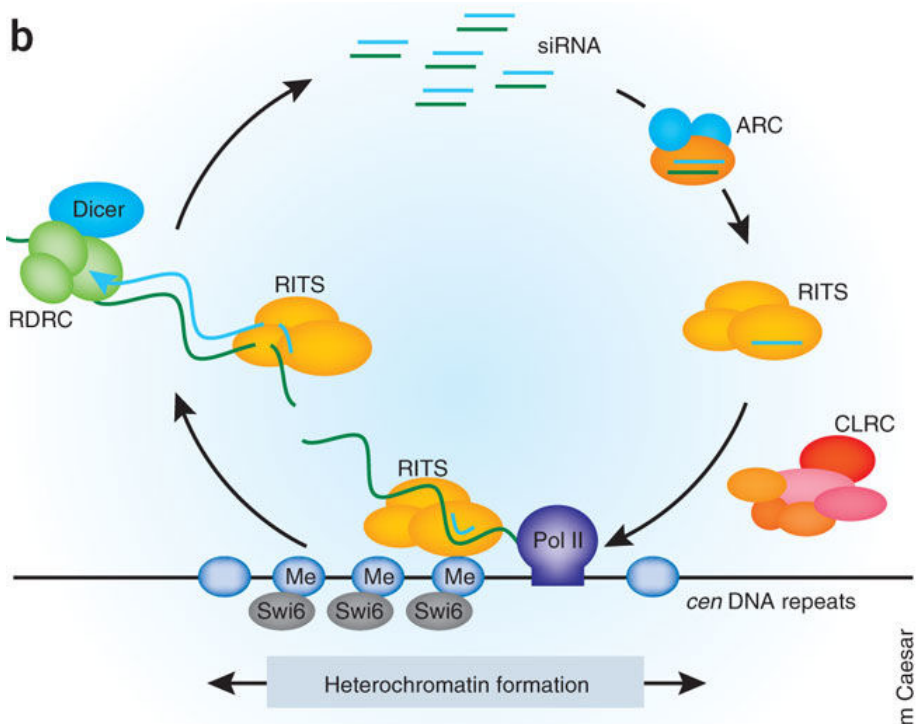
Heterochromatin is important in centromere function and the organization of chromosomes in the nucleus. A role on nuclear RNAi was indentified in *S. pombe,* in which it is required for the formation of constitutive heterochromatin at pericentromeric regions. These regions are highly enriched for H3K9me and are composed of varying numbers of repeat units that are bidirectionally transcribed to form dsRNA, cenRNA, that is then processed by Dcr1 into siRNAs



RNAi-mediated heterochromatin assembly in fission yeast

The **RITS complex** mediates heterochromatin formation by associating with nascent transcripts via siRNA base-pairing, and with methylated H3K9 via the chromodomain of its **Chp1** subunit. dsRNA synthesis and siRNA generation occur in association with specific chromosome regions and may underlie *cis* restriction of siRNA-mediated silencing. After the RITSC has been localized to repeat loci, it facilitates H3K9 methylation by recruiting thecryptic loci regulator complex (CLRC), which containsClr4, the sole H3K9 methyltransferase. The chromosome-associated siRNA synthesis loop is essential for the spreading of H3K9 methylation and silencing at the centromere. The coupling of the siRNA synthesis loop to H3K9 methylation forms a stable feedback loop that epigenetically maintains heterochromatin.





RNAi and transposones

Types of transposable elements

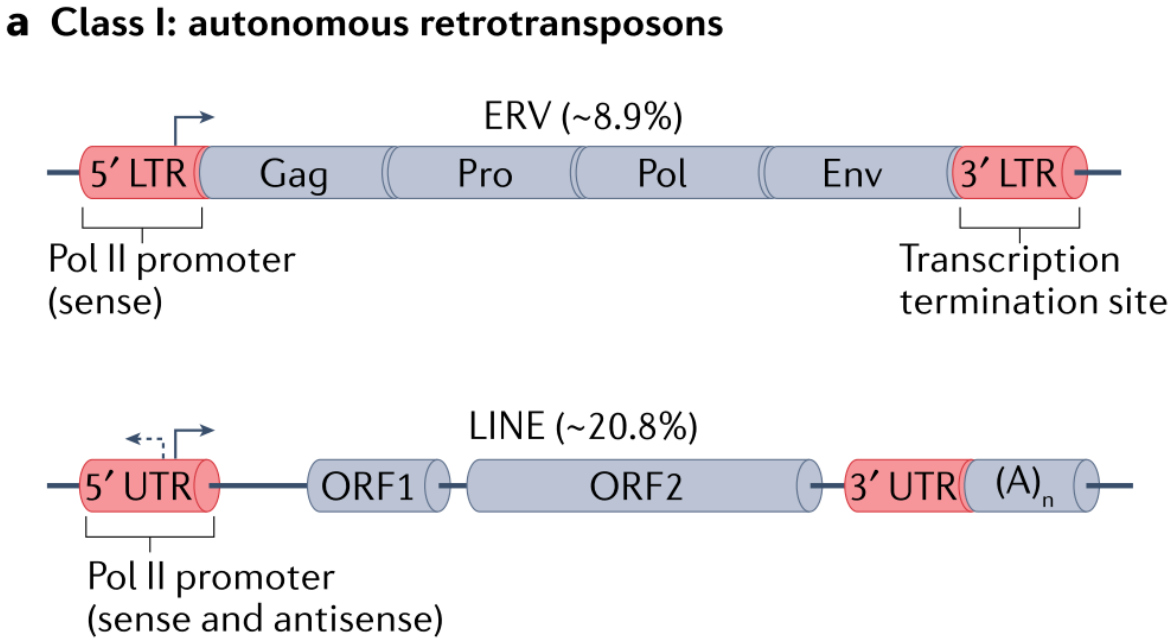
Transposable elements (TEs) are divided into Class I and Class II depending on their transposition mechanism:

- Class I elements are called **retrotransposons** because they use RNA as an intermediate that is reverse transcribed into DNA and integrated in the genome. Retrotransposomes are further divided in **autonomous retrotransposons** and **non-autonomous retrotransposons**.

- Class II **DNA transposons** encode a transposase that is required for their excision and insertion through a 'cut- and- paste' mechanism.

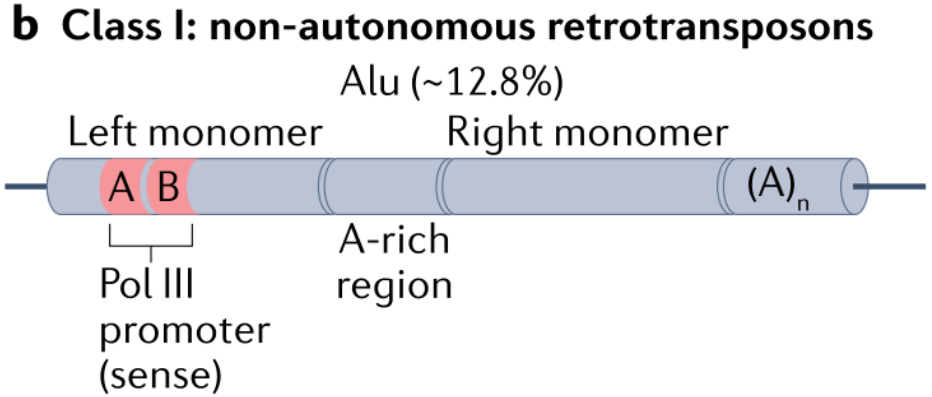
Types of transposable elements

Autonomous retrotransposons encode all the required proteins for their retrotransposition. Endogenous retroviruses (ERVs) consist of two long terminal repeats (LTRs) flanking the open reading frames (ORFs) that encode the viral proteins. During evolution, ERVs are often reduced to a single LTR, or 'solo LTR', which renders them incapable of retrotransposition. Long interspersed nuclear elements (LINEs), such as L1, contain two ORFs that encode proteins required for their retrotransposition, which are flanked by untranslated regions (UTRs). At the 3′ end, they possess an adenines tail of variable length.



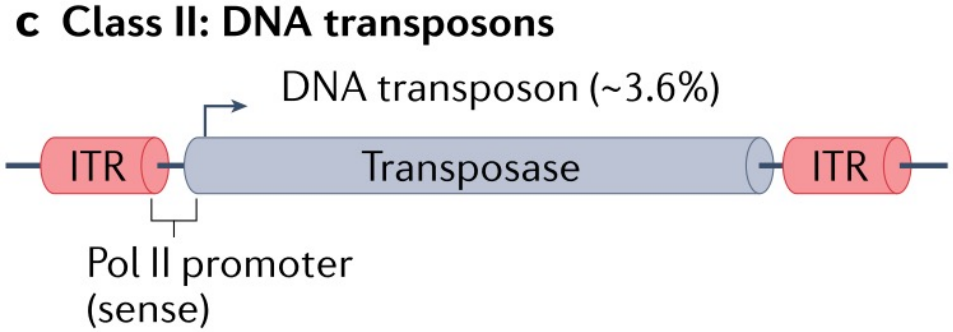
Types of transposable elements

Non-autonomous retrotransposons comprise those TEs that require the machinery encoded by autonomous retrotransposons to be mobilized. Alu elements are primate-specific short interspersed nuclear elements (SINEs), and their structure consists of two monomers derived from the 7SL non-coding RNA, flanking an adenine (A)-rich region. At the 3′ end, they possess an adenines tail of variable length. On the left monomer, boxes A and B indicate a bipartite promoter for RNA polymerase III (Pol III).



