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Hide and seek: how chromatin-based pathways silence retroelements in the mammalian germline

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Abstract

Mobile elements comprise a major fraction of most mammalian genomes. To protect their fitness and stability, hosts must keep mobile elements in check in their germline. In most tissues mobile element insertions are decorated with chromatin modifications suggestive of transcriptional silencing. However, germline cells undergo massive chromatin reprogramming events, which erase repressive chromatin marks and necessitate *de novo* re-establishment of silencing. How do host genomes achieve the discrimination necessary for this *de novo* silencing? A series of recent studies have revealed aspects of the multi-pronged strategy that mammalian genomes use to identify and silence mobile elements. These strategies include the use of small RNA-guides, of specialized DNA-binding protein adaptors and of proteins that repair chromatin discontinuities caused by retroelement insertions. Genetic analyses reveal the importance of these mechanisms of protection, each of which specializes in silencing mobile elements of different evolutionary ages. Together, these strategies allow mammalian genomes to withstand the high burden of their parasites.

Retroelement expression and silencing in the germline

Retroelements, selfish genetic elements that mobilize via an RNA intermediate, comprise a large fraction of mammalian genomes. The most successful mammalian retroelements are non-LTR retrotransposons of the LINE-1 family (Long Interspersed Nuclear Element), which occupy ~20% and ~17% of the mouse and human genome respectively. LINE-1 also help propagate non-autonomous SINE (Short Interspersed Nuclear Element) retroposons, which comprise 8% and ~11% of the mouse and human genome respectively. The final types of retroelements commonly found in mammalian genomes are LTR-retrotransposons, or endogenous retroviruses (ERVs), which are largely derived from infectious retroviruses. ERVs constitute ~10% of both mouse and human genomes. Together, these three elements dwarfs the total protein-coding gene compendium (roughly 1.2%). By over-replicating themselves via “copy-paste” retrotransposition cycle, retroelements selfishly increase their

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copy number within the host genomes. This is essential for them to stave off mutational extinction, but can be deleterious to the host.

The mobilization of active retroelements is inherently mutagenic to host genomes, which use a variety of strategies to prevent mobilization. In this review, we focus on repression of retroelement transcription, the earliest step in their mobilization. In differentiated cells, most retroelement insertions are decorated by chromatin modifications incompatible with transcription initiation, or heterochromatin, such as the methylation of lysines 9 and 27 of histone H3 (H3K9me and H3K27me respectively) and DNA methylation, whereas they are depleted for chromatin marks associated with transcriptional activation such as histone acetylation [1]. Thus proteins that mediate these DNA or histone modifications i.e., DNA methyl-transferases (DNMTs), histone methyl-transferases (HMTs) or histone deacetylases (HDACs), are crucial for the maintenance of retroelement repression.

From an evolutionary perspective, retrotransposition in differentiated somatic cells is not an effective strategy for the retroelement since it might deleteriously affect host fitness without increasing retroelement copy numbers in genomes of the host's descendants. Instead, evolutionarily successful retroelements ensure their retrotransposition in germline cells, which transmit their genomes to subsequent generations. Intriguingly, mammalian germline cells undergo unique chromatin transitions that could make them vulnerable to retroelement mobilization.

The mammalian germline includes not only germ cells (or reproductive cells) but also cells of the early zygote and the inner cell mass (from which embryonic stem (ES) cells are derived). Together these cells transmit to their descendants all the information necessary to complete embryogenesis. This unique developmental potency is reset at each generation in a process that is referred to as 'epigenetic reprogramming'. Once during pre-implantation development, and again following the induction of primordial germ cell (PGCs), repressive chromatin marks that typically repress retroelements are erased genome-wide and have to be *de novo* reestablished (reviewed in [2,3]). This lowering of the guard could transiently allow transcription and thereby proliferation of retroelements.

Indeed, there is abundant evidence for increased retroelement transcription in germline cells. For instance, adult oocytes and early mouse embryos have a high degree of ERV transcription [4–6]. Similarly, retroelement transcript and proteins have been observed in developing mouse germ cells [7,8], a finding nicely corroborated by recent transcriptome analysis of sorted mouse and human primordial germ cells [9,10]. Furthermore, transcriptome analyses revealed that discrete sets of LTR retrotransposon families were sequentially activated during human pre-implantation development indicative of a high degree of adaptation to this environment [11]. This activation is so stereotypical that ERV transcripts expressed during human and mouse embryogenesis can be used as precise markers of toti- and pluri-potent cells both *in vitro* as well as *in vivo* [12–15].

