

saRNA-guided Ago2 targets the RITA complex to promoters to stimulate transcription

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Small activating RNAs (saRNAs) targeting specific promoter regions are able to stimulate gene expression at the transcriptional level, a phenomenon known as RNA activation (RNAa). It is known that RNAa depends on Ago2 and is associated with epigenetic changes at the target promoters. However, the precise molecular mechanism of RNAa remains elusive. Using human *CDKN1A* (*p21*) as a model gene, we characterized the molecular nature of RNAa. We show that saRNAs guide Ago2 to and associate with target promoters. saRNA-loaded Ago2 facilitates the assembly of an RNA-induced transcriptional activation (RITA) complex, which, in addition to saRNA-Ago2 complex, includes RHA and CTR9, the latter being a component of the PAF1 complex. RITA interacts with RNA polymerase II to stimulate transcription initiation and productive elongation, accompanied by monoubiquitination of histone 2B. Our results establish the existence of a cellular RNA-guided genome-targeting and transcriptional activation mechanism and provide important new mechanistic insights into the RNAa process.

Keywords: RNAa; promoter; transcription; RNAP II; Argonaute; Ago2; saRNA; promoter-targeted RNA; RITA

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Introduction

Transcription is the first step to producing gene products and also a major control point for regulating gene expression. It is now known that most genes experience transcription initiation even when they are not being actively expressed [1, 2]. In such cases, transcription is curtailed due to promoter proximal pausing of RNA polymerase II (RNAP II) and failure of productive elongation [1, 2]. Thus, promoter proximal pausing is a rate-limiting step and also a target for manipulating gene expression [3]. Many strategies based on the use of DNA or RNA oligonucleotides and peptide nucleic acid

have been tested in efforts to achieve targeted regulation of gene transcription [4-6]. However, so far, attaining sequence-specific and effective transcriptional manipulation has remained a challenge.

Small double-strand RNAs (dsRNAs) can trigger an evolutionarily conserved posttranscriptional gene silencing mechanism known as RNAi [7]. Transcriptional and epigenetic modulation of gene expression guided by small RNAs has also been unveiled, including transcriptional/epigenetic gene silencing (TGS) [8-12] and activation (RNAa) [13-18]. In these phenomena, the function of almost all small RNAs depends on members of the highly evolutionarily conserved Argonaute protein family, which consists of the Ago and Piwi subfamilies and serves as an RNA-programmable homology search engine [19].

Whereas TGS has been well characterized in several lower eukaryotes such as fission yeast and *Drosophila* [20, 21], the molecular mechanism for dsRNA-induced RNAa in mammalian cells remains poorly understood.

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RNAa involves targeting of select sequences in gene promoters by short dsRNAs, also known as saRNA (small activating RNA), and has been demonstrated in mammalian species [13, 14, 22–24]. Surprisingly, RNAa also depends on Ago2 [13, 22, 24, 25], a core component of the RNA-induced silencing complex (RISC). It is believed that, in RNAa, Ago2 fulfills the same function as in RNAi, namely initial RNA duplex loading and strand processing [22, 25, 26]. Nevertheless, RNAa seems to possess unique kinetics characterized by a 24 h to 48 h delay in the onset of gene induction and its prolonged persistence thereafter [13, 14, 22], suggesting that RNAa is likely a phenomenon distinct from RNAi. Despite the demonstrated potential of RNAa both as a research tool [27, 28] and therapeutics [24, 29–31], generalized and efficient use of RNAa has been hindered by a lack of understanding of its detailed mechanism. Questions concerning how transcription is induced in RNAa, what other proteins besides Ago2 are required, and how saRNA binds to its target sequence at promoters remain unanswered.

In the present study, we performed detailed biochemical, proteomic and functional analysis using a saRNA-inducible gene, *CDKN1A* (*p21*), as a model. We provide concrete evidence that Ago2-loaded saRNAs strongly associate with their intended promoter targets, indicative of an RNA-guided genome-targeting mechanism. We further show that the saRNA-Ago2 complex interacts with RNAP II to stimulate both the initiation and elongation steps of transcription. We unveil critical components of the RNA-induced transcriptional activation (RITA) complex, which comprises at least saRNA-loaded Ago2, RHA and CTR9. Together, our results provide further support for, and new insights into, an RNA-guided and Ago2-dependent gene activation mechanism.