Types of transposable elements

Class II DNA transposons encode a transposase that is required for their excision and insertion through a 'cut- and- paste' mechanism. The transposase ORF is flanked by two inverted terminal repeats (ITRs).



Silencing mechanisms that suppress TEs

A range of chromatin modifications suppress TE transcription, including modifications of histone tails, DNA methylation and alterations in chromatin packing and condensation.

Nucleosomes that are associated with TEs are enriched for **methylation of H3K9**, which is a signal for transcriptionally repressive chromatin

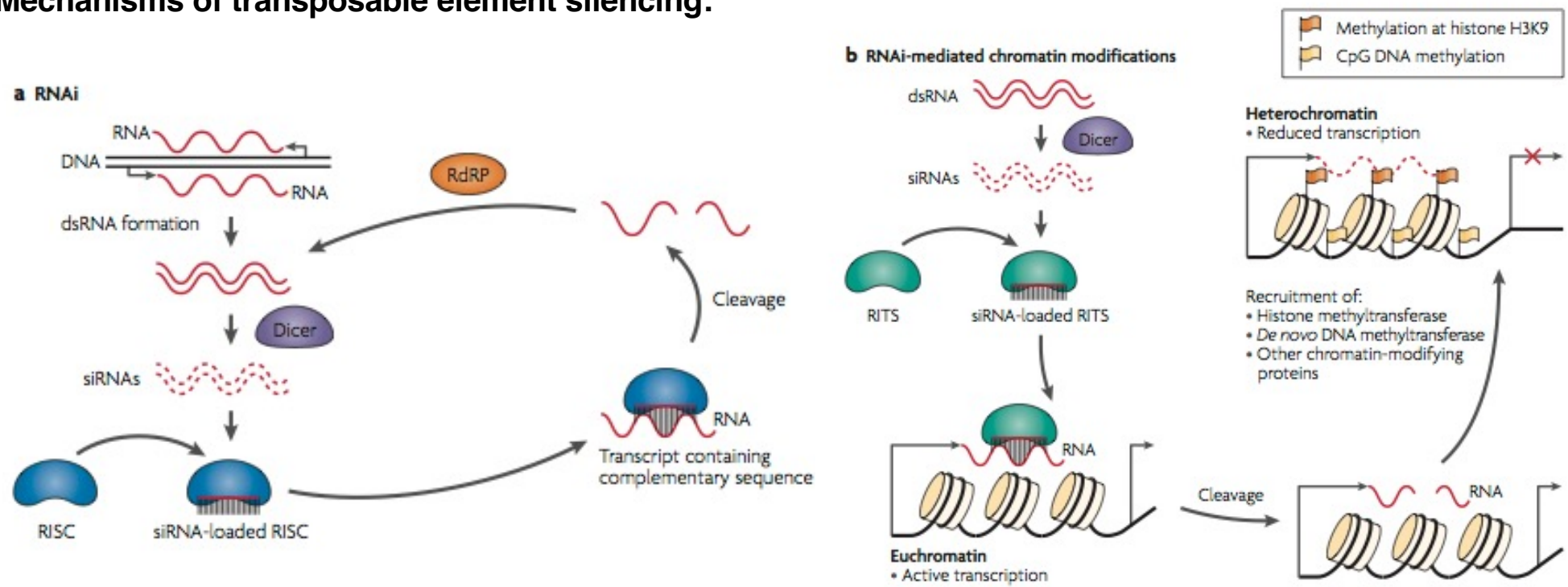
DNA methylation on cytosine residues is another important signal that represses TE transcription. In both plants and mammals, DNA methylation is copied to the new DNA strand upon DNA replication, providing a mechanism for inheritance of TE silencing.

De novo DNA methylation is also required for the epigenetic silencing of TEs at specific stages of mammalian development.

RNA-interference-mediated transposon silencing in the germline

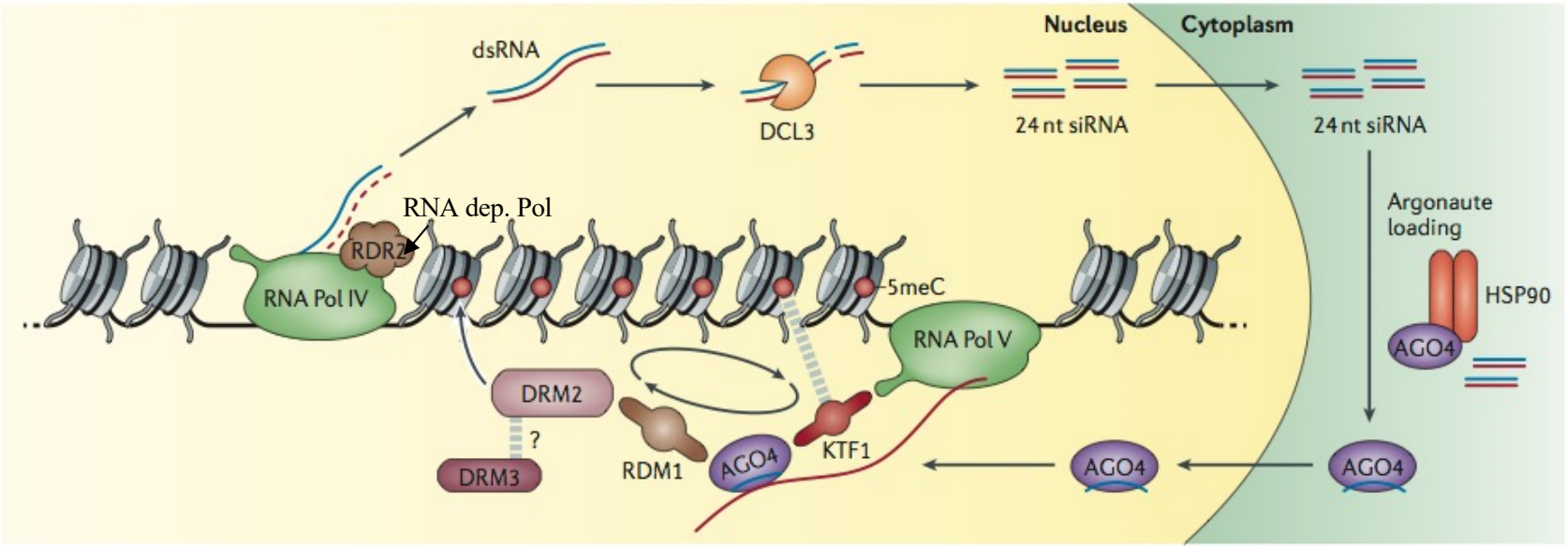
Nuclear RNAi, **in the form of the piRNA pathway** in animals and various **siRNA pathways** in plants, is a front-line defence against transposable element mobilization. These elements have the ability to move and/or to multiply themselves to new positions in the genome, thereby posing a threat to the genomic stability of an organism.

Mechanisms of transposable element silencing:



