Chapter 18

Effective Gene Knockdown in the *Drosophila* Germline by Artificial miRNA-Mimicking siRNAs

Hailong Wang, Haidong Huang, and Dahua Chen

Abstract

Gene knockdown using double-stranded RNA (dsRNA) is a powerful tool to characterize gene function in *Drosophila*. The *Drosophila* germline provides an elegant model to study the regulation of numerous processes, such as stem cell fate, gametogenesis, piRNA, and piRNA-related gene functions. However, for unknown reasons, traditional dsRNA gene knockdown has not been successful in the germline. Here, we establish a simple gene silencing method for the *Drosophila* germline by the generation of artificial miRNA-mimicking siRNAs. This method, which is different from that of classical dsRNA, mimics natural miRNA biogenesis and enables the analysis of gene functions specifically in the *Drosophila* germline.

Key words miRNA, siRNA, Knockdown, Gene silencing, Germline

1 Introduction

In 1998, Fire and Mello discovered that double-stranded RNAs (dsRNAs) trigger posttranscriptional-gene silencing (PTGS) in *Caenorhabditis elegans* [1]. This discovery initiated the new technology of RNA interference (RNAi) to determine gene function via specific silencing; RNAi is now one of the most popular and efficient tools to study gene function. Long dsRNAs are cleaved into 21–23 nt small RNAs, named small interfering RNAs (siRNAs) [2–4], which are the final effectors loaded onto the RNA-induced silencing complex (RISC) to achieve RNAi. Another small RNA group consists of microRNAs (miRNAs) that are ~23 nt long and play a significant role in posttranscriptional regulation and are necessary for developmental control. These two different small RNAs have distinct biogenesis pathways in Drosophila. siRNAs are processed by Dicer2 and miRNAs are processed by Dicer1. Two research groups have achieved RNAi using artificial miRNAs/siRNAs that mimic the natural microRNA biogenesis pathway [5, 6]. These studies shed a light on how to perform RNAi using endogenous pathways.

Mikiko C. Siomi (ed.), *PIWI-Interacting RNAs: Methods and Protocols*, Methods in Molecular Biology, vol. 1093,
DOI 10.1007/978-1-62703-694-8_18,

The *Drosophila* germline system is an excellent system for studying many biological processes, such as gametogenesis, stem cell behavior, germ cell meiosis, and other developmental regulation mechanisms. Studying loss-of-function is one of the best approaches to determine a gene's role, and the roles of many genes have been unveiled by large-scale genetic screening approaches using ethyl methanesulfonate (EMS) [7, 8] and P-element mutagenesis [9, 10]. However, analysis of a gene's role mainly depends on phenotype analysis; therefore, the effects of many genes have not yet been determined because of loss of function lethality. Consequently, given this limitation of traditional forward genetics, it is necessary to develop other ways to remove a gene's function in a tissue-specific manner to bypass lethality.

After the discovery of RNAi, many long dsRNA screens were applied to *Drosophila* and other metazoans to discover new gene function or to expand the known role of a gene [11, 12]. Compared with the traditional time-consuming screening protocols, dsRNA can target any gene product in a rapid and highly efficient way. Moreover, combined with the powerful UAS/Gal4 system, gene knockdown can be performed in a tissue-specific manner. However, dsRNA-mediated gene silencing is ineffective in the female germline because of an unknown reason; therefore, obstacles still exist in the study of germline-specific genes, for example piwi or other piRNA-related genes [13, 14]. Also, mutants of genes that function in the germline, often cause sterility, which masks their real roles. To achieve RNAi specifically in the germline, we asked whether there was any other way to knockdown gene expression without the introduction of dsRNAs. A natural RNAi method had previously been applied in somatic fly cells [15]; therefore, we applied artificial miRNAs/siRNAs to the germline to establish an effective gene silencing method. This method enables the knockdown of a gene of interest in the germline, and provides a powerful tool for systematic reverse genetic screening enabling the identification of hitherto undiscovered genes involved in GSC regulation and germline development.

