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Part 1: The PIWI-piRNA Pathway Is an Immune-Like Surveillance Process That Controls Genome Integrity by Silencing Transposable Elements

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Abstract

PiRNAs [P-element-induced wimpy testis (PIWI)-interacting RNAs] represent the most frequent but the least well-investigated subtype of small ncRNAs and are characterized by their interaction with PIWI proteins, a subclass of the Argonaute family. PiRNAs and PIWI proteins maintain integrity of the genomic structure and regulate gene expression in germline and somatic cells. The PIWI-piRNA pathway primarily constitutes a conserved immune-like surveillance process that recognizes self and nonself. This axis controls genome integrity of germline cells and nonaging somatic cells by silencing and suppressing propagation of transposable elements through epigenetic and posttranscriptional mechanisms. However, mounting evidences indicate that the PIWI-piRNA pathway has broader implications in both germinal and somatic cells in various physiological and pathological processes. It modulates mRNAs levels of expression, stability, turnover, and translation and interacts directly with many transcription factors and signaling pathways molecules. PIWI proteins and piRNAs play pivotal roles in germline stem cell maintenance and self-renewal, fertilization and development, genes and proteins expression, genome rearrangement, and homeostasis.

Keywords: piRNA, PIWI proteins, transposable element (TE), transcriptional and posttranscriptional silencing, piRNA cluster, heterochromatin, DNA methylation, ping-pong cycle, nuage

1. Introduction

Cancer is an extremely complex disorder characterized genetically, epigenetically, and histologically by highly heterogeneous proliferative cellular subpopulations, including cancer

stem cells (CSCs) and progenies. These cells harbor chromosomal abnormalities, alterations of suppressor genes (TSG) and oncogenes, and aberrant transcriptomic profiles generated by genetic and epigenetic alterations [1, 2]. These cancer cells are in close relationship with a tumor microenvironment (TME), composed of immune and nonimmune stromal cells and modified extracellular matrix. Reciprocal interactions between tumor cells and TME are pivotal in cancer progression, allowing remodeling of TME and reprogramming of cancer cells that develop adaptive strategies to adjust their phenotype to unfavorable environmental conditions. Recently, CSCs were implicated in a new paradigm accounting for tumor heterogeneity [3]. CSCs have the property of self-renewal, lack senescence, maintain an undifferentiated state, and proliferate rapidly. These properties are controlled by epigenetic mechanisms that induce changes in gene expression profiling of tumor cells. Opposite to aging cells that increase genomic and chromosomal instability during adulthood, nonaging immortal cells, such as germline, somatic, and cancer stem cells, harbor a genomic instability triggered by unrepaired mutations with either no or only limited number of genomic alterations [4]. Epigenetic abnormalities are early events in cancer progression, resulting from various environmental injuries, and associate heterogeneity of DNA methylation, posttranscriptional modifications of histones, and deregulation of noncoding RNAs (ncRNAs). Global DNA hypomethylation results in chromosomal instability, overexpression of oncogenes, and reactivation of transposable elements (TEs) [5]. Localized (genes promoters) or wide (>1 Mb) DNA hypermethylation initiates repression of TSGs and modification of epigenetic marks through histone alterations, resulting in occurrence of an aberrantly stemlike state of CSCs. These alterations of the genomic methylation during carcinogenesis allow reprogramming of atypical proliferative cells into highly malignant cells characterized by unlimited proliferation, epithelial-mesenchymal transition (EMT), invasion, and prometastatic properties [6].

Until recently, RNAs were considered as epigenetic regulators and mediators of gene expression, functioning as intermediates of translation in the flow of genetic information from DNA to proteins [7]. Large-scale genomic technologies have provided an astonishing insight into human genome and transcriptome. Next-generation sequencing techniques combined with bioinformatics have revealed that more than 50% of mammalian genomes were composed of TEs and that more than 98% of the human genome was actively transcribed [8]. However, only 1.1% of the genome encodes proteins, and a majority of genes are noncoding RNAs (ncRNAs) [9]. NcRNAs play pivotal roles in developmental and homeostatic processes, and their alterations are implicated in the pathogenesis of many diseases, by modulating expression of numerous genes at epigenetic, transcriptional, and posttranscriptional levels [10]. Most importantly, ncRNAs are frequently deregulated in cancer and have crucial roles in tumor initiation, progression, and metastatic spread. NcRNAs are classified into housekeeper ncRNAs (rRNAs, tRNAs, and snoRNAs) and regulatory ncRNAs. Regulatory ncRNAs are divided into several subfamilies, depending on their size, biogenesis, and biological functions. Small ncRNAs are composed of transcripts shorter than 200 nucleotides (nt), whereas long noncoding RNAs (lncRNAs) comprise transcripts longer than 200 nt [11]. Small ncRNAs also differ by their precursor structure and their mechanisms of biogenesis. They comprise microRNAs (miRNAs), small interfering RNAs (siRNAs), and PIWI-interacting RNA (piRNAs) [12–15].

MiRNAs and siRNAs are generated from double-stranded precursors, whereas piRNAs are processed from long single-stranded precursors. The endoribonuclease Dicer is pivotal in the maturation of miRNAs and siRNAs, but not in the piRNAs processing [16]. Regulatory

functions of small ncRNAs are insured by Argonaute (AGO) protein family, which is a very well-conserved master component of RNA silencing complexes in all organisms [17]. The least well-investigated small ncRNAs are the piRNAs, which were first identified in 2006 in mouse and rat germ cells as ncRNAs interacting with PIWI proteins, a subclass of the Argonaute proteins [18–21]. PiRNAs actually constitute the largest and most diverse class of ncRNAs [16]. PiRNAs and PIWI proteins were initially implicated in epigenetic regulation of germline cells and their overexpressions have been more recently observed in various cancers through aberrant DNA methylation.

This review will provide an overview of the PIWI-piRNA pathway, focusing mainly on origin, biochemical properties, biogenesis, functions, and mechanisms of action in germline and somatic tissues. Furthermore, we will discuss emerging implications of piRNAs in carcinogenesis and highlight their potential clinical utilities as diagnostic/prognostic biomarkers and therapeutic tools.

