Altered Expression of PIWI RNA in the Rat Brain After Transient Focal Ischemia

Ashutosh Dharap, MS; Venkata Prasuja Nakka, PhD; Raghu Vemuganti, PhD

**Background and Purpose**—The PIWI-interacting RNA (piRNA) is the most predominant RNA species in eukaryotes. The piRNA are a class of noncoding RNAs that bind and degrade the RNA formed by the transposons to control the transposon-induced gene mutations. The role of piRNA after focal ischemia is not yet evaluated.

**Methods**—We profiled 39,727 piRNAs in the cerebral cortex of adult rats subjected to transient focal ischemia using microarrays. The RT targets of stroke-responsive piRNAs were identified with bioinformatics. To understand how piRNAs are controlled, we analyzed the transcription factor binding sites in the putative promoters of 10 representative stroke-responsive piRNAs.

**Results**—In the ipsilateral cortex of ischemic rats, 105 piRNAs showed altered expression (54 up- and 51 downregulated; >2.5-fold) compared with shams. Twenty-five of those showed a >5-fold change. A bioinformatics search showed that the transposon targets of the highly stroke-responsive piRNAs are distributed among the 20 autosomal chromosomes and there is a redundancy in the targets between the piRNAs. Furthermore, the transposon targets were observed to be highly repetitive for each piRNA across the chromosome length. Of the 159 transcription factors observed to have binding sites in the piRNA gene promoters, 59% belonged to 20 major families indicating that transcription factors control stroke-responsive piRNAs in a redundant manner.

**Conclusions**—The present study is the first to show that many piRNAs are expressed in adult rodent brain and several of them respond to focal ischemia. (*Stroke. 2011;42:1105-1109.*)

Key Words: bioinformatics • brain damage • expression profiling • noncoding RNA • stroke • transposons

In eukaryotes, approximately 40% of the genome is comprised of transposons, which are transcribed into RNA, reverse-transcribed into double-stranded DNA, and inserted into new locations in the genome.1,2 As transposition mutates the protein-coding genes, a class of small noncoding (nc) RNA called PIWI-interacting RNA (piRNA; 26 to 31 nt long) selectively target and silence the RNAs formed by transposons.3 Thus, piRNA balances the fitness of the genome to maintain the genetic equilibrium. Interestingly, thousands of piRNA are known to be produced from disrupted transposons in genome regions biased toward heterochromatin.4,5

Very few studies to date evaluated the significance of ncRNA in ischemic brain damage. We and others showed that micro-RNA expression profiles alter extensively after focal ischemia and modulating specific micro-RNAs induces neuroprotection.6–10 Although these studies indicate the role of ncRNA in ischemic pathophysiology, the significance of other ncRNA like piRNA is not evaluated yet. To fill this void, we profiled the expression of 39,727 piRNAs in the brains of adult rats subjected to transient middle cerebral artery occlusion. Using bioinformatics, we identified the transposon targets of representative stroke-responsive piRNAs. Although piRNAs control transposons, the mechanisms that control piRNA are not precisely known. A plethora of transcription factors (TFs) controls the transcription of protein coding as well as nc genes, and many TFs are known to modulate ischemic brain damage.11–15 Hence, we analyzed the putative promoters of representative stroke-responsive piRNA genes to identify TF binding sites.

**Methods**

**Focal Ischemia**

Adult, male, spontaneously hypertensive rats (280 to 320 g; Charles River, Wilmington, MA) used in these studies were cared for in accordance with the Guide for the Care and Use of Laboratory Animals, US Department of Health and Human Services Publication number 86–23 (revised 1986). Transient middle cerebral artery occlusion was induced under isoflurane anesthesia by the intraluminal suture method as described earlier.6,13