RNA-directed DNA methylation (RdDM) in *A.thaliana*

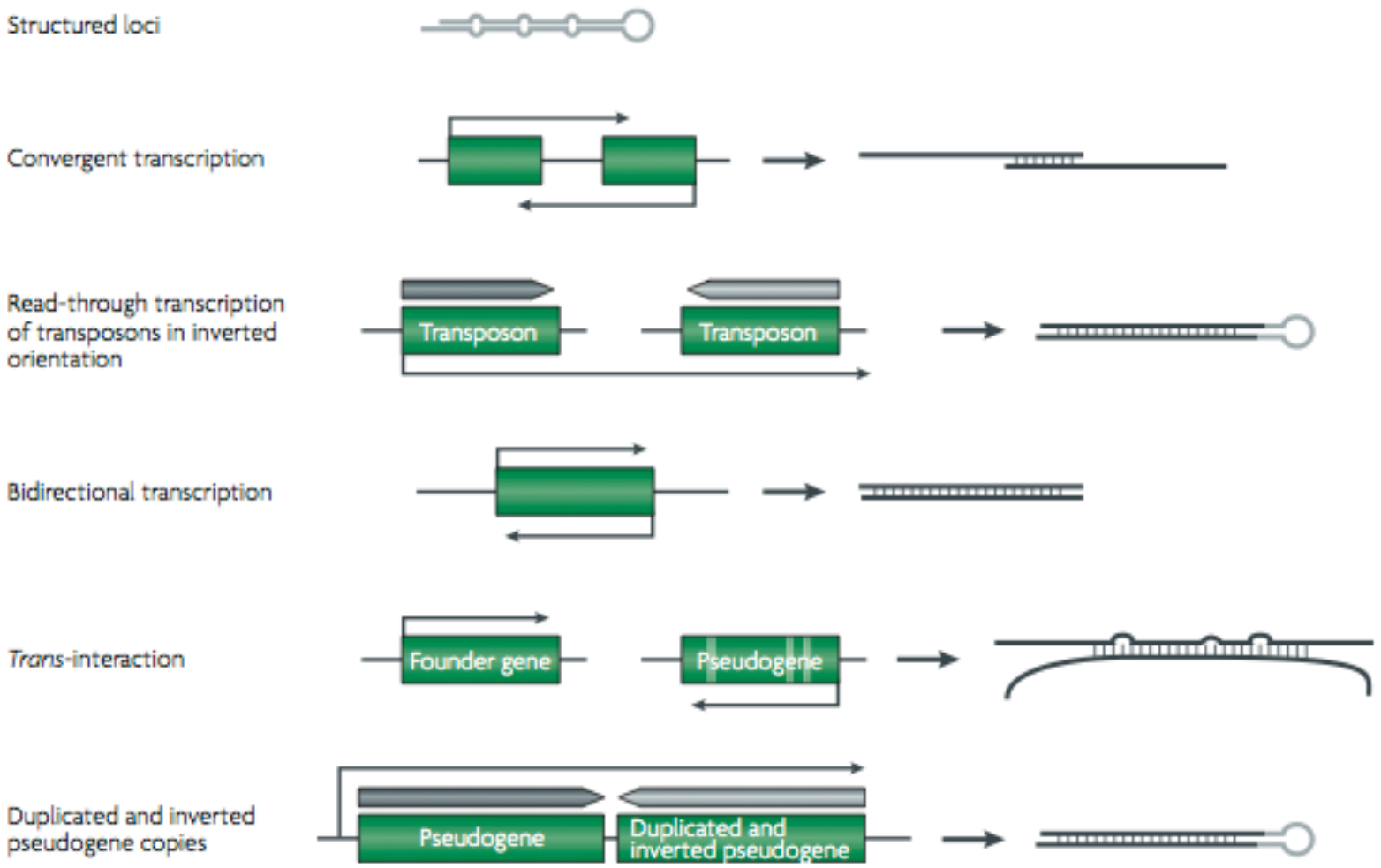
This co-transcriptional silencing by RNAi leads to the deposition of repressive cytosine methylation at loci transcribed by ***RNA Pol IV***. At least 3 of the 10 Argonautes found in *A. thaliana* are involved in RdDM, but AGO4 was the first to be identified. The initial templates for RNA Pol IV will be subject to RdDM (RDR2), which produces dsRNAs from transcripts. The dsRNAs are processed into 24 nt siRNAs (casiRNA) by DICER-LIKE 3 (DCL3) and exported into the cytoplasm, where they are loaded into an Argonaute complex. AGO4 is imported into the nucleus and guided to complementary ***RNA Pol V*** intergenic non-coding transcripts through siRNA-target base pairing. AGO4 recruitment is aided by direct protein-protein interactions. *De novo* cytosine methylation is catalysed by the DNA methyltransferase DRM2 at loci targeted by RdDM.



cis-acting siRNAs (casiRNAs) originate from transposons, repetitive elements and tandem repeats

Genomic sources of dsRNA triggers for endogenous small interfering RNAs (endo-siRNAs)

Endo-siRNAs con be produced independently from RdRP activity:



Germline nuclear RNAi in *A.thaliana*

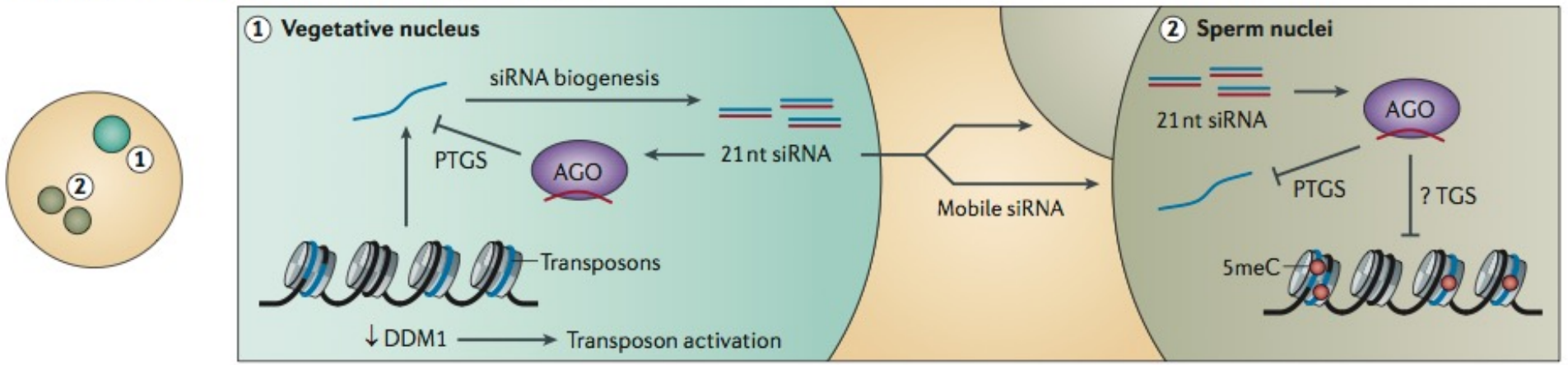
In plants, germline cells arise late in development from somatic stem cells (unlike in animals, in which the germ line is specified early in development), and so transposons must be extensively silenced throughout development.

In somatic cells, both the RdDM pathway and maintenance DNA methyltransferases keep transposons silent. However, siRNAs are produced in the **companion cells** of the germ line. These are the **vegetative nucleus** in the male germ line and the **central cell** in the female germ line. The central cell is fertilized to produce the endosperm that acts as a supportive tissue to the developing embryo.

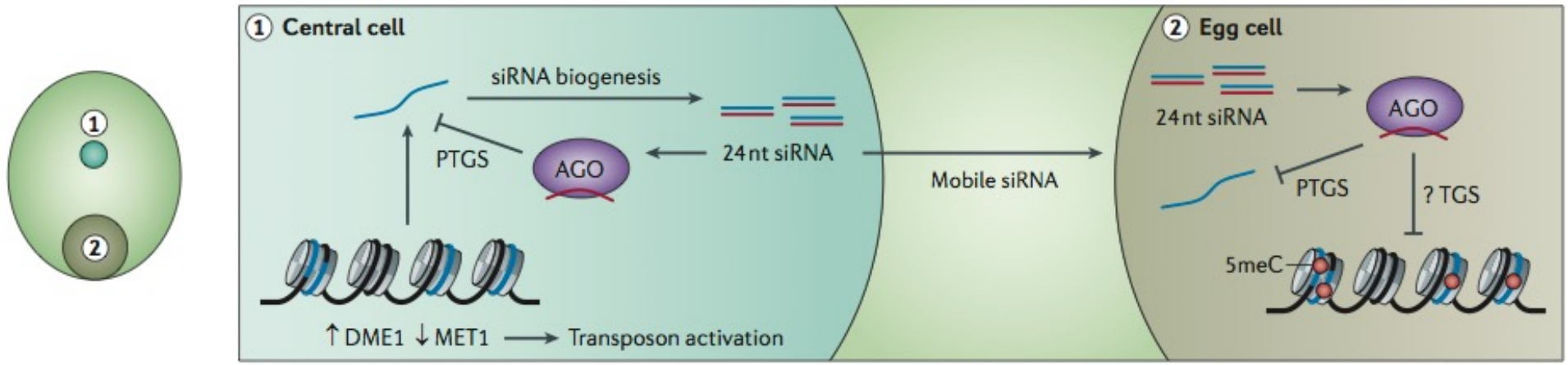
Germline nuclear RNAi in *A.thaliana*

Companion cells produce siRNAs that act non-cell-autonomously to silence retroelements in germline cells. Transposons are revealed in companion cells and are then used to generate small RNAs that enforce transposon silencing in the germ cells however, it is not known whether they can also direct TGS through nuclear RNAi

The heterochromatin remodeller DDM1 is a master regulator of transposons and is downregulated in the supportive vegetative nucleus, leading to transposon mobilization and to the production of 21 nt



