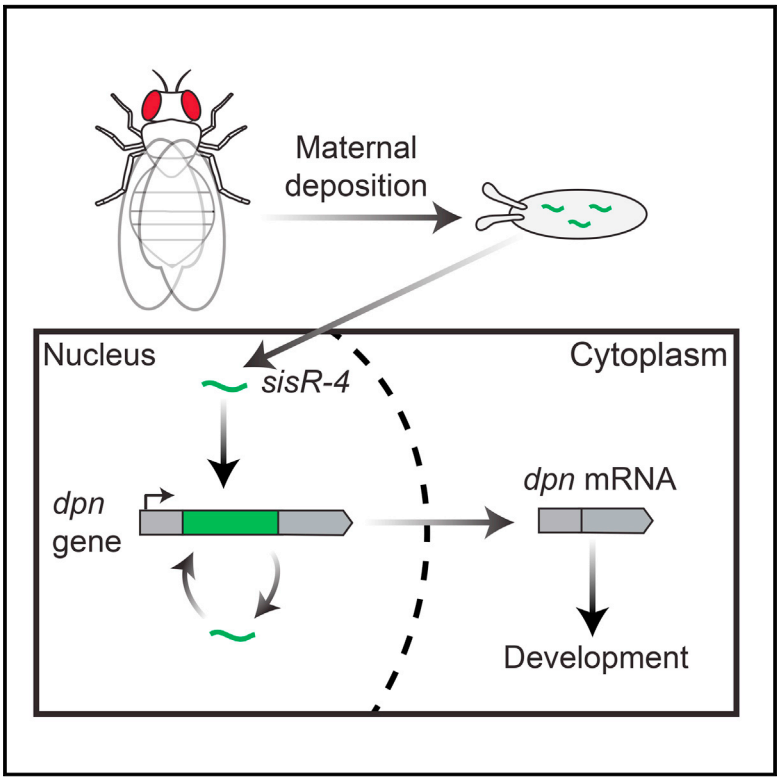
Report

Current Biology

# Maternally Inherited Stable Intronic Sequence RNA Triggers a Self-Reinforcing Feedback Loop during Development

## Graphical Abstract




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## In Brief

Stable intronic sequence RNAs are maternally deposited in frogs and fruit flies, suggesting that they may regulate gene expression. Tay and Pek show that the deadpan intron encodes a maternally inherited intronic RNA that promotes embryonic development by enhancing host gene transcription in fruit flies.

## Highlights

- Deadpan intron encodes a sisRNA sisR-4
- Maternally inherited sisR-4 is required for development
- sisR-4 promotes transcription of its host gene deadpan

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Current Biology
Report

# Maternally Inherited Stable Intronic Sequence RNA Triggers a Self-Reinforcing Feedback Loop during Development

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## SUMMARY

**Maternally inherited noncoding RNAs (ncRNAs) can regulate zygotic gene expression across generations [1–4]. Recently, many stable intronic sequence RNAs (sisRNAs), which are byproducts of pre-mRNA splicing, were found to be maternally deposited and persist till zygotic transcription in *Xenopus* and *Drosophila* [5–7]. In various organisms, sisRNAs can be in linear or circular conformations, and they have been suggested to regulate host gene expression [5–10]. It is unknown whether maternally deposited sisRNAs can regulate zygotic gene expression in the embryos. Here, we show that a maternally inherited sisRNA (*sisR-4*) from the *deadpan* locus is important for embryonic development in *Drosophila*. Mothers, but not fathers, mutant for *sisR-4* produce embryos that fail to hatch. During embryogenesis, *sisR-4* promotes transcription of its host gene (*deadpan*), which is essential for development. Interestingly, *sisR-4* functions by activating an enhancer present in the intron where *sisR-4* is encoded. We propose that a maternal sisRNA triggers expression of its host gene via a positive feedback loop during embryogenesis.**

## RESULTS AND DISCUSSION

To look for maternally inherited circular stable intronic sequence RNAs (sisRNAs) in *Drosophila*, we treated RNA from 0 to 2 hr embryos with RNase R, a 3′ to 5′ exoribonuclease that degrades linear RNA, leaving behind circular RNA [6, 9, 10]. By performing strand-specific deep sequencing, we identified five RNase R-resistant sisRNAs (Figures 1A and S1; Table S1). On the genome browser, the position of the peaks within the introns display characteristics of circular sisRNAs derived from splicing. The 5′ ends of the peaks are close to the 5′ splice sites, whereas the 3′ ends coincide with the branch points (Figures 1A and S1) [6, 9]. Circular conformation was confirmed by RT-PCR using outward-pointing primers detecting the most abundant sisRNA from the *CG9775* locus (Figure S2A). Next, we confirmed that two of these sisRNAs from the *CG9775* and *deadpan* (*dpn*) loci are maternally deposited by their presence in unfertilized eggs (Figures 1A, 1B, and S2B).

We focused our attention on the sisRNAs from the *dpn* intron. The *dpn* locus encodes a conserved basic helix-loop-helix transcription factor essential for development [11–14]. On the genome browser, two peaks are present in the *dpn* intron in the RNase R treated sample (Figure 1A), suggesting two circular molecules as confirmed by RT-PCR following RNase R treatment (Figure 1C). The abundance of *dpn* sisRNAs is too low to perform RT-PCR using outward pointing primers because reverse transcription across the branch-point is a very inefficient process. As a proof-of-principle that RNase R-resistant sisRNAs are circular, we had used the most abundant circular sisRNA of *CG9775* (Figure S2A). The pattern of the intronic peaks is consistent with the idea that these circular sisRNAs are generated by recursive splicing [15]. A recursive splice site (RSS; AG/GT) is present right at the valley of the two peaks, and two branch points are present just before the 3′ splice sites (Figure 1A). The expression of *dpn* sisRNAs is high in early (2–14 hr) embryos and drops during late (14–24 hr) embryogenesis, correlating with the expression of the *dpn* mRNA (Figure 1D). Incidentally, the *dpn* locus also contains an antisense long noncoding RNA (ncRNA) *CR44275* that overlaps at the 3′ end of the *dpn* 3′ sisRNA, hereafter called *sisR-4* (Figure 1E). *CR44275* is expressed in early and late embryos but hardly detectable in unfertilized eggs by strand-specific RT-PCR (Figures 1F and 1G). The *dpn* second intron, which encodes sisRNAs, is generally more highly conserved than the first intron. Within the second intron, the region that encodes for the *dpn* 5′ sisRNA is more highly conserved than *sisR-4*, suggesting that it may also be functionally important. The 5′ and 3′ ends of *sisR-4* are highly conserved, but not the middle portion. *CR44275* is highly conserved because it overlaps largely with the exonic region of *dpn* and 3′ end of *sisR-4* (Figure 1E).