**piRNA Microarray Analysis**

From each rat, the brain was sliced in a rat brain matrix to generate 1-mm sections. One section from the coordinates between +1 mm...
and −1 mm was quickly stained with 2-3-5-triphenyl tetrazolium chloride to confirm infarction. From the adjacent sections, the ischemic core region was dissected from the ipsilateral cortex. Cerebral cortex from sham-operated rats served as a control. Total RNA was extracted from 100 mg of each sample with the RNeasy kit (Qiagen, Valencia, CA), treated with DNase, and the RNA quality and integrity were confirmed. RNA was labeled with Cy-3 and hybridized to Rat RN34 piRNA Expression Oligo microarrays (ArrayStar, Rockville, MD) that contained probes for 39 727 piRNAs selected from the National Center for Biotechnology Information database and mapped to the RN34 genome sequence using UCSC BLAST. After hybridization, the arrays were scanned with an Agilent microarray scanner. The array quality was maintained by confirming that the spot centroids were located properly at 4 corners of the array by checking the spatial distribution of the population and nonuniformity outliers distributed across the array by running net signal statistics to confirm the dynamic range of the signal for noncontrol probes by generating histogram of signals plots to confirm the level and the shape of the signal distribution with negative control stats (the average and SD of the net signals; mean signal−scanner offset and the background-subtracted signals), correcting for local background inliers, and checking reproducibility statistics (percent coefficient of variation replicated probes). A transcript was considered detectable only if the signal intensity was higher than 3 times the maximal background signal and the spot coefficient of variation (SD/signal intensity) was <0.5. The expression data files obtained by the Agilent Feature Extraction Software were imported into the GeneSpring GX software, data sets from different arrays were quantile normalized, and the differentially expressed piRNAs were identified by fold-change screening with a threshold of ≥2.5-fold. Statistically significant differences between the groups were identified by the statistical measures built in the GeneSpring, based on the t test probability value method with a high stringency (fold change cutoff of ≥2.5 and a probability value of <0.001 to decrease the false-positives).

Bioinformatics
We analyzed the targets of the top 4 stroke-responsive piRNAs (2 upregulated and 2 downregulated) using a modified version of miRanda in piRNABank (Institute of Bioinformatics and Applied Biotechnology, Bangalore, India). Each piRNA was searched for repeat sequence targets against the transposon database of the 20 autosomal rat chromosomes (RGSC 3.4) across a representative 1 million base stretch (position 1 to 1 million) with a mean free energy maximum of −20.0 kcal/mol and a very stringent score threshold of 140. In addition, to obtain a chromosome-wide target distribution and to search for target redundancy, each piRNA was scanned against the entire length of a randomly selected autosomal chromosome in multiple 1 million base stretches with an interval of 20 million bases. The same threshold parameters as mentioned previously were applied. We also analyzed the promoters of 10 representative stroke-responsive piRNA genes for conserved TF sites. To locate the promoters, the genomic locus for each piRNA was entered into the UCSC genome browser and scanned for the nearest promoter upstream to that particular locus (within 10 kb upstream to Transcription start site [TSS]). For each piRNA, a 1-Kb sequence was analyzed for overrepresented TF binding sites using the Genomatix RegionMiner software (Genomatix Software GmbH). All TF matrices with a Z-score of ≥2 (representing P<0.05) were considered statistically significant.

Results

Stroke-Induced Changes in piRNAome
In the cerebral cortex of normal rats, 9.7% (3885±296) of the 40 000 piRNAs analyzed obtained a statistically significant present call (Supplemental Figure I; available at http://stroke.ahajournals.org). After 1-hour transient middle cerebral artery occlusion and 24 hours reperfusion, 105 piRNAs showed altered expression (54 up- and 51 downregulated) compared with sham (n=3 in each case). The complete list of piRNAs up- and downregulated by >2.5-fold in the ischemic brain are given in Supplemental Tables I and II. Table 1. Top 24 Ischemia-Responsive piRNAs

<table>
<thead>
<tr>
<th>piRNA NCBI No.</th>
<th>Δ-Fold*</th>
</tr>
</thead>
<tbody>
<tr>
<td>pIR-177729 DQ762407</td>
<td>36.01</td>
</tr>
<tr>
<td>pIR-143106 DQ727784</td>
<td>31.26</td>
</tr>
<tr>
<td>pIR-173369 DQ758047</td>
<td>12.90</td>
</tr>
<tr>
<td>pIR-64423 DQ614312</td>
<td>8.78</td>
</tr>
<tr>
<td>pIR-176691 DQ761369</td>
<td>6.83</td>
</tr>
<tr>
<td>pIR-64425 DQ614313</td>
<td>6.23</td>
</tr>
<tr>
<td>pIR-71756 DQ628212</td>
<td>5.14</td>
</tr>
<tr>
<td>pIR-87058 DQ619946</td>
<td>5.06</td>
</tr>
<tr>
<td>pIR-62321 DQ602209</td>
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<tr>
<td>pIR-62320 DQ602208</td>
<td>4.43</td>
</tr>
<tr>
<td>pIR-88241 DQ621129</td>
<td>4.43</td>
</tr>
<tr>
<td>pIR-62318 DQ602206</td>
<td>4.29</td>
</tr>
</tbody>
</table>

