Appendix Figures and Tables

Piwi/piRNAs control food intake by promoting neuropeptide F expression in locusts

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Appendix Figure S1 - Uncropped blots of all the antibodies that we used.

A, B Western blotting analysis of the protein extracts from brains and testes with anti-Piwi1 antibodies allow detection of proteins of the expected size (102 kDa).

C Immunoprecipitation (IP) assays were performed on the protein extracts from brains using anti-Piwi1 antibodies. IgG served as a negative IP control. Immunoprecipitates were analyzed by immunoblotting with anti-Piwi1 antibodies.

D Western blotting analysis of the protein extracts from testes with anti-Piwi2 antibodies allow detection of proteins of the expected size (96 kDa).

E Western blotting analysis of the protein extracts from brains with anti-Ago3 antibodies allow detection of proteins of the expected size (105.8 kDa).

F Western blotting analysis of the protein extracts from brains with anti-hnRNP F/H antibodies allow detection of proteins with the expected size (50.76 kDa).

G Western blotting analysis of the protein extracts from brains with U2AF65 antibodies allow detection of proteins with the expected size (50.76 kDa).
Appendix Figure S2 - Verification of RNAi efficiency.

A Verification of monoclonal antibodies using RNAi assays. Western blotting analysis of the protein extracts with anti-Piwi1, anti-Piwi2 and anti-Ago3 antibodies allow detection of proteins of the expected size (Piwi1, 102 kDa, in brains; Piwi2, 96 kDa, in testes; Ago3, 105.8 kDa, in brains).

B Verification of the mRNA expression of PIWI genes. The ribosomal protein RP49 was used as an endogenous control (In brain, dsPiwi1, n=6 biological replicates; dsPiwi2, n=4 biological replicates; dsAgo3, n=6 biological replicates. In testis, dsPiwi2, n=4 biological replicates).

Data information: The data are shown as the mean ± SEM (Student’s t-test, *P< 0.05, ***P< 0.001).
Appendix Figure S3 - Differential expression analysis of *dsPiwi1* and *dsGFP* in fat body transcriptomes using the DESeq2 method.

A Scatterplot of Benjamini-Hochberg adjusted *P* values in the log10 scale versus fold changes in the log2 scale.

B Scatterplot of gene expression (basemean in log2 scale) versus Benjamini-Hochberg adjusted *P* values in the log10 scale.

Basemean represents the average of the normalized count values that was divided by size factors across all samples. Plot visualization was achieved using R packages ggplot2.
Appendix Figure S4 - Logarithmic fold changes the expression of genes enriched in certain GO terms in dsPiwi1-treated locusts.

A Gene ontology enrichments for biological processes in the upregulated genes in the fat body following dsPiwi1 treatment.

B Gene ontology enrichments for biological processes in the upregulated genes in the brain following dsPiwi1 treatment.

The heatmap shows the gene ratio percentage and the number of differentially expressed genes for a given GO term/the number of all locust genes for a given GO term.
Appendix Figure S5 - Representative overall images of *dsPiwi1*-treated locusts and *dsGFP*-treated locusts on the fourth day of the fourth instar.
Appendix Figure S6 - Differential expression analysis of *dsPiwi1* and *dsGFP* in brain transcriptomes using the DESeq2 method.

A Scatterplot of Benjamini-Hochberg adjusted *P* values in the log10 scale versus fold changes in the log2 scale.

B Scatterplot of gene expression (basemean in log2 scale) versus Benjamini-Hochberg adjusted *P* values in the log10 scale.

Basemean represents the average of the normalized count values that was divided by size factors across all samples. Plot visualization was achieved using R packages ggplot2.
Appendix Figure S7 - qPCR expression analyses of *NPF1* (A), *CG10621* (B) and *MFS3* (C). The mRNA expression levels for these three genes were measured by qPCR after the dsPiwi1 treatments (n=6 biological replicates).

Data information: The data are shown as the mean ± SEM (Student’s t-test, *P*<0.05, **P**<0.01).
Appendix Figure S8 - Examination of candidate genes RNAi efficiency.

A Examination of the *NPF1* gene RNAi efficiency (n=6 biological replicates). The ribosomal protein RP49 was used as an endogenous control.

B Examination of the *CG10621* gene RNAi efficiency (n=6 biological replicates). The ribosomal protein RP49 was used as an endogenous control.

C Examination of the *MFS3* gene RNAi efficiency (n=6 biological replicates). The ribosomal protein RP49 was used as an endogenous control.