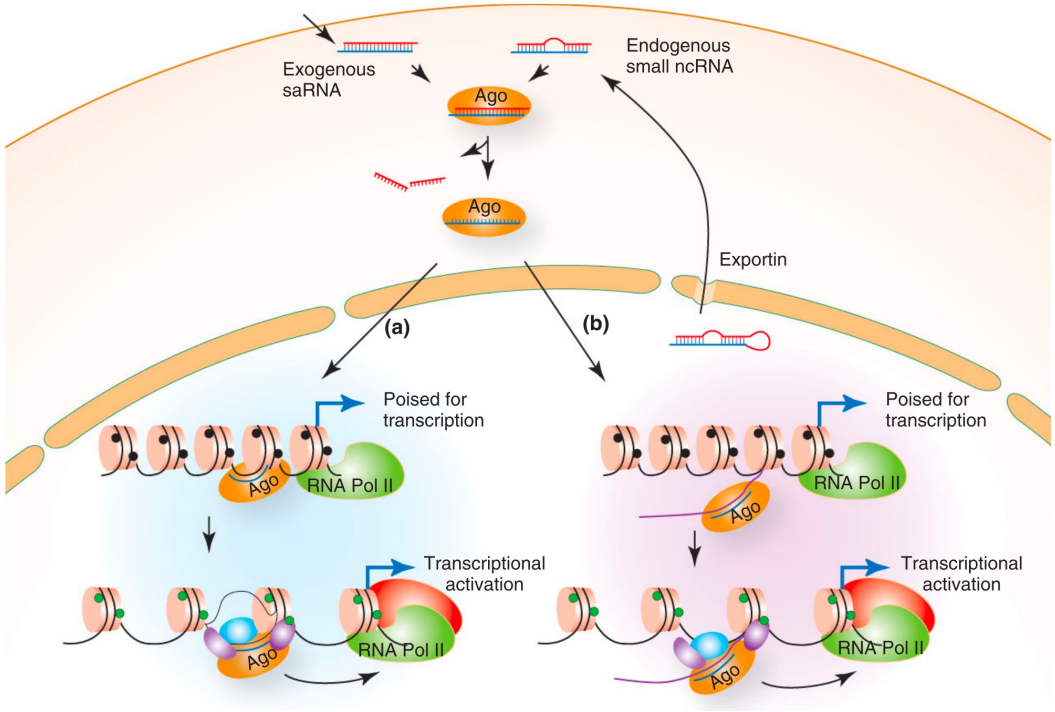
the maintenance DNA methyltransferase MET1 is downregulated, whereas the DNA glycosylase DEMETER is expressed



Small RNA and transcriptional upregulation

There is growing evidence that small RNAs can also serve as **activators** of gene expression by targeting gene regulatory sequences. This novel mechanism, known as **RNA activation** (**RNAa**), appears to be conserved in at least mammalian cells and triggered by artificially designed small RNAs. RNAa depends on Argonaute proteins, but possesses kinetics distinct from that of RNAi. Epigenetic changes are associated with RNAa and may contribute to transcriptional activation of target genes, but the underlying mechanism remains elusive.

It has been proposed that tha Ago-RNA complex may bind to (a) complementary DNA sequences or (b) nascent cognate transcripts in promoters or 3′ flanking regions and further recruit histone modifiers, leading to an open chromatin structure and active transcription.



saRNA = small activating RNAs

small activating RNAs in clinical trial

Since its discovery in mid 2000s, improvements of saRNA design, synthetic chemistry and understanding of the biology have matured the way to apply RNAa. Indeed, MiNA therapeutics Ltd has conducted the first RNAa clinical trial for advanced hepatocellular carcinoma (HCC) patients with promising outcomes. A second company, Ractigen, recently started preclinical trial with saRNAs for bladder cancer and SMA.

Drug	Company	Indication	Mechanism	Status
MTL-CEBPA plus sorafenib	MiNA	HCC	saRNA targeting *CEBPA*	Phase I/Ib
RAG-01	Ractigen	Bladder cancer	saRNA targeting p21 gene	Preclinical
RAG-06	Ractigen	SMA	saRNA targeting *SMN2*	Preclinical

PIWI-interacting RNAs (piRNAs)

PIWI-interacting RNA (piRNA)

- **PIWI-interacting RNAs (piRNAs)** are small, 21–30 nt single-stranded RNAs that associate with PIWI proteins in various organisms. It is their association with PIWI, but not AGO, proteins and their independence from Dicer that distinguishes piRNAs from siRNAs.

- **PIWI** proteins are a clade within the larger family of Argonaute proteins that is mostly specifically expressed in the germ line.

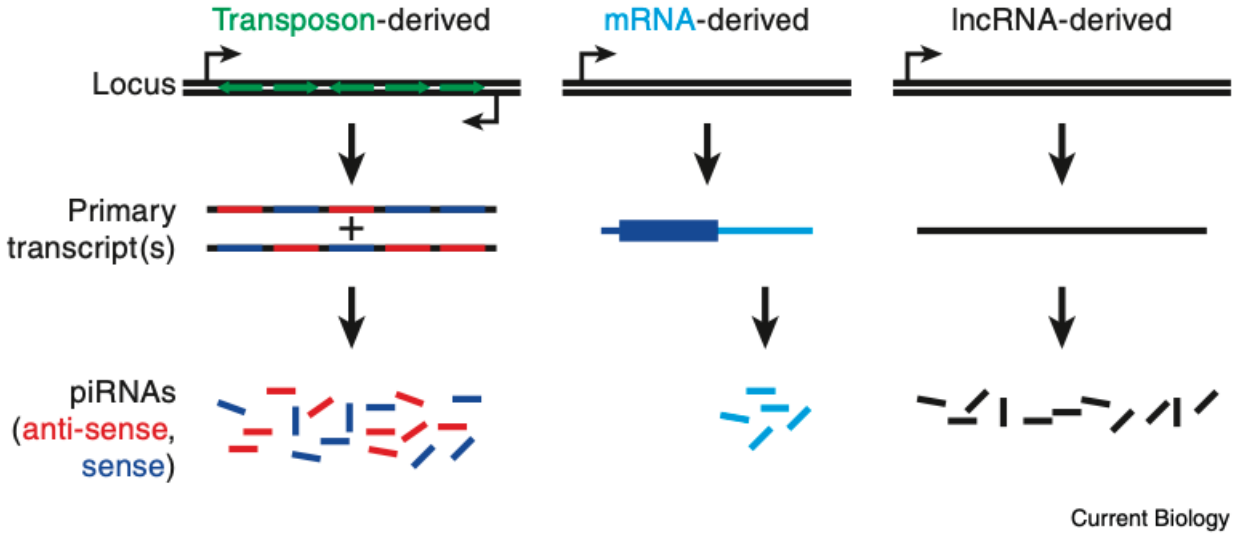
- In most animal species studied, PIWI proteins have been shown to repress 'non-self' sequences, such as transposable elements. However, non-transposon-related PIWI targets and piRNAs derived from regular mRNAs have been described as well.

- Some PIWI proteins act in the cytoplasm and may trigger degradation of mRNAs. However, some PIWI proteins also translocate to the nucleus on piRNA loading, where they can silence their targets at the transcriptional level.

- Some invertebrates use piRNAs to tackle viral infection

piRNA classification

piRNAs can originate from different sources, but piRNA function is only well understood for transposon-derived piRNAs.

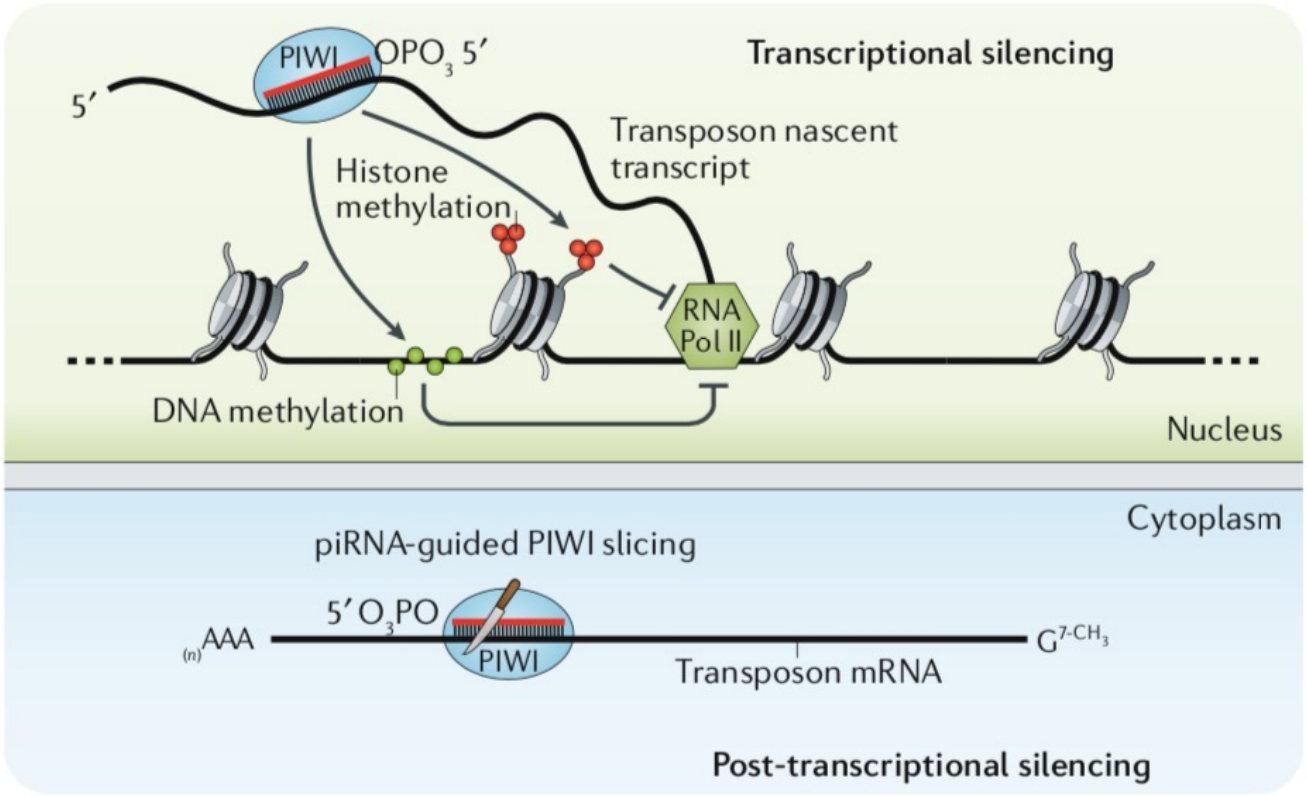


Several features of piRNAs in mice and humans have been reported.

