The activity of transposable elements is epigenetically suppressed by both transcriptional and post-transcriptional mechanisms. We recently identified a direct connection between the small RNA-mediated post-transcriptional mRNA degradation of actively transcribing transposable elements and the de novo methylation of transposable element DNA, providing a mechanistic link between these two established pathways of transposable element silencing. Here we provide a model for the initiation, establishment and epigenetic maintenance of transposable element silencing that incorporates recent data in this rapidly emerging field.

Transposable Element Silencing by Small Interfering RNAs

In plants, transposable elements (TEs) are epigenetically repressed through transcriptional gene silencing (TGS) via the establishment of RNA-directed DNA methylation (RdDM) (reviewed in refs. 1 and 2). Current models of RdDM rely on transcription of silenced TEs by RNA Polymerase IV (PolIV) and this transcript is quickly acted upon by particular RNA-dependent RNA polymerase and Dicer-like family proteins (RDR2 and DCL3) to generate specifically 24 nucleotide (nt) small interfering RNAs (siRNAs). This size class of siRNA is incorporated into Argonaute4 (AGO4) or AGO6 to target a nascent scaffolding transcript generated by RNA Polymerase V (PolV) (reviewed in ref. 1). Effective targeting of PolV transcripts results in establishment of DNA and histone methylation, reinforcing the marking of the TE as silenced heterochromatin.

Transcriptionally reactivated TEs are additionally repressed by post-transcriptional gene silencing (PTGS) via mRNA degradation. Like RdDM, PTGS uses RNA-dependent RNA polymerases and Dicer-like proteins, however the individual proteins are different members of these protein families. The RDR6 protein initiates PTGS and the Poll transcript is degraded by DCL2 and DCL4 to generate 21–22 nt siRNAs. These siRNAs are incorporated into AGO1 and target complementary TE mRNAs for cleavage. The role of RDR6 is therefore to supply the mRNA degradation cycle known as RNAi with amplified secondary 21-22 nt siRNAs.

How the TGS and PTGS pathways are interconnected and more specifically how RdDM and TGS can be initiated from an actively transcribing TE, has remained an unanswered question. Within the last year, studies have uncovered a new role for 21–22 nt siRNAs (previously associated with PTGS) in DNA methylation. The production of TE 21–22 nt siRNAs is dependent on RDR6 and therefore we refer to this newly identified pathway of DNA methylation as RDR6-RdDM to distinguish it from PolIV-RdDM. We recently investigated the role of both RDR6-RdDM and PolIV-RdDM in the epigenetic maintenance of TE TGS, the initiation of TE silencing and the corrective reestablishment of silencing for recently activated TEs. Our data suggests a model of TE silencing that distinctly utilizes both of these individual pathways to
**Proposed Model of Initiation, Establishment and Maintenance of TE Methylation and Silencing**

Similar to genes, epigenetically active TEs are transcribed by RNA Polymerase II (PolII) into mRNAs and produce proteins that enable the TE to transpose. This transcriptional activity represents the “default” epigenetic state of TEs. An active TE in a genome may be tolerated for a variable length of time, which depends on how mutagenic and disruptive that TE is. At some point in time, the active TE will be targeted for silencing, which can be triggered through several different mechanisms, depending on if the TE has homology to other silenced TEs in the genome (see below).

One route to trigger silencing of a TE is its recognition by RDR6 and the RNAi pathway. The production of 21–22 nt siRNAs from actively transcribing TEs is dependent on RDR6, DCL2, DCL4 and AGO1 (Fig. 1A). Incorporation of the DCL2 and DCL4-dependent 21–22 nt TE siRNAs into AGO1 results in the cycle of RNAi and effective PTGS (Fig. 1A). What specific feature of the TE mRNA attracts the RNAi pathway is currently unknown and remains a major focus of future investigation. DCL1 may also have some contribution to the production of these 21–22 nt siRNAs, but this cleavage would likely be RDR6-independent.