*The Δ-fold is mean (with <20% SD in each case) in comparison to the sham group (n=3 in each case). The complete list of piRNAs up- and downregulated by >2.5-fold in the ischemic brain are given in Supplemental Tables I and II. NCBI indicates National Center for Biotechnology Information.

Targets of Stroke-Responsive piRNAs
The major function of the piRNAs is to silence the RNAs formed by transposons to negate their mutagenic effects. The transposons are highly repetitive in nature and several piRNAs can target a single transposon as well as a piRNA can target multiple transposons. We conducted a bioinformatics prediction of the targets of 2 piRNAs upregulated (pIR-177729 and pIR-143106) and 2 piRNAs downregulated (pIR-169523 and pIR-70903) in the ischemic brain. For each piRNA, targets were searched in 201 million base stretches (1 to 1 million bases of each of the 20 autosomal chromosomes). The number of targets of the 4 piRNAs ranged from 6 to 31 with an average of 11.08 per million bases (Table 2). Surprisingly, all the transposon targets of these 4 piRNAs identified in the present study exclusively belonged to the class retrotransposons (RTs). There is a high level of redundancy in the RTs targeted by the 4 piRNAs. The 4 piRNAs targeted 51 classes of transposons spread among the 20 chromosomes, of which 26 classes were conserved among at least 3 of the 4 piRNAs analyzed (Table 3). Furthermore, 11 of those RTs were repeated from 4 to 15 times in each of the chromosomes for each piRNA (Table 3). The redundancy of target RTs was also confirmed by the observation that the 80 individual RTs targeted by pIR-177729, 61 are repeats of 12 classes (76%; Table 3). A similar 67% to 70% redundancy was also observed for pIR-143106 (122 of 181 are repeats of...
Table 2. No. of Target RTs of 4 Stroke-Responsive piRNAs Distributed in Bases 1 to 1 Million of the 20 Autosomal Chromosomes

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>piR-177729</th>
<th>piR-143106</th>
<th>piR-169523</th>
<th>piR-70903</th>
</tr>
</thead>
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<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>2</td>
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<td>0</td>
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<td>3</td>
<td>6</td>
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<td>14</td>
<td>20</td>
</tr>
<tr>
<td>4</td>
<td>13</td>
<td>0</td>
<td>14</td>
<td>21</td>
</tr>
<tr>
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<td>17</td>
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<td>14</td>
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</tr>
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<tr>
<td>20</td>
<td>0</td>
<td>18</td>
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<td>20</td>
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</tbody>
</table>

12 RT classes), piR-169523 (151 of 227 are repeats of 14 classes), and piR-70903 (160 of 227 are repeats of 15 classes; Table 3). When RT targets were searched in the entire length of representative chromosomes (in 1 million base stretches at an interval of 20 million bases in each case) for the 4 piRNAs tested, we observed an average of 12.2 RTs/million bases (Table 4). Consistent with the RT redundancy observed across chromosomes, RT targets are also highly repetitious within in each chromosome for all the 4 piRNAs (data not shown). For piR-169523, 74 of 116 RTs (64%) are 3 to 5 repeats of 17 RT classes (Table 5).

TF Binding Sites in the piRNA Gene Promoters

In the promoters of 10 stroke-responsive piRNA genes, we observed binding sites for 159 TFs (Supplemental Table III) and 93 (59%) of them belonged to 20 families of TFs (Supplemental Table IV). Of these 13, TF families targeted the 10 piRNA genes in a redundant manner (Figure). The zinc finger family and the Kruppel family targeted 7 of the 10 piRNA gene promoters and the E2FF family targeted 5 of the 10 piRNAs (Figure). The promoter of the piR-173369 gene showed the maximum of 46 TF binding sites (Supplemental Table V).