An insertion mutant $dpn^{MI00051}$, which disrupts *sisR-4*, was used to investigate the function of *sisR-4* (Figure 2A). The $dpn^{MI00051}$ line contains a Minos transposon insertion at a conserved region of *sisR-4* and greatly reduces *sisR-4* expression and to a lesser extent *CR44275* (Figure 2B). We investigated whether *sisR-4* is required for embryonic development by examining $dpn^{MI00051}$ homozygous mutant embryos. Mated female flies were allowed to lay eggs for 10 hr before the eggs were collected and allowed to develop for another 14 hr. Thereafter, hatching of embryos was examined at 2 hr intervals starting at the 2 hr time point (Figure 2C). The hatching rate for $dpn^{MI00051}$ embryos was significantly lower than that of the

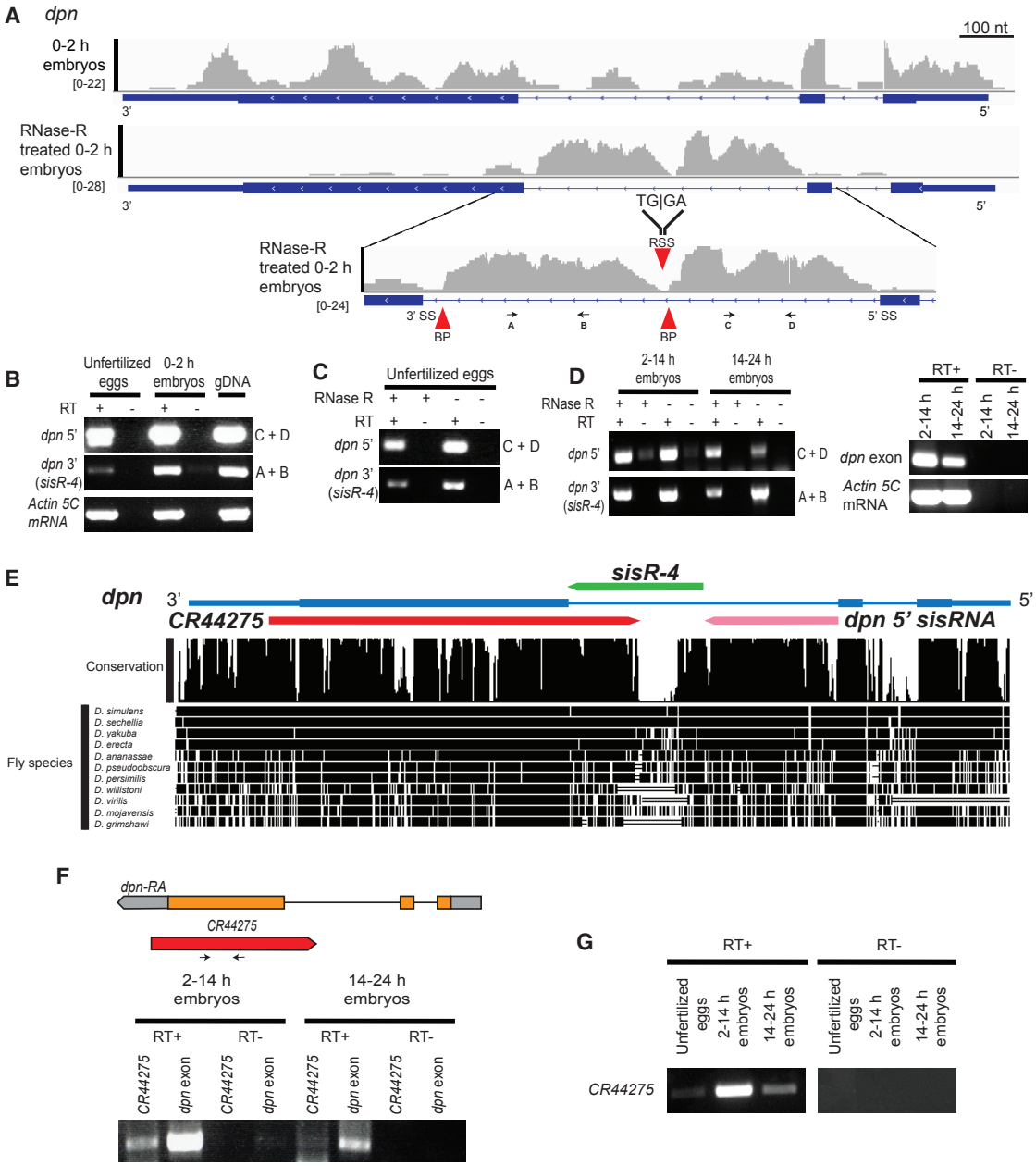



**Figure 1. sisRNAs from the *dpn* Intron Are Maternally Deposited and Expressed during Embryogenesis**

(A) A genome browser view of the *dpn* locus in untreated [7] and RNase-R-treated 0–2 hr embryos. BP, branch point; RSS, recursive splice site. Arrows labeled A–D depict the primers used for PCR.

(B) RT-PCR showing the expression of *dpn* sisRNAs (both 5′ and 3′) in unfertilized eggs and 0–2 hr embryos. Genomic DNA (gDNA) was used as a positive control.

(C) RT-PCR showing the presence of RNase-R-resistant *dpn* sisRNAs in unfertilized eggs.

(D) RT-PCR showing the expression of *dpn* sisRNAs (both 5′ and 3′) and *dpn* mRNA in 2–14 hr and 14–24 hr embryos.

(E) Conservation of the *dpn* locus in different species of *Drosophila*.

(F) Strand-specific RT-PCR showing the expression of *CR44275* and *dpn* mRNA in 2–14 hr and 14–24 hr embryos.

(G) Strand-specific RT-PCR showing the expression of *CR44275* in unfertilized eggs, 2–14 hr embryos, and 14–24 hr embryos.

See also Figures S1 and S2 and Table S1.

controls (Figures 2D and 2E), indicating that *sisR-4* is required for embryonic development. Next, we investigated whether maternal *sisR-4* is required for embryonic development. Indeed, when we crossed $dpn^{MI00051}$ females with *y w* males, the resulting embryos had a very low hatching rate as compared to crossing *y w* females with $dpn^{MI00051}$ males (Figures 2F and 2G). Embryos derived from maternal $dpn^{MI00051}$ mutants expressed lower levels of *sisR-4* compared to those derived from paternal $dpn^{MI00051}$ mutants (Figure 2H). These results suggest that maternally deposited *sisR-4* is required for embryonic development.