The focus of publications in the last year has been on understanding how the RNAi cycle described in Figure 1A initiates RdDM. We have discovered that some TE 21–22 nt siRNAs generated by RDR6, DCL2, DCL4 and AGO1 can act in the nucleus to initiate de novo DNA methylation through the RDR6-RdDM pathway (Fig. 1B). The role of these siRNAs in DNA methylation and the dependence of RDR6-RdDM pathway on PolII transcription are key discoveries toward initiation of TE silencing and form the basis of the following working model. This de novo DNA methylation may depend on 21–22 nt siRNAs being incorporated into other AGO proteins that direct DNA methylation, such as AGO2 or AGO6. Wu et al. demonstrated that DNA methylation at non-TE loci triggered by 21–22 nt siRNAs depends on these siRNAs being incorporated into the AGO6 protein, which is normally associated with 24 nt siRNAs. However, RDR6-RdDM could act through a different or multiple AGO proteins. RDR6-RdDM is dependent on a PolV scaffolding transcript and the de novo DNA methyltransferase DRM2 (Fig. 1B and C). This aspect of the RDR6-RdDM pathway is identical to the downstream components and requirements of the PolIV-RdDM pathway (Fig. 1B and C). It is not known how PolIV is initially recruited to TEs and future research needs to focus on how particular loci are selected to be transcribed by PolIV (potentially targeting only them for RdDM), or if all
regions are targeted by low-level or transient PolIV transcription.

After the initial round of DNA methylation via RDR6-RdDM, partially methylated TE loci are now a target for PolIV-RdDM (Fig. 1C). As described above, this pathway reinforces methylation at the TE locus through the activity of PolIV transcription, RDR2, DCL3, 24 nt siRNA production and targeting of DNA methylation through AGO4, AGO6, POLV and DRM2 (Fig. 1C and reviewed in ref. 1). The TE may go through multiple rounds of PolIV-RdDM, increasing the DNA methylation at each cycle. In contrast to the initiation phase of DNA methylation and TGS instigated by RDR6-RdDM, PolIV-RdDM represents the establishment phase of dense DNA methylation and TGS (Fig. 1B and C).

After increased PolIV-RdDM methylation, TE loci may exit the RdDM cycle with a highly methylated status. The CHH methylation established by RdDM will be lost or reduced over time as only CG and CHG (symmetrical) DNA methylation are required to maintain silencing of the these TE loci (Fig. 1D). MET1, CMT3 and DDM1 are essential proteins for faithful propagation of symmetrical DNA methylation and TGS (Fig. 1D).

Long TEs and TEs located in heterochromatic regions of the genome comprise this category of deeply silenced TEs. Most Arabidopsis TEs produce 24 nt siRNAs. However, mutations in PolIV-RdDM component proteins result in transcriptional reactivation of only a small percentage of the TEs that are reactivated when symmetrical DNA methylation is lost, indicating that many TEs are still targets of PolIV-RdDM without depending on its function. Alternatively, short TEs that reside near genes are dependent on PolIV-RdDM for constant DNA methylation reinforcement to achieve TGS.

The example of a transcriptionally active TE entering the genome helps illustrate the robustness of the model described above. The incoming TE can be one of two exclusive categories. Either the TE enters the genome via horizontal transfer, in which case the sequence of the new TE might be completely unique compared with existing TEs (Fig. 1E), or through cross-hybridization with a closely related species and hence the TE shares homology with some existing silenced TEs in the genome (Fig. 1F). In the case of homologous TE invasion (Fig. 1F), 24 nt siRNAs matching the TE will recognize and quickly target the TE for silencing via PolIV-RdDM and this cycle will establish dense DNA methylation (Fig. 1C). RDR6-RdDM may also target this incoming active TE, but the cell can more quickly or easily rely on the existing homologous 24 nt siRNAs that act as a cellular surveillance to silence any matching TEs. In the case of the horizontal transfer of a unique TE (Fig. 1E), the plant must rely on RDR6 to recognize the TE mRNA and initiate transcriptional gene silencing via RDR6-RdDM as described above (Fig. 1B). This process may take substantially more time than silencing by existing homologous PolIV-dependent 24 nt siRNAs and therefore the new unique TE may experience generations of activity and proliferation before it can be tamed by RDR6-RdDM and converted into a 24 nt siRNA generating locus that protects the genome from future invasions of similar TEs.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Acknowledgments
This work was supported by grant MCB-1020499 to R.K.S. from the U.S. National Science Foundation.