Discussion

In brief, the present study shows that focal ischemia significantly influences piRNA expression in rat brain. The downstream transposon targets of the stroke-responsive piRNAs are highly repetitious and distributed throughout the genome.

Furthermore, the promoters of the stroke-responsive piRNA genes contain several TF binding sites and there is a redundancy in the TF families that target these piRNAs.

Protein-coding genes represent <2% of the eukaryotic genome because approximately 98% of the transcriptional output is ncRNA that include micro-RNA, piRNA, small interfering RNA, and long ncRNA.16 Because ncRNAs are the master controllers of the transcription and translation that decides the organ-specific and cell-specific protein repertoire, any disruption in their function could lead to severe compromises in cellular homeostasis.17,18 Recent studies demonstrated that stroke profoundly alters miRNAome and modulating specific micro-RNAs induce neuroprotection.11–15 The present study shows that stroke also influences other ncRNAs like piRNA.

Bioinformatics analysis showed that the targets of the stroke-responsive piRNAs are highly repetitious. This redundancy of function is similar to that is known to exist for micro-RNAs and their target mRNAs. Bioinformatics analysis further showed that the TF families that might control the stroke-responsive piRNAs are also redundant. For example,
zinc finger family of TFs was observed to have binding sites in the gene promoters of 7 of 10 piRNAs evaluated.

The role played by piRNA in maintaining the normal cellular homeostasis is enormous. It is estimated that the eukaryotic genome contains >2.5 million interspersed repeat elements and most of them are highly active transposable RTs. Because piRNAs silence the transposable elements, any disruption in the piRNAome can be disastrous to the cell. The micro-RNAs are the most studied ncRNAs and recent studies showed that cerebral micro-RNAs respond quickly to focal ischemia and the effect of ischemia is more extensive on micro-RNAsome than observed previously for mRNAome (40% micro-RNAs versus <5% mRNAs alter after focal ischemia).6,7,11,19 Furthermore, modulating individual micro-RNAs was reported to have profound effects on postischemic brain damage.9,10,15 In rodents, the number of piRNAs identified so far exceeds the number of known micro-RNAs by severalfold and thus the network of permutations and combinations of piRNAs and their targets could be much higher than the micro-RNAs and their targets. As per bioinformatics predictions, a piRNA can target multiple transposons that contain the consensus binding sites throughout the genome and a transposon can contain multiple binding sites for several piRNAs. Because the transposon-induced mutagenesis is a major disabler of protein coding genes, this redundancy might be nature’s adaptation for a proper physiological

### Table 4. No. of Target RTs of 4 Stroke-Responsive piRNAs Distributed Across the Length of Representative Chromosomes*

<table>
<thead>
<tr>
<th>Position in CHR</th>
<th>No. of Targets/Million Bases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Start Base</td>
<td>End Base</td>
</tr>
<tr>
<td></td>
<td>pIR-177729 (CHR 10)</td>
</tr>
<tr>
<td>1</td>
<td>1 000 000</td>
</tr>
<tr>
<td>21 000 000</td>
<td>22 000 000</td>
</tr>
<tr>
<td>42 000 000</td>
<td>43 000 000</td>
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<tr>
<td>168 000 000</td>
<td>169 000 000</td>
</tr>
<tr>
<td>Average</td>
<td></td>
</tr>
</tbody>
</table>

*The chromosome lengths (in million bases) obtained from the National Center for Biotechnology Information database are 106, 169, 127, and 87 for CHR 10, CHR 3, CHR 7, and CHR 11, respectively.

### Table 5. RT Targets of piR-169523 Repeated in the Length of CHR 7*

<table>
<thead>
<tr>
<th>RT</th>
<th>1 to 1 Million</th>
<th>21 Million to 22 Million</th>
<th>62 Million to 63 Million</th>
<th>105 Million to 106 Million</th>
<th>126 Million to 127 Million</th>
<th>No. of Repeats</th>
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</table>

*The length of chromosome 7 is 127 million bases.
P indicates present. No targets were observed in stretches 42 to 43 million and 84 to 85 million.
balance at the genomic level. Our present studies on piRNAs together with the previous studies on micro-RNAs indicate that ncRNAs need to be factored in to understand the molecular mechanisms of ischemic brain damage.