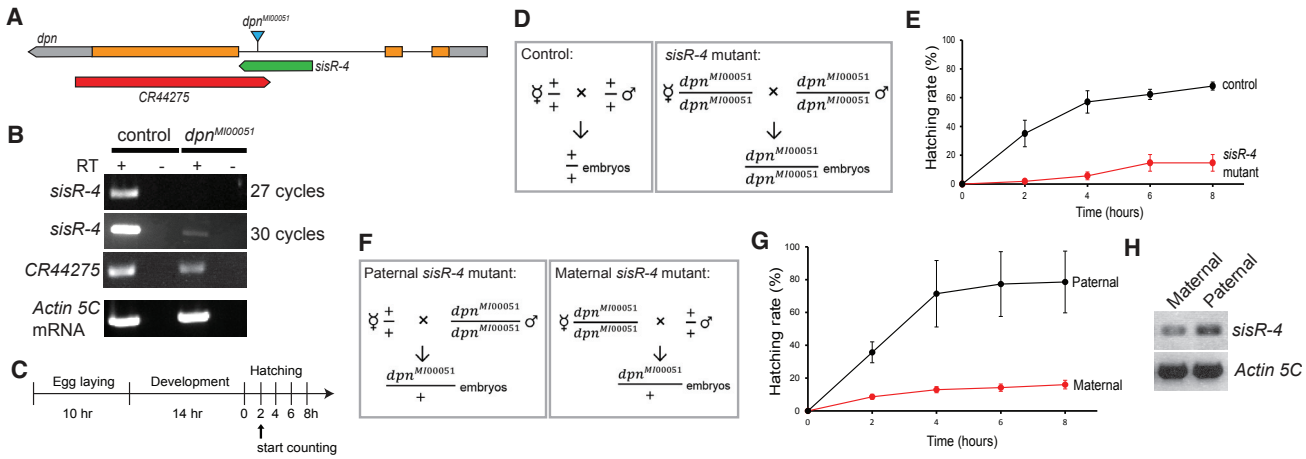



**Figure 2. Maternally Deposited *sisR-4* Is Required for Embryonic Development**

(A) Gene locus showing the position of the Minos transposon insertion in the *sisR-4*-encoded intron.

(B) RT-PCR showing the expression of *sisR-4* and *CR44275* in *y w* control and $dpn^{MI00051}$ 2–14 hr embryos. *Actin5C* mRNA was used as a loading control.

(C) Experimental scheme for hatching rate analyses.

(D) Cross scheme for generating $dpn^{MI00051}$ mutant embryos.

(E) Chart showing the hatching rates of *y w* control and $dpn^{MI00051}$ mutant embryos. Error bars depict SD from three biological replicates.

(F) Cross scheme for generating maternal or paternal $dpn^{MI00051}$ mutant embryos.

(G) Chart showing the hatching rates of embryos from maternal or paternal $dpn^{MI00051}$ mutants. Error bars depict SD from three biological replicates.

(H) RT-PCR showing the relative expression of *sisR-4* in $dpn^{MI00051}$/+ 2–14 hr embryos from $dpn^{MI00051}$ mutant fathers (paternal) and $dpn^{MI00051}$ mutant mothers (maternal).

To confirm whether the embryonic developmental phenotype of $dpn^{MI00051}$ is indeed due to the lack of *sisR-4*, we performed a rescue assay by restoring *sisR-4* expression in $dpn^{MI00051}$ mutants. We generated transgenic flies harboring *UASt-dsRed-sisR-4-encoding intron-myc* (Figure 3A), a method that was previously used to express sisRNAs [7]. Expression of this transgene led to an increase in circular (RNase-R-resistant) *sisR-4* expression in 2–14 hr embryos (Figures 3B and 3C).

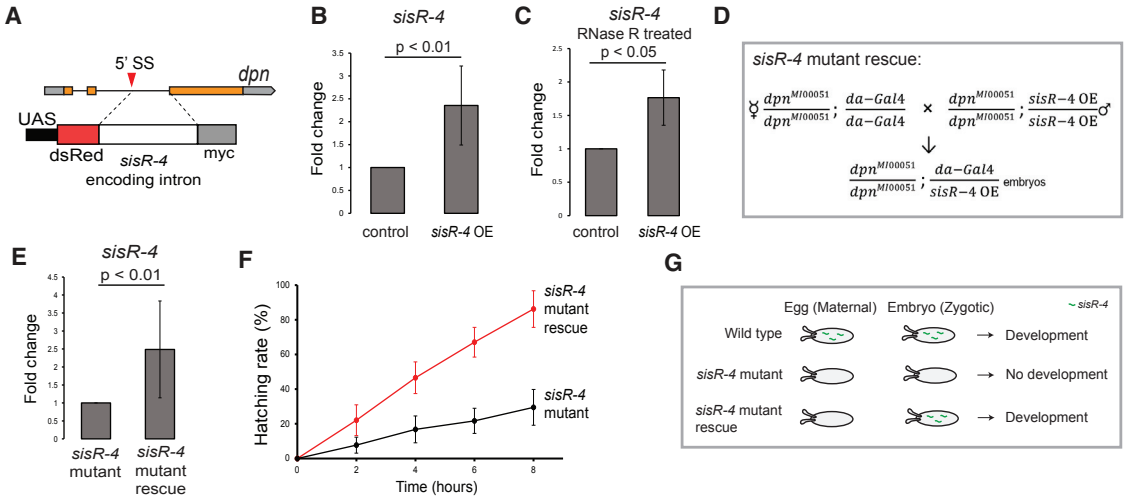



**Figure 3. Rescue of $dpn^{MI00051}$ Mutants by Zygotic Expression of *sisR-4***

(A) Construct used to express *sisR-4*. The intronic region used to express *sisR-4* is also shown.

(B and C) qRT-PCR showing the relative expression of *sisR-4* in *da-Gal* control and *da-Gal4>sisR-4* overexpression 2–14 hr embryos. Total RNA (B) and RNase-R-treated RNA (C) are shown. Two-tailed t test was performed.

(D) Cross scheme for zygotic expression of *sisR-4* in $dpn^{MI00051}$ mutants.

(E) qRT-PCR showing the relative expression of *sisR-4* in $dpn^{MI00051}$ and $dpn^{MI00051}$; *da-Gal4>sisR-4* overexpression 2–14 hr embryos. Two-tailed t test was performed.

(F) Chart showing the hatching rates of $dpn^{MI00051}$ mutant and $dpn^{MI00051}$; *da-Gal4>sisR-4* embryos. Error bars depict SD from three biological replicates.

(G) Diagram showing that maternally inherited *sisR-4* is required during embryogenesis.