1) are mainly present in germ cells;
2) are expressed in a regulated manner (PIWI protein has regulated expression);
3) map to the genome via clusters;
4) show a strong tendency for uridine (U) at the 5′ end;
5) have both sense and antisense directions;
6) are missing stem loop structures and have 5′-phosphate and 3′-OH groups;
8) biogenesis is independent from Dicer;
9) interact with PIWI proteins.

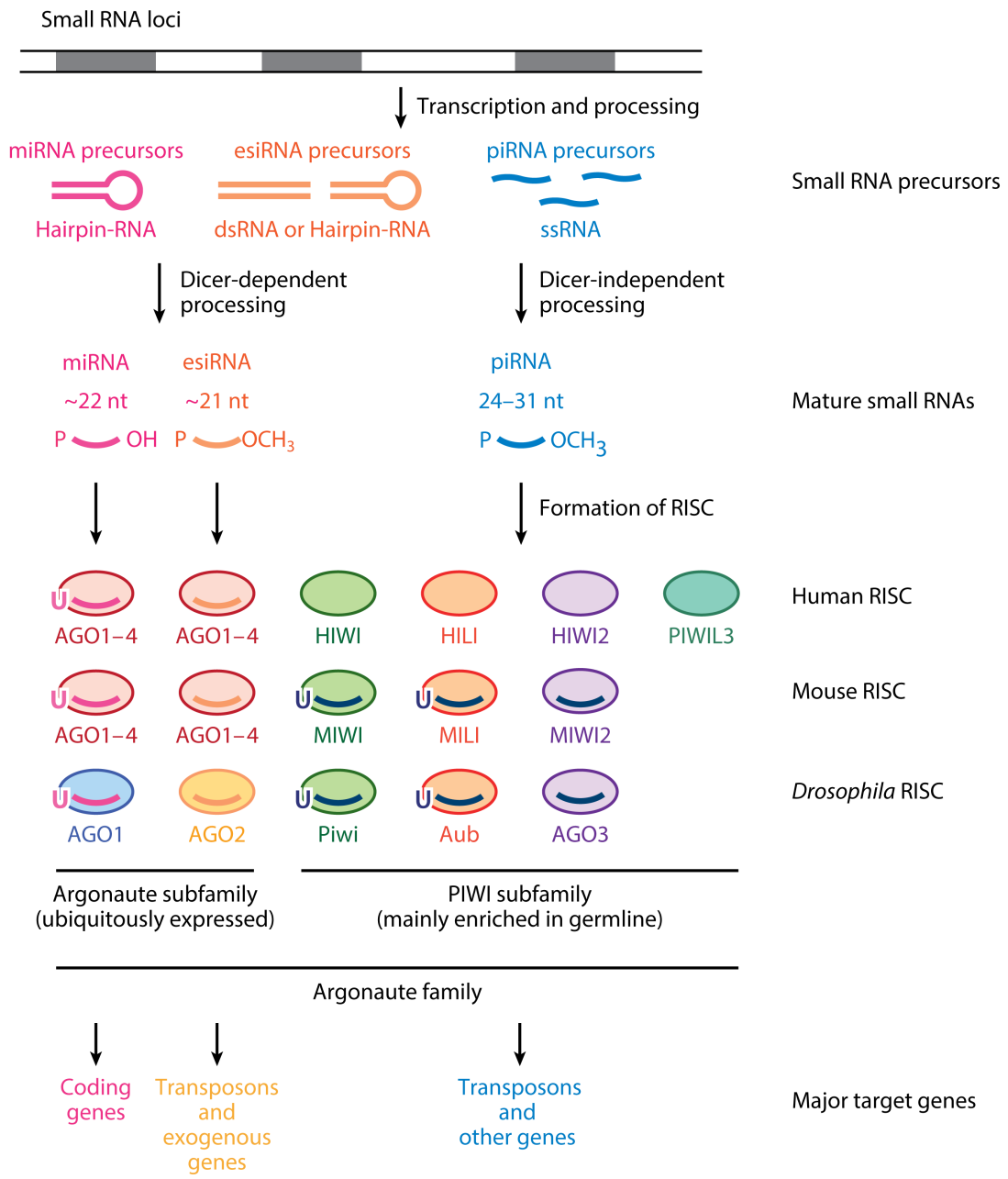
They are one of the most abundantly expressed small RNAs, with every human spermatid containing approximately 1 million piRNAs.

piRNAs silence transposons transcriptionally and post-transcriptionally



piRISC

PIWI subfamily proteins (PIWI proteins) are expressed mainly in germline cells and form specific RISCs with a small RNA population known as PIWI-interacting RNAs (piRNAs); these RISCs are termed piRISCs.



Iwasaki YW, et al. 2015.
Annu. Rev. Biochem. 84:405–33

Transcription of piRNA clusters

- piRNAs are produced from specific genomic loci termed ‘piRNA clusters’ that are transcribed by RNA Pol II as long non-coding transcripts before they are processed into piRNAs.

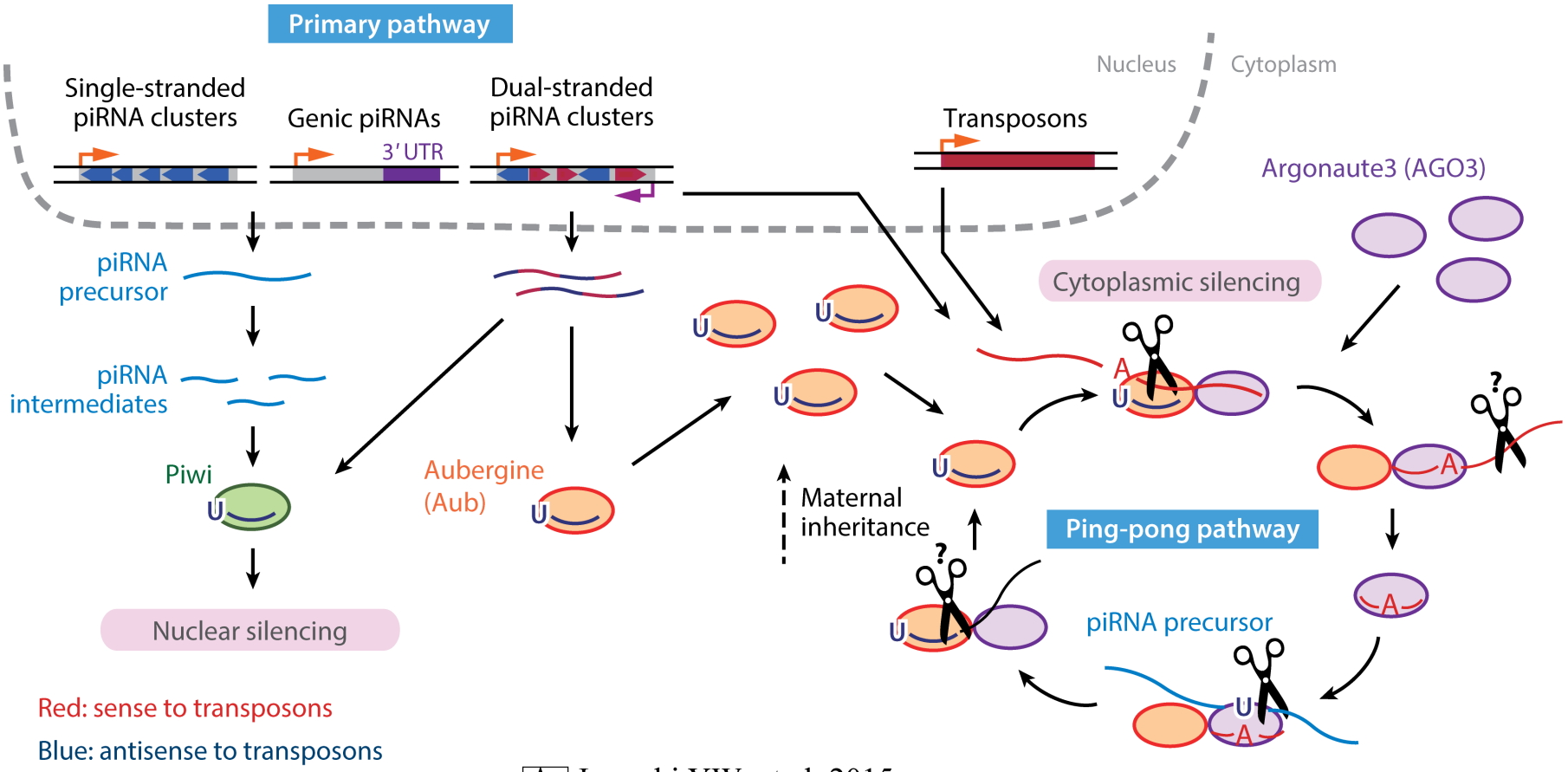
- Most piRNA clusters are bidirectional, lack clear promoter regions and carry H3K9 trimethylation marks that are generally associated with transcription repression.

