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Small RNAs: Artificial piRNAs for Transcriptional Silencing

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Technologies have been developed in animal germ cells that produce artificial piRNAs from transgenes in piRNA clusters to silence target genes by cleaving their transcripts. A new study provides a simple way to generate artificial piRNAs to direct *de novo* DNA methylation in mice.

It is now known that although chromatin modifications stabilize silencing of transposable elements (TEs), a genetic reinforcement loop based on germline-specific small RNAs, PIWI-interacting RNAs (piRNAs), is at the heart of epigenetic regulation in animal gonads [1–3]. piRNAs are loaded onto PIWI proteins, a germline-specific clade of the Argonaute family, to form effector complexes, termed piRNA-induced silencing complexes (piRISCs). piRISCs are guided to TEs by means of base-pairing and direct posttranscriptional silencing by cleaving their transcripts in the cytoplasm or by mediating the deposition of repressive chromatin modifications including methylation of histone H3 lysine-9 (H3K9me) and DNA methylation at the target TE loci to induce heterochromatization. The cleavage process involves small RNA-directed endonuclease or Slicer activity of PIWI proteins, whereas some PIWI proteins form nuclear piRISCs with other proteins to direct and maintain the epigenetic chromatin modification that suppresses transcription. These silencing mechanisms act in *trans* as well as in *cis*. Disruption of the piRNA pathway very often disturbs germline development, thereby leading to sterility. Thus TE regulation by piRNAs has a profound effect on reproduction.

Single-stranded precursors, transcribed mostly from genomic loci termed piRNA clusters, are processed to generate primary piRNAs by a Dicer-independent mechanism. In some cases, they further initiate a Slicer-mediated feed-forward amplification loop, termed the ping-pong cycle, to produce secondary piRNAs. The piRNA clusters mostly comprise various types of TEs and their remnants with sizes ranging from a few kilobases (kb) to more than 200 kb. It has been proposed that piRNA clusters act as TE traps [4]; once a TE inserts into a piRNA cluster by chance, it can become fixed by evolutionary selection and can start to produce corresponding piRNAs that base-pair with other homologous elements to regulate them in *trans* in germ cells. This model implies that the greater their movement activity, the higher the chance that a TE will jump into a piRNA cluster, thereby steering piRNA production towards highly expressed and transpositionally active TEs. piRNA clusters also acquire processed pseudogenes, which in turn become piRNA sources and may adapt to regulate cognate functional genes [5]. piRNA clusters in *Drosophila* are mostly located in heterochromatin and proximal heterochromatin–euchromatin boundary zones [6]. Synteny of piRNA cluster genomic locations is highly conserved among mammals, although the primary sequence of each piRNA shows no apparent similarity [5,7–9]. These findings suggest that the relative chromosomal position has specific features that allow TE insertion and the production of piRNAs. However, a new study by Itou

et al. [10] reported in this issue of *Current Biology* now demonstrates that piRNAs can be produced in mice to mediate target silencing through DNA methylation, independently of piRNA clusters.

Recent studies have started to elucidate how a specific locus turns into a piRNA-producing site. In addition to TE-containing clusters, piRNAs are also produced from the 3'UTRs of protein-coding genes, which are referred to as genic piRNA clusters [11,12]. Only a few 3'UTRs give rise to piRNAs, suggesting that specific primary sequences and/or secondary structures may exist in genic piRNA clusters to direct piRNA production. BAC clones containing piRNA clusters can be inserted into euchromatic sites in flies and mice and corresponding piRNAs are produced, showing that piRNA clusters can function even when separated from their native genomic locations [13]. In addition, transcription of a piRNA cluster named *42AB* in *Drosophila* germline cells is initiated by Rhino (Rhi), an HP1a family protein [14]. Rhi forms a complex with Deadlock and Cutoff, which is anchored to H3K9me3-marked chromatin [15,16]. The association of the complex appears to allow the cluster to be transcribed. It also protects the transcripts from transcription termination and suppresses splicing of the cluster transcript. Thus these findings indicate that the *cis*-elements that funnel their RNA products into the piRNA-generating pathway must reside within the clusters themselves. These *cis*-elements in piRNA cluster chromatin and/or their RNA products must be recognized by *trans*-factors, which in turn distinguish piRNA clusters and their transcripts from cellular counterparts.