2. The PIWI-piRNA pathway

RNA interference (RNAi) is a widely conserved small-RNA-mediated gene-silencing mechanism involved in crucial homeostatic events of most eukaryotes [22–24]. Small regulatory RNAs of 20–32 nt, such as endogenous siRNAs, miRNAs, and piRNAs, modulate transcriptional and posttranscriptional repression through complementary RNA or DNA recognition by interacting with well-conserved proteins of 95 kDa belonging to the Argonaute family that cleave their targets [25, 27]. AGO proteins include a PAZ domain [P-element-induced wimpy testis (PIWI)-AGO-Zwille] located in the N terminal region, which binds small ncRNAs and a PIWI domain in the C-terminal region that functions as double-strand-specific RNA endonuclease [28]. Based on sequence homology and functional domains in different species, the AGO family of proteins is divided into three subfamilies: AGO proteins (homologous to *Arabidopsis thaliana* AGO1), PIWI proteins (homologous to *Drosophila melanogaster* Piwi), and WAGO (worm-specific Argonaute clade). The number of AGO family proteins varies considerably between species. Eight different proteins were present in humans, whereas 27 proteins were identified in *C. elegans* and only one protein was observed in fission yeast [29]. AGO proteins interact functionally with siRNAs and miRNAs, which are small single-stranded RNAs of 20–22 nt in length processed in a Dicer-dependent manner from double-stranded precursors, to induce posttranscriptional gene silencing in the cytoplasm [30–32]. Conversely, PIWI proteins are implicated in biogenesis of piRNAs and in their main function through transcriptionally and posttranscriptionally repressing TEs in the nucleus and the cytoplasm [33]. PIWI proteins and piRNAs edify ribonucleoproteins named PiRNA-induced silencing complexes (pi-RISCs). Pi-RISCs specificity is determined by piRNA sequence, whereas Argonaute PIWI protein mediates its effector function. PiRNAs associate with PIWI proteins and guide piRISCs to recognize complementary targets and achieve RNA silencing at transcriptional and posttranscriptional levels (**Figure 1**). Cytoplasmic PIWI-piRNA complexes silence their targets posttranscriptionally *via* piRNA-directed cleavage and the “ping-pong” amplification cycle, whereas nuclear PIWI proteins and piRNAs silence gene transcriptionally through epigenetic changes, including DNA methylation, implementation of H3K9me3 repressive marks, interactions with Mael and HP1 proteins, and repression of Pol II.

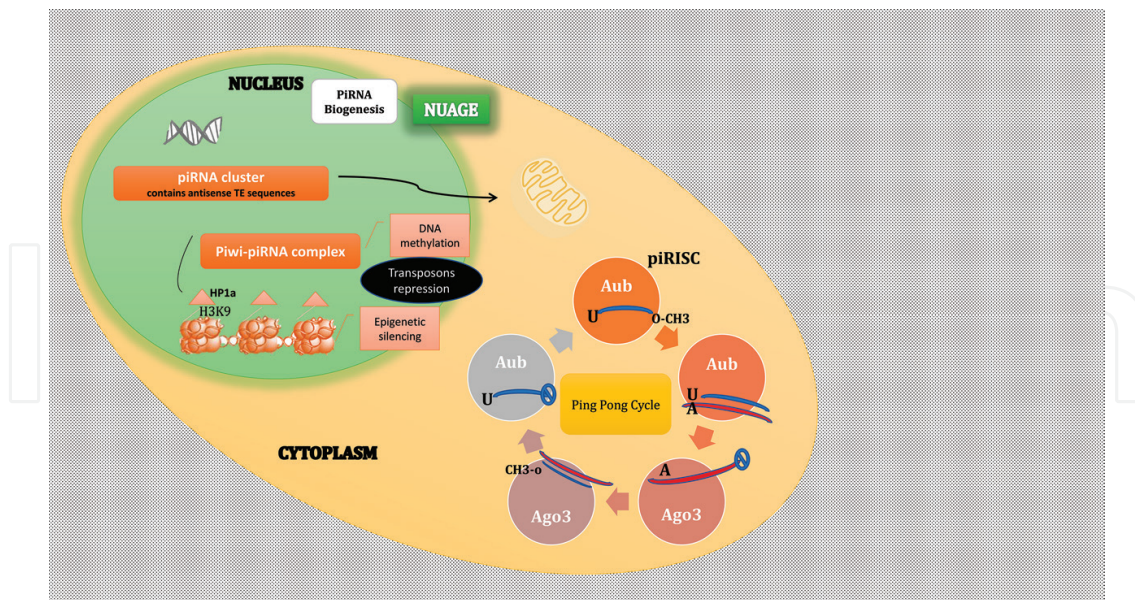


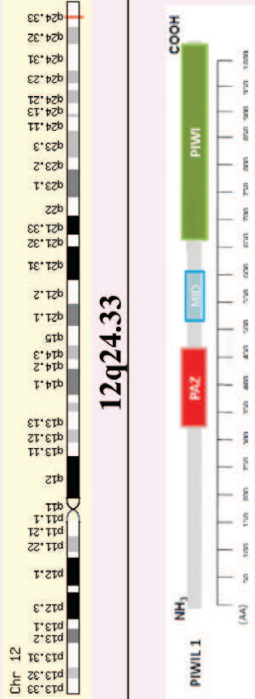
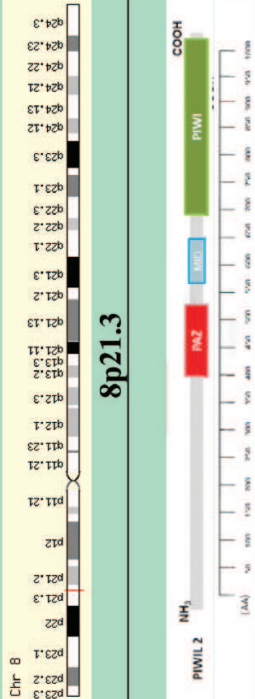
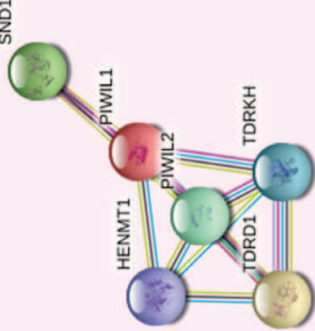
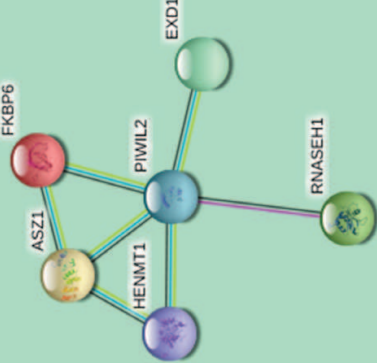
Figure 1. Biogenesis of piRNA and PIWI-piRNA pathways and their function in maintaining genome integrity through transposable element (TE) in germline cells at transcriptional and posttranscriptional level. In *Drosophila* ovaries, the primary pathway (in the nucleus) operates in both germline and surrounding somatic cells, whereas the “ping-pong” cycle (in the cytoplasm) operates only in germline cells. In the nucleus, PIWI-piRNA complex can regulate HP1, H3K9 methylation, and DNA methylation to influence transposons.