Many studies have shown differences between miRNA and siRNA biogenesis pathways, in which miRNAs prefer to load into Ago1 and siRNAs assemble on Ago2 [16] (reviewed in [17, 18]). However, Ago1- and Ago2-loading compete with each other; the final loading results depend on the mature RNA duplex and its intrinsic structure [19]. A duplex containing more mismatches preferentially loads onto Ago1, while a duplex with fewer mismatches preferentially loads onto Ago2. The artificial siRNA in this protocol mimics *dme-miR-1*, which only has two mismatches, and the mature product is perfectly complementary to the target; therefore, loading onto Ago2 [20] (and unpublished data), the core RNAi effector, is favored to achieve gene silencing.

2 Materials

2.1 Vector Modification

1. UASp vector [21].
2. Synthesized vector adaptors to yield UASp-KN, UASp-NB, and UASp-BX vectors, i.e., *Kpn*I/*Not*I-s and *Kpn*I/*Not*I-as adaptors to yield UASp-KN vector, *Not*I/*Bam*HI-s and *Not*I/*Bam*HI-as adaptors to yield UASp-NB vector, and *Bam*HI/*Xba*I-s and *Bam*HI/*Xba*I-as adaptors to yield UASp-BX vector (the adaptor sequences are shown in Table 1).
3. Restriction enzymes: *Kpn*I, *Not*I, *Bam*HI, and *Xba*I.
4. Agarose gel DNA isolation kit.
5. T4 DNA ligase.
6. Competent *E. coli* cells.
7. LB medium.
8. LB plates containing 100 ng/ml Ampicillin.
9. DNA preparation kit.

2.2 Artificial dme-miR-1-Mimicking siRNA Design and Expressing Vector Construction

1. Sense and antisense-strand oligos encoding artificial *dme-miR-1*-mimicking siRNAs. Each oligo is dissolved in ddH_2O to a final concentration of 100 μM.
2. 10× Taq polymerase buffer.
3. PCR instrument.
4. UASp-KN, UASp-NB, and UASp-BX vectors from Subheading 3.1.
5. Restriction enzymes: *Nhe*I and *Eco*RI.

Table 1
UASp vector adaptors

*Kpn*I/*Not*I-s	C ATCCCATATTCAGCC GCTAGC AGTCCACT GAATTC GGGCGAGACATCGGAG GC
*Kpn*I/*Not*I-as	GGCCGC CTCCGATGTCTCGCCC GAATTC AGTGGACT GCTAGC GGCTGAATATGGGAT GGTAC
*Not*I/*Bam*HI-s	GGCCGC ATCCCATATTCAGCC GCTAGC AGTCCACT GAATTC GGGCGAGACATCGGAG G
*Not*I/*Bam*HI-as	GATCC CTCCGATGTCTCGCCC GAATTC AGTGGACT GCTAGC GGCTGAATATGGGAT GC
*Bam*HI/*Xba*I-s	GATCC ATCCCATATTCAGCC GCTAGC AGTCCACT GAATTC GGGCGAGACATCGGAG T
*Bam*HI/*Xba*I-as	CTAGA CTCCGATGTCTCGCCC GAATTC AGTGGACT GCTAGC GGCTGAATATGGGAT G

Underlined bases are restriction enzyme sites, *Nhe*I or *Eco*RI

6. T4 DNA ligase.
7. Competent *E. coli* cells.
8. LB medium.
9. LB plates containing 100 ng/ml Ampicillin.
10. DNA preparation kit.
11. Restriction enzymes: *Not*I, *Bam*HI, and *Xba*I.

2.3 Transgene Engineering

1. Fly stocks: W^{1118}, sp/Cy0; *bam*Δ^{86}/TM3, sp/Cy0; *nosP-gal4vp16*/TM3.
2. Injection buffer: mix 20 μl of 100 mM PIPES, 15 μl of glycerol, 10 μl of 10 mM EDTA, and 55 μl of ddH_2O.
3. Egg laying plates containing 20 % sucrose, 20 % apple cider, and 2.4 % agar powder.
4. Micro-injection platform (Olympus).
5. Carl-Zeiss LSM7 laser confocal microscopy.