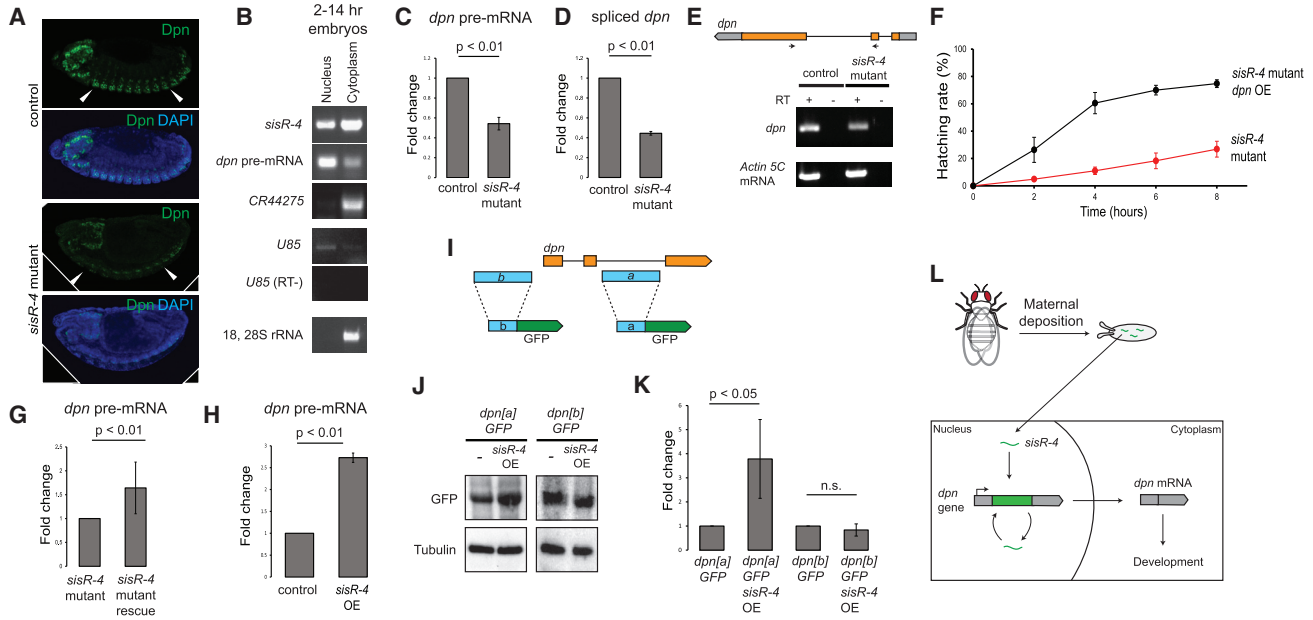



**Figure 4. *sisR-4* Promotes *dpn* Transcription via an Intronic Enhancer**

(A) Confocal sections showing expression patterns of Deadpan (green) and DAPI (blue) in early embryos. Arrowheads point to ventral neuroblasts.

(B) RT-PCR showing the presence of *sisR-4* in both nuclear and cytoplasmic fractions of 2–14 hr embryos. *CR44275* localizes to the cytoplasm. The *dpn* pre-mRNA and *U85* were used as nuclear controls, and 18, 28S rRNA was used as cytoplasmic control.

(C and D) qRT-PCR showing the relative expression of (C) *dpn* pre-mRNA and (D) spliced *dpn* mRNA in control and $dpn^{MI00051}$ 2–14 hr embryos.

(E) RT-PCR showing the fidelity of splicing in $dpn^{MI00051}$ 2–14 hr embryos.

(F) Chart showing the hatching rates of $dpn^{MI00051}$ mutant and $dpn^{MI00051}$; *da-Gal4>dpn* embryos. Error bars depict SD from three biological replicates.

(G) qRT-PCR showing the relative expression of *dpn* pre-mRNA in $dpn^{MI00051}$ and $dpn^{MI00051}$; *da-Gal4>sisR-4* overexpression 2–14 hr embryos. Two-tailed t test was performed.

(H) qRT-PCR showing the relative expression of *dpn* pre-mRNA in control and *da-Gal4>sisR-4* overexpression 2–14 hr embryos. Two-tailed t test was performed.

(I) The *dpn* gene locus showing the regions of the enhancer used for GFP reporter constructs in (J). Not drawn to scale.

(J) Western blots showing the abundance of GFP in 2–14 hr embryos containing *dpn*[*a*]*GFP* or *dpn*[*b*]*GFP* transgenes, with and without overexpression of *sisR-4*. Tubulin was used as a loading control.

(K) Graph showing the relative expression of GFP normalized to tubulin shown in (J). Error bars depict SD from three biological replicates. Two-tailed t test was performed.

(L) A proposed model of how maternally inherited *sisR-4* can trigger a self-amplification loop in the expression of *dpn* to achieve robust embryonic development in *Drosophila*.

See also Figures S3 and S4 and Table S2.

Importantly, zygotic expression of *sisR-4* rescued the embryonic developmental phenotype of $dpn^{MI00051}$ embryos (Figures 2D and 3D–3F). These data indicate that zygotic expression of *sisR-4* is sufficient to rescue the lack of maternal *sisR-4* (Figure 3G), suggesting that maternal *sisR-4* plays a role by regulating gene expression during embryonic development. We did not manage to generate functional transgenic flies to knockdown or overexpress *CR44275*, precluding our analysis on *CR44275*. Nevertheless, maternal *CR44275* is hardly detectable as compared to *sisR-4*, implicating a major contribution of maternal *sisR-4* during early development.

Because the *sisR-4* host gene *dpn* is essential for development [11–14], and circular sisRNAs have been shown to positively regulate the transcription of their host genes [9, 16], we hypothesized that *sisR-4* may regulate embryonic development, at least in part, by promoting its host gene transcription. In *sisR-4* mutants, the expression pattern of Dpn protein was perturbed, especially in the ventral neuroblasts (Figure 4A, arrowheads), suggesting that *sisR-4* regulates embryonic development, at least in part, by promoting Dpn expression. During early embryogenesis, *sisR-4* is present in both the nucleus and cytoplasm (Figure 4B), similar to what was seen in *Xenopus* oocytes [6]. On the other hand, *CR44275* is mainly detected in the cytoplasm. In $dpn^{MI00051}$ 2–14 hr embryos, the levels of *dpn* pre-mRNA were decreased by ~50% compared to controls (Figure 4C). This was accompanied by a decrease of a similar degree in spliced *dpn* mRNA (Figure 4D), suggesting a transcriptional, but not a splicing, defect. It may also suggest that *sisR-4* regulates the stability of *dpn* pre-mRNA. Furthermore, the Minos insertion did not interfere with the splicing of *dpn* pre-mRNA (Figure 4E), excluding the possibility that the drop in *dpn* pre-mRNA in $dpn^{MI00051}$ embryos was due to degradation of improperly spliced Minos-containing *dpn* pre-mRNA. Importantly, overexpression of *dpn* rescued the lethality phenotype of *sisR-4* mutants (Figure 4F), indicating that *sisR-4* regulates development, at least in part, by promoting *dpn* expression. It is possible that *sisR-4* may regulate other genes that act together with *dpn* to control embryogenesis.

Expression of *sisR-4* in $dpn^{MI00051}$ embryos was sufficient to restore the levels of *dpn* pre-mRNA (Figure 4G), supporting the idea that *sisR-4* promotes *dpn* transcription. To corroborate the idea that *sisR-4* regulates *dpn* transcription, we next asked whether overexpression of *sisR-4* in the wild-type background alone is sufficient to induce *dpn* pre-mRNA expression. Indeed, overexpression of *sisR-4* led to an increase in *dpn* pre-mRNA expression (Figure 4H). Several enhancer sequences had been found to control *dpn* transcription. Interestingly, one of them is the intron that encodes for *sisR-4* [17–19]. This intronic enhancer is active and adopts an open chromatin structure (high sensitivity to DNase) during early embryonic development (Figure S3) [18, 19]. To test whether *sisR-4* regulates *dpn* transcription via the intronic enhancer, we examined two GFP reporter lines that are controlled by either the intronic enhancer [a] or an upstream enhancer [b] (Figure 4I) [17]. Overexpression of *sisR-4* in 2–14 hr embryos led to an upregulation of GFP expression in the *dpn*[*a*]*GFP* line, but not in the *dpn*[*b*]*GFP* line (Figures 4J and 4K), showing that *sisR-4* promotes *dpn* transcription by regulating the *dpn* intronic enhancer. Although this study focuses on a nuclear role of *sisR-4* in regulating parental gene transcription, the observation that both *sisR-4* and *CR44275* also localize to the cytoplasm suggests that *sisR-4* and *CR44275* may have additional regulatory roles.