Despite these observations, there remains limited evidence about whether epigenetic reprogramming directly causes retroelements activation due to erasure of repressive chromatin marks. With some exceptions [16,17], global retrotransposon reactivation

displayed when disrupting HMTs or DNMTs could be the result of broad pleiotropic effects on chromatin patterns. However, recent studies in induced pluripotent stem cells (iPSCs), or embryonic stem cells (ESCs) do suggest that the germline chromatin state is more permissive to retroelements activity than in somatic cells. For instance, upon reprogramming to a more germline-like state, iPSCs dramatically upregulate LINE-1 expression and retrotransposition relative to the parental cell lines they were derived from [18].

Retroelement control: recognizing the enemy

In somatic cells, the propagation and replenishment of repressive chromatin likely maintains retroelement silencing. The findings that retroelements are activated in germline cells might suggest that the germline is a fairly promiscuous environment for retroelement transcription. However, that would be an over-simplification. Closer analyses of published transcriptomes suggest that only a selected number of retroelement subfamilies are actually activated, whereas the vast majority is silent. How is retroelement silencing achieved in germline cells in spite of the wholesale erasure of chromatin marks? How are the repressive chromatin marks established following this erasure?

Ultimately, the challenge in maintaining retroelement control is about discriminating potentially harmful retroelements from host sequences. Any silencing mechanism has also to take into account that retroelements can evolve rapidly to adapt to host transcription factor repertoires and become integral parts of cell-type specific regulatory networks. Indeed, retroelements account for a large portion of bound sites for some transcription factors [19–22]. For example, it was recently shown that the primate specific LTR7 element (belonging to HERVHs ERVs) provide multiple OCT4, NANOG and LBP9 binding-sites that are essential for the maintenance of human pluripotent stem cells in culture [15]. Mammalian genomes therefore have the challenging task of distinguishing and silencing threatening retroelement but not those that have been coopted as host sequences.

Retroelement families, which flourished in our distant ancestors, have been under the control of silencing pathways for more generations and acquired mutations leading them to pose less of an imminent threat to the germline than more recently active families. Pathways silencing older elements will typically involve passive spreading or maintenance of chromatin modifications; they are part of the canonical regulatory toolkit of our cells. In contrast, pathways monitoring recently integrated copies of actively replicating retrotransposon families need to use targeting mechanisms to deposit silencing modifications to the correct locations *de novo*. In response, retroelements are under severe evolutionary pressure to devise ‘escape strategies’. Thus, not only do host strategies need to be efficient and discriminative, they also need to be evolutionarily flexible to adapt to new threats to the germline. Here, we highlight three different strategies that mammalian germline cells use to achieve this control (Figure 1).

Finger on the pulse: the piRNA pathway mediates silencing of the youngest retroelements in germ cells

The discovery of the role of piwi proteins and their associated piRNAs (PIWI interacting RNAs) in *Drosophila* and in mice revealed a widespread mechanism of germline defense conserved in most animal species. Studies in these two model organisms uncovered the key steps that allow this RNA-based silencing mechanism to detect retroelements, but also some important differences. For instance, whereas flies rely on transposon graveyards (piRNA clusters) to combat young and old transposon invasions in the ovary, mammalian piRNAs adapt to new transposons independently of piRNA cluster formation. In developing mammalian male germ cells, abundantly expressed retroelements are processed into a pool of small RNAs that, once bound to PIWI proteins, guide these complexes to complementary target sequences [23,24]. Mouse PIWI proteins MILI (PIWIL2) and MIWI2 (PIWIL4) begin their expression soon after epigenetic reprogramming; this expression pattern is conserved in humans [10]. Mice knocked out for either MILI or MIWI2 are fully sterile and display retrotransposon derepression [25–27].

The earliest indication that the piRNA pathway contributes to transcriptional silencing came from studies showing that piRNAs, in complex with MIWI2, translocated to the nucleus of PGCs [23]. Furthermore, DNA methylation patterns were compromised in germ cells of mutant animals [23–25,28]. High-throughput sequencing of MILI mutant germ cells revealed that DNA methylation and H3K9 methylation are unaltered over the bulk of retroelement insertions but specifically lost over the promoters of a small number of retroelements, which were members of the youngest and most active subfamilies [29,30]. These data indicate that piRNAs are absolutely required to detect threatening retroelement insertions and target them for *de novo* heterochromatin formation in PGCs, although there are still unknowns about how information from piRNAs is relayed back to chromatin modification. Aside from its role in male germ cells, PIWI proteins have been reported to affect human L1 regulation in induced pluripotent stem cells [31]. However, its role during the embryonic stages of the germline cycle or in female germ cells remains mysterious [32]. It is likely that other pathways regulate retroelement silencing in these tissues.

