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Noncoding Transcription by RNA Polymerase Pol IVb/Pol V Mediates Transcriptional Silencing of Overlapping and Adjacent Genes

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SUMMARY

Nuclear transcription is not restricted to genes but occurs throughout the intergenic and noncoding space of eukaryotic genomes. The functional significance of this widespread noncoding transcription is mostly unknown. We show that *Arabidopsis* RNA polymerase IVb/Pol V, a multisubunit nuclear enzyme required for siRNA-mediated gene silencing of transposons and other repeats, transcribes intergenic and noncoding sequences, thereby facilitating heterochromatin formation and silencing of overlapping and adjacent genes. Pol IVb/Pol V transcription requires the chromatin-remodeling protein DRD1 but is independent of siRNA biogenesis. However, Pol IVb/Pol V transcription and siRNA production are both required to silence transposons, suggesting that Pol IVb/Pol V generates RNAs or chromatin structures that serve as scaffolds for siRNA-mediated heterochromatin-forming complexes. Pol IVb/Pol V function provides a solution to a paradox of epigenetic control: the need for transcription in order to transcriptionally silence the same region.

INTRODUCTION

Nuclear transcription in eukaryotes is not restricted to messenger RNAs (mRNAs), ribosomal RNAs (rRNAs), transfer RNAs (tRNAs), or genes required for their processing. In humans, such conventional genes account for less than 2% of the genome, yet ~90% of the genome is transcribed (Kapranov et al., 2007; Prasanth and Spector, 2007; Willingham et al., 2006). Much of the noncoding RNA (ncRNA) pool corresponds to intergenic sequences or antisense transcripts of unknown function. However, the potential for noncoding RNAs (ncRNAs) to epigenetically regulate adjacent genes is increasingly clear (Prasanth and Spector, 2007). Long ncRNAs that regulate adjacent genes include the *Xist* and *Tsix* RNAs involved in X chromosome inactivation in mammals (Masui and Heard, 2006; Yang and Kuroda, 2007), the H19 and Air ncRNAs involved in imprinting at mouse and human Igf2 and Igf2r loci, respectively (Pauler et al., 2007), and the *roX* ncRNAs involved in X chromosome dosage compensation in flies (Bai et al., 2007). The persistence of Xist and roX transcripts at affected loci indicates a role in the assembly of repressive or activating chromatin states, respectively (Bai et al., 2007; Herzing et al., 1997). Likewise, at the *Drosophila Ultrabithorax* (*Ubx*) locus, intergenic ncRNAs serve as scaffolds for the recruitment of Ash1, a histone methyltransferase that modifies the adjacent chromatin to switch on *Ubx* transcription (Sanchez-Elsner et al., 2006).

In diverse eukaryotes, establishment of DNA methylation and/or repressive heterochromatic histone modifications are ncRNA-directed processes (Buhler et al., 2007; Grewal and Elgin, 2007; Zaratiegui et al., 2007). In plants and fission yeast, small interfering RNAs (siRNAs) of 20–25 nt that are generated from long double-stranded RNA (dsRNA) precursors by dicer endonuclease(s) bind to argonaute (AGO) proteins and guide chromatin modifications to homologous DNA sequences (Baulcombe, 2006; Brodersen and Voinnet, 2006; Peters and Meister, 2007). Noncoding transcripts in fission yeast serve at least two functions: acting as precursors of siRNAs and as scaffolds to which siRNAs bind in order to recruit the chromatin-modifying machinery (Buhler et al., 2006, 2007; Irvine et al., 2006). AGO-mediated slicing of scaffold transcripts coupled with RNA-dependent RNA polymerase-mediated dsRNA production generates additional siRNAs, thereby perpetuating heterochromatin formation (Irvine et al., 2006; Locke and Martienssen, 2006). RNA-mediated heterochromatin formation requires that an affected region be transcribed (Buhler et al., 2006; Djupedal et al., 2005; Irvine et al., 2006; Kato et al., 2005), presenting an intriguing paradox as to how transcription and transcriptional silencing can occur at the same locus (Grewal and Elgin, 2007).

The paradox of transcription-dependent gene silencing in plants might be explained by the existence of two structurally and functionally distinct plant-specific RNA polymerases: RNA polymerases IVa/Pol IV and Pol IVb/Pol V (Herr et al., 2005; Kanno et al., 2005; Onodera et al., 2005; Pontier et al., 2005). Pol IVa/Pol IV and Pol IVb/Pol V are not essential for viability in *Arabidopsis* but participate in multiple small RNA-mediated gene silencing pathways (Pikaard et al., 2008). Pol IVa/Pol IV and Pol IVb/Pol V have distinct largest subunits that have been named either NRPD1a and NRPD1b (Herr et al., 2005; Onodera et al., 2005) or RPD1 and RPE1 (Luo and Hall, 2007). The latter terminology has been adopted, in modified form, to allow the naming of Pol IVa/Pol IV subunits using the Nuclear RNA polymerase D (NRPD) gene symbol and Pol IVb/Pol V subunits using the Nuclear RNA polymerase E (NRPE) prefix. The transition to the

Pol IV and Pol V nomenclature in place of Pol IVa and Pol IVb has been made necessary by the need for a systematic nomenclature defining their numerous subunits (T. Ream and C.S.P., unpublished data) and reflects the fact that the two activities are functionally nonredundant as well as structurally distinct. Therefore, we refer to Pol IVa and Pol IVb as Pol IV and Pol V for the remainder of this paper. The revised nomenclature denotes the largest subunits of Pol IV and Pol V as NRPD1 and NRPE1. Pol IV and Pol V both utilize a second-largest subunit that is encoded by a single gene bearing the synonymous names *NRPD2* or *NRPE2*. In the siRNA-directed DNA methylation pathway, Pol IV is required for siRNA production, whereas Pol V acts primarily downstream of siRNA production (Kanno et al., 2005; Mosher et al., 2008; Pontes et al., 2006; Pontier et al., 2005; Zhang et al., 2007). Pol IV or Pol V transcripts have not been identified in vivo or in vitro, but the catalytic subunits of Pol IV and Pol V have amino acids that are invariant at the active sites of multisubunit RNA polymerases and are essential for Pol IV and Pol V biological functions (J.R.H. and C.S.P., unpublished data).

By pursuing the hypothesis that Pol IV and/or Pol V might synthesize ncRNAs required for transcriptional gene silencing, we identified intergenic regions where Pol V-dependent transcripts are detectable by RT-PCR. Pol V (Pol IVb) physically associates with loci that give rise to these transcripts and also physically associates with the RNA transcripts themselves. Moreover, production of the Pol V-dependent transcripts is lost upon mutation of the conserved active site of NRPE1/NRPD1b, suggesting that the RNAs are Pol V transcripts. The putative chromatin remodeler DRD1 is required for Pol V to physically associate with intergenic loci and generate transcripts that suppress adjacent transposons via the establishment of repressive heterochromatin. Importantly, Pol V transcription alone is not sufficient for transposon silencing; instead, the combination of Pol V transcription and siRNA production is required. Collectively, our data indicate that Pol V (Pol IVb) transcription occurs independently of siRNA biogenesis and support a model whereby Pol V transcripts serve as scaffolds for the binding of siRNAs that guide heterochromatin formation. Pol V's role in gene silencing provides a solution in plants to the paradox of how transcription can be required for transcriptional gene silencing.

RESULTS

Identification of Pol V-Dependent Transcripts in Intergenic Noncoding Regions

A heterochromatic knob, or chromomere, on the northern arm of *A. thaliana* chromosome 4 is a well-characterized interval rich in transposons and other heterochromatic repeats (Fransz et al., 2000; Lippman et al., 2004). Within this domain are intergenic noncoding (IGN) regions at which RNA transcripts have not been detected using tiling DNA microarrays (Lippman et al., 2004). Nonetheless, siRNAs and DNA hypermethylation often map to these regions (Kasschau et al., 2007; Lippman et al., 2005; Lister et al., 2008), suggesting that low-abundance transcripts might serve as siRNA precursors. Therefore, we used RT-PCR to search for IGN RNAs present in wild-type plants but missing in Pol IV or Pol V mutants. Of 14 IGN regions examined, six had RNAs that were lost or reduced in Pol V mutants (Figures 1 and S1 available online). For instance, at intergenic noncoding regions 5 and 6 (*IGN5* and *IGN6*) (Figures 1A and 1B), transcripts detected in wild-type (ecotype Col-0) and *nrpd1* mutants are depleted in *nrpe1* (*nrpd1b-11*) or *nrpd2* mutants (Figure 1E, top three rows), indicating that Pol V, but not Pol IV, is required for their production. However, *AtSN1* family retrotransposons are derepressed (activated) in both the Pol IV and Pol V mutants (Figure 1E, fourth row from the top). Actin 2 mRNA abundance is unaffected by the mutations (Figure 1E).