Results

RNAa occurs at the transcriptional level of gene regulation and is an “on-target” effect

Previous chromatin immunoprecipitation (ChIP) analyses revealed enrichment of RNAP II and association of specific epigenetic marks such as methylation of histone 3 at lysine 4 (H3K4) at saRNA target promoters [25, 32–34]. These observations argue for a transcriptional mechanism of gene induction by saRNAs. To determine the transcription rate of saRNA-inducible genes, we performed nuclear run-on (NRO) assays using two previously identified saRNAs, saP21 and saEcad, which induce expression of the *p21* and *E-cadherin* genes, respectively [13]. saP21 and saEcad target the *p21* and *E-cadherin* promoters at –322 and –215 relative to

each gene’s transcription start site (TSS; Figure 1A and Supplementary information, Table S1). We transfected the saRNAs or a control dsRNA (saControl) into PC-3 cells (prostate carcinoma) and harvested the transfected cells for nuclei isolation 48 h later. To isolate nascent RNA transcripts, we performed the NRO reaction in the presence of 5-bromouridine 5-triphosphate (BrUTP) followed by immunoprecipitation and affinity purification of BrUTP-labeled RNA transcripts. Purified NRO RNA was reverse transcribed and analyzed by quantitative RT-PCR (RT-qPCR) using primers that amplify the 5’ end of either *p21* or *E-cadherin* mRNA (Figure 1A). Compared with mock transfection, we detected significantly higher levels of nascent transcripts for both the *p21* and *E-cadherin* genes in cells transfected with the corresponding saRNAs. As shown in Figure 1B and 1C, saP21 and saEcad caused a 9.3- and 28.3-fold increase in BrUTP-labeled nascent *p21* and *E-cadherin* transcripts, respectively, relative to mock transfection. Transfection with saControl did not significantly alter the transcription rate of either gene (Figure 1B and 1C). These results indicate that RNAa occurs at the transcriptional level to increase transcription rates and is largely independent of an effect on mRNA stability, as measured by mRNA decay kinetics (Supplementary information, Figure S1).

Small duplex RNAs are known to trigger sequence-specific and nonspecific off-target effects. To exclude the possibility that RNAa is an off-target effect, we used the CRISPR technique to mutate the saP21 target on the *p21* promoter and obtained a subline of PC-3 cells (A-D7), which carried a 12- and 13-nt deletion in each of the two alleles within the saP21 target site (Figure 1D–1F). In wild type PC-3 cells (WT), saP21 induced *p21* mRNA expression in a dose-dependent manner, whereas mutation of saP21 target in A-D7 cells almost abolished *p21* mRNA induction by saP21 at all concentrations (Figure 1E). Western blotting analysis revealed that saP21 was still able to stimulate *p21* protein level in A-D7 cells, but to an extent much less than that in WT cells (Figure 1F). The *p21* protein induction by saP21 in A-D7 cells seemed to have occurred at the translational or post-translational levels through a mechanism unrelated to RNAa (Supplementary information, Data S1). These results indicate that the *p21* promoter-targeted saRNA is able to induce *p21* transcription through an on-target effect.

RNAa induces both transcription initiation and elongation

To further understand at which step of transcription RNAa acts, we determined the binding profile of RNAP II and the phosphorylation status of RNAP II C-terminal