- Most piRNA precursor transcripts lack classical mRNA features (that is, splicing and polyadenylation). They also use a specific variant of the nuclear export adaptor complex to reach the cytoplasm.

- piRNA precursor transcripts are transported to cytoplasmic perinuclear RNA granules called ‘*nuage*’, where they are processed into mature piRNAs.

Processing of piRNAs

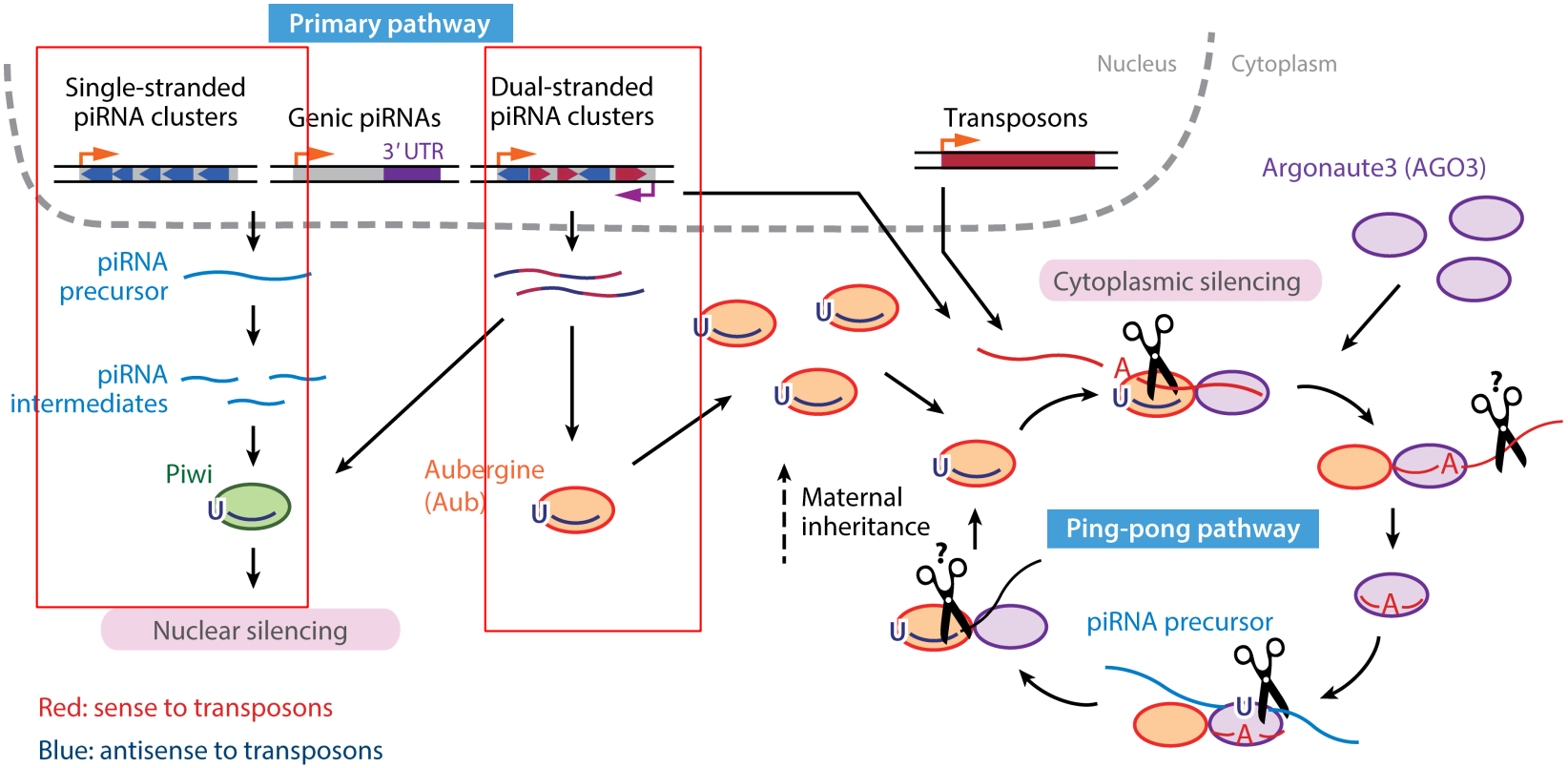
Two major pathways generate piRNAs: the **primary processing pathway** and the **ping-pong cycle** that amplifies secondary piRNAs. In *Drosophila* ovaries, the primary pathway operates in both germline and surrounding somatic cells, whereas the ping-pong cycle operates only in germline cells.



Iwasaki YW, et al. 2015.
Annu. Rev. Biochem. 84:405–33

Biogenesis of piRNAs

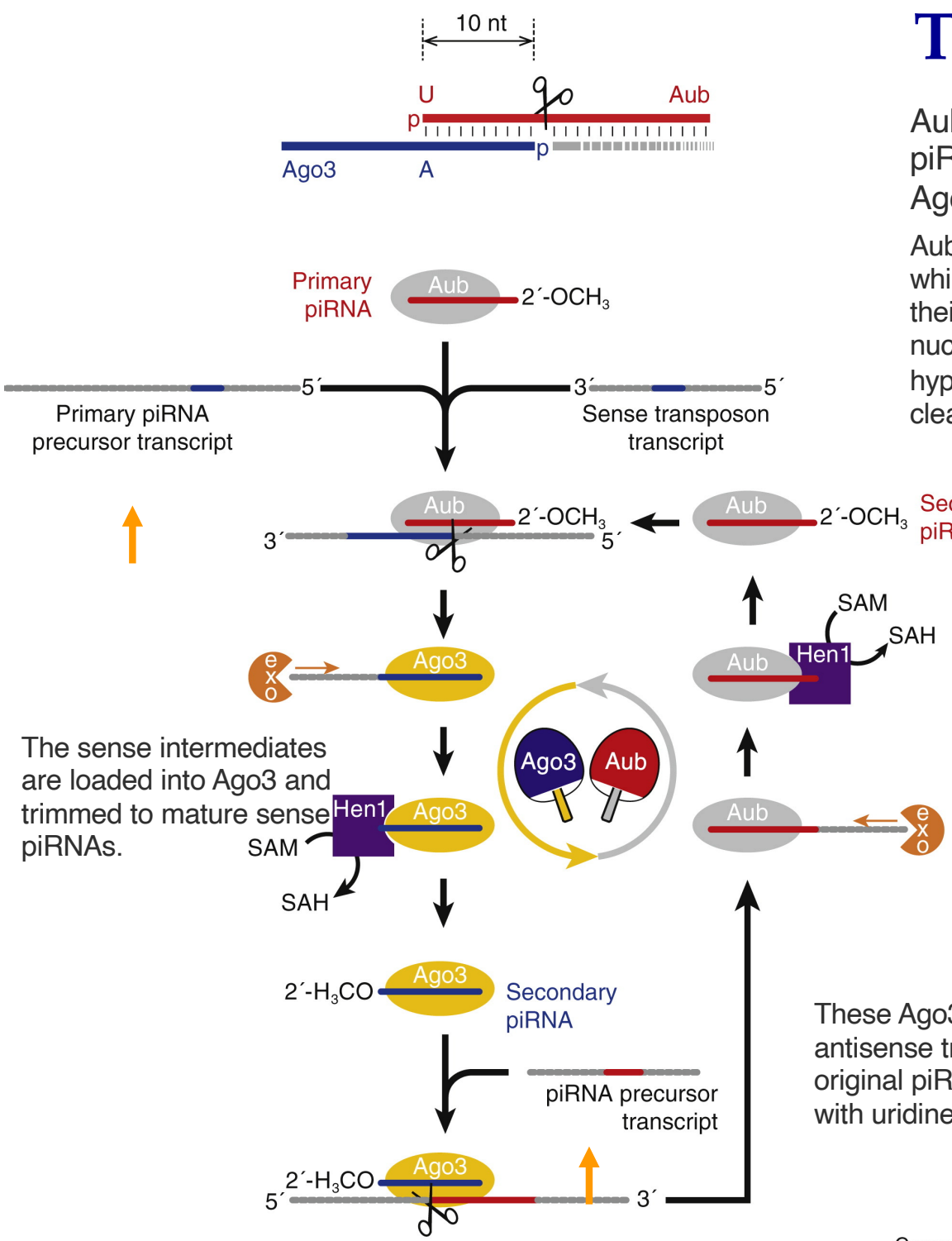
In the primary pathway, piRNAs are transcribed from genomic regions called piRNA clusters, processed, and loaded onto Piwi or Aub. Silencing takes place both in the cytoplasm and nucleus. Piwi performs transcriptional gene silencing in the nucleus. Together with AGO3, the Aub–piRNA complex serves as a trigger to start the ping-pong amplification pathway. The ping-pong pathway silences the target transposon sequence and amplifies the piRNA sequence at the same time. Note that some Aub–piRNA complexes are also maternally inherited.