IGN5 and *IGN6* are located in regions rich in transposon-derived elements, siRNA production, and DNA hypermethylation (Lister et al., 2008), all characteristic of heterochromatic domains. Pol V-dependent transcripts are also detected at *IGN7* and *IGN17* (Figure 1F), which are located in pericentromeric heterochromatic regions (Figure S1). However, *IGN10* and *IGN15* are present in gene-rich environments with relatively few transposon-related repeats (Figures 1C and 1D) yet also give rise to Pol V-dependent transcripts (Figure 1F). Collectively, these data suggest that Pol V contributes to IGN transcription in both heterochromatic and euchromatic environments.

Characterization of Pol V-Dependent Transcripts

To determine whether Pol V-dependent RNAs initiate at specific sites, we performed 5′ RACE at *IGN5* and *IGN6* (Figures 2A–2C). Resulting PCR-amplified RACE products yielded distinct bands upon agarose gel electrophoresis (Figure 2C), but excising the bands and cloning and sequencing of the cDNAs revealed heterogeneity at the 5′ ends. At *IGN5*, top-strand clones initiated at two sites seven nucleotides apart (Figures 2A and S2). An *IGN5* bottom-strand-specific primer yielded five different 5′ ends spanning a 33 nt interval (Figures 2A and S2). At *IGN6*, clones derived from the gel-purified upper and lower bands collectively revealed four distinct 5′ ends spanning a 94 nt interval (Figures 2B and S2). Bottom-strand-specific transcripts were not detected at *IGN6*.

It is noteworthy that the 5′ terminal nucleotides of all RACE products were adenosine or guanosine (Figure S2), given that transcripts of eukaryotic Pol I, II, III, and bacterial RNA polymerase typically begin with purines (Smale and Kadonaga, 2003; Sollner-Webb and Reeder, 1979; Zecherle et al., 1996). To test whether RACE 5′ ends represent transcription start sites or cleavage sites, we exploited the fact that initiating nucleotides have 5′ triphosphate groups (Pol I, Pol III) or 7-methylguanosine caps (Pol II). By contrast, cleaved RNAs have 5′ monophosphate or hydroxyl groups. Terminator exonuclease (Epicentre Biotechnologies) is a 5′→3′ exonuclease that degrades RNAs having 5′ monophosphates, but not RNAs that have 5′ triphosphate groups, 5′ hydroxyl groups, or 7-methylguanosine caps. Total RNA treated with Terminator endonuclease was subjected to RT-PCR using *IGN5*-specific primers (Figure 2D; interval A is depicted in Figure 2A). In agreement with Figure 1, *IGN5* transcripts were detected in wild-type (Col-0) plants but were absent in the Pol V mutant (*nrpe1*/*nrpd1b-11*). Terminator exonuclease treatment prior to RT-PCR caused an ~70% reduction in the Pol V-dependent *IGN5* transcript signal, suggesting that the majority of the transcripts amplified by PCR are 5′ monophosphorylated; however, the remaining transcripts are resistant to the exonuclease (Figure 2D). Treatment of the RNA with Tobacco Acid

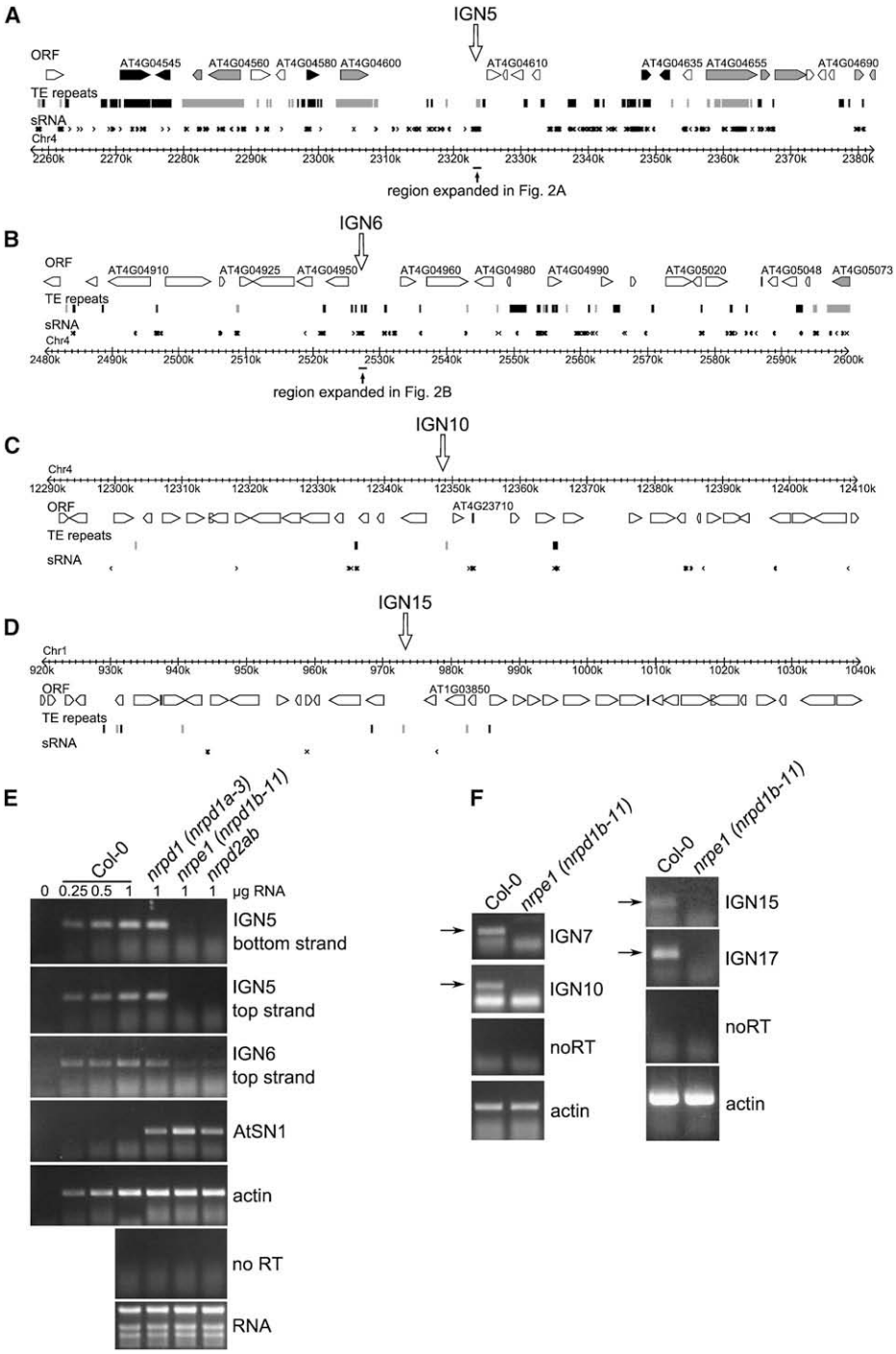

Figure 1. Detection of Intergenic Pol V-Dependent Transcripts

(A–D) Chromosomal contexts of intergenic regions *IGN5*, *IGN6*, *IGN10*, and *IGN15*. Open reading frames (ORF), transposable element (TE)-derived repeats, and small RNAs (sRNA) in the MPSS database (http://mpss.udel.edu/at/) are shown. Single-copy genes are marked in white; retrotransposons, in gray; and DNA transposons, in black. Diagrams derive from http://chromatin.cshl.edu/cgi-bin/gbrowse/arabidopsis5/.

(E) Strand-specific RT-PCR analysis of *IGN5*, *IGN6*, and *AtSN1* transcripts in wild-type (ecotype Col-0), *nrpd1a-3*, *nrpe1* (*nrpd1b-11*), and *nrpd2a-2 nrpd2b-1* mutants. Actin RT-PCR products and ethidium bromide-stained rRNAs resolved by agarose gel electrophoresis serve as loading controls. Dilutions of Col-0 RNA show that PCR results are semiquantitative. To control for background DNA contamination, a reaction using *IGN5* top-strand primers, but no reverse transcriptase (no RT), was performed. No RNA (0 µg) controls are provided for all primer pairs.

(F) RT-PCR analysis of Pol V-dependent transcripts at intergenic regions *IGN7*, *IGN10*, *IGN15*, and *IGN17* in wild-type (Col-0) and *nrpe1* mutants.