PiRNAs are small single-stranded ncRNAs of 25–33 nt identified in various organisms ranging from sponges to higher vertebrates [34]. Experimental and bioinformatics studies have shown that piRNAs are the most abundant small ncRNAs expressed in mammalian species [35]. They are derived from long single-strand RNA precursors in a Dicer-independent manner. The human genome comprises more than 30,000 piRNAs in which 80% originate from intergenic sequences and 20% from introns and exons of pre-mRNAs [36]. Unlike miRNAs and endosRNAs, production of piRNAs is not carried out in a precise manner and single strands of long primary precursor transcripts generate numerous piRNAs without a conserved sequence [37]. They comprise Uracil at their 5' end and methylated 2'-O group at their 3' end [38, 39]. PiRNAs were first identified in *Drosophila melanogaster* and were named repeat-associated small-interfering RNAs (rasiRNAs) because of their repetitive elements and TEs suppressing activity. RasiRNAs were later found to interact with Argonaute PIWI proteins and ultimately renamed piRNAs in 2006 [40–42]. PiRNAs were further investigated in *Caenorhabditis elegans*, zebrafish, mice, and more recently in humans. Their vast number present in numerous locations of the genome suggests that piRNAs may have potential crucial implications in the control of major biological processes. Indeed, PIWI-piRNA complexes silence TEs and control expression and activity of genes and proteins. They are also instrumental in genome rearrangement, germ stem cell maintenance, reproduction and fertility regulation, embryogenesis, and homeostasis [26, 43–48]. At the opposite of miRNAs and endosRNAs, piRNAs function only through binding with PIWI proteins and harbor tissue-specific expression in various organs such as prostate and thyroid [49].

PIWI proteins were also initially identified in *Drosophila melanogaster* in which they play crucial roles in germline stem cell maintenance and self-renewal [50]. These proteins contain

three functional domains: the PIWI-Argonaute-Zwille (PAZ) domain recognizing the 3' end of the RNA, the middle domain (MID) providing a binding pocket for the 5' end of guide strand RNA, and the PIWI domain containing catalytic residues that cleave target transcripts [47]. Unlike proteins of the AGO subclass, PIWI proteins comprise posttranslationally dimethylated arginine-rich motifs that allow interactions with Tudor proteins. These last proteins have pivotal role in functional activities of PIWI proteins by providing a scaffold for edification of higher-order molecular complexes located in *Drosophila* germ cells and mouse testis perinuclear granules named "nuage," similar to P-bodies [51–53]. The PIWI protein family is conserved in numerous organisms, including jellyfish, sponge, *planaria*, zebrafish (Ziwi, Zili), *Caenorhabditis elegans* (Prg1, Prg 2), *Drosophila melanogaster* (Piwi, Aub, Ago3), mouse (MIWI, MILI, MIWI2), and human (PIWIL1, PIWIL2, PIWIL3, PIWIL4) [51] (Table 1). PIWIL3 is observed only in human, and its functions are actually largely unknown. PIWI proteins expression is identified in a majority of organs such as liver, lung, heart, brain, pancreas, and kidney [54, 55]. PIWI proteins and piRNAs were first implicated in development, differentiation, and maintenance of germline cells [18, 56, 57]. However, mounting evidence has revealed that the PIWI-piRNA pathway is also instrumental in controlling gene expression both in germinal and somatic cells [58].

This pathway has pivotal roles at all steps of oogenesis and spermatogenesis, but also in somatic cells such as ovary and testis of *Drosophila* [15]. This axis also controls, although at lower levels of expression, numerous biological processes implicated in homeostasis, including brain maturation [59] pancreatic function [55], fat metabolism [60], and regeneration [61]. Indeed, this pathway was initially studied in gonads and implicated in gene silencing of germinal cells [43]. Loss of function studies performed in zebrafish, *Drosophila*, *Caenorhabditis*, and mice have confirmed that the PIWI-piRNA pathway is involved in germline development, spermatogenesis, and maintenance of germline stem cells. Mutations in this pathway resulted in expansive TEs mobility, genomic instability, and sterility [44]. PIWI proteins have nonredundant functions in cell compartments. *Drosophila* PIWI proteins Aub and Ago3 cleaved TEs in the cytoplasm, whereas Piwi inactivated TEs in the nucleus [62, 63]. All mouse PIWI proteins MIWI, MILI, and MIWI2 were expressed during spermatogenesis, whereas only MILI was weakly expressed in female germinal cells [64, 65]. These mouse PIWI proteins not only silenced TEs posttranscriptionally but also inactivated TEs genes transcriptionally through CpG DNA methylation on TEs loci. Homozygous MIWI, MILI, and MIWI2 knockout male mice models were associated with propagation of LINE1 sequences, depleted spermatogenesis, and apoptosis of germinal cells [66]. Particularly, *Drosophila* PIWI mutants were correlated with derepression of TEs, absence of germline stem cell renewal, and depletion of gametes [67–69]. Actually, the main function of this pathway is maintaining germline and somatic genome integrity by silencing TEs at transcriptional and posttranscriptional levels [70]. However, only 20% of piRNAs are localized in TEs and other repeat genomic regions, suggesting that this pathway may have additional biological functions. In germinal cells, the PIWI-piRNA pathway prevents genomic instability of the next generation and sterility. In somatic nonaging cells, this pathway is pivotal in self-renewal, differentiation and maturation of stem cell, embryonic development, and whole body regeneration. In somatic tissues, the PIWI-piRNA pathway is implicated in chromosomal conformation, memory-related synaptic plasticity, transcriptional regulation of mRNAs with deadenylation, and transgenerational inheritance to preserve the memory of self and nonself [71–73].