To our knowledge, this is the first study on cerebral piRNAome in experimental stroke. The pathophysiological significance of piRNAs in stroke-induced brain damage is not known presently. Although the present studies indicate that stroke alters piRNAome, we do not have any evidence to show that these changes mediate ischemic brain damage. However, we predict that stroke might also influence transposons and altered piRNAome is a response to counter the increased transposon activity to control mutagenesis in the ischemic brain. On the other hand, altered piRNA levels might lead to a disruption of the normal transposon network leading to pathophysiological changes after stroke. Future functional studies with knockin/knockout technologies might clarify these issues as well as might show if altered piRNAome and/or altered transposon activity contribute to ischemic brain damage. Our studies are the first to indicate that stroke alters piRNAome and designing drugs to manipulate piRNA and perhaps other ncRNA might be essential to counter the stroke-induced mortality and morbidity.

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**Disclosures**

None.
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Supplemental Material

Altered expression of PiRNA in rat brain following transient focal ischemia

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Running Head: PiRNA in Stroke
**Detailed method of focal ischemia:** Adult, male, spontaneously hypertensive rats (SHR; 280-320g; Charles River, Wilmington, MA) used in these studies were cared for in accordance with the *Guide for the Care and Use of Laboratory Animals*, U.S. Department of Health and Human Services Publication number 86-23 (revised 1986). The Research Animal Resources and Care Committee of the University of Wisconsin-Madison approved the surgical procedures. Transient MCAO was induced with an intraluminal suture. In brief, a rat was anesthetized with halothane, placed in a stereotaxic frame fitted with a nose cone with 2% isoflurane anesthesia. A craniotomy (4 mm in diameter, 2-4 mm lateral and 1-2 mm caudal to bregma) was performed with extreme care over the MCA territory using a trephine. The dura was left intact and a laser Doppler flow-meter probe (model PD-434; Vasamedics, LLC, St Paul, MN, USA) was placed on the surface of the ipsilateral cortex and fixed to the periosteum with a 4-0 silk suture. The probe was connected to a laser flowmeter device (Laserflow blood perfusion monitor BPM 403A; TSI Inc., St Paul, MN) for continuous monitoring of regional cerebral blood flow (rCBF). The left femoral artery was cannulated for continuous monitoring of arterial blood pressure and to obtain the measurements of pH, $P_aO_2$, $P_aCO_2$, hemoglobin and blood glucose concentration (i-STAT; Sensor Devices, Waukesha, WI). The rectal temperature was controlled at 37.0 ± 0.5°C during surgery with a feedback-regulated heating pad. After a midline skin incision, the left external carotid artery (ECA) was exposed, and its branches were coagulated. A 3-0 surgical monofilament nylon suture, blunted at the end, was introduced into the ECA lumen and gently advanced to the internal carotid artery until rCBF was reduced to 10 to 16% of the baseline (recorded by laser Doppler flowmeter). After a 1h occlusion, the suture was withdrawn to restore the blood flow (confirmed by laser Doppler). Rats were killed at 24h of reperfusion. After suturing the wound, the rat was allowed to recover from anesthesia and returned to the cage with *ad libitum* access to food and water. During the MCAO, $P_aO_2$ (100 to 200 mm Hg) and $P_aCO_2$ (30 to 40 mm Hg) were maintained at physiological levels. Sham-operated rats served as control.
Supplementary Fig. 1: The piRNA microarray experiment showed that in the normal rat cerebral cortex an average of 3,885 (± 296) piRNAs of the ~40,000 piRNAs (9.7%) analyzed obtained a statistically significant present call. On a scale of 1 to 900,000 units, 132 piRNAs were expressed at a very high level (100,000 to 800,000 units), 155 at a high level (10,000 to 100,000 units), 421 at a moderate to high level (1,000 to 10,000 units), 311 at a moderate level (500 to 1,000), 492 at a low to moderate level (250 to 500), 1160 at a low level (100 to 250) and 1,241 at a very low level (<100 units).
Supplementary Table 1: Stroke-responsive piRNAs upregulated by >2.5 fold in rat cerebral cortex