3 Methods

3.1 Vector Modification

For artificial *dme-miR-1*-mimicking siRNA cloning, three adaptors are individually inserted into original UASp vector [21] to construct three different modified UASp vectors, namely UASp-KN, UASp-NB, and UASp-BX. KN means that *Kpn*I and *Not*I sites are used as the adaptor insertion sites. Likewise, the adaptor insertion sites of UASp-NB and UASp-BX vector are *Not*I and *Bam*HI, and *Bam*HI and *Xba*I, respectively. Each adaptor contains *Nhe*I and *Eco*RI cloning sites for artificial *dme-miR-1*-mimicking siRNA insertion (Fig. 1).

1. To yield UASp-KN vector, digest ~1 μg of UASp vector with *Kpn*I and *Not*I, according to the manufacturer's instruction. To yield UASp-NB and UASp-BX vectors, *Kpn*I and *Not*I are substituted with *Not*I and *Bam*HI, and *Bam*HI and *Xba*I, respectively.
2. Mix 0.1 μg of *Kpn*I/*Not*I-s adapter with 0.1 μg of *Kpn*I/*Not*I-as adaptor and 2μl 10× Tag buffer in an Eppendorf tube and adjust the final volume to 20 μl by adding ddH_2O. Incubate the tube at 95 °C for 5 min and put it on bench to cool down to room temperature to Anneal a *Kpn*I and *Not*I double cohesive ends KN adaptor. Likewise, double-stranded cohesive ends NB and BX adaptors are produced by annealing *Not*I/*Bam*HI-s and *Not*I/*Bam*HI-as adaptors, and *Bam*HI/*Xba*I-s and *Bam*HI/*Xba*I-as adaptors, respectively. Resultant adaptor fragments are agarose gel-purified using a commercially available kit, according to the manufacturer's instruction.

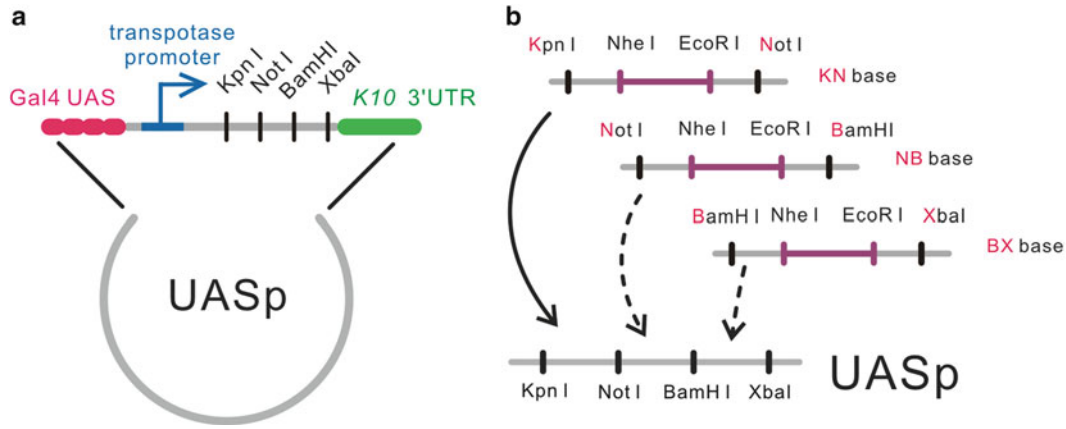

Fig. 1 Schematic diagram of vector modification. (**a**) The UASp vector, redrawn from Rørth (1998) [21]. (**b**) UASp vector modifications. Three independent adaptors bearing *Nhe*I and *Eco*RI were ligated into the UASp MCS and formed three different vectors: UASp-KN, UASp-NB, and UASp-BX. An artificial microRNA can then be integrated into the vector

3. Ligate gel-isolated double-stranded KN adaptor with UASp vector digested with *Kpn*I and *Not*I (from **step 1** in Subheading 3.1). To an Eppendorf tube, add 0.01 μg of digested vector, 0.05 μg of annealed adaptor, 1 μl of 10× T4 DNA buffer, 1 μl of T4 DNA ligase, and make the total volume to 10 μl. Gel-isolated NB and BX adaptors are ligated with UASp vector digested with *Not*I and *Bam*HI, and *Bam*HI and *Xba*I, respectively.
4. Transform *E. coli* with the reaction mixture and grow them on LB plates containing Ampicillin at 37 °C overnight.
5. Inoculate 2 ml of LB medium containing 100 ng/ml Ampicillin with each transformant and isolate plasmid DNA from *E. coli* using a DNA preparation kit according to the manufacturer's instruction.
6. Digest plasmid DNAs with *Nhe*I or *Eco*RI restriction enzymes, and sequence them, to confirm if modified vectors are obtained, as expected.