Indeed, targeted or random knockin of a GFP reporter into a piRNA cluster gives rise to piRNAs corresponding to the GFP reporter in flies, silkworm and mice [13,17,18] (Figure 1A–C). The resulting piRNAs, when they are derived from an antisense GFP sequence, can repress the expression of GFP genes integrated elsewhere in the genome by cleaving GFP mRNA. These findings are also consistent with the model in which piRNA clusters act as TE traps; by being inserted into piRNA clusters, new elements become incorporated into the piRNA repertoire to repress homologous elements in *trans.* This implies that this knockin system can be used to express artificial piRNAs to repress the expression of a gene of interest in animal germ cells. Artificial piRNAs can also be expressed in fly germ cells using a transgenic LacO–LacI system to tether a factor required for piRNA production to an ectopic locus [16] (Figure 1D). Tethering of Rhi, as a LacI fusion protein, to a transgene encoding a GFP fusion protein is sufficient to trigger piRNA production from a *trans* combination of transgenic GFP reporters that express complementary transcripts. This suggests that, although it is not known how Rhi is specifically recruited to piRNA clusters, Rhi binding has a function in differentiating clusters or cluster transcripts from cellular genes and producing piRNA precursors.

Itou *et al.* introduce a simple transgenic system in the embryonic mouse testis to produce artificial piRNAs that mediate