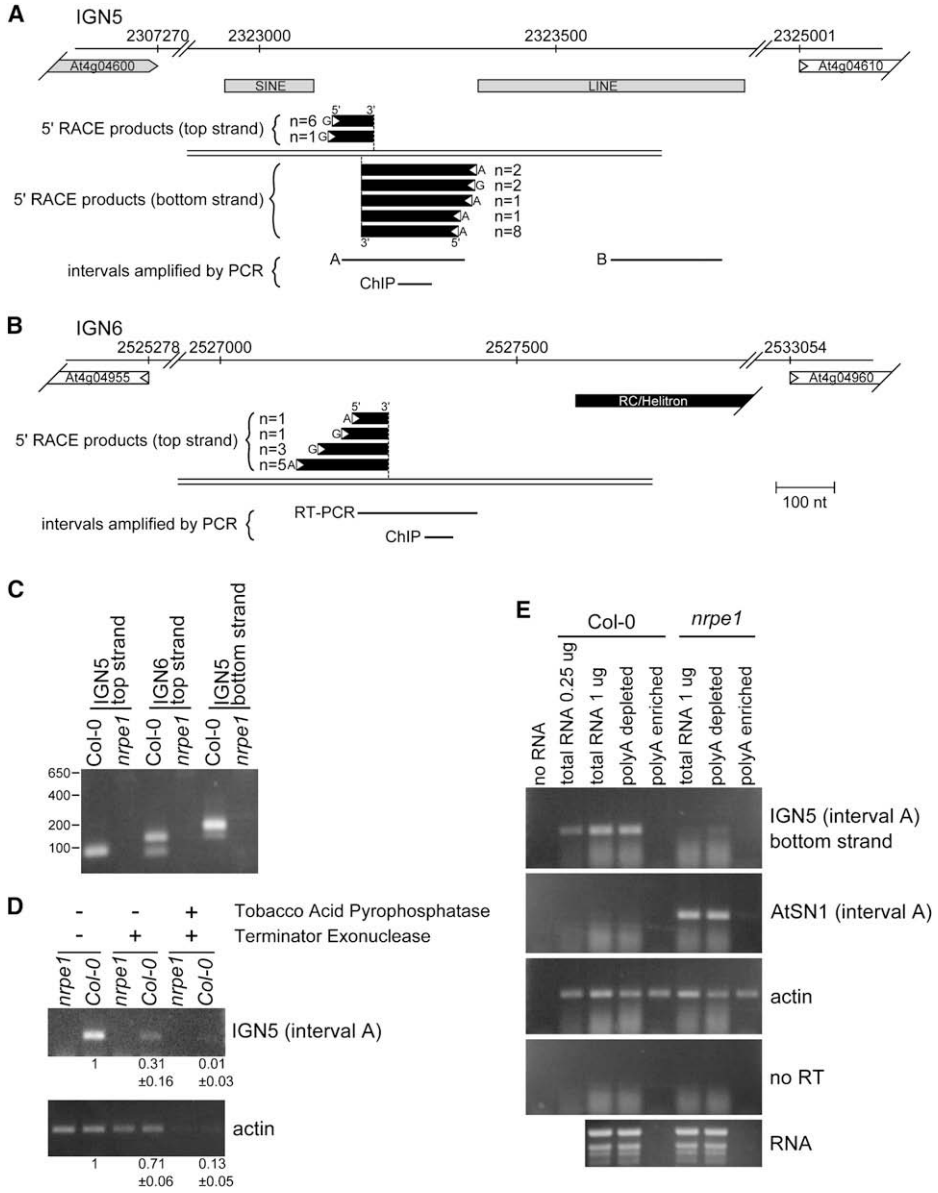

Figure 2. Characterization of Pol V-Dependent Transcripts

(A and B) Local contexts of *IGN5* (A) and *IGN6* (B), showing neighboring genes or transposons, 5′ RACE products, and intervals amplified by PCR. Color coding of annotated genes and TE elements is the same as in Figure 1. For RACE products, the 5′ terminal nucleotide and number of clones (n) sharing that 5′ end are shown.

(C) Ethidium bromide-stained agarose gel of 5′ RACE products.

(D) 5′ end analysis for Pol V-dependent *IGN5* transcripts. RT-PCR was performed on total RNA or RNA treated with Terminator exonuclease, Tobacco Acid Pyrophosphatase, or both enzymes. Numbers below the panels are relative densitometric band intensities relative to the untreated control. The mean and standard deviation resulting from three independent experiments is shown.

(E) Pol V-dependent transcripts are not polyadenylated. Poly A-enriched and poly A-depleted RNA fractions were subjected to RT-PCR using *IGN5*, *AtSN1*, and actin primer pairs followed by agarose gel electrophoresis and ethidium bromide staining. Controls include no RT (*IGN5* bottom-strand primers) and no RNA (all primer pairs) reactions.

Pyrophosphatase, which removes 7-methylguanosine caps or triphosphates and leaves a 5′ monophosphate, rendered the *IGN5* transcripts and actin control fully susceptible to Terminator exonuclease digestion. Therefore, *IGN5* transcripts that require Tobacco Acid Pyrophosphatase in order to be made Terminator susceptible are deduced to be triphosphorylated or capped (Figure 2D), indicative of transcription start sites. It is noteworthy that 5′ RACE requires a 5′ monophosphate for adaptor ligation. RACE products were only obtained upon treating RNA with Tobacco Acid Pyrophosphatase, but not upon treating RNA with

T4 polynucleotide kinase and ATP (data not shown), which would have converted 5′ hydroxyls to phosphates and allowed their cloning. Collectively, our observations suggest that the 5′ ends detected by RACE are transcription start sites. However, much of the RNA detected by RT-PCR consists of processed RNAs.

To test whether Pol V-dependent transcripts are polyadenylated, total RNA was fractionated using oligo d(T) magnetic beads. *IGN5* transcripts were detected in total RNA and poly A-depleted fractions of wild-type Col-0 but were not detected in poly A-enriched RNA (Figure 2E), unlike *Actin 2* mRNA. *AtSN1* transcripts produced in *nrpe1* (*nrpb1b-11*) mutants were present in total and poly A-depleted, but not poly A-enriched, RNA, consistent with Pol III transcription of *AtSN1* (see below).

Collectively, the assays of Figure 2 suggest that Pol V-dependent transcripts can be at least ~200 nt in size, can initiate from multiple sites, have triphosphates or 7meG caps at their 5′ ends, and lack poly A tails.

Evidence that Pol V Synthesizes IGN Transcripts

The largest subunits of Pol IV and Pol V include sequences that are invariant among DNA-dependent RNA polymerases, including a DFDGD at the active site (metal A site) that coordinates a magnesium ion essential for nucleoside polymerization (Cramer, 2004). We tested the importance of the presumptive NRPE1 metal A site by analyzing *nrpe1* (*nrpd1b-11*) mutants transformed with a wild-type *NRPE1* transgene or a transgene in which the invariant aspartates were changed to alanines (active site mutant [ASM]) (Figure 3A). Both transgenes utilized the native *NRPE1* promoter, included their full complement of introns and exons, and were similarly expressed, as shown by immunoblot detection of the FLAG epitope tags added to their C termini (Figure 3B, bottom row). Moreover, the wild-type and ASM mutant proteins both coimmunoprecipitate NRPD2/NRPE2, the second-largest subunit of both Pol IV and Pol V, suggesting that the ASM mutation does not disrupt Pol V subunit assembly (J.R.H. and C.S.P., unpublished data). The wild-type *NRPE1* transgene restored Pol V-dependent *IGN5* and *IGN6* transcripts in the *nrpe1* (*nrpd1b-11*) mutant background, but the *NRPE1*-ASM transgene did not (Figure 3B), indicating that synthesis of Pol V-dependent transcripts requires the conserved active site.

To determine whether NRPE1 physically interacts with loci giving rise to Pol V-dependent transcripts, we performed chromatin immunoprecipitation (ChIP) of FLAG-tagged NRPE1 as well as FLAG-tagged NRPB2, the second-largest subunit of RNA polymerase II (Figure 3C). Subsequent quantitative real-time PCR showed that NRPE1 physically associates with *IGN5*, whereas NRPB2 does not. A retrotransposon-derived solo long terminal repeat (LTR) shown to be silenced in a Pol V-dependent manner (Huettel et al., 2006) is also occupied by NRPE1. The *solo LTR* most likely programs Pol II transcription, and Pol II is detected at this locus above background (defined as ChIP signals obtained with Col-0 plants that lack a FLAG-tagged transgene) but at lower levels than at the *actin 2* gene locus *At3g18780*. Collectively, the ChIP data indicate that Pol V is present at loci that give rise to Pol V-dependent RNAs.

We next asked whether Pol V-dependent RNAs could be immunoprecipitated (IPed) in association with NRPE1. Formaldehyde-crosslinked chromatin preparations of nontransgenic Col-0 or *nrpe1* (*nrpd1b-11*) lines expressing FLAG-tagged NRPE1 were IPed using anti-FLAG antibody. Following DNase I treatment, samples were tested by RT-PCR (Figure 3D). *IGN5*, *IGN6*, *AtSN1*, and *solo LTR* RNAs were all enriched in IP fractions of NRPE1-FLAG plants compared to nontransgenic Col-0 controls that were also subjected to anti-FLAG IP (Figure 3D). Background levels of abundant actin mRNA were equivalent in Col-0 and NRPE1-FLAG IP fractions, indicating that the enrichment of the IGN and transposon RNAs in NRPE1-FLAG IP fractions compared to Col-0 reflects specific interaction of these RNAs with Pol V. Because Pol V-dependent transcripts require the presumptive NRPE1 active site, NRPE1 physically associates with loci giving rise to these transcripts, and NRPE1 physically associates with the transcripts themselves, we deduce that Pol V synthesizes the transcripts.