The ping-pong cycle

Aub and Piwi seem to preferentially bind piRNAs in the antisense orientation while Ago3 tends to associate with sense piRNAs

Aub-bound antisense piRNAs typically start with a 5′ uridine while Ago3-bound sense piRNAs often have adenosine as their tenth nucleotide. Complementarity between the first ten nucleotides of Aub- and Ago3-bound piRNAs led to the hypothesis that sense piRNAs are generated by target cleavage directed by antisense piRNAs, and vice versa



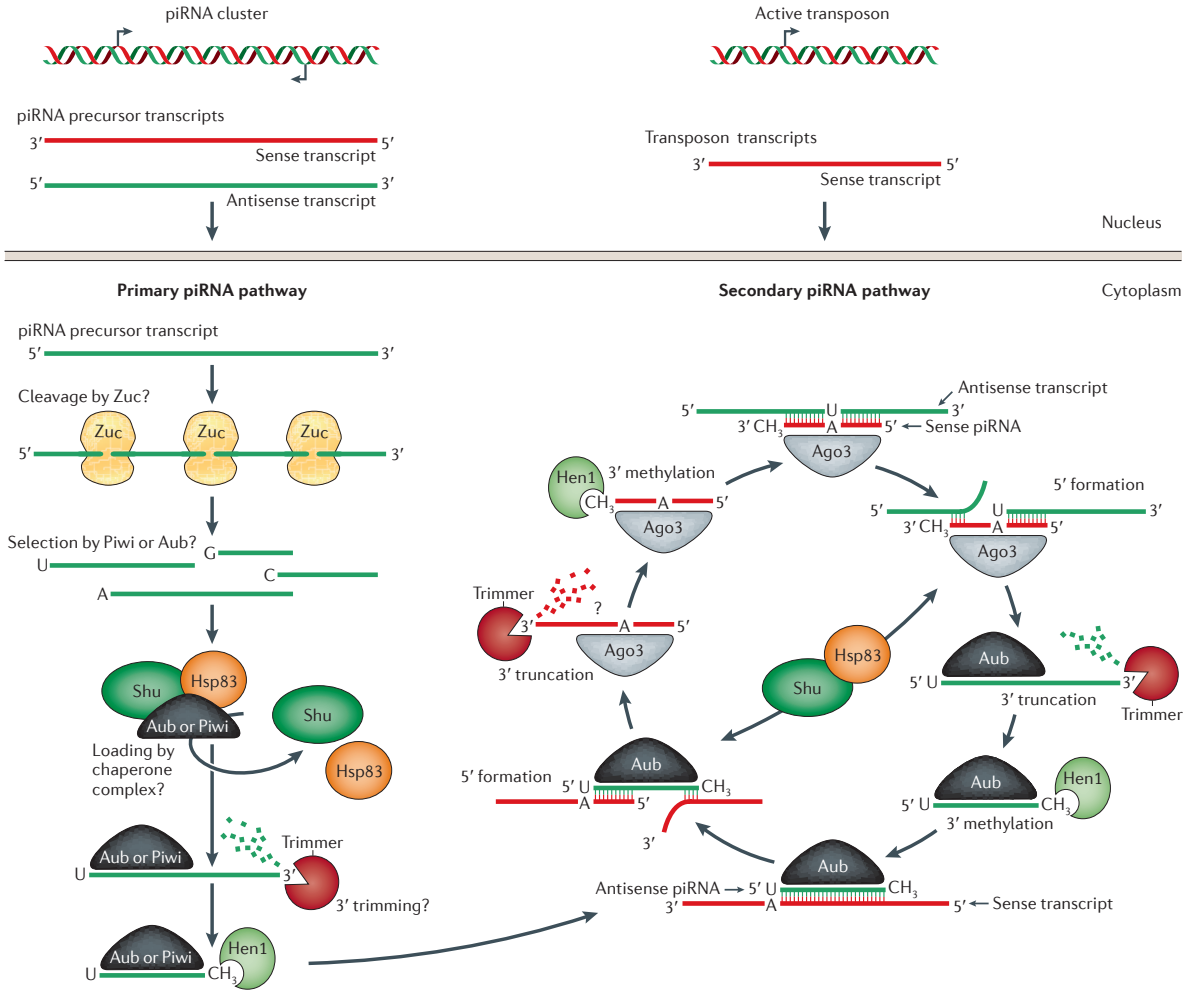
Aub-bound, antisense piRNAs initiate the Ping-Pong cycle by cleaving transposon mRNA transcripts and generating sense-oriented piRNA intermediates.

The sense intermediates are loaded into Ago3 and trimmed to mature sense piRNAs.

These Ago3-bound, sense piRNAs can then bind and cleave the antisense transposon sequences present in the transcripts of the original piRNA cluster, producing piRNA intermediates that begin with uridines

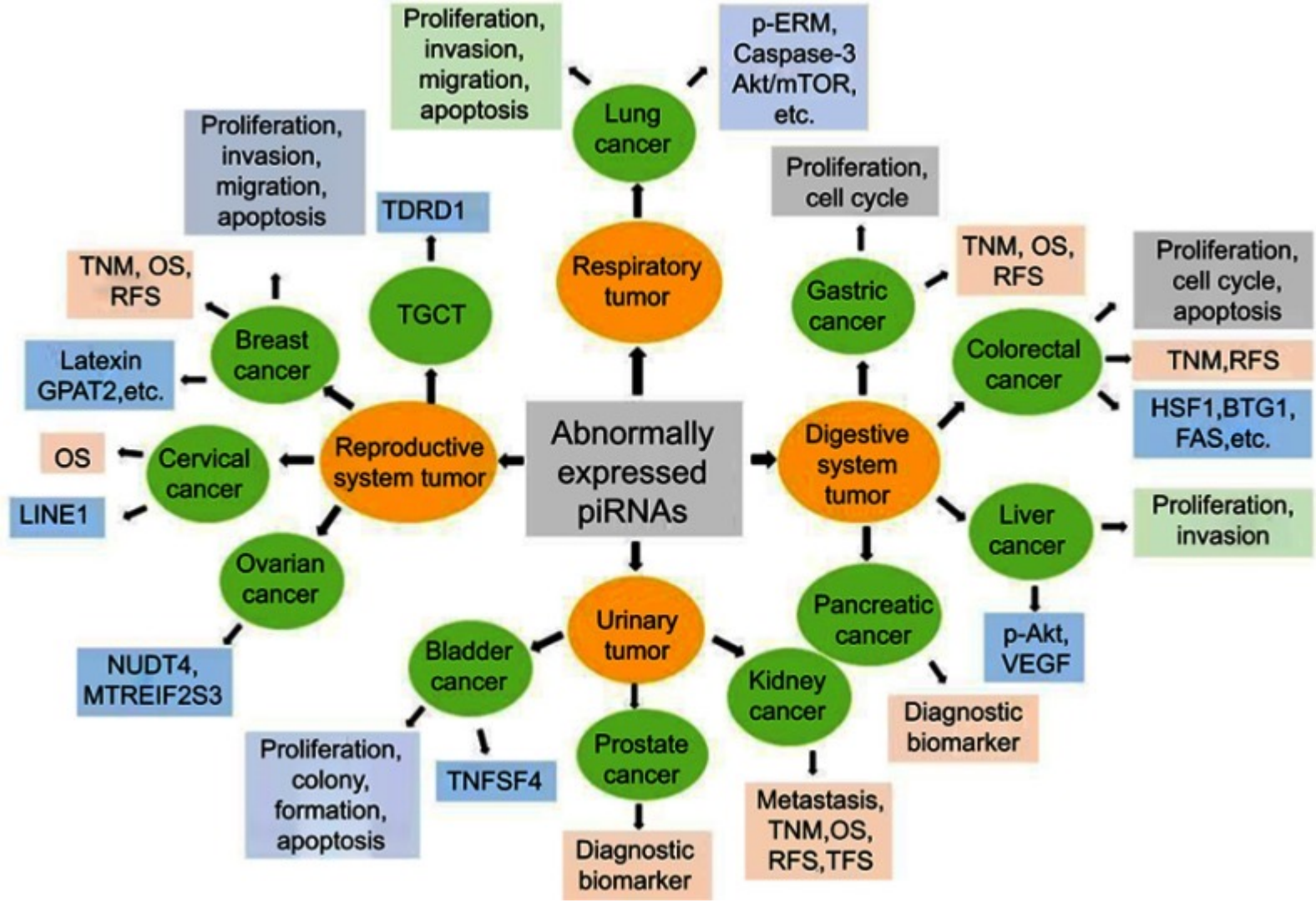
Piwi pathways of Drosophila melanogaster

In the primary pathway, the precursors are cleaved by an endonuclease, Zucchini (Zuc). Next, cleaved fragments are incorporated into Piwi or Aubergine (Aub) with the help of Shutdown (Shu) and Heat shock protein 83 (Hsp83). Fragments with a 5′U may be heavily selected for at this step. After loading into the Piwi protein, the Trimmer enzyme trims the 3′ end to fit the Piwi protein, after which Hen1 methylates the mature 3′ end. This completes primary biogenesis. Aub, but not Piwi, can then enter the secondary pathway. Aub can recognize a cognate transcript and cleave it. The 3′ cleavage fragment of the targeted RNA can then be taken up by another Piwi protein named Argonaute 3 (Ago3), again with the help of Shu69. Further downstream, steps are probably identical to primary biogenesis. In turn, Ago3 may assist in loading more Aub protein with secondary piRNAs.