3.2 Artificial *dme-miR-1*-Mimicking siRNA Design and Expressing Vector Construction

Artificial *dme-miR-1*-mimicking siRNAs are designed through websites using siRNA design tools, such as Gene Link shRNA Explorer (http://www.genelink.com/sirna/shRNAi.asp) and DSIR (http://biodev.extra.cea.fr/DSIR/DSIR.html). An optimal siRNA sequence has a 40–60 % GC content, excludes repeats sequentially appearing more than three times, and is located within a region of the coding sequence that is within 50–100 nucleotides of the ATG start codon and within 50–100 nucleotides from the termination codon. Since the backbone of artificial siRNAs is *miR-1*, they should begin with a 5′-T to mimic the endogenous 5′ uracil

of *miR-1* (*see* **Notes 1** and **2**). Selected siRNA sequences should also be Blast-searched against miRBase (http://www.mirbase.org/) and FlyBase (http://flybase.org/) to eliminate off-target effects (*see* **Note 3**). When necessary, it should be ensured that artificial siRNAs target all possible isoforms of their target genes (*see* **Note 4**).

Since miRNA-mimicking siRNAs mature from the hairpin-type pre-miRNA structure, flanking sequences [23] and mismatched bases are essential for siRNA maturation and should be included in the resultant artificial siRNAs to mimic the natural *miR-1*. We chose the same online tool used by Haley [15] (http://flybuzz.berkeley.edu/cgi-bin/constructhairpin.cgi). The pNE option was selected then the reverse complementary sequence of the siRNA designed above was entered into the "input sequence" textbox and the "create" button clicked. The resultant 71-nt oligos are then synthesized.

3.2.1 Single Hairpin Artificial dme-miR-1-Mimicking siRNA Construction

1. Anneal sense-strand and antisense-strand oligos by mixing 2 μl of 100 μM sense-strand oligo with 2 μl of 100 μM antisense-strand oligo, 1 μl of 10× Taq polymerase buffer in an Eppendorf tube and bring the final volume to 10 μl by adding ddH_2O. Then, incubate the mixture at 95 °C in a PCR instrument for 5 min, and then cool down to room temperature by following 20 min.
2. Dilute annealed oligo 10 times by adding 90 μl of ddH_2O.
3. Digest ~1 μg of UASp-KN vector with *Nhe*I and *Eco*RI, according to the manufacturer's instruction. UASp-NB and UASp-BX vectors can alternate UASp-KN vector.
4. Ligate an annealed oligo with UASp-KN vector digested with *Nhe*I and *Eco*RI to yield a plasmid to express *dme-miR-1*-mimicking siRNA, termed *UASp artmiR-X*. Different oligos can be inserted to other UASp-originating vectors, for instance, oligo 1 is inserted into UASp-KN, while oligo 2 is inserted into UASp-NB or UASp-BX vector. The construction is confirmed by PCR and/or sequencing (*see* **Note 5**).

3.2.2 Tandem Hairpin Construction

A single artificial *dme-miR-1*-mimicking siRNA may have a limited knockdown efficiency of the target gene; therefore, the use of two or three hairpins can ensure effective knockdown (*see* **Note 6**). Here, we show how to link three tandem hairpins.

1. Construct individually three independent single hairpin vectors as indicated in **step 2** in Subheading 3.1.
2. Excise the NB-hairpin fragment inserted into the UASp-NB vector by digesting the construct with *Not*I and *Bam*HI restriction enzymes (*see* **Note 7**).