Pol V Transcription Is Necessary in Order to Silence Overlapping and Adjacent Genes

Transcriptional silencing of *AtSN1* retroelements requires both Pol IV and Pol V (see Figure 1E). *AtSN1* family elements are short interspersed nuclear elements (SINEs) that possess A box and B box elements (see diagram in Figure 4A) typical of the internal promoters of Pol III-transcribed genes (Myouga et al., 2001). In wild-type (Col-0) plants, *AtSN1* elements are silenced, but, in *nrpe1* (*nrpd1b-11*) mutants, they are derepressed (Figures 1E and 4C, interval A). *AtSN1* silencing is restored in *nrpe1* mutants by the full-length *NRPE1* transgene, but not by the active site mutant *NRPE1-ASM* transgene (Figure 4C, top row), indicating that Pol V transcription is required for *AtSN1* silencing. In the intergenic region and overlapping the expected Pol III transcription start site (see Figures 4A and S3), IGN transcripts corresponding to both DNA strands can be detected by RT-PCR. These transcripts, within intervals B and C, are readily detected in wild-type plants but are absent, or much reduced, in *nrpe1* mutants (Figure 4C, rows 2–5). The interval B and C transcripts are restored in *nrpe1* mutants by the wild-type *NRPE1* transgene, but not by the *NRPE1-ASM* transgene. Collectively, the data indicate that *AtSN1* transcripts are only generated if Pol V transcripts are absent.

Like *AtSN1*, a long interspersed nuclear element (LINE), *At5g27845*, which overlaps the *solo LTR* (see Figure 4B), is silenced in a Pol V (Pol IVb)-dependent manner (Huettel et al., 2006). Transcription of this LINE is low in wild-type plants but increases substantially in the *nrpe1* (*nrpd1b-11*) mutant (Figure 4D, RT-PCR interval A). Silencing is restored by the wild-type *NRPE1* transgene, but not by the *NRPE1-ASM* transgene (Figure 4D).

In wild-type plants, transcripts are detected from both strands upstream of the LINE and *solo LTR* (interval B), including intergenic sequences and overlapping an adjacent transcription unit, At5g27850 (see Figure 4B). These RNAs in wild-type plants might be Pol V transcripts. However, unlike the intergenic region adjacent to *AtSN1*, where transcripts disappear in *nrpe1* (*nrpd1b-11*) mutants, suggesting that Pol V is the sole polymerase transcribing the region, transcript abundance in the region adjacent to the *solo LTR* increases dramatically in *nrpe1* or *NRPE1-ASM* transgenic plants (Figure 4D). This increased transcription is attributable to RNA polymerase II, as shown by ChIP (Figure 4F). Whereas Pol II occupancy of the locus is low in wild-type plants,

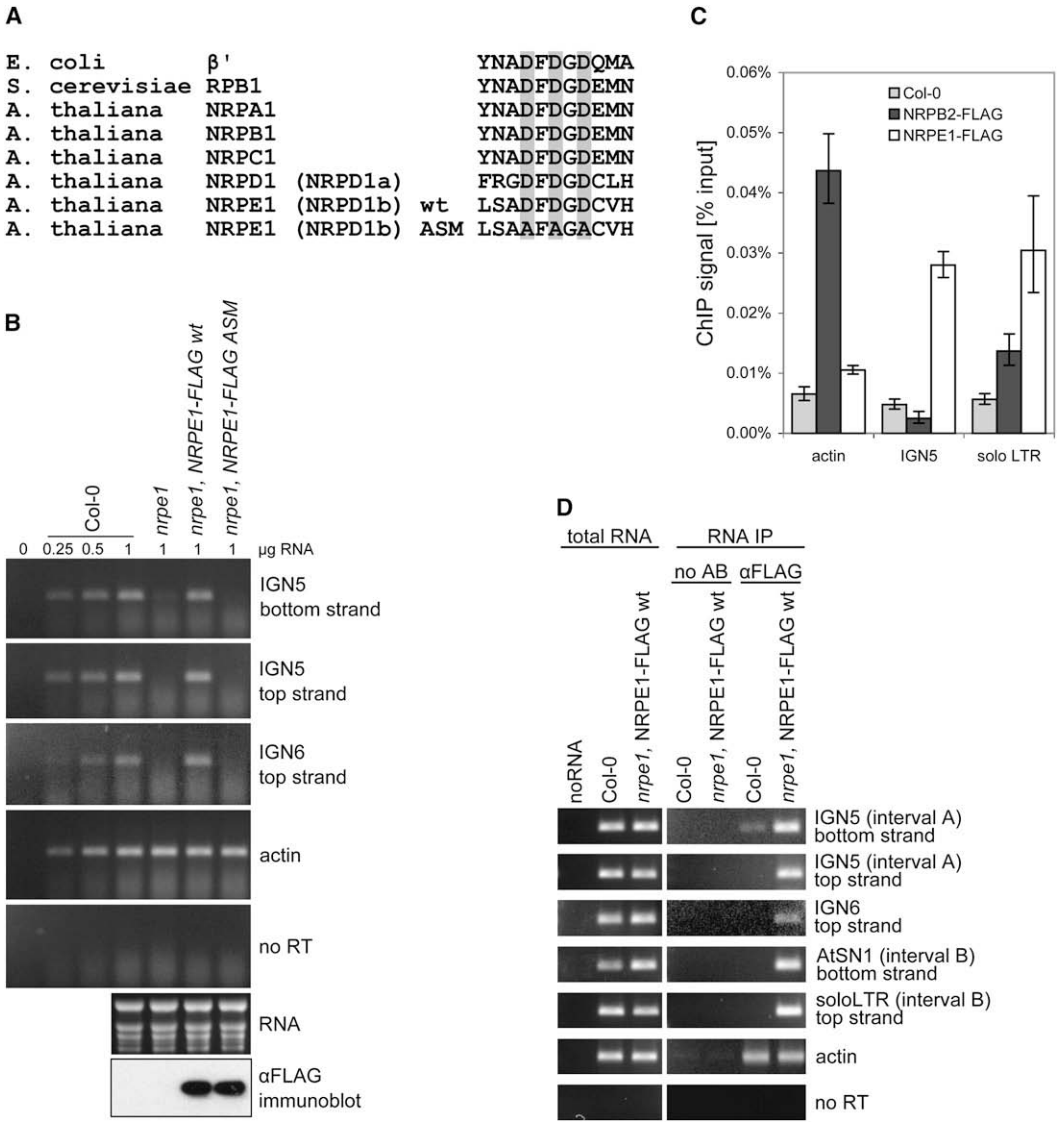

Figure 3. Evidence that Pol V Synthesizes IGN Transcripts

(A) Multiple alignments of DNA-dependent RNA polymerase largest subunits surrounding the metal A active site. Invariant aspartates are marked in gray. (β′) Largest subunit of *E. coli* polymerase; (RPB1) Largest subunit of yeast Pol II; (NRPA1) Largest subunit of *Arabidopsis* Pol I; (NRPB1) Largest subunit of *Arabidopsis* Pol II; (NRPC1) Largest subunit of *Arabidopsis* Pol III; (NRPD1) Largest subunit of *Arabidopsis* Pol IV (also known as NRPD1a); (NRPE1 WT) Largest subunit of *Arabidopsis* Pol V (also known as NRPD1b); (NRPE1-ASM) Active site mutant of NRPE1.

(B) Strand-specific RT-PCR analysis of *IGN5* and *IGN6* transcripts in Col-0 wild-type, *nrpe1* (*nrpd1b-11*), and *nrpe1* mutants transformed with a wild-type (WT) FLAG-tagged *NRPE1* transgene or the *NRPE1*-ASM transgene. Actin RT-PCR reactions and ethidium bromide-stained rRNAs serve as loading controls. Dilutions of Col-0 wild-type RNA demonstrate that PCR results are semiquantitative. No RT (*IGN5* top-strand primers) and no RNA (all primer pairs) controls are included. Equal expression of transgenic wild-type and active site mutant NRPE1 was verified by immunoprecipitation followed by αFLAG immunoblot detection (bottom row).

(C) ChIP of FLAG-tagged Pol II or Pol V at the *actin 2* gene, *IGN5*, or a solo retroelement LTR silenced by Pol V. Wild-type Col-0 plants or plants expressing FLAG-tagged NRPB2 or FLAG-tagged NRPE1 were subjected to ChIP using anti-FLAG antibody followed by real-time PCR. Histograms show mean values ± SD obtained for three independent PCR amplifications.

(D) RNA immunoprecipitation. Wild-type (nontransgenic) Col-0 and *nrpe1* (*nrpd1b-11*) mutants expressing the NRPE1-FLAG transgene were subjected to RNA-IP using anti-FLAG antibody. Following DNase treatment, *IGN5*, *IGN6*, *AtSN1*, *solo LTR*, or *actin 2* RNAs were detected by RT-PCR. *AtSN1* and *solo LTR* PCR-amplified intervals are shown in Figure 4; *IGN5* and *IGN6* PCR-amplified intervals are shown in Figure 2. Total RNA controls, assayed prior to immunoprecipitation, show that the RNAs are present in equivalent amounts in wild-type Col-0 and *NRPE1-FLAG* transgenic plants. No RT controls used *IGN5* top-strand primers. No signals were obtained following RNA IP in the absence of anti-FLAG antibody (no AB columns). Background signal for actin RNA shows that equal RNA amounts were tested.

it increases dramatically in the *nrpe1* mutant. Transformation of *nrpe1* with the wild-type *NRPE1* transgene reduces Pol II occupancy of the locus, whereas the *NRPE1-ASM* mutant is ineffective (Figure 4F). Taken together, the data indicate that derepression of Pol II transcription in the *solo LTR* region occurs in the absence of Pol V transcription.