We propose that maternally inherited *sisR-4* functions to promote transcription of *dpn* during early embryonic development. Transcription of *dpn* in turn produces more *sisR-4*, which triggers an amplification loop. This process results in a boost in the production of the *dpn* gene product that is required for embryonic development (Figure 4L). This paradigm of a maternally deposited ncRNA mediating an amplification loop is similar to that for Piwi-interacting RNAs (piRNAs) in the *Drosophila* germline. In one case, maternal piRNAs enter the ping-pong cycle to generate more secondary piRNAs [4], whereas in another case, a maternal piRNA bound to Piwi promotes the transcription of the piRNA precursor, thus generating more piRNAs, leading to a self-amplification loop [20].

In *Drosophila* and *Caenorhabditis elegans*, maternal piRNAs had been shown to activate gene expression in the progeny [2, 3, 20]. Another example of host gene activation by maternal RNA is the *fem-1* RNA in *C. elegans* [1]. The mechanism for *fem-1* RNA-mediated epigenetic gene licensing appears to be different from that of *sisR-4* because the intronic sequence of *fem-1* is not required for activation. Maternally inherited sisRNAs may play a general role in promoting host gene expression. Half of the previously identified maternal sisRNAs in total RNA samples (15/29, including the *fem-1* sisRNA) [7] map to regions of high DNase sensitivity during early development (Figure S4; Table S2). In general, sisRNAs may provide a potential mechanism for the phenomenon of intron-mediated enhancement seen in plants and animals [21]. Recent studies have suggested that long ncRNAs can function to recruit transcription factors to regulate gene expression [22, 23]. Thus, it is conceivable that sisRNAs may function to recruit transcription factors to intronic enhancers in a sequence-specific manner or establish a permissive chromatin structure for transcription. With growing evidence of enhancer RNAs derived from introns [24], the promotion of zygotic gene activity by maternally deposited sisRNAs may be a conserved mechanism in metazoans and plants.

## ACCESSION NUMBERS

The accession number for the deep-sequencing data reported in this paper is GEO: GSE89496.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2017.02.040.

## AUTHOR CONTRIBUTIONS

J.W.P. conceived the project. M.L.T. and J.W.P. performed the experiments and wrote the paper.

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## REFERENCES

1. Johnson, C.L., and Spence, A.M. (2011). Epigenetic licensing of germline gene expression by maternal RNA in C. elegans. Science *333*, 1311–1314.
2. Seth, M., Shirayama, M., Gu, W., Ishidate, T., Conte, D., Jr., and Mello, C.C. (2013). The C. elegans CSR-1 argonaute pathway counteracts epigenetic silencing to promote germline gene expression. Dev. Cell *27*, 656–663.
3. Wedeles, C.J., Wu, M.Z., and Claycomb, J.M. (2013). Protection of germline gene expression by the C. elegans Argonaute CSR-1. Dev. Cell *27*, 664–671.
4. Brennecke, J., Malone, C.D., Aravin, A.A., Sachidanandam, R., Stark, A., and Hannon, G.J. (2008). An epigenetic role for maternally inherited piRNAs in transposon silencing. Science *322*, 1387–1392.
5. Gardner, E.J., Nizami, Z.F., Talbot, C.C., Jr., and Gall, J.G. (2012). Stable intronic sequence RNA (sisRNA), a new class of noncoding RNA from the oocyte nucleus of Xenopus tropicalis. Genes Dev. *26*, 2550–2559.
6. Talhouarne, G.J., and Gall, J.G. (2014). Lariat intronic RNAs in the cytoplasm of Xenopus tropicalis oocytes. RNA *20*, 1476–1487.
7. Pek, J.W., Osman, I., Tay, M.L., and Zheng, R.T. (2015). Stable intronic sequence RNAs have possible regulatory roles in Drosophila melanogaster. J. Cell Biol. *211*, 243–251.
8. Osman, I., Tay, M.L., and Pek, J.W. (2016). Stable intronic sequence RNAs (sisRNAs): a new layer of gene regulation. Cell. Mol. Life Sci. *73*, 3507–3519.
9. Zhang, Y., Zhang, X.O., Chen, T., Xiang, J.F., Yin, Q.F., Xing, Y.H., Zhu, S., Yang, L., and Chen, L.L. (2013). Circular intronic long noncoding RNAs. Mol. Cell *51*, 792–806.
10. Lu, Z., Filonov, G.S., Noto, J.J., Schmidt, C.A., Hatkevich, T.L., Wen, Y., Jaffrey, S.R., and Matera, A.G. (2015). Metazoan tRNA introns generate stable circular RNAs in vivo. RNA *21*, 1554–1565.
11. Bier, E., Vaessin, H., Younger-Shepherd, S., Jan, L.Y., and Jan, Y.N. (1992). deadpan, an essential pan-neural gene in Drosophila, encodes a helix-loop-helix protein similar to the hairy gene product. Genes Dev. *6*, 2137–2151.

12. Southall, T.D., and Brand, A.H. (2009). Neural stem cell transcriptional networks highlight genes essential for nervous system development. EMBO J. *28*, 3799–3807.
13. Younger-Shepherd, S., Vaessin, H., Bier, E., Jan, L.Y., and Jan, Y.N. (1992). deadpan, an essential pan-neural gene encoding an HLH protein, acts as a denominator in Drosophila sex determination. Cell *70*, 911–922.
14. Barbash, D.A., and Cline, T.W. (1995). Genetic and molecular analysis of the autosomal component of the primary sex determination signal of Drosophila melanogaster. Genetics *141*, 1451–1471.
15. Burnette, J.M., Miyamoto-Sato, E., Schaub, M.A., Conklin, J., and Lopez, A.J. (2005). Subdivision of large introns in Drosophila by recursive splicing at nonexonic elements. Genetics *170*, 661–674.
16. Li, Z., Huang, C., Bao, C., Chen, L., Lin, M., Wang, X., Zhong, G., Yu, B., Hu, W., Dai, L., et al. (2015). Exon-intron circular RNAs regulate transcription in the nucleus. Nat. Struct. Mol. Biol. *22*, 256–264.
17. Babaoğlan, A.B., Housden, B.E., Furriols, M., and Bray, S.J. (2013). Deadpan contributes to the robustness of the notch response. PLoS ONE *8*, e75632.
18. Kvon, E.Z., Kazmar, T., Stampfel, G., Yáñez-Cuna, J.O., Pagani, M., Schernhuber, K., Dickson, B.J., and Stark, A. (2014). Genome-scale functional characterization of Drosophila developmental enhancers in vivo. Nature *512*, 91–95.
19. Jenett, A., Rubin, G.M., Ngo, T.T., Shepherd, D., Murphy, C., Dionne, H., Pfeiffer, B.D., Cavallaro, A., Hall, D., Jeter, J., et al. (2012). A GAL4-driver line resource for Drosophila neurobiology. Cell Rep. *2*, 991–1001.
20. Yin, H., and Lin, H. (2007). An epigenetic activation role of Piwi and a Piwi-associated piRNA in Drosophila melanogaster. Nature *450*, 304–308.
21. Gallegos, J.E., and Rose, A.B. (2015). The enduring mystery of intron-mediated enhancement. Plant Science *237*, 8–15.
22. Lee, N., Moss, W.N., Yario, T.A., and Steitz, J.A. (2015). EBV noncoding RNA binds nascent RNA to drive host PAX5 to viral DNA. Cell *160*, 607–618.
23. Sigova, A.A., Abraham, B.J., Ji, X., Molinie, B., Hannett, N.M., Guo, Y.E., Jangi, M., Giallourakis, C.C., Sharp, P.A., and Young, R.A. (2015). Transcription factor trapping by RNA in gene regulatory elements. Science *350*, 978–981.
24. Li, W., Notani, D., and Rosenfeld, M.G. (2016). Enhancers as non-coding RNA transcription units: recent insights and future perspectives. Nat. Rev. Genet. *17*, 207–223.