	<p style="text-align: center;">PIWIL1</p>  <p style="text-align: center;">Chr: 12 12q24.33</p>	<p style="text-align: center;">PIWIL2</p>  <p style="text-align: center;">Chr: 8 8p21.3</p>
<p>Chromosome NCBI</p>	<p>Molecule *Bamezai <i>et al</i>, 2012</p>	<p>8p21.3</p>
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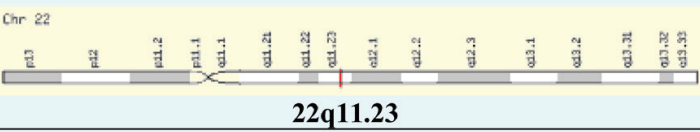
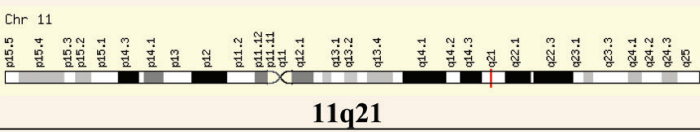

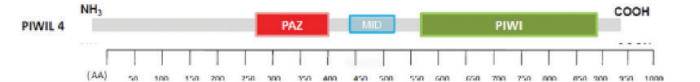
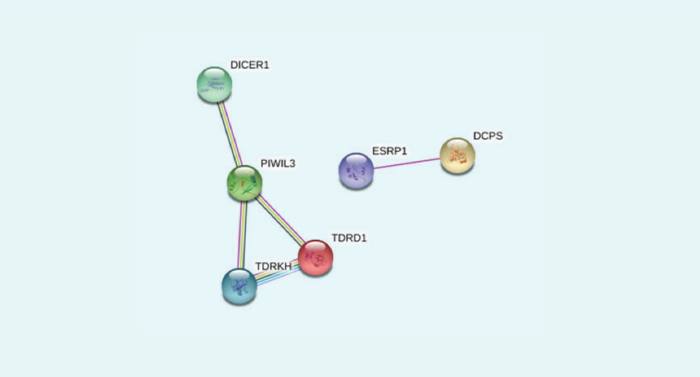
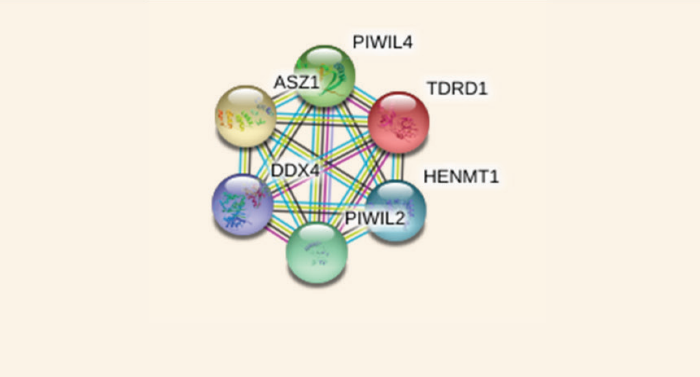
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Chromosome NCBI	 <p>Chr 22 22q11.23</p>	 <p>Chr 11 11q21</p>
Molecule Bamezai et al, 2012	 <p>PIWIL 3 NH₂ PAZ MID PIWI COOH (AA)</p>	 <p>PIWIL 4 NH₂ PAZ MID PIWI COOH (AA)</p>
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Table 1. This table shows the structure and the network of human PIWI proteins. The human PIWI protein family includes PIWIL1, PIWIL2, PIWIL3, and PIWIL4. The general structure of Argonaute proteins depicting the PAZ domain (red) with the MID domain (blue), and PIWI domain (green). NCBI (<http://www.ncbi.nlm.nih.gov>) [74].

3. Origin and biogenesis

Understanding of piRNA origin and biogenesis results principally from studies in *Drosophila* and mice [75]. PiRNAs can be classified according to their origin in three subgroups: transposon-derived piRNAs, mRNA-derived piRNAs, and lncRNAs-derived piRNAs. Transposon-derived piRNAs are produced from both genomic strands and generate sense and antisense piRNAs, whereas RNA-derived piRNAs are transcribed from 3' untranslated regions (UTRs) of mRNAs and lncRNAs-derived piRNAs originate from the entire transcript [76]. Unlike miRNAs and siRNAs, which are derived from stem-loop and double-stranded precursors that are processed by the RNase III Dicer, piRNAs are predominantly transcribed as large up to 200 kb single-stranded precursors independently from Dicer [43]. Furthermore, piRNAs do not possess secondary structures [77, 78].

The piRNA pathway is composed of PIWI proteins that interact with piRNAs, whose precursors are transcribed from piRNA clusters, cleaved by PIWI proteins, and secondary amplified in the cytoplasm through a sequence-complementary-dependent “ping-pong” cycle. Mature piRNAs are thus derived from two major pathways, the primary pathway and the “ping-pong” cycle that amplifies secondary piRNAs. In germline cells, molecules implicated in biogenesis of the PIWI-piRNA pathway are located at a perinuclear organelle called the “nuage” [79, 80]. Various components of the “nuage” colocalize with mitochondria [81]. In *Drosophila*, the primary pathway was observed in both germline and somatic cells, whereas the “ping-pong” cycle was identified only in germline cells.

3.1. Primary piRNA biogenesis

Deep sequencing of piRNAs recently revealed millions of distinct piRNAs [29]. However, they were usually located to discrete genomic loci, called piRNA clusters [31]. In the primary piRNA biogenesis, piRNAs provide from long single-strand RNA precursors originating from these clusters. These transcriptional units are highly enriched in dysfunctional remnants of TEs and other repetitive elements and are mainly located in pericentromeric and subtelomeric heterochromatin [41, 70, 82]. PiRNA clusters constitute the basis of immunity against TEs dissemination. Primary piRNAs derived from these clusters include uridine (U) at their 5' nucleic acid and are mostly antisense to TEs mRNA sequences, functioning as guides for PIWI proteins to inactivate TE transcripts through complementary base pairing [40–42]. In the female *Drosophila* germline, these loci are either unidirectionally transcribed (unistrand clusters generating antisense piRNAs) or bidirectionally transcribed (dual-strand clusters generating both sense and antisense piRNAs), producing piRNAs that map to one genomic strand and both strands, respectively [41]. Transcription of unistrand clusters is performed through the canonical polymerase II, whereas dual-strand clusters transcription is generated *via* the noncanonical rhinoadlock-cutoff (RDC) complexes that are also recruited by PIWI proteins and piRNAs through an intricate feedback loop [34, 83–86]. In female flies, piRNA clusters are expressed in germline cells (oocytes and nurse cells) and somatic cells (follicular cells). Interestingly, germline clusters are transcribed bidirectionally, whereas somatic clusters are transcribed unidirectionally, producing piRNAs antisense to TE coding regions in flies. In mouse spermatogenic cells, one class

of piRNA clusters is transcribed during embryonic development and defends the germline against TEs, whereas a second class of clusters is expressed in adolescent mice during the first division of meiosis. The transcription factor A-MYB regulates expression of pachytene piRNA clusters and regulates their transcription through the PIWI-piRNA pathway in mouse [88–97].