Data information: The data are shown as the mean ± SEM (Student’s t-test, **P<0.01, ***P<0.001).
Appendix Figure S9 - The influence of starvation on NPF1 gene expression in the brain. NPF1 expression was elevated in the locust brain after fasting for 12 h (n=6 biological replicates). The ribosomal protein RP49 was used as an endogenous control.

Data information: The data are shown as the mean ± SEM (Student’s t-test, *P< 0.05).
Appendix Figure S10 - Expression levels of piRNAs located within the flanking region of the *NPF1* gene in the *dsPiwi1*-treated locusts and *dsGFP*-treated control locusts (n=5 biological replicates). U6 was used as an endogenous control.

Data information: The data are shown as the mean ± SEM, ns means not significant (Student’s t-test).
Appendix Figure S11 - *NPF1* expression in the *in vivo* gain- and loss-of-function experiments.

A The mRNA expression level of *NPF1* in locusts injected with piRs-1 mimics and the negative control (n=5 biological replicates), NC means negative control. U6 was used as an endogenous control.

B The mRNA expression level of *NPF1* in locusts injected with piRs-2 inhibitors and the negative control (n=5 biological replicates), NC means negative control. U6 was used as an endogenous control.

C The mRNA expression level of *NPF1* in locusts injected with piRs-3-I1 mimics and the negative control (n=5 biological replicates), NC means negative control. U6 was used as an endogenous control.

Data information: The data are shown as the mean ± SEM, ns means not significant (Student’s t-test).
Appendix Figure S12 - Injection of piRs-3-I2 and piRs-3-I3 disrupts the normal metabolic functions of the fat body.

A Representative images and quantification of Nile red staining of fat body lipid droplets in locusts injected with piRs-3-I2 and piRs-3-I3 inhibitors and the negative control (n=7 biological replicates). The scale bars represent 50 µm.

B Measurement of TAG levels in the fat bodies of locusts injected with piRs-3-I2 and piRs-3-I3 inhibitors and the negative control (n=5 biological replicates), NC means negative control.

Data information: The data are shown as the mean ± SEM (Student’s t-test, *P<0.05).
Appendix Figure S13 - Read mapping summary of piRs-3-I3 variants. Only two mismatches were allowed. The piRNA variants of which read number are less than 15 are not shown.
Appendix Figure S14 - piRNAs can enter the nuclei of brain cells.

A, B Relative levels of anti-piRs-3-I2 and anti-piRs-3-I3 in the nucleus and cytoplasm after injecting inhibitors of piRs-3-I2 and piRs-3-I3 (n=4 biological replicates), NC means negative control. U6 was used as endogenous control.

Data information: The data are shown as the mean ± SEM (Student’s t-test, **P < 0.01, ***P < 0.001).
Appendix Figure S15 - Relative expression of piRs-3-I2 and piRs-3-I3 generated from total smRNA-seq libraries (n=3 replicate libraries).
Appendix Figure S16 - Verification of the hnRNP F/H monoclonal antibody.
Appendix Figure S17 - The fluorescence signals of proteins hnRNP F+H (A) and U2AF65 (B) were determined in locust brains. Hocheft33342 was used to label cell nuclei in locust brains. The scale bars represent 50 µm.
Appendix Figure S18 - Schematic organization of the branch site and its flanking regions. The stem #1 region (mutated pre-NPF1) was used for random mutational assays because the mutated nucleotides could not abolish the recognition sites of recursive splicing. The representative sequence of recursive splicing sites in fruit-fly long introns was inferred by the dominant nucleotides of position frequency matrix (PFM) retrieved from a previous study (Burnette et al., 2005).
Appendix Figure S19 - CUT&Tag qPCR analysis of the flanking regions of piRs-3-I3 binding site (A) and LM-Gypsy1 (B) in H3K9me3 immunoprecipitated DNAs from brain tissue extracts (n=3 biological replicates). Normal mouse IgG was used as a negative control.

Data information: The data are shown as the mean ± SEM (Student’s t-test, **P<0.01).
Supplementary Note