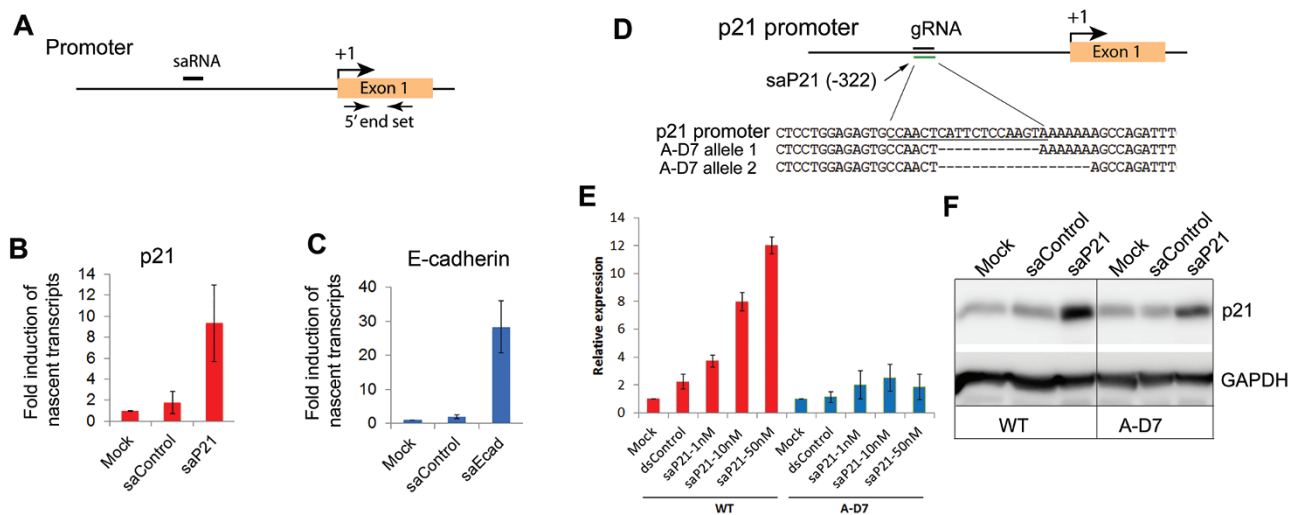


Figure 1 RNAa occurs at the transcriptional level and is an on-target effect. **(A)** Schematic of the nuclear run-on (NRO) assay showing the location of the primers used to detect nascent transcripts. **(B, C)** RT-qPCR analysis of the 5' ends of run-on purified transcripts transcribed from the *p21* **(B)** and *E-cadherin* **(C)** genes. Results were normalized to U2 snRNA and plotted as fold changes relative to mock transfection. Background from samples without addition of rNTPs was also measured and subtracted from the results. Results are means \pm SD of two independent experiments. **(D)** Mutation of saP21 target on *p21* promoter using the CRISPR technique. Top is a schematic representation of *p21* promoter with saP21 and gRNA targets indicated. Bottom is the alignment of genome reference sequence for *p21* promoter (saP21 target is underlined), mutated alleles of *p21* promoter from A-D7 cells. **(E)** Loss of *p21* mRNA induction in A-D7 cells. Wild-type PC-3 cells (WT) and A-D7 cells were transfected with the indicated saRNAs at the indicated concentrations for 72 h. Mock samples were transfected in the absence of saRNA. *p21* mRNA level was determined by RT-qPCR and plotted as level relative to mock transfection (mean \pm SD of three independent experiments). **(F)** Cells were transfected as in **E** with 10 nM of the indicated saRNA. *p21* protein expression was assessed by immunoblotting assay using a *p21* antibody. Detection of GAPDH serves as a control for protein loading.

domain (CTD) at serine 5 (Ser5) and serine 2 (Ser2) in response to saP21 treatment. RNAP II CTD phosphorylated at Ser5 and Ser2 corresponds to a pausing and elongating polymerase respectively. To accomplish this, we performed scanning ChIP assays on the *p21* gene using antibodies that recognize either the initiating (non-phosphorylated) form of RNAP II or the phosphorylated RNAP II at CTD residues Ser5 (Ser5P) or Ser2 (Ser2P). A scanning ChIP assay for Ago2 was also performed. The resulted ChIP DNA was amplified by quantitative PCR (ChIP-qPCR) using six sets of primers in *p21* gene ranging from -1.6 kb to $+5.4$ kb relative to *p21* TSS (Figure 2A). A further upstream primer set (-3 861/ -3 777) served as a control for background binding (Figure 2A). As shown in Figure 2B, in saP21-transfected cells, a significant enrichment of Ago2 binding was detected in a region (-417 / -1) surrounding the saP21 target site with its peak centered on saP21 target, suggesting specific Ago2 binding guided by saP21. Further, RNAP II ChIP showed that, in mock-treated PC-3 cells, there was a minimal enrichment of the initiating RNAP II surrounding the TSS (Figure 2C, black line); however, upon saP21

treatment, we observed a massive increase in RNAP II binding surrounding the saRNA target site and the core promoter of *p21* gene (Figure 2C, red line), consistent with the increased transcription rate from the *p21* promoter as measured in the NRO experiments (Figure 1B) and increased Ago2 binding at the same location (Figure 2B). This result suggests that saRNA-Ago2 targeting of promoters may facilitate formation of the transcription initiation complex by associating with the initiating form of RNAP II and possibly by contributing to stabilization of the complex. Next, scanning ChIP analysis of RNAP II Ser5P and Ser2P found localized RNAP II Ser5P accumulation surrounding the TSS, but not in the gene body, in both mock- and saP21-treated cells (Figure 2D). In contrast, RNAP II Ser2P accumulation at the *p21* gene in mock-treated cells was moderate (Figure 2E, black line), indicating potential promoter pausing and the absence of productive transcription elongation under non-RNAa conditions. Upon saP21 treatment, however, we observed significantly higher accumulation of RNAP II Ser2P starting from the 5' end of and extending throughout the transcribed region of *p21* (Figure 2E, red line).