In *Drosophila*, nuclear primary transcripts are processed into cytoplasmic mature primary piRNAs (**Figure 1**). These transcripts are resolved of secondary structures by the RNA helicase Armitage and then cleaved by the mitochondria-associated endonuclease Zucchini to generate pre-piRNAs with a characteristic 5' monophosphate [93–97]. Pre-piRNAs are then loaded on PIWI proteins and their 3' ends trimmed to a final length by the 3'–5' exonuclease Nibbler [98, 99]. The 2' hydroxy group at the 3' end is then methylated by the small-RNA 2'-O-methyltransferase Hen1 that increases PIWI binding affinity and piRNA stability, while the 5' end residue of the piRNA incorporated in PIWI shows a strong bias for uridine residues [100–103]. After processed into final length, piRNAs bind PIWI proteins and edify piRNA/PIWI ribonucleoprotein effector complexes (piRISCs) located into the cytoplasmic perinuclear “nuage” [104–106]. PiRISCs migrate back to the nucleus and reach their target genes to epigenetically repress their transcription. Through complementary base pairing of piRNAs and DNA, piRISCs induce transcriptionally heterochromatin formation by establishing a repressive H3K9me3 chromatin state mark on chromatin at target TEs loci and adjacent genes, in order to induce their silencing [107, 108]. H3K9me3 repressive marks are deposited by SETDB1 and Su(var)3–9 methyltransferases and heterochromatin protein 1 (HP1) [109, 110]. In *Drosophila*, the nuclear protein Panoramix is an adaptor allowing interactions between the PIWI-piRNA pathway and the general silencing machinery. Panoramix is implicated with its nuclear partner Asterix, in amplification of the piRNA-dependent TEs silencing [111]. In this way, piRNAs constitute transcriptional regulators that act mainly on TE sequences by recruiting histone methyltransferases, which will lead to establishment of transcriptionally silent heterochromatin [26].

In *Drosophila*, primary piRNAs accumulating in the cytoplasm are amplified by the “ping-pong” cycle [19]. They interact with Ago3 or Aub proteins to form piRNA/Ago or piRNA/Aub complexes, which contain complementary sequences to each other. PiRNA/Ago complexes generate sequences of RNA functioning as substrates for the generation of new piRNAs, which can load Aub proteins. Resulting piRNA/Aub complexes will generate additional RNA substrates to edify new piRNA/Ago3 complexes. The “ping-pong” amplification cycle is mainly observed in early evolutionary species, including *sponges*, *zebrafish*, and *D. melanogaster* [34].

3.2. Secondary piRNA biogenesis

Secondary piRNAs are generated from mRNA transcripts of active TEs [79]. They are primed in the cytoplasmic “nuage” by primary piRNAs (**Figure 1**) that guide their associated PIWI proteins to cleave target TE transcripts based on sequence complementarity [112]. Cleaved TEs are loaded on another PIWI protein and modified to give rise to multiplied secondary piRNAs in an amplification loop, called the “ping-pong” cycle. This posttranscriptional mechanism associates TEs silencing with piRNAs biogenesis by modifying TEs transcripts to give rise to secondary piRNAs [52]. Cleavage of TEs transcripts by PIWI proteins leads to destruction of TEs message, generation of secondary piRNAs, and concomitant amplification of these

defensive sequences targeting active TEs [44]. This process is highly conserved through species and characterized by 5' U bias of primary piRNAs, 10th adenosine bias of secondary piRNAs, and 10-nt overlap between the 5' ends of primary and secondary piRNAs [113–115]. The secondary piRNA biogenesis cycle may constitute an adaptive system to TEs propagation by increasing piRNAs production after incorporation of new TEs into piRNA clusters [116, 117].

3.3. Cellular localization and mechanisms of action

PIWI proteins and piRNAs are located in the nucleus and the cytoplasm of cells expressing this pathway. Loading of piRNAs onto PIWI proteins is localized into the cytoplasm, and PIWI-piRNA complexes generated are required for trafficking of PIWI proteins to the nucleus [90]. Several cytoplasmic organelles, including mitochondria and the “nuage,” are instrumental in functional activity of the PIWI-piRNA axis by controlling piRNA precursor processing [118]. PIWI-piRNA complexes control gene expression through two different mechanisms of action functioning at transcriptional and posttranscriptional levels.

At transcriptional level, nuclear PIWI-piRNA complexes control TEs and gene expression by promoting epigenetic modifications of the chromatin structure and histone proteins through combining DNA and histone methylation. PIWI proteins and piRNAs regulate expression and activity of three active DNA methyltransferases (DNMT1, DNMT3A, and DNMT3B), which normally repress initiation of transcription through methylation of CpG islands in promoter sites of target genes. When PIWI-piRNA complexes recognize TEs and target transcripts, they directly upregulate expression of these DNA methyltransferases and prevent binding of transcription factors through methylation of promoter regions. PIWIL1 induces overexpression of DNMT1 and DNMT3a [119], and piR-823 upregulates DNMT3A and DNMT3B [120]. PIWIL2 and PIWIL4 promote overexpression of DNMT1, DNMT3A, and DNMT3B, which silence TEs and target genes. Experimental loss of PIWIL2 and PIWIL4 induces downregulation in DNA methylation of promoter regions [121]. The PIWI-piRNA complexes also control methylation of histone lysine residues H3K and H4K through recruiting and interacting with histone methyltransferases (HMTs) such as Suv39H1 and SETDB1, which upregulate the histone H3 lysine 9 methylation (H3K9me). Furthermore, these complexes bind with different isoforms of HP1 and guide them to interact with H3K9me in target regions, which is a gene repressive mark. Accumulated methylation of H3K9 induces a heterochromatin state that allows segregation of chromosomes during cell division and prevents accessibility of TEs and genes to transcription factors [122]. Thus, PIWI-piRNAs complexes promote gene repression by using epigenetic mechanisms that allow HP1 α recruitment to TEs loci, heterochromatin edification, and transcription silencing state [123]. PIWIL2 and PIWIL4 increase H3K9 methylation [124]. PIWIL4 recruits SUV39H1 or SETDB1 and promotes H3K9 methylation in promoter region of *CD1A* in monocytes, resulting in recruitment of HP1 α and repression of gene transcription [125]. In leukemias, cell cycle-related piRNAs hsa-piR_014637 and hsa_piR_011186 are implicated in edification of molecular complexes combining DNMT1 and HMTs Suv39H1 and EZH2 that induce H3K9 and H3K27 methylation in the *CDKN2B* gene and inhibition of its transcription by DNMT1-induced CpG methylation in promoter region. In *Drosophila*, PIWI proteins also interact with subunits of the polycomb repressive complex 2 (PRC2). They

maintain integrity of the ovary germline stem cells genome through preventing binding of PRC2 to HP1 α at target gene sites and inhibition of H3K27 di- and trimethylation, a repressive mark upregulated on facultative heterochromatin [126]. Reduction of HP1 α interactions with H3K27me3 promotes maintenance of constitutive heterochromatin, which is pivotal for accurate chromatin segregation and repression of developmentally regulated genes [127].