A LINE element located to the right of *IGN5* is expressed at low levels in wild-type plants but is derepressed in the *nrpe1* mutant (Figure 4E). Silencing is restored by the wild-type *NRPE1* transgene, but not by the *NRPE1-ASM* mutant transgene. Collectively, the data of Figure 4 indicate that intergenic Pol V transcription plays a direct role in suppressing transcription from overlapping or adjacent LINE and SINE transposons.

Pol V Transcription Is Necessary for Heterochromatin Formation at Affected Loci

We next examined histone modifications and cytosine methylation at Pol V affected loci (Figure 5). ChIP using an antibody specific for histone H3 lysine 27 monomethylation (H3K27me1), a heterochromatic mark previously shown to be dependent on Pol V (Pol IVb) (Huettel et al., 2006), resulted in significant enrichment of *IGN5*, the *solo LTR* region, and *AtSN1* relative to the actin gene control (Figure 5A). Decreased H3K27me1 at the *IGN5*, *solo LTR*, and *AtSN1* loci in *nrpe1* (*nrpd1b-11*) was restored by the *NRPE1* transgene, but not the *NRPE1*-ASM transgene (Figure 5A). ChIP controls in which antibody was omitted yielded negligible background signals (Figure S4). ChIP using an antibody specific for dimethylated histone H3 lysine 9 (H3K9me2), also a heterochromatic mark, showed association of *IGN5* and the *solo LTR* region that was reduced in *nrpe1* and rescued by the wild-type *NRPE1* transgene, but not the *NRPE1-ASM* transgene (Figure 5B). Interestingly, Pol V mutations did not significantly affect H3K9me2 at *AtSN1* despite their pronounced effect on H3K27me1 at the locus.

Diacetylation of histone H3 on lysines 9 and 14 (abbreviated H3Ac2) is a characteristic of active, euchromatic genes, such as actin (Figure 5C). At the *solo LTR*, H3Ac2 levels increased significantly in the *nrpe1* (*nrpd1b-11*) mutant and were restored by the wild-type *NRPE1* transgene, but not the *NRPE1-ASM* transgene (Figure 5C). These results parallel increased Pol II occupancy of the locus in the absence of functional NRPE1 (see Figure 4F). H3Ac2 levels at *IGN5* and *AtSN1* were not influenced by NRPE1. Differences in histone hyperacetylation at the loci may reflect the different RNA polymerases transcribing them; *IGN5* is transcribed by Pol V, and *AtSN1* is presumably transcribed by Pol III, whereas Pol II transcribes the *solo LTR*.

We assayed *IGN5*, *IGN6*, and *solo LTR* DNA methylation status based on *Mcr*BC endonuclease sensitivity (Figure 5D). *Mcr*BC specifically cleaves methylated DNA, preventing its subsequent amplification by PCR. In wild-type Col-0, methylcytosine levels are high at *IGN5*, *IGN6*, and the *solo LTR*, such that *Mcr*BC digestion reduces their PCR amplification by ~80% (Figure 5D). At *IGN5* and the *solo LTR*, DNA methylation is significantly reduced in the *nrpe1* (*nrpd1b-11*) mutant and in a null mutant for RNA-dependent RNA polymerase 2 (RDR2), a protein required for 24 nt siRNA biogenesis (Xie et al., 2004). In the *nrpe1* mutant background, *IGN5* and *solo LTR* methylation are restored by the wild-type *NRPE1* transgene, but not by the *NRPE1-ASM* transgene. The data indicate that Pol V transcription, like RDR2, is needed for siRNA-directed DNA methylation at these loci.

Unlike *IGN5* and the *solo LTR*, DNA methylation at *IGN6* does not require Pol V or RDR2 but does require DDM1 (decrease in DNA methylation 1), a SWI/SNF family chromatin remodeler that acts primarily in the maintenance, rather than RNA-mediated establishment of cytosine methylation (Jeddeloh et al., 1999). DDM1 also affects maintenance methylation at *IGN5* but has no appreciable effect at the *solo LTR*, which may rely exclusively on RNA-directed DNA methylation.

Loss of DNA methylation at the *AtSN1*, *IGN5*, and *solo LTR* loci in the *nrpe1* (*nrpd1b-11*) mutant was also demonstrated using methylation-sensitive restriction endonucleases (Figures 5E and 5F). Methylation of *Hae*III or *Alu*I recognition sites blocks the enzymes from cutting the DNA, allowing PCR amplification of the region. However, unmethylated sites are cleaved such that PCR amplification fails. DNA methylation was lost at *Hae*III or *Alu*I sites of the *AtSN1*, *IGN5*, and *solo LTR* loci in the *nrpe1* mutant and was restored by the wild-type *NRPE1* transgene, but not by the *NRPE1-ASM* transgene (Figures 5E and 5F). At *IGN6*, no effect of *nrpe1* was observed on methylation of the sole *Alu*I site tested (Figure 5F). Collectively, the data indicate that Pol V mediates the establishment of heterochromatic histone modifications and DNA methylation changes that correlate with the silencing of Pol II- or Pol III-transcribed genes that overlap the Pol V-transcribed regions.

Pol V-Dependent Transcription Does Not Require Small RNA Biogenesis

Because Pol V is required for siRNA-dependent DNA methylation, we asked whether mutations in genes required for siRNA biogenesis, RNA-directed gene silencing, or DNA methylation affect Pol V transcription (Figure 6A). At *IGN5* and *IGN6*, Pol V transcripts lost in *nrpe1* (*nrpd1b-11*) and *nrpd2* mutants were unaffected by mutation of the four dicers that process double-stranded RNA precursors into siRNAs, including a quadruple mutant that combines a hypomorphic *dcl1* allele with null alleles of *dcl2*, *dcl3*, and *dcl4*. Pol V-dependent transcripts were also unaffected in mutants defective for RNA-dependent RNA polymerases (*rdr2*, *rdr1*, and *rdr6*) implicated in generating siRNA precursors or in mutants affecting cytosine methylation (*drm2*, *met1*, and *ddm1*). However, many of these mutants interfere with *AtSN1* silencing, including the dicer quadruple mutant, *rdr2*, *nrpd1a*, *drm2*, and *drd1* (Figure 6A, row 4). Collectively, the results reveal that Pol V transcription occurs independently of small RNA biogenesis, de novo cytosine methylation (*drm2*), or maintenance cytosine methylation (*met1*, *ddm1*). However, Pol V and siRNA biogenesis are both required for *AtSN1* silencing.

DRD1 Facilitates the Association of Pol V with Chromatin

As shown in Figure 6A, Pol V transcripts are lost in *drd1-6* mutants. DRD1 is a member of the SWI2/SNF2 family of ATP-dependent chromatin remodelers and was identified in a genetic screen that also identified *nrpe1* (*nrpd1b*) and *nrpd2* alleles, suggesting that DRD1 and Pol V act in collaboration (Huettel et al., 2007). ChIP of FLAG-tagged NRPE1 in wild-type or *drd1* mutant backgrounds was conducted to determine whether