**Current Biology, Volume *27***

## Supplemental Information

# Maternally Inherited Stable Intronic Sequence RNA Triggers a Self-Reinforcing Feedback Loop during Development

**Mandy Li-Ian Tay and Jun Wei Pek**

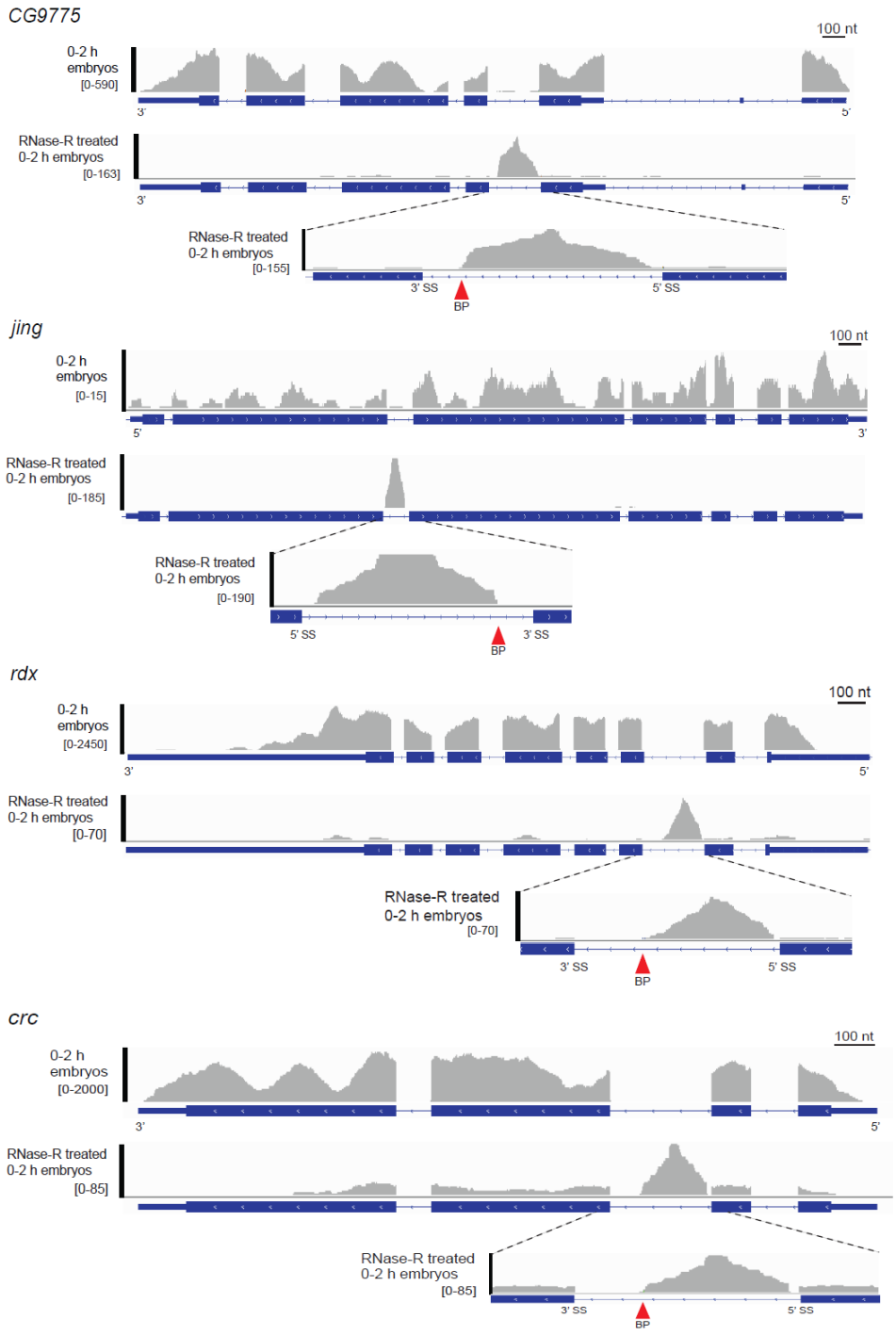



**Figure S1. Identification of candidate circular sisRNAs in 0-2 hr embryos. Related to Figure 1.** Genome browser views of the *CG9775*, *rdx*, *jing* and *crc* loci in untreated and RNase R-treated 0-2 hr embryos. BP: branch point.

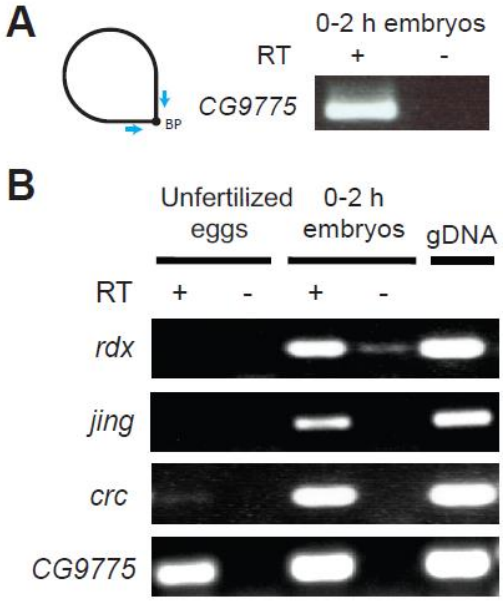



**Figure S2. Characterization of circular sisRNAs. Related to Figure 1.** (A) RT-PCR using outward pointing primers to detect circular sisRNA from *CG9775* intron. (B) RT-PCR showing the expression of *CG9775* sisRNAs in unfertilized eggs and 0-2 hr embryos. Genomic DNA (gDNA) was used as a positive control.