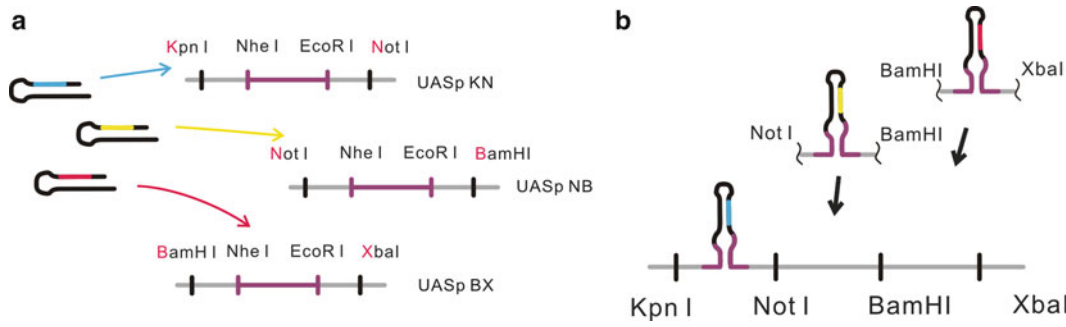

Fig. 2 Illustration of tandem hairpin creation. (**a**) By using appropriate restriction sites, different hairpins can be ligated into one vector. (**b**) Tandem hairpin procedure

3. Digest the UASp-KN vector containing the KN-hairpin fragment with *Not*I and *Bam*HI restriction enzymes.
4. Ligate ~100 bp NB-hairpin fragment into the linearized UASp-KN vector containing the KN-hairpin fragment. This is to link two hairpins in tandem in one vector. Insertion of the hairpin is confirmed by PCR and sequencing (*see* **Note 5**).
5. The third hairpin fragment bearing *Bam*HI and *Xba*I sites at 5′ and 3′ ends, respectively, can be inserted into the two hairpin-containing vector digested with *Bam*HI and *Xba*I (*see* Fig. 2).

3.3 Transgene Engineering

The micro-injection protocol is based on P-element [22] insertion.

1. Mix 2 μg/μl artificial *dme-miR-1*-mimicking siRNA expression plasmid and 1 μg/μl helper plasmid (pπ25.7 Δ2–3 wc) in Injection buffer. It is recommended that these plasmids are purified using a Qiagen Plasmid Midi Kit.
2. Collect fresh wild-type fly (w^{1118}) embryos (0–60 min) from an egg laying plate.
3. Inject the plasmid mix into the embryos via a micro-injection platform.
4. Incubate the injected embryos in a 25 °C growth cabinet for 36–48 h.
5. Transfer the larvae to fresh medium for continued growth.
6. Collect hatching adults and separate the sexes.
7. Cross them with mapping lines (we choose $bam\Delta^{86}$) to obtain red eye offspring.
8. The insertions are then mapped and stable or balanced lines set up. The choice of crossing fly and balancers depends on individual preference.

9. To ensure the transgenes obtained are correct, the insert is amplified and sequenced.

3.4 Checking Phenotype for Knockdown Efficiency

The UAS/Gal4 system can be driven by different drivers and combined with the artificial *dme-miR-1*-mimicking siRNA; thus, theoretically, any gene can be knocked down in a tissue-specific manner. We tested the knockdown efficiency in the germline. *Bam* is a key factor controlling germline stem cell differentiation [24–26]; therefore, we generated a *UASp 3xart-bam* transgene line to silence *Bam* expression [27]. When knockdown was driven by *nosP*-gal4VP16 in the germline, the *UASp 3xart-bam* line phenocopied the *bam* mutant (immunohistochemistry procedure is described in [28]). The mRNA level, evaluated by real-time PCR, was highly reduced as expected. We subsequently targeted different genes and obtained similar knockdown effects [27]. These data indicate that two hairpins used together are capable of efficient gene silencing.