Non-specific effects of piRNA inhibitors

In siRNA/miRNA-based regulation, mature mRNAs are silenced via RNA-induced silencing complexes (RISCs) in cytoplasm. Because the piRs-3-I3 inhibitors are antisense to the intron region of pre-NPF1 transcripts, the complementary regions of piRs-3-I3 inhibitors are absent (intron removing) in the NPF1 transcript once they are exported to cytoplasm. Thus, siRNA/miRNA loaded RISCs is not able to binds the complementary regions of piRs-3-I3 inhibitors. Furthermore, we verified that the piRs-3-I3 inhibitors could enter the nuclei of brain cells (Appendix Figure S13). Our experimental results also showed that, although piRNA inhibitors are antisense to the intron region of pre-NPF1 transcripts, they were impossible to affect NPF1 expression in other conventional manners (for example, in siRNA/miRNA-based regulation). After the injections of the inhibitors piRs-3-I2 and piRs-3-I3, the expression levels of pre-NPF1 and mature NPF1 should be decreased in the siRNA/miRNA-based regulation, inconsistent with our experimental results in Fig 7C. In addition, the NPF1 expression was unaffected after the piRNA inhibitor treatments for the sense piRNAs located in the first intron region of NPF1 (Appendix Figure S10). Taken together, these results suggested the expression regulation based on piRNA inhibitors is less likely to be dependent on conventional siRNA/miRNA/piRNA-based regulation in cytoplasm.

Although a large majority of Ago protein studies focuses on their cytoplasmic functions, the nuclear Ago proteins do have been reported to serve multi-functional roles: Ago-mediated chromatin modification processes and RNA-mediated alteration of splicing (Huang and Li, RNA Biol, 2014). Thus, the nuclear piRNA inhibitors that are tethered to Ago proteins may affect gene expression independent of the interactions of piRNAs. The Ago-mediated chromatin modification processes can activate and inhibit transcription at the targeted promoter through altering chromatin marks, respectively. The binding site of piRs-3-I3 in locusts is located at intron region and is far away from promoter region. This excludes the possibility that our proposed
piRNA-mediated mechanism is achieved by the Ago-mediated chromatin modification processes. In RNA-mediated alteration of splicing, the regulation of alternative splicing by endogenous small RNAs is dependent on H3K9me3 mark deposition through the interactions with other chromatin components (Huang and Li, RNA Biol, 2014; Alló, Nat Struct Mol Biol, 2009). However, our results showed the binding site of piRs-3-I3 was devoid of H3K9me3 signals in the brains of locusts (Appendix Fig S1A-B), inconsistent with the observations in RNA-mediated alteration of splicing. Splice-switching oligonucleotides that are hybridized to pre-mRNA sequences can modulate alternative splicing by blocking access to the transcript by splicing factors (Bauman, Nucleic Acids Res, 2010). However, splice-switching oligonucleotides are more likely to only modulate alternative splicing but not to affect the gene expression (Figure 2A in the Bauman paper). This observation is contrary to our results that the administration of piRNA inhibitors resulted in the significant reduction of NPF1 expression. Taken together, these data suggest that the observed effects of piRNA inhibitors are not likely due to the mechanisms that have been reported previously.

CUT&Tag qPCR analysis

CUT&Tag experiments were performed as described previously (Kaya-Okur et al, 2019). The brains were isolated and immobilized to concanavalin A-coated beads (Novoprotein). The beads-bound cells were incubated with primary antibodies by rotating overnight. The resulting cells were incubated with secondary antibodies at room temperature for 1 h and then were washed 3 times with dig-wash buffer. Then cells were resuspended in dig-300 buffer (300 mM NaCl) with pA/G-Tn5 adapter complex at room temperature by rotating, followed by tagmentation at 37°C for1 h. Using these DNAs as templates, the levels of the flanking regions of piRs-3-I3 binding site and LM-Gypsy1 in the immunoprecipitates were measured as the relative enrichments in the anti-H3K9me3 (Abcam ab8898) antibody-treated sample compared with the anti-IgG antibody-treated sample. The transposable element LM-Gypsy1 was used for positive control.
## Appendix Tables

### Appendix Table S1. The sequences of differentially expressed piRNAs following *dsPiwi1* and *dsGFP* treatment.

| piRNA name | Sequence *(5’-3’)* |
|------------|--------------------|
| piRs-1-P1  | CGTTCGCGCAGACTCGGCC |
| piRs-1-P2  | AATGTTGCACTTGTTTTTTATCTC |
| piRs-1-P3  | TATAGTGACTAAGTGCAATGTTG |
| piRs-1-P4  | TTAATCTCAGCTGGCAACTG |
| piRs-1-P5  | TGGGCGGCGAGTGCGGGCA |
| piRs-1-P6  | GCTGACGAGTGCGGCGGACGG |
| piRs-1-P7  | TTGAATGTGATGATTGAATT |
| piRs-1-P8  | TTACAGAGAAAGAGCTCAACACATTGTA |
| piRs-1-P9  | TGTAGACATTTGACAGCAATAGACAA |
| piRs-2-I1  | TACACACAACCTCAACCGCTCTTAAGCAGT |
| piRs-2-I2  | TAGTTTCAGCACGGCAGCGCAAGAGGTGCTCT |
| piRs-2-I3  | TCAAGAGTAGACTGGGATATGATGACC |
| piRs-2-I4  | TCAACACTGAAAGAGCAAGCAAGACTGATGACCA |
| piRs-2-I5  | TCAACGTTGGGTGCTCTGACTATTGAGC |
| piRs-2-I6  | TAAGCAGTATTTCCCTGAGACGCTAG |
| piRs-2-I7  | TGTTGTGATACAGCTGACGA |
| piRs-3-I1  | TGAGATCATTTGAAAGCTGTAA |
| piRs-3-I2  | TCTGTGTGGGCTCTCTATCTCCTGATT |
| piRs-3-I3  | TGAAGTCAAACCAAGCTTTGATGGGGGGG |
| piRs-3-I4  | TTTTCTGGGCTGCCAACAAATGCTTGCTG |
| piRs-3-I5  | GGGGCGCTAGCTCAGATGGCC |

