Primal RNAs: The End of the Beginning?

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The amplification of small RNAs and the assembly of heterochromatin are mutually dependent processes in fission yeast. But which comes first? Halic and Moazed (2010) propose that primal small RNAs initiate the amplification of small interfering RNAs that drive heterochromatin formation and chromatin silencing.

RNA interference (RNAi) was originally described as a silencing phenomenon initiated by the experimental introduction of double-stranded RNA (dsRNA). Genetic and biochemical studies have revealed that the enzyme Dicer processes dsRNAs into small interfering RNAs (siRNAs), which interact with the enzyme Argonaute and direct target destruction through base pair interactions. In some cases, RNA-dependent RNA polymerase generates dsRNA intermediates important for the amplification of siRNAs and the maintenance of chromatin silencing. RNAi-related pathways are now recognized to have multiple endogenous triggers and to participate in essential cellular functions ranging from the regulation of translation to chromosome maintenance (Carthew and Sontheimer, 2009).

The fission yeast *Schizosaccharomyces pombe* provides an exquisite model for investigating the interaction between RNAi and heterochromatin formation (Moazed, 2009, and references therein). In *S. pombe*, the core components of the RNAi machinery are Argonaute (Ago1), RNA-dependent RNA polymerase (Rdp1), and Dicer. These components cooperate in a positive feedback loop that is important for heterochromatin formation within pericentric and subtelomeric DNA repeats and at the mating-type locus (Cam et al., 2005), which share *dg* and *dh* repeat sequence elements. Conversely, the biogenesis of siRNAs and localization of RNAi components to heterochromatin is dependent on factors associated with heterochromatin, including the H3K9 methyltransferase, Clr4, and the heterochromatin protein 1 (HP1) homolog, Swi6. The codependence of RNAi and heterochromatin factors has been a long-standing

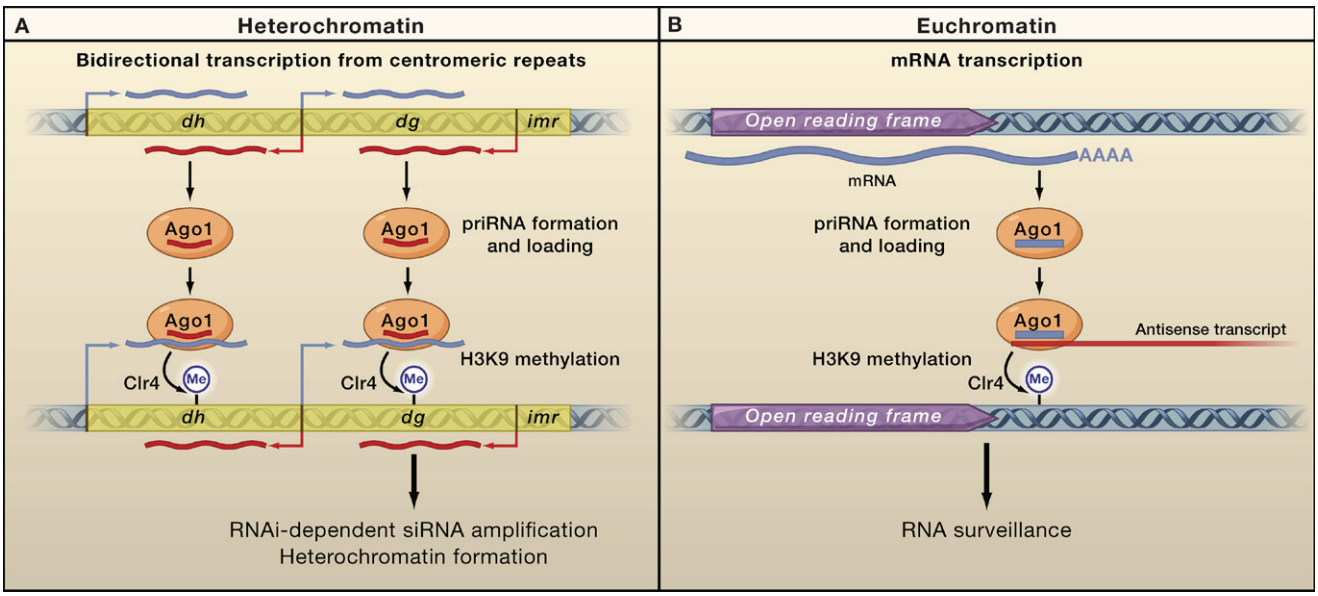

Figure 1. Primal RNAs in Fission Yeast
(A) At heterochromatin-associated repeats in the DNA of fission yeast, primal small RNAs or priRNAs are derived from sense (blue) and antisense (red) transcripts. PriRNAs are then sampled by the Argonaute protein (Ago1) and associate with Ago1. This complex targets RNA transcripts of the opposite polarity resulting in low-level methylation of repeat regions mediated by H3K9 methyltransferase (Clr4). This initial targeting is proposed to nucleate the amplification of small interfering RNAs (siRNAs) at *dg* repeats, which then promotes heterochromatin formation (Halic and Moazed, 2010).
(B) In euchromatic DNA, priRNAs are believed to have a different function, namely RNA surveillance. In euchromatin, priRNAs map primarily to the 3′ UTRs of mRNAs (blue) and may suppress read-through of antisense transcripts (red) with help from Clr4 and Ago1.