4 Notes

1. Since the synthesized oligos mimic the pre-*miR-1* processing, the intrinsic duplex structure should also mimic the natural *miR-1*. Thus, the thermodynamic properties of the duplex terminal are important for the sorting and strand selection by RISC. The mature miRNA duplex generally has thermodynamic asymmetry; the guide strand 5′ end has a lower thermodynamic energy than the passenger strand [29, 30]. Therefore, the siRNA 5′ T (T-A base pairing) accompanied with the *miR-1* 5′ U has a lower thermodynamic energy, which is compatible for loading onto RISC.
2. If the target CDS is too short to generate a siRNA, you can reduce the restrictions, such as using a wider GC content range or extending the target sequence to include the 3′ UTR.
3. To eliminating off-target effects, blast search the siRNA against annotated genes in FlyBase to exclude targeting other genes. MiRNA target recognition is dependent on its "seed" sequence (nucleotides 2–8 from the miRNA's 5′ end) [31]. Thus, exclude 21 nt siRNAs that have the same miRNA "seed" sequence by searching *Drosophila* miRNAs in mirBase.
4. For a rescue experiment, the 3′ UTR sequence should be the only target sequence, but ensure the *dme-miR-1*-mimicking siRNA can target all isoforms.
5. Inserted hairpin identification, PCR is a feasible method to identify the insertion(s). Synthesized oligos have cohesive termini and are ~100 bp long. We designed a pair of primers near the insertion site (see sequence below) to amplify the inserted hairpin(s). Blank UASp vector yields a PCR product of ~250 bp, thus one inserted hairpin yields a product

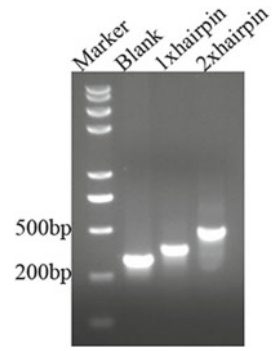

Fig. 3 PCR identification of insertions (1.5 % agarose gel). Addition of one hairpin yields PCR products that are shifted by ~100 bp in agarose gel electrophoresis

100 bp longer (*see* Fig. 3). Agarose gel electrophoresis of a 1.5 % agarose gel can identify vectors with different numbers of hairpins.

Primer/s: catttatgtaaacaataacgtgactgtgcg
Primer/as: accatgggtttaggtataatgttatcaagctc

6. Knockdown efficiency can be measured by phenotype, real-time PCR or Western blot. If the knockdown effect is not desirable, choose a different siRNA and link three hairpins.
7. In the tandem hairpin procedure, the digested fragments were too small to be detected; therefore, more than 20 μg of hairpin plasmid should be digested to ensure enough digestion fragment product.

References

1. Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC (1998) Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature 391(6669):806–811
2. Hammond SM, Bernstein E, Beach D, Hannon GJ (2000) An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. Nature 404(6775):293–296
3. Zamore PD, Tuschl T, Sharp PA, Bartel DP (2000) RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. Cell 101(1):25–33
4. Elbashir SM, Lendeckel W, Tuschl T (2001) RNA interference is mediated by 21-and 22-nucleotide RNAs. Gene Dev 15(2):188–200
5. Dickins RA, Hemann MT, Zilfou JT, Simpson DR, Ibarra I, Hannon GJ, Lowe SW (2005) Probing tumor phenotypes using stable and regulated synthetic microRNA precursors. Nat Genet 37(11):1289–1295
6. Silva JM, Li MZ, Chang K, Ge W, Golding MC, Rickles RJ, Siolas D, Hu G, Paddison PJ, Schlabach MR, Sheth N, Bradshaw J, Burchard J, Kulkarni A, Cavet G, Sachidanandam R, McCombie WR, Cleary MA, Elledge SJ, Hannon GJ (2005) Second-generation shRNA libraries covering the mouse and human genomes. Nat Genet 37(11):1281–1288
7. Alonso AGD, Gutierrez L, Fritsch C, Papp B, Beuchle D, Muller J (2007) A genetic screen identifies novel polycomb group genes in Drosophila. Genetics 176(4):2099–2108
8. Bokel C (2008) EMS screens: from mutagenesis to screening and mapping. Methods Mol Biol 420:119–138
9. Spradling AC, Stern D, Beaton A, Rhem EJ, Laverty T, Mozden N, Misra S, Rubin GM (1999) The Berkeley Drosophila Genome Project gene disruption project: single P-element insertions mutating 25% of vital Drosophila genes. Genetics 153(1):135–177