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## Supplementary Table 2: Stroke-responsive piRNAs down-regulated by >2.5 fold in rat cerebral cortex

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## Supplementary table 3: Comprehensive list of all TFs and TF families showing hits on the 10 piRNA promoters

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**Legend:**
- CRE-binding protein 2/c-Jun heterodimer
- cAMP-responsive element binding proteins
- NKX homeodomain factors
- Krueppel like transcription factors
- Mouse Krueppel like factor
- Abdominal-B type homeodomain transcription factors
- GC-Box factors SP1/GC
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<td>SP2.01</td>
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<td>E2F.03</td>
<td>E2F, involved in cell cycle regulation, interacts with Rb p107 protein</td>
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<td>E2F-myc activator/cell cycle regulator</td>
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<td>PAX-PAX-2 binding sites</td>
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<td>PAX5.01</td>
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<td>PAX-BAX-5 binding sites</td>
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<td>CKROX.01</td>
<td>Collagen krox protein (zinc finger protein 67 - zfp67)</td>
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<td>EGR-EGR/nerve growth factor induced protein</td>
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<td>Wilms Tumor Suppressor</td>
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<td>EGR1.02</td>
<td>EGR1, early growth response 1</td>
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<td>PTX1.01</td>
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<td>Fork head homologous X binds DNA with a dual sequence specificity (FHXA and FHXB)</td>
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<td>STAT6.01</td>
<td>Signal transducer and activator of transcription</td>
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<td>STAT3.01</td>
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<td>ATF6.01</td>
<td>Member of b-zip family, induced by ER damage/stress, binds to the ERSE in association with NF-Y</td>
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<td>PTATA.02</td>
<td>Plant TATA box</td>
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<td>transcription factors below show no family affiliation, or if a family</td>
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<td>Ubiquitous GLI - Krueppel like zinc finger involved in cell cycle</td>
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<td>GAGA-Box</td>
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<td>AML1/CBFA2 Runt domain binding site</td>
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<td>MyoD/E47 and MyoD/E12 dimers</td>
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<td>Chicken ovalbumin upstream promoter (COUP-TF), DR0 sites</td>
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<td>Octamer binding protein</td>
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<td>Pdx1 (IDX1/IPF1) pancreatic and intestinal homeodomain TF</td>
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<td>Promyelocytic leukemia zink finger (TF with nine Krueppel-like zink fingers)</td>
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<td>C2H2 zinc finger protein PLZF</td>
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<td>Peroxisome proliferator activated receptor homodimers</td>
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<td>PRDI-BF1 and RIZ homologous (PR) domain proteins (PRDM)</td>
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<td>Ras-responsive element binding protein 1, Special AT-rich sequence-binding protein 1, predominantly expressed in thymocytes, binds to matrix attachment regions (MARs)</td>
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<td>keratinization and thymus epithelium differentiation</td>
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The TF matrix, TF family and TF family descriptions are presented according to the nomenclature of MatBase (Genomatix, GmbH). The number of hits for each transcription factor is presented in addition to the total number of hits for each TF family, which is obtained by combining the number of hits for each individual transcription factor in the family.
Supplementary Table 4: The top 20 TF families showing three or more promoter hits per family in a redundant manner

<table>
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<tr>
<th>TF family</th>
<th># of piRNA promoter hits</th>
<th># of individual piRNAs</th>
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<td>MYBL family</td>
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The TF family names are presented according to the nomenclature of MatBase (Genomatix, GmbH). The number of piRNA promoter hits for each family corresponds to the number of hits of all the family members combined, for all 10 promoters that were scanned. The third column corresponds to the total number of promoters out of 10 that showed hits by all members combined of a particular family. The highest and lowest number of promoters targeted by a single TF family is 7 and 1 respectively.
Supplementary Table 5: List of individual TFs that bind each of the 10 piRNA promoters

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The TF family names are presented according to the nomenclature of MatBase (Genomatix, GmbH).