conundrum. Do sense and antisense transcripts form dsRNAs that are recognized by Dicer, thus triggering siRNA production? Or does low-level methylation of lysine 9 on histone H3 (H3K9) in DNA repeat regions lead to heterochromatin formation and recruitment of the RNAi machinery, which results in siRNA amplification and stabilization of heterochromatin? In this issue, Halic and Moazed (2010) attempt to answer these questions by combining the awesome power of yeast genetics with the equally awesome sensitivity of deep-sequencing technology. They provide compelling evidence that Dicer-independent transcriptome degradation products, referred to as primal small RNAs or priRNAs, interact with Ago1 to drive the initial amplification of siRNAs by the RNAi machinery in the absence of heterochromatin. Their findings argue that the initial amplification of siRNAs is what nucleates heterochromatin formation.

Although siRNAs are dramatically reduced in mutant yeast strains that lack the machinery required to establish H3K9 methylation and therefore lack heterochromatin, Halic and Moazed show that Ago1 nevertheless continues to interact with siRNAs. Moreover, they show that many siRNAs are derived from the heterochromatin-associated *dg* and *dh* repeats, arguing against the notion that repeat-associated heterochromatin is necessary to drive the initial accumulation of siRNAs at these target sites. Interestingly, siRNAs derived from *dh* repeats are more strongly depleted in the absence of heterochromatin than are siRNAs derived from *dg* repeats. The higher levels of *dg* repeat small RNAs in the absence of heterochromatin are dependent on Rdp1, Dicer, and, importantly, the slicer activity of Ago1. These findings indicate that the *dg* repeats nucleate the amplification of repeat-derived siRNAs even in the absence of heterochromatin (Figure 1A).

But what is the origin of the heterochromatin-independent and Dicer-independent species of *dg* siRNAs (the priRNAs)? Halic and Moazed rule out both Rdp1 and the slicer activity of Ago1 as culprits. Instead, although priRNAs bind to Ago1 and resemble conventional siRNAs in both size and their first nucleotide, they appear to be derived from the degradation products of transcripts that randomly associate with Ago1 (Figure 1A). Consistent with this possibility, priRNA levels tend to correlate with the expression levels of their corresponding transcripts and are partially dependent on a functional exosome pathway, which is important for RNA degradation. The authors propose that bidirectional transcription within DNA repeat regions provides targets for the otherwise sense-oriented priRNAs and explains their ability to initiate RNAi-dependent amplification of siRNAs from *dg* repeats. Perhaps most importantly, the authors provide evidence that Ago1 is required for the previously observed low-level H3K9 methylation in the absence of siRNA amplification (Noma et al., 2004; Sadaie et al., 2004) and show that the level of RNAi-independent H3K9 methylation correlates with the ability of Ago1 to bind to priRNAs. These findings raise the distinct possibility that priRNAs represent the initial triggers of siRNA amplification and heterochromatin formation.

The findings of Halic and Moazed significantly advance our understanding of the role of RNAi in heterochromatin assembly and reveal complexities that were not previously appreciated in this system. Furthermore, the study suggests that careful consideration should be given to how random noise is defined in large data sets. As with any comprehensive study, there are as many new questions as there are answers. We consider two. First, what are the features that distinguish *dg* from *dh* repeats? Although both *dg* and *dh* repeats generate priRNAs, heterochromatin-independent siRNA amplification occurs only within *dg* repeats. If priRNAs do indeed trigger siRNA amplification, one might expect siRNA amplification to be triggered at both *dg* and *dh* repeats. Perhaps, as the authors suggest, this difference is defined by structural differences between the *dg* and *dh* transcripts. Alternatively, there may be other distinguishing chromatin marks or proximal sequences (e.g., *imr*) that help to define *dg* repeats as sites for the initiation of heterochromatin formation and siRNA amplification. The authors liken the sampling of priRNAs by Ago1 to the sampling of peptides for presentation by molecules of the major histocompatibility complex (MHC). Drawing on this analogy, it is tempting to speculate that a secondary feature associated with the *dg* locus could act much like a costimulator in the MHC-mediated activation of T cells (Abbas and Janeway, 2000).

Second, how are priRNAs generated? The authors suggest that priRNAs resemble piwi-interacting RNAs or piRNAs in that they are Dicer-independent and their 3′ end formation is likely to be mediated by an exoribonuclease activity. Presumably, an endo- or exoribonuclease activity is required for 5′ end formation, as the slicer activity of Ago1 is not required for priRNA production. It is difficult to imagine a completely random process where RNAs dissociate from the turnover machinery and associate with Ago1. Instead, perhaps Ago1 associates with the RNA turnover machinery to allow sampling of 5′ ends. It will be interesting to learn whether priRNA biogenesis is a regulated process.