At posttranscriptional level, cytoplasmic PIWI-piRNA complexes principally govern degradation of TEs transcripts through the “ping-pong” amplification cycle. Apart from their implication in repression of TEs transcripts, the cytoplasmic functions of the PIWI proteins are mostly independent of their partner piRNAs. PIWI proteins modulate functions of many intracellular signaling proteins and receptors through degradation of mRNAs, inhibition of translation, and posttranslational modifications. PIWI proteins inhibit gene expression through mRNAs degradation by interacting with deadenylation complexes (Trf4-Air2-Mtr4 polyadenylation complex or CCR4 complex), resulting in shortening of poly-A tails. The PIWIL4-piR30840-Ago4 complex induces degradation of pre-mRNAs through binding to the Trf4-Air2-Mtr4 polyadenylation complex in human T lymphocytes [128]. They repress translation by interacting with translation initiation factors (eIF3a, eIF4E, eIF4F), preventing ribosomal subunits binding to 5' cap of mRNAs. In mouse, Miwi interacts with eIF4E, while Mili binds to eIF3a, eIF4E, and eIF4F [129]. These proteins also regulate activity and stability of numerous molecules belonging to major signaling pathways by controlling posttranslational modifications such as phosphorylation and ubiquitination. PIWIL2 and PIWIL4 interact with the transcription factor STAT3 and upregulate its phosphorylation and activity. At the opposite, PIWIL4 binds to p53 and prevents serine 15 phosphorylation, inhibiting its functions [130]. PIWI proteins can also upregulate stability of target molecules by preventing their ubiquitination-dependent degradation. Interaction of PIWIL1 with Stathmin 1 inhibits its phosphorylation, resulting in prevention of PIWIL1 degradation by the ubiquitin ligase RLIM. Likewise, PIWIL2 binding to cytokeratin 8 promotes its phosphorylation and upregulates its stability by preventing its ubiquitination-derived degradation [131].

3.4. Biological functions

Up to now, biological functions of piRNAs have been only partially identified, due to the wide variation in piRNA sequences and mechanisms of action over species. However, a great majority of piRNAs are not complementary to mRNAs of target genes and are mainly implicated in epigenetic regulation rather than posttranscriptional modulation of biologic processes. PiRNAs have been implicated in TEs silencing, epigenetic, genes and proteins regulation, genome rearrangement, fertilization, germline and somatic stem cell self-renewal, embryogenesis, and maintenance of homeostasis.

3.4.1. Maintenance of genome stability and integrity

The PIWI-piRNA pathway maintains integrity and stability of the general organization of the genome, including regulation of genes, through recognition of self and nonself and prevention

of TEs propagation. During interphase, the genome of eukaryotic cells is organized into various spatial three-dimensional topologically associating domains (TADs) edifying functional subcompartments implicated in pivotal cellular activities [132]. It has been recently observed in somatic cells of *Drosophila* ovaries PIWI-interacting chromosomal domains overlapping with genomic regions bound by nuclear pore complexes (NPCs). Furthermore, a third of protein-coding genes have been identified in the PIWI-interacting domains. PIWI proteins stochastically interact with nascent transcripts of genes and TEs and scan them through complementarity with piRNAs. Although perfect complementarity allows transcriptional silencing of TEs, imperfect complementarity leads to maintenance of PIWI proteins interactions with transcripts in the mRNPs after their detachment from the sites of transcription until dissociation from mRNPs in the nucleoplasm [133].

3.4.2. Retrotransposons silencing

TEs, first identified in maize by Barbara McClintock in the 1940s, constitute genetic units that can move and propagate within the genome [70]. More recently, genome-sequencing techniques have revealed that TEs occupy 15–22% of the genome of *Drosophila melanogaster* and 55% of the human genome. TEs of the human genome are divided into two major classes. Class II comprises DNA transposons that are generally inactive genetic elements representing less than 2% of the human genome and depending on transposases for their mobilization. They do not need transcription to propagate and use a “cut and paste” mechanism to excise and insert into new genomic sites without increasing their copy number. Conversely, class I retrotransposons are usually active genetic elements propagating through a “copy and paste” mechanism that allows retrotranscription into cDNA by a reverse transcriptase encoded by the retrotransposon and insertion into new genomic sites *via* these RNA transposition intermediates [134]. Retrotransposons are composed of three subclasses: (1) the long interspersed elements 1 and 2 (LINE-1/L1 and LINE-2/L2) are about 6 kb long and encode the two proteins ORF1p and ORF2p. ORF1p is implicated in edification of the retrotransposon particle, and ORF2p allows the enzymatic activities required for retrotransposition such as reverse transcriptase and endonuclease. Analysis of transgenic mice has demonstrated presence of L1 transcripts in gametes, but rare genomic insertion, suggesting posttranscriptional mechanisms allowing preservation of genomic integrity in germline. Conversely, genomic insertions of L1 sequences were mostly identified in somatic tissues during the early phases of embryogenesis [135]; (2) the short interspersed elements (SINEs) belong to the SINE-Alu and SVA classes. Whereas LINES are autonomous sequences encoding a reverse transcriptase, SINEs are dependent on two proteins encoded by LINES for their replication and integration [136]. Non-LTR families L1, SVA, and Alu were found to be upregulated in breast, ovarian, colon, and hematological cancers [137]; (3) the third subclass is composed of inactive LTR retrotransposons resulting from ancient germline retroviral infections. Within the human genome, only 80–100 TEs among LINE sequences are competent for the entire retrotransposition activity [8]. In the germline, TEs represent pivotal actors implicated in the shaping of genomes during evolution, and presence of retrotransposition in numerous somatic cells indicates that TEs contribute to edification of mosaicism. TEs have important role in edifying genetic diversity but are also a major source of genetic instability through mutations, chromosomes rearrangements, and epigenetic/genetic deregulations [138].