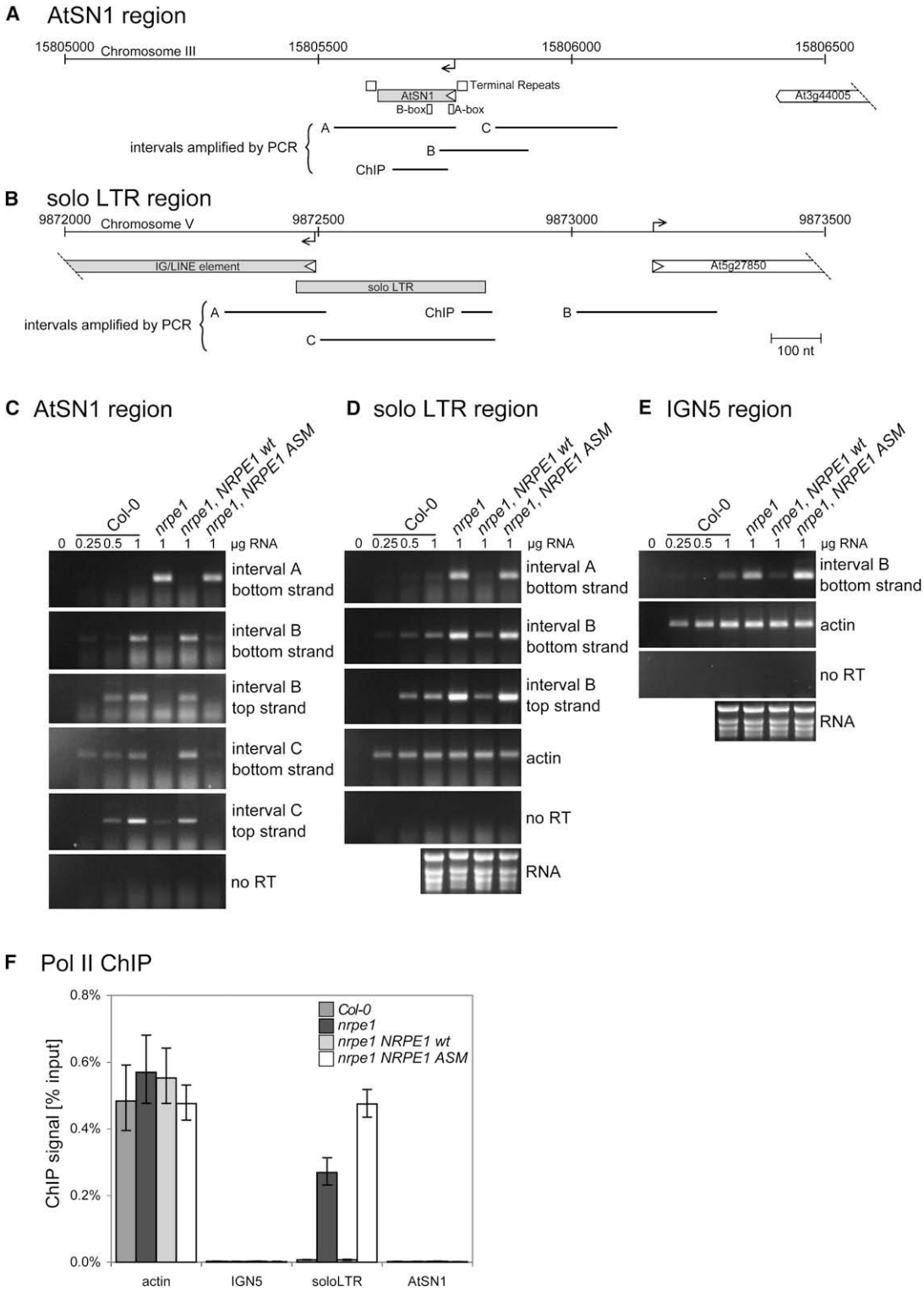

Figure 4. RNA Polymerase Activity of Pol V Is Necessary for Silencing Adjacent Transposons and Repetitive Elements

(A and B) *AtSN1* (A) and *solo LTR* (B) regions, including neighboring genes, repetitive elements, and regions amplified by PCR. The diagram for the *solo LTR* region is based on analysis of transcription units by Huettel et al. (2006).

(C) Strand-specific RT-PCR analysis of transcription from the *AtSN1* region in Col-0 wild-type, *nrpe1* (*nrpd1b-11*), and the *nrpe1* mutant expressing a wild-type *NRPE1* transgene or the *NRPE1-ASM* transgene. Intervals amplified by RT-PCR are depicted in (A). No RT (interval A bottom-strand primers) and no RNA controls (all primer pairs) are included.

DRD1 regulates Pol V association with chromatin (Figure 6B). NRPE1-FLAG protein levels were similar in both genetic backgrounds (Figure 6C). In *nrpe1* plants that are wild-type at the *DRD1* locus, the NRPE1-FLAG protein physically associates with *IGN5*, *IGN6*, and the *solo LTR* locus (Figure 6B). However, in the *drd1* mutant background, NRPE1 association with these loci is reduced to background levels resembling the actin gene control (Figure 6B). We conclude that DRD1 mediates Pol V recruitment to chromatin.

DISCUSSION

Polymerase Activity of Pol V

RNA polymerase activity has not yet been demonstrated for Pol IV or Pol V in vitro. However, our study provides in vivo evidence for Pol V polymerase activity by demonstrating the existence of Pol V-dependent transcripts, by showing that these RNAs require the conserved polymerase active site, by showing that Pol V physically associates with DNA loci corresponding to Pol V-dependent transcripts, and by showing that Pol V physically associates with the transcripts themselves. The most parsimonious explanation for the results is that Pol V transcribes DNA into RNA, which fits with the crosslinking of Pol V to both DNA and RNA and with the requirement for the putative chromatin remodeler DRD1 in order for Pol V to associate with transcribed loci. DRD1 and Pol V do not appear to physically interact, based on coIP experiments (T. Ream, A.T.W., and C.S.P., unpublished data), suggesting that DRD1 functions upstream of Pol V, presumably by remodeling chromatin to facilitate Pol V recruitment to the DNA. If Pol V were to utilize RNA templates, a prediction is that Pol V-dependent transcript abundance would increase in accord with the abundance of RNAs serving as templates. However, mutations that derepress transposons, including *rdr2*, *drm2*, *met1*, or *ddm1*, have no effect on Pol V transcript abundance. Likewise, Pol V transcripts do not decrease in mutants for the major RNA-directed RNA polymerases, *rdr2* or *rdr6*, which could potentially generate RNA templates for Pol V.

Detection of multiple Pol V transcript 5′ ends using RACE suggests that Pol V may initiate transcription in a promoter-independent fashion. How sites of Pol V initiation are chosen is unclear. One hypothesis is that specific DNA methylation patterns or histone modifications recruit Pol V. However, Pol V transcripts are detectable in both heterochromatic, transposon-rich regions as well as gene-rich, presumably euchromatic environments. Moreover, mutants affecting siRNA production or DNA methylation have no effect on Pol V transcript abundance. An alternative possibility, which we favor, is that Pol V initiates transcripts throughout the genome, both in silenced and nonsilenced regions, and these transcripts are necessary, but not sufficient, for gene silencing. Instead, we envision that Pol V transcription renders a locus competent for silencing, but silencing only occurs if siRNAs complementary to the locus are also produced (see below).

The Role of Pol V Transcription in Transcriptional Gene Silencing

ncRNAs originating in intergenic regions are prevalent in eukaryotes, including *Arabidopsis*, but their functional significance is mostly unknown. Our results indicate that Pol V-transcribed ncRNAs play direct roles in silencing overlapping or adjacent genes. At the *AtSN1* locus, Pol V transcripts and retrotransposon transcripts presumably generated by Pol III are mutually exclusive, suggesting that Pol V transcription prevents Pol III transcription. Likewise, at the *solo LTR* locus, Pol II association is low in wild-type plants but increases 35-fold in *nrpe1* mutants. Similar increases in transcription of the LINE element adjacent to *IGN5* occur in *nrpe1* mutants. Collectively, the data indicate that Pol V transcription facilitates the silencing of overlapping genes as a result of repressive chromatin modifications, including H3K9 methylation, H3K27 methylation, and cytosine hypermethylation.

Pol V transcription is necessary, but not sufficient, to silence *AtSN1* and *solo LTR* elements. Other necessary proteins include Pol IV, RDR2, one or more DCL proteins, AGO4, DRD1, and DRM2 (see Figure 6), which are components of the 24 nt siRNA-directed DNA methylation pathway. Because mutants that disrupt siRNA biogenesis (e.g., *nrpd1*, *rdr2*, dicer) have no effect on the production of Pol V-dependent transcripts, our results suggest that Pol V transcription and siRNA production occur independently but collaborate in gene silencing. This hypothesis fits with the observation that Pol V is not required for siRNA production at the majority of the ~4000 loci giving rise to 24 nt siRNAs (Mosher et al., 2008), including the *AtSN1*(Kanno et al., 2005; Pontes et al., 2006) and *solo LTR* (Huettel et al., 2006) loci we have examined. At other endogenous repeat loci giving rise to siRNAs, all of which require Pol IV, Pol V is apparently required (Mosher et al., 2008). However, this does not necessarily imply that Pol V transcripts serve as siRNA precursors. Instead, Pol V-dependent heterochromatin formation may stimulate Pol IV-dependent production of siRNAs in a positive feedback loop that enforces gene silencing (Li et al., 2006; Pontes et al., 2006).

In our alternative models (Figure 7), we envision that chromatin remodeling by DRD1 is required for Pol V transcription initiation. In parallel, siRNAs produced by the combined actions of Pol IV, RDR2, and DCL3 are incorporated into AGO4. Our favored model is that Pol V transcripts base pair with siRNAs that are associated with AGO4 (Figure 7A), similar to the way that Pol II transcripts reading through silenced fission yeast pericentromeric regions are proposed to interact with the siRNA-AGO moiety of the RNA-induced transcriptional silencing (RITS) complex (Buhler et al., 2006; Irvine et al., 2006). The interaction of the siRNA with the nascent transcript might then direct the silencing machinery, including the de novo cytosine methyltransferase DRM2 and/or

(D) Strand-specific RT-PCR analysis of transcription at the *solo LTR* region. No RT (interval B bottom-strand primers) controls are included.

(E) Strand-specific RT-PCR analysis of transcription from a LINE element flanking *IGN5*. Figure 2A shows the location of interval B amplified by PCR. No RT (interval B bottom-strand primers) controls are included.