The sequence in black is the sense strand of the *NPF1* gene. The sequence in red is the antisense strand of the *NPF1* gene.
Appendix Table S2. Primers used in the qPCR and RNAi experiments.

| Gene name | Forward primer (5’-3’) | Reverse primer (5’-3’) |
|-----------|------------------------|-----------------------|
| GFP RT    | GTAAACCCGAAAGGGAATTGA  | GAAGAAACTGCATGGGCAAT  |
| GFP RNAi  | TAATACGACTCACTATAGG    | TAATACGACTCACTATAGG   |
| Piwi1 RT  | CAAAGTTCAGCGTGTCCG     | GTCACCTTGATGCGCTTC    |
| Piwi1 RNAi| TAATACGACTCACTATAGG    | TAATACGACTCACTATAGG   |
| Piwi2 RT  | CGAAACCACGAGAAAGAAGAG  | TCTGCAAAATGATCCAGCAT  |
| Piwi2 RNAi| TAATACGACTCACTATAGG    | TAATACGACTCACTATAGG   |
| Ago3 RT   | CCAGCAGCAGCAACCTACTTGCT| ACCGCAATGTAGTGTAATAGCT|
| Ago3 RNAi | TAATACGACTCACTATAGG    | TAATACGACTCACTATAGG   |
| NPF1 RT   | CGGCAAAAGCTGGAGAG     | CTTTCAATACGACAGCAATCC|
| NPF1 RNAi | TAATACGACTCACTATAGG    | TAATACGACTCACTATAGG   |
| CG10621 RT| ACGGCAACCCGGCTTGGA   | GACGCTCGCTTGATGTGTT  |
| CG10621 RNAi | TAATACGACTCACTATAGG | TAATACGACTCACTATAGG |
| MFS3 RT   | ACCGATGTGGGGCTACCT   | GCTGCTCAAACCCGGTC    |
| MFS3 RNAi | TAATACGACTCACTATAGG    | TAATACGACTCACTATAGG   |
| pri-NPF1 RT| ATTTACGGTGTTAAGGT    | AGCAATCAGCCACAGCAT   |
| pri-NPF1 RNAi | TACCTAGGGTCTCAGCGAGAATG | ATAGGGCAACCAGAGGGAGG |
| tRNA RT   | CGGTAATGCCTGGAGGAGGCT| GGAATGACGTCATCGACG    |
| RNase III | CATCAGATGACTGAAAGTAAA| TCATCAGAAGGAACTTGTAAT|

The sequence in red is the T7 promoter sequence.
Appendix References

Kaya-Okur HS, Wu SJ, Codomo CA, Pledger ES, Bryson TD, Henikoff JG, Ahmad K, Henikoff S (2019) CUT&Tag for efficient epigenomic profiling of small samples and single cells. *Nat Commun* 10: 1930.

Burnette JM, Miyamoto-Sato E, Schaub MA, Conklin J, Lopez AJ (2005) Subdivision of large introns in *Drosophila* by recursive splicing at nonexonic elements. *Genetics* 170: 661-674.

Huang V, Li LC (2014) Demystifying the nuclear function of Argonaute proteins. *RNA Biol* 11: 18-24.

Allo M, Buggiano V, Fededa JP, Petrillo E, Schor I, de la Mata M, Agirre E, Plass M, Eyras E, Elela SA et al (2009) Control of alternative splicing through siRNA-mediated transcriptional gene silencing. *Nat Struct Mol Biol* 16: 717-724.

Bauman JA, Li SD, Yang A, Huang L, Kole R (2010) Anti-tumor activity of splice-switching oligonucleotides. *Nucleic Acids Res* 38: 8348-8356.