Although mobilization of class I retrotransposons may be considered as beneficial by promoting biological variability within the genome, existence of an active insertional mutagenesis can induce genomic instability in aging cells, leading to human genetic diseases, degenerative pathologies, and cancer [39]. Class I retrotransposons propagating through their “copy and paste” mechanism result in an increased copy of TEs number, which may become a source of endogenous mutagenesis by producing insertion-mediated deletions with cell cycle arrest and nonhomologous recombination [139]. Gradual release of TEs induces molecular alterations in DNA repair processes, autophagy, chaperones, and ubiquitin-proteasome system [140, 141].

During evolution, organisms have adopted molecular systems to contain expansion of TEs activity. Among them, PIWI proteins and piRNAs constitute a small-RNA-based innate immune-like system mainly expressed in gonads. Upon new expansion, TEs propagate into different regions in the genome, can be trapped into piRNA clusters, and leave traces of their sequences in these TEs traps. By falling into these clusters, novel piRNAs targeting TEs are generated and amplified through the two biogenesis pathways [87]. These pathways are highly conserved in eukaryotes and mainly implicated in protection of the genome integrity and normal gametogenesis by silencing TEs [62]. Within the germline, TEs inactivation is performed by both PIWI-piRNA and siRNA pathways. Propagation of TEs is controlled by the PIWI-piRNA pathway, of which the PIWI proteins are the executive components. The nuclear PIWI proteins allow transcriptional silencing of TEs by recognizing nascent transcripts through perfect complementarity with loaded piRNAs and are assisted by the RNA-binding protein Asterix. Recognition of multiple complementary sites in nascent TE transcripts by Asterix-PIWI-piRNA complexes favors interaction with the adaptor protein Panoramix, resulting in recruitment of the cell silencing machinery that represses TEs transcription. Moreover, introns containing remnants of TEs or genes located in proximity of TEs can be repressed by the PIWI-piRNA axis. Current studies indicated that the high mobility group protein Maelstrom (Mael) may act downstream of Piwi and histone methylation. In mouse, both Mili and Miwi2 promote TEs silencing and a heterochromatin state in mice through DNA and histones methylation. Decreased expression of PIWI proteins and piRNAs is associated with upregulation and propagation of active TEs. However, unlike siRNAs, which are active in both gonadal and somatic aging cells, the PIWI-piRNA pathway predominantly operates in nonaging cells of gonads [142, 143]. This pathway could be part of a mammalian recognition system of coding and non-coding self-genes and non-self-TEs and repeat sequences by using characteristic TEs mobility.

3.4.3. Epigenetic activation

Mounting evidence suggests that PIWI proteins and piRNAs can function as epigenetic activators. In *Drosophila*, Piwi protein increases chromosome 3R telomere-associated sequence (3R-TAS) expression [144].

3.4.4. Genes and proteins regulation

PiRNAs control levels of expression of genes where they are localized. PiR_015520, located in intron 1 of the human melatonin receptor 1A gene (*MTNR1A*), is upregulated in prostate

cancer and represses *MTNR1A* gene by directly interacting with its genomic site [145]. PiRNAs also modulate stability of their PIWI partners by promoting direct molecular interactions with specific proteins. During late mouse spermatogenesis, piRNAs regulate ubiquitination of Miwi through its binding to APC/C complex [146]. Furthermore, piRNAs can modify activity and expression of many distant genes. PiRNA-36026 interacts with suppressor proteins Serpin peptidase inhibitor, clade A, member 1 (SERPINA1), and lecithin retinol acyltransferase (LRAT). However, the PIWI-piRNA pathway is also present at lower levels in somatic pluripotent stem cells to differentiated cells [147, 148]. In adult somatic cells of *Drosophila melanogaster*, this pathway is active in ovarian follicle cells, in salivary glands, and in the brain [149]. The pathway is principally observed in stem cells with pluripotent capacities, including mesenchymal and hematopoietic stem cells, but rarely in adult stem cells with limited differentiation capacity [150]. Furthermore, the PIWI-piRNA pathway seems also to regulate protein-coding genes. The first piRNAs identified in *Drosophila melanogaster* were transcribed from the Suppressor of Stellate locus located on the Y chromosome and targeted the protein-coding gene Stellate on the X chromosome [151]. More recently, genome-wide mapping techniques have demonstrated that genic piRNAs derive from TEs and 3' UTRs of coding genes [56]. Mounting evidence suggests that germline genes could have ancestral implication in regulating stemness. The “nuage” is located in lower metazoan stem cells but restricted to germline cells in upper metazoans [152]. The PIWI-piRNA pathway is expressed in stem cells of metazoans with partial or whole-body regeneration capabilities [153].

3.4.5. Differentiation

PIWI proteins play pivotal roles in cell differentiation during early embryogenesis. In *Drosophila* ovary, self-renewal of differentiated germline stem cells is located in niches composed of different types of cells, including escort cells (ECs). Experimental deregulation of PIWI proteins expression in EC cells was associated with reduction of EC cell population and predominance of undifferentiated germline stem cells. PIWI proteins induced germline cell differentiation by promoting direct interaction between germline stem cells and escort cells through repression of the TGF β signaling and bone morphogenetic protein (BMP) pathway by preventing edification of Smad complexes. PIWIL2 is the major PIWI protein implicated in cell differentiation through inhibition of the TGF β signaling pathway. PIWIL2 directly interacts with Smad4 and HSP90 and prevents HSP90-T β R complex formation, resulting in inhibition of the TGF β signaling pathway. Furthermore, PIWIL2 promotes degradation of TGF β receptor (T β R) and Smad by upregulating ubiquitination and degradation of T β R by the ubiquitin E3 ligase Smurf2. PIWI proteins contribute to germline stem cells differentiation by repressing c-Fos at post-transcriptional. These proteins promote piRNAs synthesis from 3' UTR region of c-Fos mRNA, resulting in c-Fos mRNA instability and repression of its translation [151–154].

3.4.6. Cell survival

The PIWI-piRNA axis promotes activation of numerous prosurvival molecules. PIWIL1-induced cell survival by upregulating expression of antiapoptotic molecule FGF8 and down-regulating expression of proapoptotic Bax and p21. In blastema cells of Mexican *axolotl*,

experimental defect of PIWIL1 and PIWIL2 promoted apoptosis by suppressing FGF8 expression at transcriptional level and prevented limb regeneration and development. PIWIL2 principally controlled p53 through direct interaction with STAT3 and c-Src by edifying a PIWIL2/STAT3/c-Src complex, resulting in repression of p53 phosphorylation and expression and inhibition of Fas-mediated apoptosis. PIWIL2 - induced activation of STAT3 also upregulated expression of the antiapoptotic Bcl-XL [125].