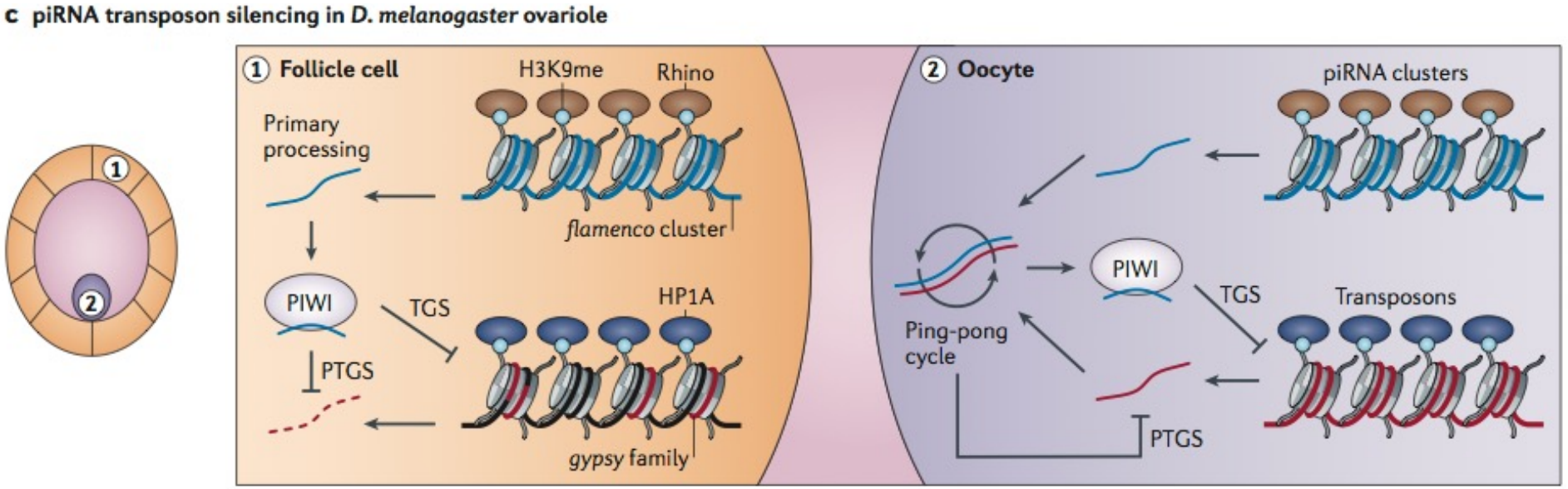
piRNAs in cancer

A study showed that piRNA expression differs significantly across human somatic tissues. Variable degrees of heterogeneity in expression patterns are observed in different kinds of tissues. It has been reported that aberrant expression of piRNAs is a potential cancer-specific signature and can be correlated with clinical features in malignant tissues, indicating an important role for piRNAs in several types of cancers.



Epigenetic silencing by piRNA in *Drosophila*

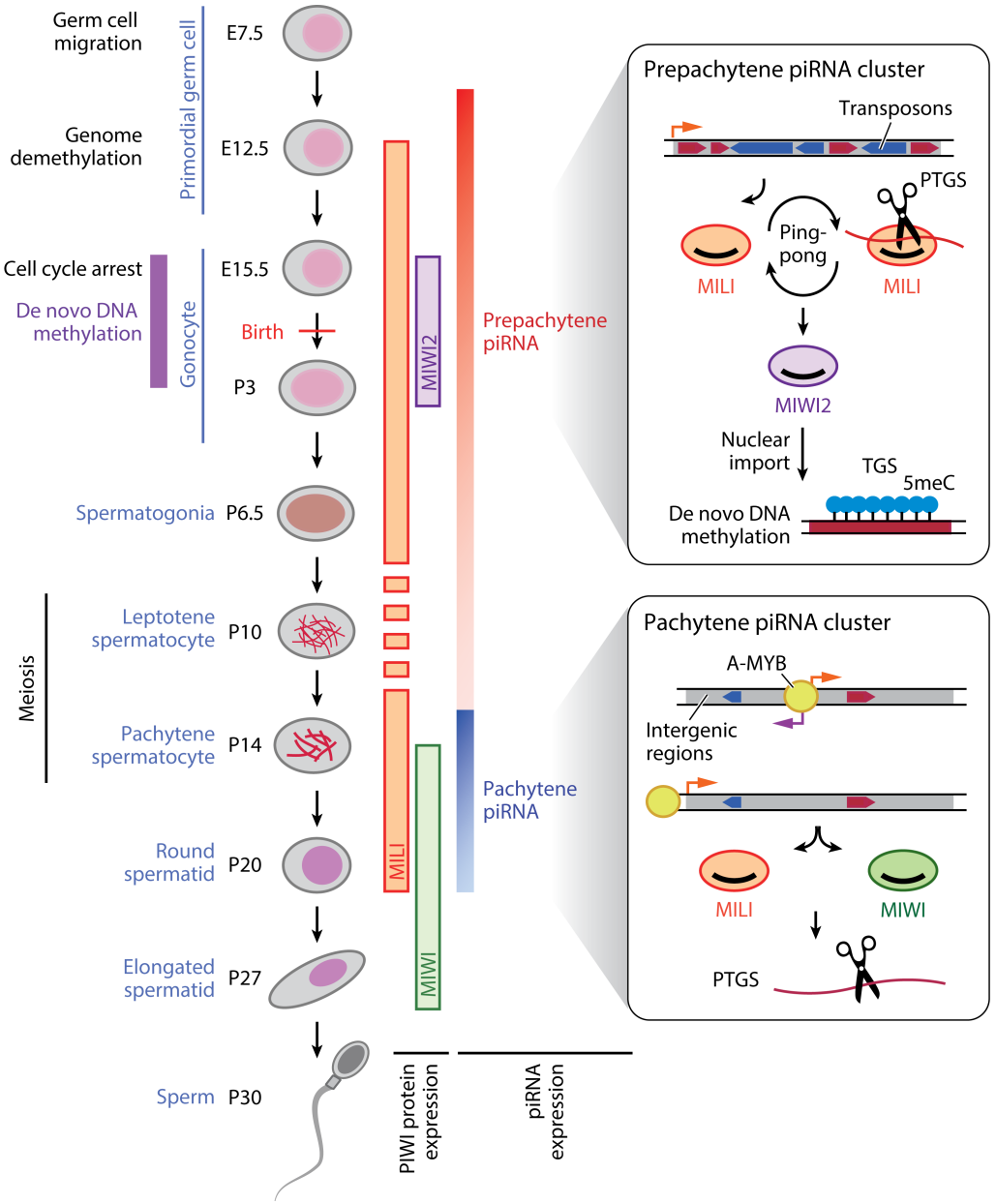
In the *D. melanogaster* ovariole, the flamenco cluster is expressed in somatic follicle cells and generates piRNAs independently of the ping-pong cycle. Loaded PIWI silences the gypsy family of retrotransposons. In oocytes and surrounding nurse cells, all piRNA clusters are expressed, and the primary transcripts enter the ping-pong cycle to produce piRNA. Active transposons are post-transcriptionally silenced, and nuclear PIWI promotes transcriptional silencing by H3K9me and HP1A localization.



The HP1A homologue **Rhino** binds to heterochromatic piRNA clusters in place of HP1A and promotes transcription.

The mouse piRNA pathway

piRNAs are classified into **pachytene piRNAs** and **prepachytene piRNAs**. In gonocytes, **MILI** and **MIWI2** bind to piRNAs from a prepachytene piRNA cluster, which consists mainly of transposons. MILI performs the ping-pong cycle to silence targets by PTGS and produce piRNAs that associate with MIWI2. MIWI2 localizes to the nucleus upon piRNA loading to accomplish nuclear silencing by de novo DNA methylation. Beyond the pachytene stage, MIWI and MILI are bound to piRNAs from pachytene piRNA clusters, a large fraction of which consists of intergenic regions. Pachytene piRNAs regulate their target genes by PTGS in the cytoplasm.



*Only MILI is expressed in female germ cells