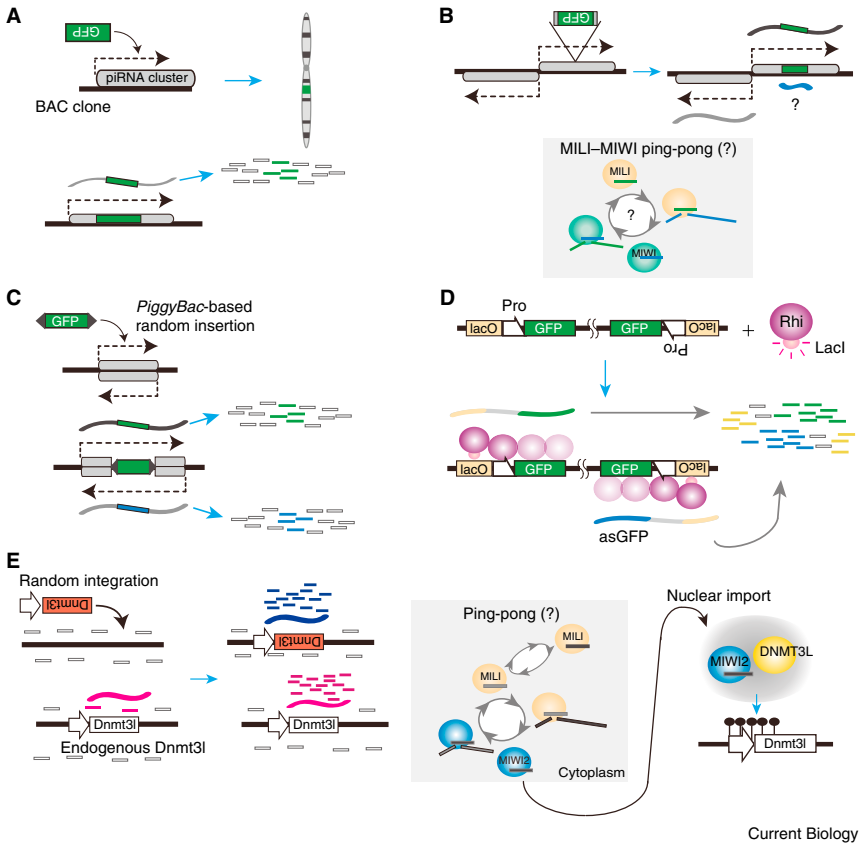

Figure 1. Transgenic systems that produce artificial piRNAs in animal germ cells.
(A) Targeted knockin of a GFP reporter into a piRNA cluster. Muerdter *et al*. used BAC clones to generate artificial piRNAs in flies and mice [13]. BAC clones containing piRNA clusters were recombineered to insert a cassette comprising a GFP sequence and a bacterial neomycin resistance gene. Then the BAC clones were inserted into genomes to produce transgenic animals. (B) Targeted knockin of a GFP reporter into a piRNA cluster. Yamamoto *et al*. introduced a targeting vector containing a GFP reporter into ES cells, which were then used to generate chimeric mice [18]. The GFP reporter was targeted into an adult/pachytene and bidirectional piRNA cluster. piRNAs corresponding to the knockin sequences were produced and they appear to be amplified in the ping-pong cycle. (C) Random knockin of a GFP reporter into a piRNA cluster. Kawaoka *et al*. introduce a GFP-containing cassette into the genome of a silkworm germ cell line, BmN4, using the *piggyBac* transposase. They then screened and selected clonal BmN4 cell lines that produce GFP-derived piRNAs [17]. (D) Tethering a piRNA cluster factor to an ectopic locus. Zhang *et al*. used a transgenic LacO–LacI system to tether Rhino (Rhi) to a locus containing a transgene with the GFP sequence [16]. Expression of antisense GFP sequences driven by a second transgene was required for piRNA production in fly ovarian germ cells. Binding of Rhi–LacI fusion to the LacO site may lead to Rhi spreading into the downstream transcription unit. (E) Expression of both sense and antisense RNAs using promoters active in the piRNA biogenesis phase of spermatogenesis. Itou *et al*. introduce a simple transgenic system to produce artificial piRNAs that leads to *de novo* DNA methylation in the gonocytes of fetal testes [10].

target silencing independently of piRNA clusters [10] (Figure 1E). In mouse testes, three PIWIs (MIWI, MIWI2, MILI) are expressed at different stages during spermatogenesis [2]. In embryonic male germ cells, MILI associates with primary piRNAs and hands secondary piRNAs to MIWI2, which in turn is imported into the nucleus to direct specific DNA methylation of TE loci [2]. Itou *et al.* produce transgenic mice that express both sense and antisense GFP transcripts driven by *Oct4* and *Miwi2* promoters, both of which are active in the piRNA biogenesis phase of spermatogenesis. These transgenes are not integrated into any known piRNA clusters, though it has not escaped our notice that these transgenes are inserted in the middle of a TE [10]. It is known that unlike MILI- and MIWI-associated piRNAs, MIWI2-associated piRNAs often arise from dispersed euchromatic TE copies [19]. Itou *et al.* find that high levels of antisense GFP transcripts correlate with DNA methylation of *Oct4* and *Miwi2* promoters [10]. This DNA methylation state also correlates with the production of sense and antisense GFP piRNAs. Thus, this finding suggests that a piRNA response for a given gene, whose locus is outside of the known piRNA clusters in male germ cells, can be initiated by simple production of sense and antisense transcripts for the gene, leading to DNA methylation and silencing. This work also adds to the current hypothesis that piRNAs guide specific *de novo* DNA methylation to silence their targets in mammals. Because endogenous *Oct4* and *Miwi2* promoters were not methylated in the transgenic mice analyzed, signals of piRNA-mediated DNA methylation may be spread from the GFP gene body to the promoter region.

In proof-of-principle experiments, Itou *et al.* demonstrate that this system can be applied to silence an endogenous gene [10]. Transgenic mice expressing antisense transcripts of *Dnmt3l* driven by the *Miwi2* promoter exhibit DNA methylation of the endogenous *Dnmt3l* gene and produce piRNAs corresponding to the gene. These findings suggest that as long as a sense transcript or mRNA is expressed in the piRNA biogenesis phase of spermatogenesis, only the production of a corresponding antisense transcript from a transgene is sufficient for piRNA production and subsequent DNA methylation of the associated locus. As mentioned above, several transgenic systems have been developed to express artificial piRNAs. However, these piRNAs are not thought to be involved in *de novo* DNA methylation. The work by Itou *et al.* opens the door to studies aimed at understanding how piRNAs mediate DNA methylation. The work, however, does not address the fundamental mechanism by which expression of sense and antisense transcripts elicits a piRNA response. Itou *et al.* find that production of an antisense transcript leads to activation of the ping-pong cycle with corresponding sense transcripts. In the mouse ping-pong cycle, MILI-associated primary piRNAs are normally sense-oriented, which initiates the production of MIWI2-associated antisense secondary piRNAs [2]. How then can the production of antisense transcripts activate the ping-pong cycle? Could pre-existing sense piRNA-like molecules function as a seed to initiate the ping-pong cycle? There is precedence from a fission yeast system for a decisive influence of Dicer-independent transcriptome degradation products, referred to as primal RNAs, to act as the initial trigger of small RNA production [20]. It will be interesting to examine if primal RNA-like molecules exist to initiate the ping-pong cycle in mice and see how these stories unfold.