Thus, priRNAs can be added to the growing list of Dicer-independent small RNAs and to the expanding functions of Ago-dependent silencing pathways. Interestingly, outside of heterochromatic repeats, priRNAs tend to be derived in greatest abundance from 3′ untranslated regions (UTRs), suggesting that the priRNA pathway may play a role in mRNA surveillance (Figure 1B). A class of 3′ UTR-directed piRNAs has recently been identified (Robine et al., 2009; Saito et al., 2009), suggesting additional connections between the biogenesis and function of piRNAs and the priRNAs described by Halic and Moazed. Finally, recent work by Grewal and colleagues implicates Clr4 and Ago1 in suppressing antisense transcripts in euchromatic regions of the genome, a role that is distinct from their function in heterochromatin formation (Zofall et al., 2009). Perhaps the euchromatic Ago1/priRNA complexes are involved in the suppression of antisense transcripts. If so, an alternative, but not mutually exclusive, explanation for the findings of Halic and Moazed is that the priRNAs associated with Ago1 represent an RNA surveillance pathway whose activity at heterochromatin repeat domains is uncovered when other silencing pathways are compromised. It will be interesting to see how these stories unfold. This is apparently just the end of the beginning for understanding the complexity and functions of RNAi-related pathways.

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Reining in H_2O_2 for Safe Signaling

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Mammalian cells use hydrogen peroxide (H_2O_2) not only to kill invading pathogens, but also as a signaling modulator. Woo et al. (2010) now show that the local inactivation of a H_2O_2-degrading enzyme ensures that the production of this oxidant is restricted to the signaling site.

The notion that hydrogen peroxide (H_2O_2) acts as a signaling molecule in mammalian cells was first proposed over a decade ago but remains controversial. Skepticism stems from the apparent paradox between the specificity that is required for a signaling molecule and the damaging properties of this oxidant, which is normally inactivated in cells by detoxifying enzymes, such as peroxiredoxins (D'Autréaux and Toledano 2007). In phagocytic cells, NADPH oxidase generates H_2O_2 for the direct purpose of killing invading microbes. So how does the mammalian cell tame the toxicity of H_2O_2 to make it useful as a signaling molecule? In this issue, Woo et al. (2010) help to resolve this paradox. They show that localized inactivation of a peroxiredoxin enzyme allows low concentrations of H_2O_2 to mediate tyrosine kinase receptor signaling in specific cellular subdomains, while preventing the toxic accumulation of H_2O_2 elsewhere in the cell.

The details of how H_2O_2 is produced and how it affects signaling after activation of receptor tyrosine kinases are becoming clearer. Engagement of a receptor tyrosine kinase with its ligand results in the production of H_2O_2, which is catalyzed by an NADPH oxidase in the plasma membrane of many types of mammalian cells (Lambeth 2004) (Figure 1). Produced close to the activated receptor tyrosine kinases, H_2O_2 helps to sustain the nascent signal because H_2O_2 inactivates nearby protein tyrosine phosphatases, which normally shut down signaling by dephosphorylating pathway components (Tonks 2006). Protein tyrosine phosphatases have an active site cysteine residue with a low pKa value, making them susceptible to oxidation by H_2O_2 and hence inactivation.

Although the involvement of H_2O_2 in membrane receptor signaling is well established, major gaps remain in the current model. First, it is difficult to fathom how a build up of H_2O_2 at the levels required for effective oxidation, and hence inactivation of protein tyrosine phosphatases, occurs in the presence of cellular peroxiredoxins, which reduce H_2O_2 to water (Figure 1). Considering that both cellular concentrations and reactivity toward H_2O_2 are orders of magnitude greater for peroxiredoxins than for protein tyrosine phosphatases, oxidation of protein tyrosine phosphatases by H_2O_2 is highly unlikely (D'Autréaux and Toledano 2007). So, how does the cell override this redox barrier while still preventing the toxic accumulation of H_2O_2?

In their new work, Woo and colleagues provide an interesting, albeit indirect, answer to this problem. They build upon the notion that H_2O_2 production begins only when the NADPH oxidase complex assembles within discrete plasma membrane subdomains near activated receptors (Ushio-Fukai 2006) (Figure 1). The authors identify peroxiredoxin PrxI as being integral to the precision of H_2O_2 signaling in mammalian cells. They show that, upon receptor engagement, this enzyme becomes phosphorylated on a tyrosine residue (Tyr^{194}) by a Src family kinase. In vitro peroxidase assays demonstrated that phosphorylation inactivates PrxI by decreasing the reactivity of its catalytic cysteine residue. They then show that PrxI phosphorylation correlates with H_2O_2 production and with the strength of the intracellular signal induced by the engaged receptor. Most importantly, the authors find that, whereas the majority of unmodified PrxI protein is found in the soluble fraction of cell extracts, phosphorylated PrxI, which accounts for about 0.3% of the total PrxI in the cell, is exclusively confined to the membrane-associated fraction that is known to contain c-Src, NADPH oxidase and receptor tyrosine