References
1. Law JA, Jacobsen SE. Establishing, maintaining and modifying DNA methylation patterns in plants and animals. Nat Rev Genet 2010; 11:204-20; PMID:20412834; http://dx.doi.org/10.1038/ng.2179
2. Haag JR, Pikard CS. Multisubunit RNA polymerases IV and V: purveyors of non-coding RNA for plant gene silencing. Nat Rev Mol Cell Biol 2011; 12:483-92; PMID:21779025; http://dx.doi.org/10.1038/nrm3152
3. Sijen T, Plasterk RHA. Transposon silencing in the Caenorhabditis elegans germ line by natural RNAi. Nature 2003; 426:310-4; PMID:14628056; http://dx.doi.org/10.1038/nature2107
4. McCue AD, Nuthikuttu S, Reeder SH, Slotkin RK. Gene expression and stress response mediated by the epigenetic regulation of a transposable element small RNA. PLoS Genet 2012; 8:e1002474; PMID:22346759; http://dx.doi.org/10.1371/journal.pgen.1002474
5. Nuthikuttu S, McCue AD, Panda K, Fultz D, Defraia C, Thomas EN, et al. The initiation of epigenetic silencing of active transposable elements is triggered by RDR6 and 21-22 nucleotide small interfering RNAs. Plant Physiol 2013; 162:116-31; PMID:23542151; http://dx.doi.org/10.1104/pp.113.216481
6. Pontier D, Picart C, Roudier F, Garcia D, Lahmy S, Azavedo J, et al. NERD, a plant-specific GW protein, defines an additional RNAI-dependent chromatin-based pathway in Arabidopsis. Mol Cell 2012; 48:121-32; PMID:22940247; http://dx.doi.org/10.1016/j.molcel.2012.07.027
7. Garcia D, Garcia S, Pontier D, Marchais A, Renou JP, Lagrange T, et al. Ago hook and RNA helicase motifs underpin dual roles for SDE3 in antiviral defense and silencing of nonconserved intergenic regions. Mol Cell 2012; 48:109-20; PMID:22940249; http://dx.doi.org/10.1016/j.molcel.2012.07.028
8. Wu L, Mao L, Qi Y. Roles of dicer-like and argonaute proteins in TAS-derived small interfering RNA-triggered DNA methylation. Plant Physiol 2012; 160:990-9; PMID:22846193; http://dx.doi.org/10.1104/pp.111.200279
9. Minouze M, Reinders J, Bucher E, Nishimura T, Schneeberger K, Ossowski S, et al. Selective epigenetic control of retrotransposition in Arabidopsis. Nature 2009; 461:427-30; PMID:19734882; http://dx.doi.org/10.1038/nature08328
10. Laubinger S, Zeller G, Henz SR, Buechel S, Sachsenberg T, Wang JW, et al. Global effects of the small RNA biogenesis machinery on the Arabidopsis thaliana transcriptome. Proc Natl Acad Sci USA 2010; 107:17466-73; PMID:20870966; http://dx.doi.org/10.1073/pnas.1012891107
11. Eun C, Lorković ZJ, Naumann U, Long Q, Havercek ER, Simon SA, et al. AGO6 functions in RNA-mediated transcriptional gene silencing in shoot and root meristems in Arabidopsis thaliana. PLoS ONE 2011; 6:e25730; PMID:21998686; http://dx.doi.org/10.1371/journal.pone.0025730
12. Zemach A, Kim MY, Hsheh PH, Coleman-Derr D, Eshed-Williams L, Thao K, et al. The Arabidopsis nucleosome remodeler DDM1 allows DNA methylation transfers to access H1-containing heterochromatin. Cell 2013; 153:193-205; PMID:23540698; http://dx.doi.org/10.1016/j.cell.2013.02.030
13. Mosher RA, Schwach F, Strahleme D, Baulcombe DC. PolIVb influences RNA-directed DNA methylation independently of its role in siRNA biogenesis. Proc Natl Acad Sci USA 2008; 105:3145-50; PMID:18287047; http://dx.doi.org/10.1073/pnas.0709632105