10. Oh SW, Kingsley T, Shin HH, Zheng Z, Chen HW, Chen X, Wang H, Ruan P, Moody M, Hou SX (2003) A P-element insertion screen identified mutations in 455 novel essential genes in Drosophila. Genetics 163(1): 195–201
11. Mathey-Prevot B, Perrimon N (2006) Drosophila genome-wide RNAi screens: are they delivering the promise? Cold Spring Harb Symp 71:141–148
12. Friedman A, Perrimon N (2004) Genome-wide high-throughput screens in functional genomics. Curr Opin Genet Dev 14(5):470–476
13. Juliano C, Wang J, Lin H (2011) Uniting germline and stem cells: the function of Piwi proteins and the piRNA pathway in diverse organisms. Ann Rev Genet 45:447–469
14. Siomi MC, Sato K, Pezic D, Aravin AA (2011) PIWI-interacting small RNAs: the vanguard of genome defence. Nat Rev Mol Cell Biol 12(4):246–258
15. Haley B, Hendrix D, Trang V, Levine M (2008) A simplified miRNA-based gene silencing method for Drosophila melanogaster. Dev Biol 321(2):482–490
16. Okamura K, Ishizuka A, Siomi H, Siomi MC (2004) Distinct roles for Argonaute proteins in small RNA-directed RNA cleavage pathways. Genes Dev 18(14):1655–1666
17. Kim VN, Han J, Siomi MC (2009) Biogenesis of small RNAs in animals. Nat Rev Mol Cell Biol 10(2):126–139
18. Ghildiyal M, Zamore PD (2009) Small silencing RNAs: an expanding universe. Nat Rev Genet 10(2):94–108
19. Tomari Y, Du T, Zamore PD (2007) Sorting of Drosophila small silencing RNAs. Cell 130(2):299–308
20. Ni JQ, Zhou R, Czech B, Liu LP, Holderbaum L, Yang-Zhou D, Shim HS, Tao R, Handler D, Karpowicz P, Binari R, Booker M, Brennecke J, Perkins LA, Hannon GJ, Perrimon N (2011) A genome-scale shRNA resource for transgenic RNAi in Drosophila. Nat Methods 8(5):405–407
21. Rorth P (1998) Gal4 in the Drosophila female germline. Mech Dev 78(1–2):113–118
22. Spradling AC, Rubin GM (1982) Transposition of cloned P elements into Drosophila germ line chromosomes. Science 218(4570): 341–347
23. Han J, Lee Y, Yeom KH, Nam JW, Heo I, Rhee JK, Sohn SY, Cho Y, Zhang BT, Kim VN (2006) Molecular basis for the recognition of primary microRNAs by the Drosha-DGCR8 complex. Cell 125(5):887–901
24. McKearin DM, Spradling AC (1990) bag-of-marbles: a Drosophila gene required to initiate both male and female gametogenesis. Genes Dev 4:2242–2251
25. Chen D, McKearin DM (2003) A discrete transcriptional silencer in the bam gene determines asymmetric division of the Drosophila germline stem cell. Development 130:1159–1170
26. Song X, Wong MD, Kawase E, Xi R, Ding BC, McCarthy JJ, Xie T (2004) Bmp signals from niche cells directly repress transcription of a differentiation-promoting gene, bag of marbles, in germline stem cells in the Drosophila ovary. Development 131(6):1353–1364
27. Wang H, Mu Y, Chen D (2011) Effective gene silencing in Drosophila ovarian germline by artificial microRNAs. Cell Res 21(4):700–703
28. Xia L, Jia S, Huang S, Wang H, Zhu Y, Mu Y, Kan L, Zheng W, Wu D, Li X, Sun Q, Meng A, Chen D (2010) The Fused/Smurf complex controls the fate of Drosophila germline stem cells by generating a gradient BMP response. Cell 143(6):978–990
29. Czech B, Zhou R, Erlich Y, Brennecke J, Binari R, Villalta C, Gordon A, Perrimon N, Hannon GJ (2009) Hierarchical rules for Argonaute loading in Drosophila. Mol Cell 36(3):445–456
30. Ghildiyal M, Xu J, Seitz H, Weng Z, Zamore PD (2010) Sorting of Drosophila small silencing RNAs partitions microRNA* strands into the RNA interference pathway. RNA 16(1):43–56
31. Bartel DP (2009) MicroRNAs: target recognition and regulatory functions. Cell 136(2):215–233