By using an RNA-based detection and targeting mechanism, the piRNA pathway is the best strategy to respond to new retroelement insertions and new subfamily invasions with little-to-no *a priori* ‘education’. The sole expression of the retroelement could suffice to trigger the response. To escape this silencing, retroelements might be under selection to rapidly alter their cis-regulatory elements to ensure they are not expressed in germ cells where they would be under surveillance, but instead expressed in other cells of the germline (Figure 1a). It is conceivable that such ‘escape strategies’ could explain the rapid diversification seen in regulatory regions of L1s (5′UTRs) and ERVs (regulatory LTR regions) during retroelement evolution [33]; such diversification could also occur during ERV evolution although this remains unexplored [34].

Stalking their prey: DNA binding proteins shadow and silence active retroelement lineages

Parallel to the RNA-based silencing of the piRNA pathway is the DNA sequence-based recognition and silencing of retroelements by host KZNF DNA-binding proteins (for Krüppel associated box (KRAB) containing C2H2-zinc-finger (ZNF)). These modular proteins recruit the universal co-repressor KAP1 (KRAB associated protein 1, also called TRIM28 or TIF1β) through their KRAB domain and bind DNA via an array of ZNFs. By virtue of sampling new DNA-binding sequences via ZNF diversification, KZNF target various subfamilies of retroelements. In turn, KAP1 recruits HMTs and DNMTs to retroelement insertions to promote the deposition of repressive marks [35⁻37].

This paradigm of KZNF-mediated retroelement silencing was elucidated by pioneering work showing how the KZNF protein ZFP809 silenced genomic insertions of the Murine Leukemia Virus (MLV) in mouse ES cells via the recognition of its primer-binding site (PBS) [38]. Recent genome-wide analyses of ZFP809-deficient mouse demonstrated how this silencing extended to other retroelements with a similar PBS [39]. Similar to ZFP809, many KZNFs are expressed during the germline cycle and have the potential to silence many retroelement families ([10›40]) although there are only a handful of examples where the association between a retrotransposon sequence and a unique KZNF has been demonstrated [41⁻44].

Unlike the more immediate piRNA response, KZNF-based targeting is evolutionarily slower to respond to new retroelement families, since it requires gene duplication and adaptive evolution of DNA-binding preferences. However, once the appropriate specificity is achieved, KZNF proteins can globally repress copies of a given subfamily. If the targeted sequence is essential for retrotransposition, as it is in the ZFP809/MLV interaction, it is more difficult for the retroelement to ‘escape’ detection [38]. KZNF genes can also effectively target intermediate aged retroelements, which are less of a threat to the host. This sets up an evolutionary arms race, in which DNA binding proteins recurrently duplicate and adapt to new retroelement features without losing control over old ones or causing deleterious ‘self’-recognition, whereas retroelements evolve to evade this recognition (Figure 1b). Indeed, the tempo of KZNF gene duplication nicely mirrors the tempo retroelement family diversification [45]. A recent illustration of this arms-race emerged from the study of specific KZNFs from ES cells that target LINE-1s and SVA (SINE-VNTR-Alu) retroelements. In this study, both gain of specific KZNF DNA-binding affinity as well as retroelement escape via target sequence deletion was shown to have occurred in recent primate history [42].

Although KAP1 coordinates the deposition of repressive chromatin marks over many retroelement families in mouse and human ES cells [46⁻50], it may recruit distinct effectors to different retroelement classes. For instance, silencing of Class I and II ERVs is strictly dependent on the HMT SetDB1 (or ESET) whereas ERVL and LINE-1 retroelements appear to be specifically regulated by the G9a and Suv39h1/2 HMTs respectively [51⁻54]. Although SETDB1 silencing has been proposed to be dependent on deposition of the histone variant H3.3 [55], the role of this variant histone in ERV silencing still remains to be fully

clarified [56]. These studies further suggest that there may be additional regulators beyond KZNF proteins that specify retroelement targeting by KAP1, for example, the transcription factor YY1 required for LTR retroelement silencing in ES cells [57].