3.4.7. Fertilization and development

Although most attention has been given to the pivotal role of the PIWI-piRNA pathway in germline TEs silencing, mounting evidence has revealed their implication in germline and somatic epigenetic and posttranscriptional regulation of gene expression [151]. This pathway is mainly implicated in the germline biology, including maintenance, differentiation, and function of *Drosophila* and murine GSCs. Furthermore, piRNAs epigenetically activate gene expression with transgenerational epigenetic effects by inducing euchromatin through activation of H3K4me3 and inhibition of H3K27me3 in subtelomeric heterochromatin [56, 73, 152–155]. The PIWI-piRNA axis is implicated in embryonic development, including cell cycle progression, nuclear division, chromatin organization, chromosome integrity during mitosis, control of mRNA translation, and embryonic sex determination [144, 156–158]. Spatial-temporal activation and regulation of PIWI proteins and piRNAs are of pivotal importance during mammalian oogenesis and spermatogenesis, early embryogenesis, organogenesis, and postbirth [159]. PIWIL2 is upregulated in germline cells and appears instrumental in maintaining genome stability, an open state of chromatin and DNA repair via silencing TEs and histones modifications, thus preventing TEs propagation, chromosome rearrangements, oncogenic mutations, and gene dysregulation [160].

3.4.7.1. Oogenesis

In *Drosophila*, PIWI-piRNA complexes promote TEs silencing at embryonic germ cell stage, mediate cellular memory of TEs repression, and thus maintain this mechanism in ovaries at the adult stage. In mouse ovary, Miwi upregulation is observed during neonatal stage and its expression is lower in adult ovaries [161]. Human PIWI proteins expression profiling is also variable, depending on the stage of development. PIWIL1 and PIWIL2 are highly upregulated in oocytes of human adult ovary that present a strong activity of TEs, whereas fetal oocytes, whose TEs propagation is lower, overexpress PIWIL2 but not other PIWI proteins [162].

3.4.7.2. Spermatogenesis

In mouse, Miwi inactivation occurs during late spermatogenesis and is induced by the anaphase promoting complex (APC)/C-26S proteasomal pathway [159]. Functional destruction box (D-box) is required for Miwi ubiquitination and degradation by (APC)/C system. A genetic analysis in mouse azoospermia showed that mutations in D-box favor Hiwi stabilization in late spermatogenesis. Stabilized mutant Hiwi interacts with RNF8 implicated in

histone ubiquitination and prevents its nuclear translocation and ubiquitin ligase activity [163]. Human PIWIL4 function is crucial in accurate spermatogenesis, and genetic polymorphisms of PIWIL4 gene are significantly correlated with defective spermatogenesis associated with spermatogenesis defect and male infertility [164].

3.4.7.3. Organogenesis

In *Drosophila*, PIWI proteins induce ovary tissue morphogenesis through c-Fos inactivation at posttranscriptional level [165]. They are implicated in development of eye color [144]. In silkworm, fempiRNA, a piRNA located on female W-chromosome, is pivotal in sex determination by repressing masculinization mRNA at posttranscriptional level [167]. At early stages of human embryonic lungs development, PIWIL1, PIWIL2, and PIWIL4 levels of expression are strongly upregulated from 6th week to 9th week and then decline [168]. In human, PIWIL1 and PIWIL2 have crucial role in neural polarization and radial migration during maturation of the cerebral cortex region of the brain [59].

3.4.8. Physiological processes

The PIWI-piRNA pathway has pivotal role in numerous physiological processes.

3.4.8.1. Brain plasticity

PIWI proteins and piRNAs are instrumental in synaptic plasticity and stabilization of long-term memory through serotonin-dependent suppression of CREB2 at transcription level that is induced by methylation of CpG islands in the promoter region of the *CREB2* gene [59]. In rodents, several piRNAs are upregulated in hippocampal neurons and Miwi associated with piRNAs control dendritic spine development and morphogenesis [149]. Mili expression is associated with anxiety and locomotory drive [54]. In humans, PIWIL1 controls cortical neuron activity through modulation of microtubule-associated proteins (MAPs) expression [169]. Furthermore, mutations of PIWIL2 and PIWIL4 are significantly correlated with autism [170].

3.4.8.2. Regeneration

PIWI proteins and piRNAs have crucial role in self-renewal, regeneration, and homeostasis. In planarian *Schmidtea mediterranea*, SMEDWI-2 and SMEDWI-3 increase division of adult stem cells to induce regeneration in injured tissues [113]. In jellyfish, Cniwi is upregulated during transdifferentiation of striated muscle into smooth muscle [171]. In humans, PIWI proteins promote hepatocyte regeneration [61] and maintain integrity of retinal cells [130].

3.4.8.3. Metabolism

The PIWI-piRNA pathway controls fat metabolism through repression of TEs, and fat metabolism inactivation is associated with depletion of lipid synthesis and storage [60]. PIWIL2 and PIWIL4 modulate pancreatic β -cells function and insulin secretion. Alterations of their levels of expression were observed in diabetic conditions [55].

4. Deregulation of the PIWI/piRNA pathway in pathological nonneoplastic disorders

Mounting evidence has revealed that many transcription factors and signaling molecules interact with the PIWI-piRNA pathway and represent downstream targets of these complexes under pathological conditions. PIWI proteins and piRNAs are deregulated, and their levels of expression are highly altered in various pathological processes. The PIWI-piRNA pathway is pivotal for regeneration after amputation in *Botrylloides leachi* [172]. In Mexican axolotl, PIWIL1 and PIWIL2 transient upregulation in limb blastemal cells induces regeneration of wounded limb [173]. In rat, PIWIL2 expression increases after 24 h of partial hepatectomy, and a set of 72 piRNAs is deregulated during 48 h of posthepatectomy [58]. In rodents, expression of more than 100 piRNAs is deregulated in brain during ischemic condition [174]. In rat, PIWIL2 enhances activity of the autophagic process in diabetic nephropathy by regulating expression of beclin 1 and LC3A study in diabetic rat kidney [175]. Pro-inflammatory cytokines IL1 β and TNF α promote PIWIL2 and PIWIL4 upregulation in synovial fibroblasts of rheumatoid arthritis [176].

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