(F) Pol II occupancy of *actin 2*, *IGN5*, *solo LTR*, and *AtSN1* loci detected using ChIP. Col-0 wild-type, *nrpe1* (*nrpd1b-11*), and *nrpe1* mutant plants transformed with the wild-type *NRPE1* transgene or the *NRPE1-ASM* transgene were subjected to ChIP using αNRPB2 antibody and detected by real-time PCR. Histograms show the means ± SD obtained from three independent amplifications.

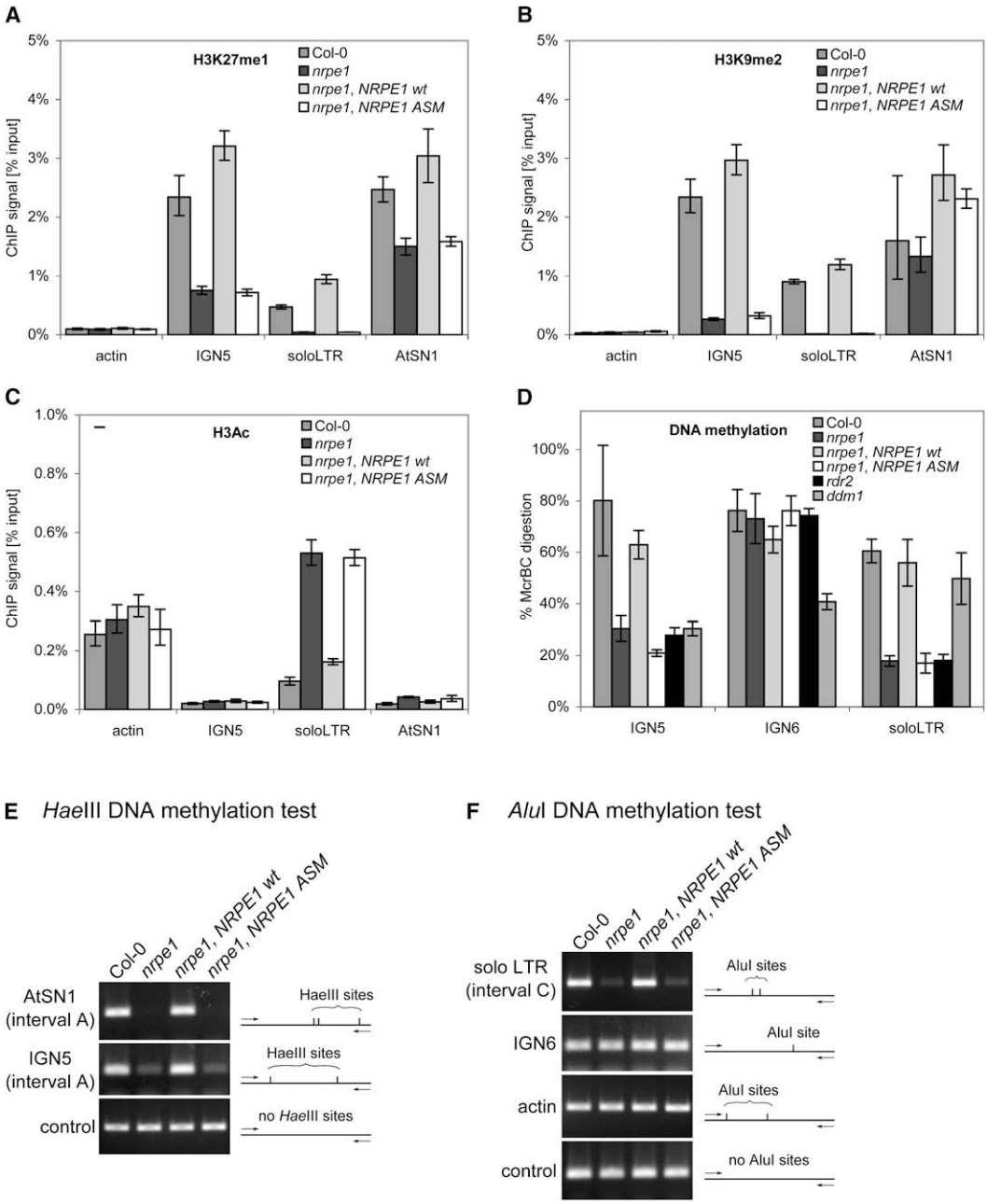

Figure 5. Pol V-Dependent Transcription Is Necessary for Heterochromatin Formation

(A–C) ChIP using αH3K27me1 (A), αH3K9me2 (B), or αH3Ac (C) antibodies and chromatin of Col-0 wild-type, *nrpe1* (*nrpd1b-11*), or *nrpe1* mutants transformed with the wild-type *NRPE1* transgene or *NRPE1-ASM* transgene. Histograms show the means ± SD from three independent amplifications.

(D) DNA methylation analysis at the indicated loci performed by digestion of genomic DNA with *Mcr*BC followed by quantitative real-time PCR. Comparison to undigested DNA allowed the fraction susceptible to *Mcr*BC to be calculated.

(E and F) DNA methylation analysis at the *AtSN1*, *IGN5*, *IGN6*, and *solo LTR* loci performed by digesting purified DNA with the methylation-sensitive restriction endonucleases *Hae*III (E) or *Alu*I (F) followed by PCR. Sequences lacking *Hae*III (actin; [E]) or *Alu*I (*IGN5* interval A; [F]) sites served as controls to show that equivalent amounts of DNA were tested in all reactions.

histone-modifying activities, to the adjacent DNA. Alternatively, Pol V transcripts may directly bind to AGO4 and stabilize siRNA-DNA interactions (Figure 7B). It is also possible that Pol V transcripts or the act of transcription itself influence structural features of heterochromatin that are required by AGO4 for efficient interactions with target loci (Figure 7C). In each of these scenarios, AGO4 recruitment is expected to be cotranscriptional and may involve direct interactions between AGO4 and the C-terminal domain of NRPE1/NRPD1b (El-Shami et al., 2007; Li et al., 2006). A prediction of all of the models is that transcriptional

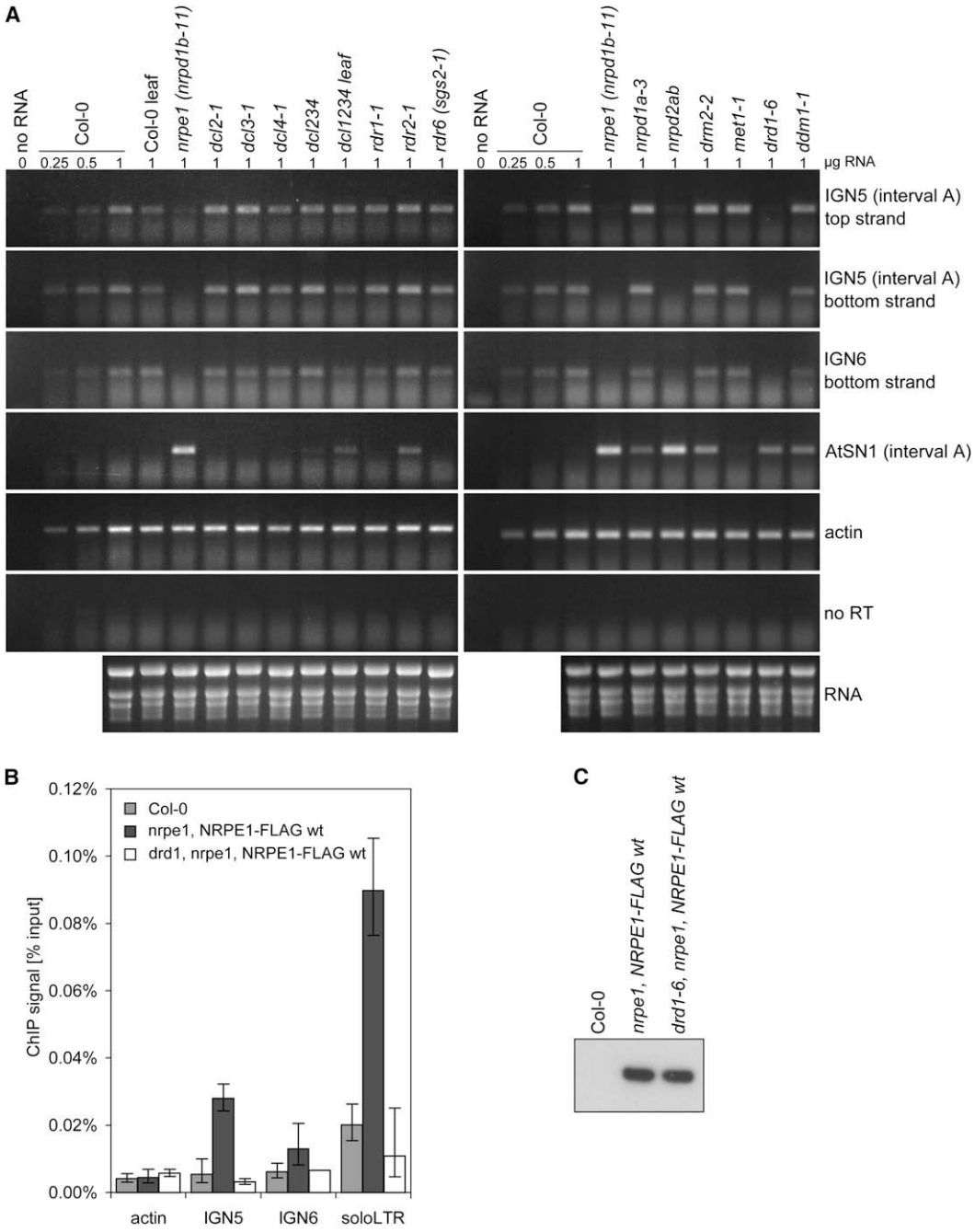

Figure 6. Pol V-Dependent Transcription Requires the Chromatin Remodeler DRD1, but Not siRNA Production or DNA Methylation