Neighborhood watch: looking for signs of recent vandalism

Both the piRNA and KZNF-based defense rely on recognizing retroelement sequence features. However, retroelement insertions also have the effect of disrupting the contiguous chromatin context into which they insert. A recent study demonstrated that this discontinuity can be recognized as a chromatin ‘scar’ and recruits a novel chromatin-associated HUSH (human silencing hub) complex that spreads repressive marks onto the newly inserted retroelement (Figure 1c) [58]. In this study, a genetic screen was carried out to find genes required for the silencing of an LTR-based reporter vector introduced in human haploid cell lines. This approach identified a complex composed of the chromodomain protein MPP8 (M-Phase Phosphoprotein 8), which interacts with G9a and DNMT3a [59], the epigenetic modifier Fam208a/TASOR (Transgene Activation SuppressOR) [60] and Periphilin 1 (PPHLN1) [61]. Through its interaction with heterochromatin, HUSH directly recruits Setdb1 to mediate the deposition of H3K9me over both new insertions of the retroelement reporter in heterochromatin, as well as over hundreds of additional genomic regions [58]. Thus, HUSH simultaneously appears to perform the dual role of repairing heterochromatin scars and spreading heterochromatin over a subset of retroelements. Interestingly, Fam208a/TASOR knockout mice are embryonic lethal and MPP8 is essential for spermatogenesis suggesting that the HUSH complex could perform its function over the entire germline cycle [60,62].

The principle of ‘chromatin scars’ as a means to identify new genomic insertions is not exclusive to the HUSH complex and might be shared among other proteins identified in recent genetic screens for establishment or maintenance of retroelement silencing [63–65]. Among these, the DAXX protein, which is a histone H3.3 chaperone, is particularly intriguing because it appears to directly engage with the integrase protein of the incoming retrovirus to mediate epigenetic silencing of the resulting provirus [66]. Additionally, the HMTs Suv39h1/2 and the heterochromatin-associated proteins of the HP1 family can interact with pre-existing heterochromatin domains and consolidate retrotransposon silencing in ES cells and germ cells [51,54,67,68]. Going even beyond heterochromatin, there might be other diagnostic ‘scars’ or chromatin discontinuities introduced by retroelement insertions, such as DNA repair-associated signatures [69] which may be utilized by host genomes to deposit specific histone or DNA modifications over subsets of retroelement insertions. For example, retroelement-directed arginine methylation of histone tails by PRMT5 [70] or retroelements DNA methylation regulated by the helicase LSH ([71]) still lack clear described targeting mechanisms in the germline.

In terms of the host-retroelement conflict, repairing chromatin ‘scars’ provides only an insertion-specific defense, but this defense is not dependent on target specificity like piRNAs or KZNFs. It is therefore not clear how retroelements could evade such silencing except to avoid inserting in certain chromatin neighborhoods.

Summary

We have discussed a few of the molecular pathways used to recognize and silence retroelement insertions in the mammalian germline. On the surface, they appear as redundant pathways, often mediating the deposition of similar chromatin modifications or targeting similar retrotransposon families. However, if one looks deeper, they each act on different evolutionary strata of retrotransposon insertions. RNA-guided pathways are tuned to the youngest evolutionary stratum of retroelements; the expression of these young, active retroelements in germ cells is also their vulnerability. In contrast, DNA-binding proteins evolve rapidly via both gene duplication and adaptive evolution to act as adaptors to target the silencing of the intermediate stratum. Finally, complexes repairing ‘chromatin scars’ also help silence new retroelement insertions independent of sequence determinants. A sobering thought is that although each of these molecular pathways has operated for hundreds of millions of years, our genomes are still vulnerable to retroelement activity, as demonstrated by the high genomic burden of retroelement insertions into mammalian genomes.