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Bioluminescence: A Fungal Nightlight with an Internal Timer

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A recent study shows that green light emission by *Neonothopanus gardneri* mushrooms, endemic to coconut forests of Northern Brazil, is controlled by a circadian clock. Furthermore, insects are attracted by the light, raising the possibility that bioluminescence functions in spore dispersal and fungal dissemination.

Enchantment and curiosity are immediately evoked when, entering humid woods under a new moon, one sees green, shining mushrooms popping up on the surface of rotten logs. The photo of a colony of *Mycena lucentipis* mushrooms (Agaricomycetes) should help the reader appreciate these rare and splendid creatures [1] (Figure 1). Seventy-one out of thousands of fungus species occurring mainly in tropical and temperate zones of the globe are documented to be bioluminescent, of which twenty-six species belong to the genus *Mycena* [1,2]. As reported in this issue of *Current Biology* by Oliveira *et al.* [3], light emission by *Neonothopanus gardneri* mushrooms found in Brazilian coconut forests is controlled by a circadian clock and serves to attract insects for spore dispersal.

Although observed all over the world and documented by Aristotle and Pliny the Elder, natural philosophers of the Ancient World [4], fundamental questions about bioluminescent fungi — how, when, and why they emit light — have not been answered. Their biochemical and biological features are still murky, partly because it is difficult to spot them in dense forests, even with dark-adapted eyes, and to collect, transport and cultivate their mycelia and mushrooms in the laboratory. The chemical mechanism and function of light production by fungi is still controversial [5,6]. Up until recently, a key question has concerned whether molecular oxygen or hydrogen peroxide oxidizes a luciferin substrate in the presence of the enzyme luciferase. Alternatively, it was possible that light emission is a byproduct of some metabolic process such as lignin degradation? Almost all bioluminescent organisms known use oxygen to produce the electronically excited product (oxyluciferin), which decays to the ground state by photon emission (see reaction scheme below). Thus, the visible and 'cold' light emission results from efficient conversion of energy from chemical bonds to light without heat dissipation.

$$L + NAD(P)H + H^+ \xrightarrow{\text{soluble reductase}} LH_2 + NAD(P)^+$$

$$LH_2 + O_2 \xrightarrow{\text{insoluble luciferase}} LO + H_2O + \text{light}$$

L, luciferin; LH_2, reduced luciferin; LO, oxidized luciferin

In contrast to fungi, the luciferin/luciferase systems of dozens of luminescent organisms — from bacteria to fishes and insects — have long been identified. Furthermore, the bioluminescence produced by such systems has been implicated in courtship and mating, prey attraction or visual localization, predator warning (aposematism), camouflage, and species recognition/grouping [6]. Various chemically and phylogenetically unrelated luciferins have been isolated, identified and synthesized since the 1950s, among them firefly luciferin (a benzothiazole), sea-pansy coelenterazine and jellyfish aequorin (imidazopyrazinones), dinoflagellate luciferin (an open chain tetrapyrrole), bacterial luciferin (flavins), annelid luciferin (an oligoamide) and the limpet luciferin (a formylated aldo-enol) [5,6]. In a number of bioluminescent reactions (e.g., fireflies, crustaceans and coelenterates), chemical electronic excitation of the light emitter involves an intermediate consisting of a highly unstable four-membered ring peroxide named dioxetanone (or α-peroxylactone), whose thermal cleavage yields CO_2 and the excited product [7].