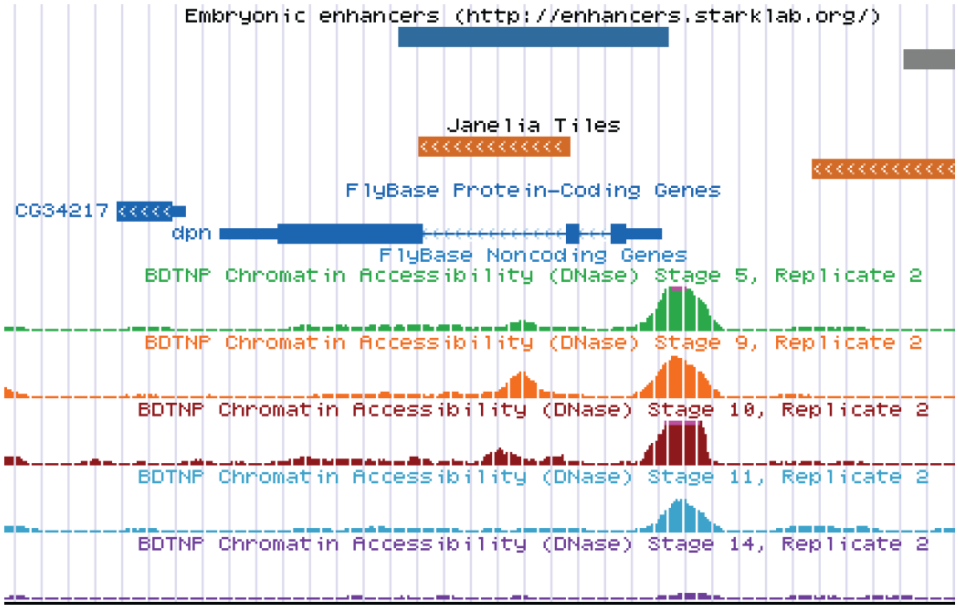



**Figure S3. An enhancer in the *dpn* intron. Related to Figure 4.** Genome browser view of the *dpn* locus showing the enhancer regions that encompass the intronic sequences identified by the Rubin and Stark laboratories. It also shows the chromatin accessibility (by DNase) during different stages of embryonic development.

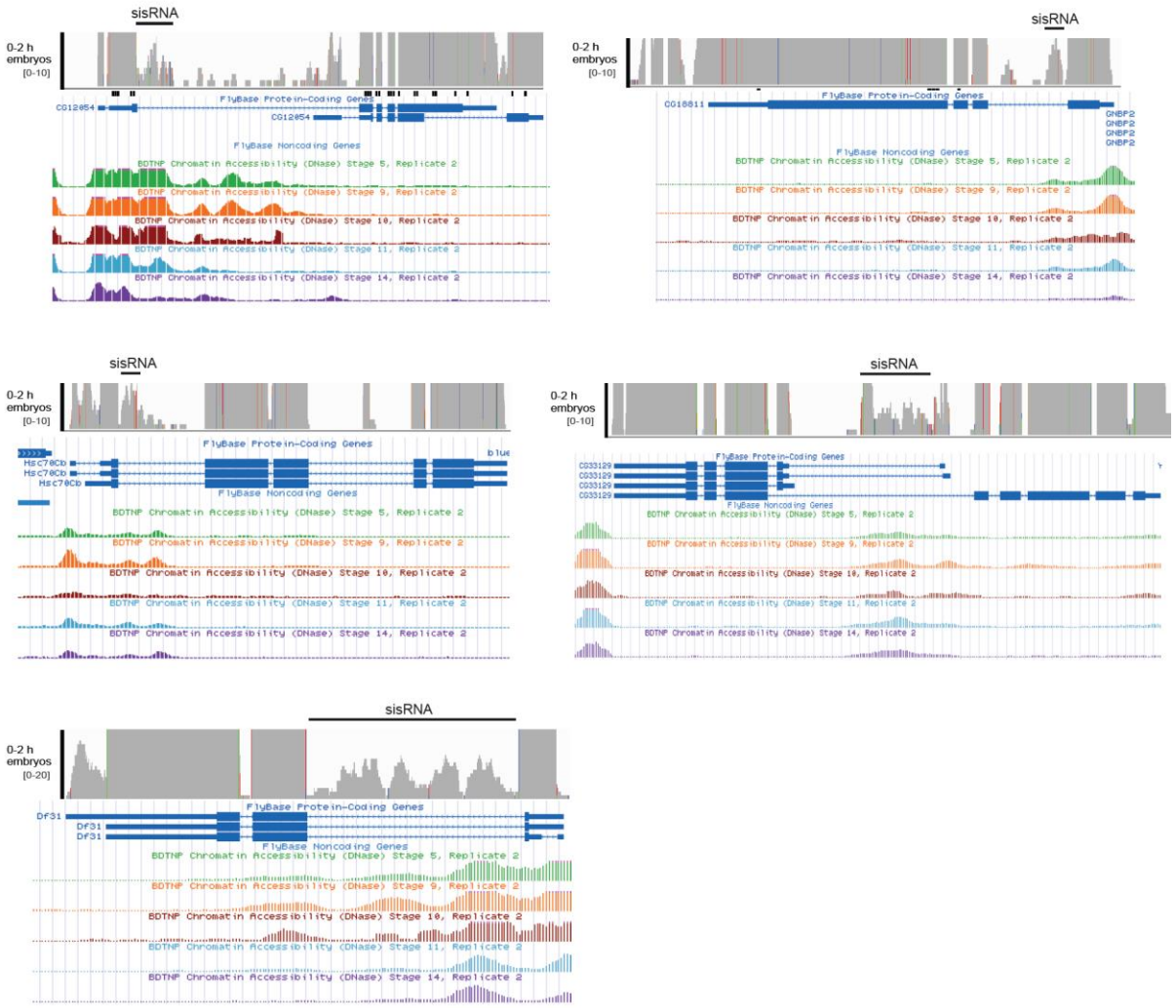



**Figure S4. Genome browser views of 0-2 hr sisRNAs (horizontal line) and the corresponding regions of high chromatin accessibility (open chromatin) during embryogenesis. Related to Figure 4.**

**Table S1. List of RNase R resistant sisRNAs identified in 0-2 hr embryos. Related to Figure 1.**

| Chromosome | Gene | Coordinates | Remarks |
|---|---|---|---|
| 3R | *rdx* | chr3R:9,796,045-9,797,096 | |
| 2R | *jing* | chr2R:2,502,933-2,503,729 | |
| 3R | *crc* | chr3R:5,455,809-5,456,334 | |
| 3R | *CG9775* | chr3R:463,508-467,716 | |
| 2R | *dpn* | chr2R:4,114,617-4,121,973 | *sisR-4* |
| 2R | *dpn* | chr2R:4,115,157-4,122,513 | *dpn 5'* |