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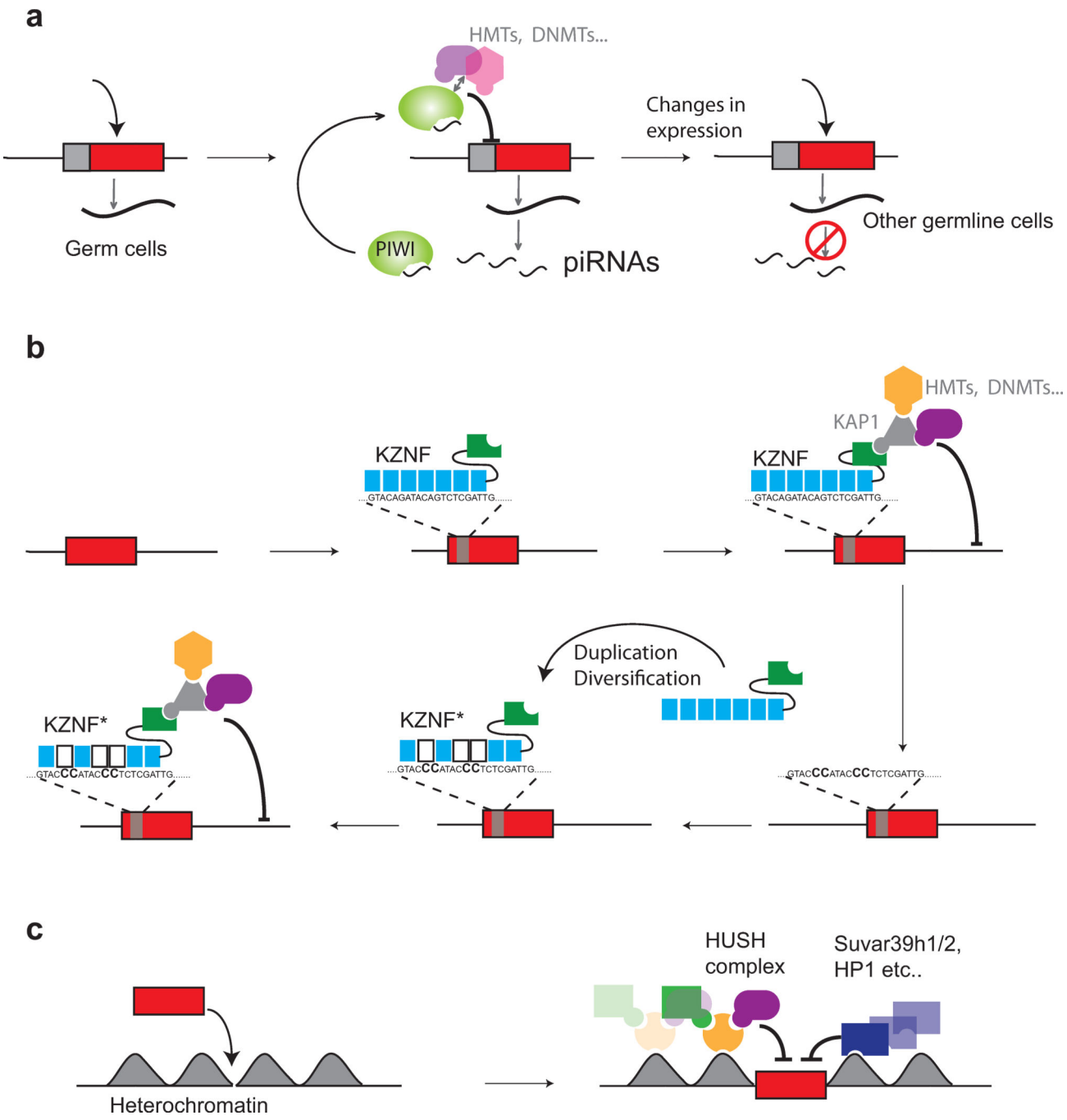

Figure 1. Three layers of retrotransposon control in the mammalian germline

(a) piRNA-mediated silencing in male germ cells. Retroelement insertions expressed in germ cells (left panel) are processed into piRNAs, which bind to PIWI proteins (middle panel). In turn, these piRNA-PIWI complexes guide the deposition of repressive chromatin marks on individual retroelement insertions. New families of retroelements can escape the piRNA pathway by changing their promoter sequences (white bars) and avoid expression in germ cells (right panel). **(b)** An evolutionary arms race between host KZNF proteins and retroelements. KZNF proteins bind to individual insertions via their ZNF domain (blue

boxes) and trigger silencing by recruiting KAP1, HMTs and DNMTs via their KRAB domain (green box). New retroelements evade KZNFs by changing (or deleting) this binding site. Upon KZNF gene duplication and ZNF diversification a new KZNF (KZNF*) can regain control over the new element. **(c)** A positioneffect-variegation (PEV) like mechanism for silencing retroelements. Upon the insertion of a retroelement into a heterochromatin domain (left panel), chromatin-binding complexes (e.g., HUSH complex proteins) can read and repair these scars by ‘spreading’ heterochromatin and establish effective silencing over the element (right panel).