(A) Strand-specific RT-PCR analysis of *IGN5* and *IGN6* transcription in mutants disrupting dicer (*dcl1*, *dcl2*, *dcl3*, *dcl4*), RNA-dependent RNA polymerase (*rdr1*, *rdr2*, *rdr6*), Pol IV (*nrpd1*, *nrpd2*), Pol V (*nrpe1*/*nrpd1b-11*, *nrpd2*) DNA methylation (*met1*, *ddm1*, *drm2*) or chromatin remodeling (*ddm1*, *drd1*) activities. Detection of *AtSN1* retroelement transcripts indicates a loss of *AtSN1* silencing. Col-0 RNA dilutions show that results are semiquantitative. No RT controls used *IGN5* top-strand primers.

(B) DRD1 is required for Pol V to interact with chromatin. ChIP with αFLAG antibody was performed using chromatin isolated from Col-0 wild-type, *nrpe1* (*nrpd1b-11*) plants expressing the *NRPE1-FLAG* transgene or *drd1 nrpe1* double mutants expressing the *NRPE1-FLAG* transgene. *Actin 2*, *IGN5*, *IGN6*, and *solo LTR* loci were detected using quantitative real-time PCR. Histograms show the means ± SD obtained from three independent amplification reactions.

(C) Immunoblot with αFLAG antibody showing that equivalent amounts of NRPE1-FLAG recombinant protein are immunoprecipitated in the *nrpe1* (*nrpd1b-11*) and *drd1 nrpe1* genetic backgrounds.

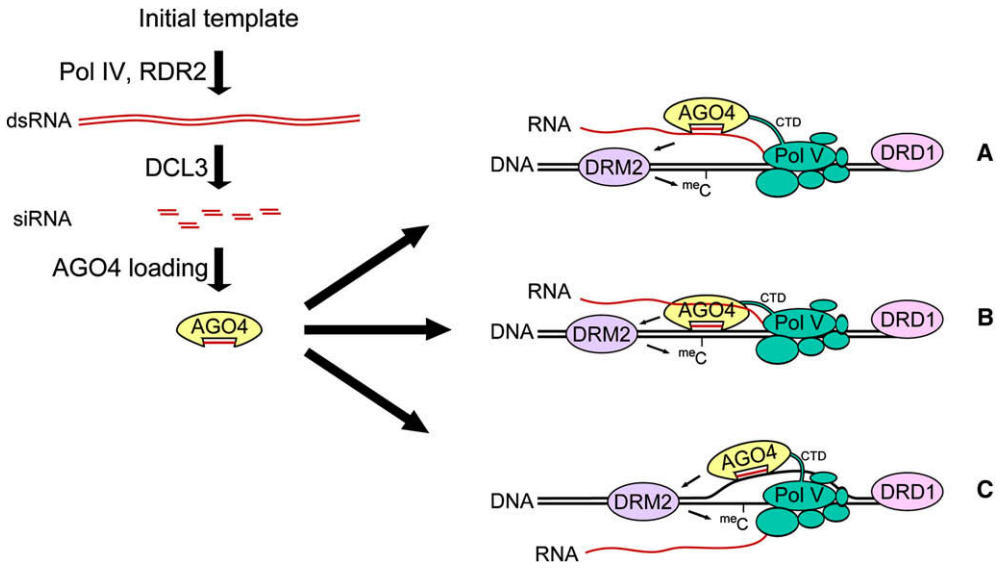

Figure 7. Possible Modes of Action for Pol V in RNA-Directed Transcriptional Silencing

Pol V transcription and siRNA production occur independently but collaborate in silencing transposons such as *AtSN1*. 24 nt siRNAs are produced by Pol IV, RDR2, and DCL3 and loaded into AGO4. Chromatin remodeling by DRD1 is required for Pol V to associate with chromatin, and physical interactions may occur between the Pol V C-terminal domain (CTD) and AGO4. In (A), which we favor, siRNAs bound to AGO4 interact with nascent Pol V transcripts, thereby recruiting chromatin-modifying activities, including histone-modifying enzymes and the de novo cytosine methyltransferase DRM2, to the adjacent DNA. In (B), AGO4 interacts with the nascent transcripts, but the siRNA base pairs with DNA. In (C), the siRNA associated with AGO4 interacts with DNA in a manner dependent upon Pol V-mediated chromatin perturbation.

silencing does not occur everywhere that Pol V transcription occurs but only at sites where Pol V transcription and siRNA production overlap. Testing this hypothesis on a whole-genome basis is a goal for future studies.

EXPERIMENTAL PROCEDURES

Plant Strains

A. thaliana nrpd1a-3 (*nrpd1*), *nrpd1b-11* (*nrpe1*), and *nrpd2a-2 nrpd2b-1* mutants were described previously (Onodera et al., 2005; Pontes et al., 2006), as were *nrdp1b-11 NRPD1b-FLAG* (*NRPE1-FLAG*) (Pontes et al., 2006) and *NRPB2-FLAG* (Onodera et al., 2008) transgenic lines. *NRPE1* mutagenesis and production of transgenic lines expressing Pol IV and Pol V active site mutants will be described elsewhere (J.R.H. and C.S.P., unpublished data). *rdr1-1*, *rdr2-1*, *dcl2-1*, and *dcl3-1* were provided by J. Carrington; *sgs2-1* (*rdr6*) and *dcl4-1* were provided by H. Vaucheret; *drd1-6* was provided by M. Matzke; *met1-1* and *ddm2-1* were provided by E. Richards; *dcl234* (*dcl2-5 dcl3-1 dcl4-2*) and *dcl1234* (*dcl1-9 dcl2-5 dcl3-1 dcl4-2*) were provided by T. Blevins; *drm2-2* (SAIL_70_E12) was provided by E. Richards.

RNA Analysis

RNA was isolated from 2-week-old plants using an RNeasy Kit (QIAGEN). The 5′ RACE was performed using a GeneRacer Kit (Invitrogen) with two nested amplification steps; see Table S1 for primers. The 5′ RACE products were gel purified and cloned into TOPO-TA (Invitrogen). Tobacco Acid Pyrophosphatase (Invitrogen) or Terminator exonuclease (Epicentre) treatments followed manufacturers' instructions. Polyadenylated RNA was purified using a FastTrack MAG Kit (Invitrogen). For RT-PCR, 1 μg of RNA digested with DNase I (Invitrogen) was reverse transcribed 30 min at 55°C using 60 units SuperScript III Reverse Transcriptase (Invitrogen), 1.5 units Platinum Taq (Invitrogen), and a gene-specific primer. After heat inactivation of reverse transcriptase, the second primer was added and PCR was performed. Alternatively, the One-Step RT-PCR Kit (QIAGEN) was used. Table S1 shows primer pairs.

ChIP and RNA-IP

ChIP was performed by adapting existing protocols (Lawrence et al., 2004; Nelson et al., 2006), as was RNA-IP (Gilbert and Svejstrup, 2006; Martianov et al., 2007). Details are provided in the Supplemental Data. All ChIP and RNA IP experiments were reproduced at least twice.

Real-Time Quantitative PCR

DNA was amplified using an Applied Biosystems model 7500 thermocycler with 0.5 units of Platinum Taq (Invitrogen), SYBR Green I (Invitrogen), and Internal Reference Dye (Sigma). Primer pairs are shown in Table S1. Results were analyzed using the comparative C_T method (Livak and Schmittgen, 2001) relative to input or undigested samples.

Antibodies

Anti-FLAG M2 mouse monoclonal and rabbit polyclonal antibodies were purchased from Sigma-Aldrich. Anti-Pol II (anti-NRPB2) was described previously (Onodera et al., 2005). Anti-H3K27me1 antibody no. 8835 (Peters et al., 2003) was provided by Thomas Jenuwein. Antibody against diacetyl-H3 (K9 and K14) was obtained from Upstate Biologicals (cat. no. 06599, lot no. 31994). Anti-H3K9me2 was obtained from Abcam (cat. no. ab7312, lot no. 133588).

SUPPLEMENTAL DATA

The Supplemental Data include Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at http://www.cell.com/supplemental/S0092-8674(08)01192-6.

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A.T.W. and C.S.P. conceived the work, A.T.W. performed all described experiments and contributed all figures, J.R.H. generated the NRPE1 active site mutant, and A.T.W. and C.S.P. wrote the paper. We thank Keith Earley and Eric Richards for helpful discussions. This work was supported by NIH grant GM077590.

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