**Table S2. List of maternally inherited sisRNAs with a corresponding presence of increased chromatin accessibility during embryogenesis. Related to Figure 4.**

| No. | Chromosome | Host gene | CG number | Coordinates of the approximate center of sisRNAs |
|---|---|---|---|---|
| 1 | X | *PPP4R2r* | CG2890 | chrX:10,373,299 |
| 2 | 2L | *Df31* | CG2207 | chr2L:21,628,530 |
| 3 | 2L | *RpS13* | CG13389 | chr2L:8,364,446 |
| 4 | 2L | | CG33129 | chr2L:10,967,648 |
| 5 | 2R | *Mlf* | CG8295 | chr2R:11,825,014 |
| 6 | 2R | *Khc* | CG7765 | chr2R:12,158,613 |
| 7 | 2R | *betaTub56D* | CG9277 | chr2R:15,338,652 |
| 8 | 2R | *Fem-1* | CG9025 | chr2R:16,553,821 |
| 9 | 2R | *zip* | CG15792 | chr2R:20,898,466 |
| 10 | 3L | *hsc70cb* | CG6603 | chr3L:14,031,059 |
| 11 | 3L | *capr* | CG18811 | chr3L:18,665,583 |
| 12 | 3R | | CG9795 | chr3R:145,807 |
| 13 | 3R | *RpS27* | CG10423 | chr3R:21,072,909 |
| 14 | 3R | | CG12054 | chr3R:27,047,539 |
| 15 | 4 | *CaMKII* | CG18069 | chr4:1,061,498 |

**Table S3. List of primer sequences. Related to Figure 1-4.**

| Primer | Sequence (5’ → 3’) |
|---|---|
| *sisR-4* intron forward primer with AscI RE site | CGATGCGGCGCGCCAGGTTGAGGGGGCGGGGC TGGC |
| *sisR-4* intron reverse primer with NotI RE site | TAGCATGCGGCCGCATCTAAAAGTATAGAAGA CGAA |
| *dpn* exon forward primer | CAGCGTTGTCCAGAAGTTCA |
| *dpn* exon reverse primer | AAACAAGCCACCACGGTATC |

| | |
|---|---|
| Spliced *dpn* forward primer | CACTGCCTCAACGAACTGAA |
| Spliced *dpn* reverse primer | ACTCAACGAATCCCGTCTTG |
| *dpn* pre-mRNA forward primer | ACGATTTCGACTGCTCCAAC |
| *dpn* pre-mRNA reverse primer | AAAACGATCGAGGGTGCTAA |
| *sisR-4* forward primer | GCGTGGGAACCTTTTGCTA |
| *sisR-4* reverse primer | GGGCCCTACAACAAGTTCAA |
| *dpn 5'* forward primer | GGCGGCAGAACTACAAAAGA |
| *dpn 5'* reverse primer | GCAGGATCACTCCTTGGAAA |

## Supplemental Experimental Procedures

### Fly strains

*y w* flies were used a controls unless otherwise stated. The following strains were used in this study: $dpn^{MI00051}$ (Bloomington #30603) [S1], *da-Gal4*, *ptc-Gal4*, *UAS-deadpan [S2]*, *dpn[a]GFP* [S3], and *dpn[b]GFP* [S3]. Transgenic flies expressing *dsRed-sisR-4-myc* was generated as previously described [S4]. Intronic fragment was amplified and sub-cloned into linearized UASt-dsRed-myc plasmid. Injection was done by BestGene Inc. Primer sequences are available in Table S3.

### RNase R treatment

RNase R treatment was performed as previously described [S4]. RNA was denatured at 65$^{o}$C for 5 min in 10X RNase R reaction buffer (Epicenter). 1 uL of RNase R was added and incubated at 37$^{o}$C overnight.

### Deep sequencing and sequence mapping

Deep sequencing was performed as previously described [S4]. Random hexamers were used as primers following the protocol in the Illumina TruSeq RNA sample preparation guide. The Illumina HiSeq 2000 sequencer was used to perform 100-bp sequencing. The resulting reads were aligned to the *Drosophila melanogaster* genome dm3. Sequence alignment was visualized using a genome browser and manually screened for the presence of peaks that

clustered in the introns and candidates were chosen based on the criteria that the 5' ends of the peak maps near the 5' splice sites and the 3' ends near the branch points.

## RT-PCR and qPCR

RT-PCR was performed as previously described [S4]. RNA was reverse transcribed using M-MLV RT (Promega) for 1 hr with random hexamers. For lariats, Superscript III (Invitrogen) was used. For qPCR, SYBR Fast qPCR kit master mix (2X) universal (Kapa Biosystems, USA) was used with addition of ROX reference dye high and carried out on the Applied Biosystems 7900HT Fast Real-Time PCR system. Primer sequences are available in Table S3.

## Nuclear-cytoplasmic fractionation

Nuclear-cytoplasmic fractionation was performed as previously described [S4]. Embryos were dechlorinated in 50% bleach for 2 min and homogenized in cold lysis buffer (100 mM potassium acetate, 0.1% Triton X-100, 50 mM Hepes, pH 7.4, 2 mM magnesium acetate, 10% glycerol, 1mM DTT, and 1X complete mini EDTA-free protease inhibit cocktail, Roche). The lysate was centrifuged at 1,300 g for 10 min at 4 $^{o}$C, and the supernatant was kept as the cytoplasmic fraction. The crude nuclear pellet was then homogenized again in cold lysis buffer and centrifuged at 1,300 g for 10 min at 4 $^{o}$C. The nuclear pellet was kept as the nuclear fraction. RNA was extracted and dissolved in equal volumes of water, and the same volumes were used for RT-PCR experiments.

## Western blotting

Western blotting was performed as previously described [S5]. The primary antibodies used were mouse anti-GFP (1:1,000, Invitrogen) and mouse anti-alpha Tubulin (1:10,000, Upstate). Western blot detection was done digitally using the ChemiDoc Touch Imaging System (BioRad) and under non-saturating conditions. Equivalent amount of protein was loaded into each lane as estimated by the number of embryos used. Quantification of band intensities was done using ImageJ software.

## Immunostaining

Embryos were dechorionated in 1:1 bleach/water for 60 s and fixed in freshly prepared 1:1 heptane/4% paraformaldehyde in PBS with shaking for 20 min. Fresh 1:1 heptane/methanol was later added with shaking for 30 s twice. Finally, only methanol was added with shaking for 15 s for three times. The embryos were then stained in parallel with guinea pig anti-deadpan (1:1,000, gift from Y. Cai) as described previously [S5]. Imaging was performed using Zeiss Upright Exciter with identical settings and processed using Photoshop.

### Supplemental References


S1. Venken, K.J., Schulze, K.L., Haelterman, N.A., Pan, H., He, Y., Evans-Holm, M., Carlson, J.W., Levis, R.W., Spradling, A.C., Hoskins, R.A., et al. (2011). MiMIC: a highly versatile transposon insertion resource for engineering Drosophila melanogaster genes. Nature methods *8*, 737-743.

S2. Wallace, K., Liu, T.H., and Vaessin, H. (2000). The pan-neural bHLH proteins DEADPAN and ASENSE regulate mitotic activity and cdk inhibitor dacapo expression in the Drosophila larval optic lobes. Genesis *26*, 77-85.

S3. Babaoglan, A.B., Housden, B.E., Furriols, M., and Bray, S.J. (2013). Deadpan contributes to the robustness of the notch response. PloS one *8*, e75632.

S4. Pek, J.W., Osman, I., Tay, M.L.-I., and Zheng, R.T. (2015). Stable intronic sequence RNAs have possible regulatory roles in Drosophila melanogaster. The Journal of Cell Biology *211*, 243-251.
S5. Pek, J.W., and Kai, T. (2011). A role for vasa in regulating mitotic chromosome condensation in Drosophila. Curr. Biol. *21*, 39-44.