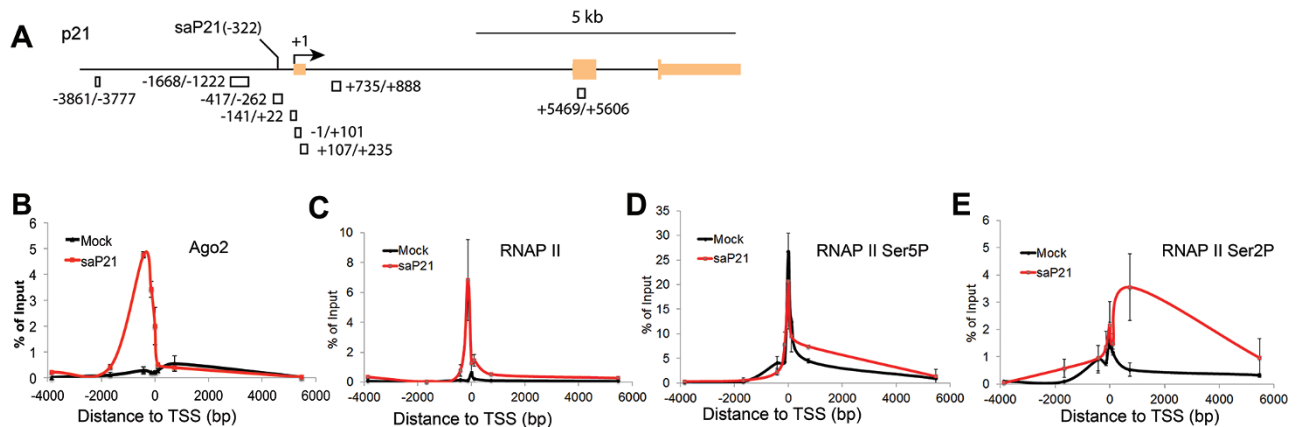


Figure 2 RNAa induces transcription at both the initiation and elongation steps. **(A)** Schematic of the *p21* gene with the locations of exons (orange blocks), TSS (+1), saRNA target (saP21) and ChIP primers (open rectangles) indicated. **(B-E)** Scanning ChIP analysis of Ago2 **(B)**, RNAP II **(C)**, RNAP II Ser5P **(D)**, and RNAP II Ser2P **(E)** following mock (black line) or saP21 (red line) treatment. Data represent the means \pm SD of at least three independent experiments and are plotted as % of input.

Furthermore, the abrupt increase in RNAP II Ser2P accumulation at the 5' end (Figure 2E) of *p21* gene coincides well with the sharp decline of RNAP II Ser5P at the same position (Figure 2D), reflecting a transition in response to saP21 treatment from RNAP II pausing (Ser5P) at the TSS to RNAP II elongating (Ser2P) across the gene. Together, our results suggest that saRNA-guided Ago2 binding to promoter can stimulate both transcription initiation and productive elongation.

Development of the chromatin isolation by biotinylated RNA pull-down assay

To address several important mechanistic questions related to RNAa, including whether saRNAs actually bind to their intended targets on promoters, which saRNA strand is the guide or activator, and which protein factors participate in RNAa, we developed a ChIP-based assay which we called chromatin isolation by biotinylated RNA pull-down (ChIbRP). ChIbRP allows us to isolate DNA and protein components in the context of target chromatin associated with an saRNA (Figure 3A). This technique can also provide important information about saRNA loading by Ago2. In ChIbRP assay, an saRNA duplex is biotinylated at the 3' end of one of the strands and transfected into cells. Following standard formaldehyde crosslinking, chromatin isolation and sonication, the chromatin is precipitated using magnetic streptavidin beads. After washing, nucleic acids or proteins associated with the biotinylated RNA are eluted from the beads, purified, and used in downstream analyses. Since previous studies have shown that Ago2 loading of duplex RNAs can be impaired by the addition of chemical groups such

as biotin to the 5' end of the guide strand, but not to the 3' end [22, 35, 36], we added a biotin group to the 3' end of either the sense or antisense strand of saP21, saEcad and saControl (Supplementary information, Table S1) and named the sense- and antisense- labeled duplexes as Bio(S) and Bio(AS) respectively. We first determined RNAa activity of these 3'-biotinylated saRNA duplexes by transfecting them individually and evaluating their target gene expression at the mRNA and protein levels. As shown in Figure 3B to 3D, compared with unlabeled saP21 and saEcad, duplex saP21 and saEcad 3'-biotinylated on either the sense or antisense strand had almost equivalent RNAa activity in inducing, respectively, *p21* (Figure 3B) and *E-cadherin* (Figure 3C) mRNA and protein levels (Figure 3D), whereas the control saRNA had no effect on the expression of either gene. We also added a psoralen group to the 5' end of the antisense strand of saP21-Bio(AS) (Supplementary information, Table S1). However, the 5' psoralen labeling of saP21-Bio(AS) abolished its activity in inducing *p21* expression (Supplementary information, Figure S2A and S2B) by blocking Ago2 loading (Supplementary information, Figure S2C). These results confirm that attaching a biotin group to the 3' end of either strand of a duplex saRNA does not affect its ability of triggering RNAa, whereas 5' end labeling prevents Ago2 from loading the duplex and abolishes its RNAa activity.

Ago2-loaded saRNA associates with its intended target promoter DNA and interacts with RNAP II

Having demonstrated that saRNAs tolerate 3' biotinylation, we next applied ChIbRP assay using bioti-

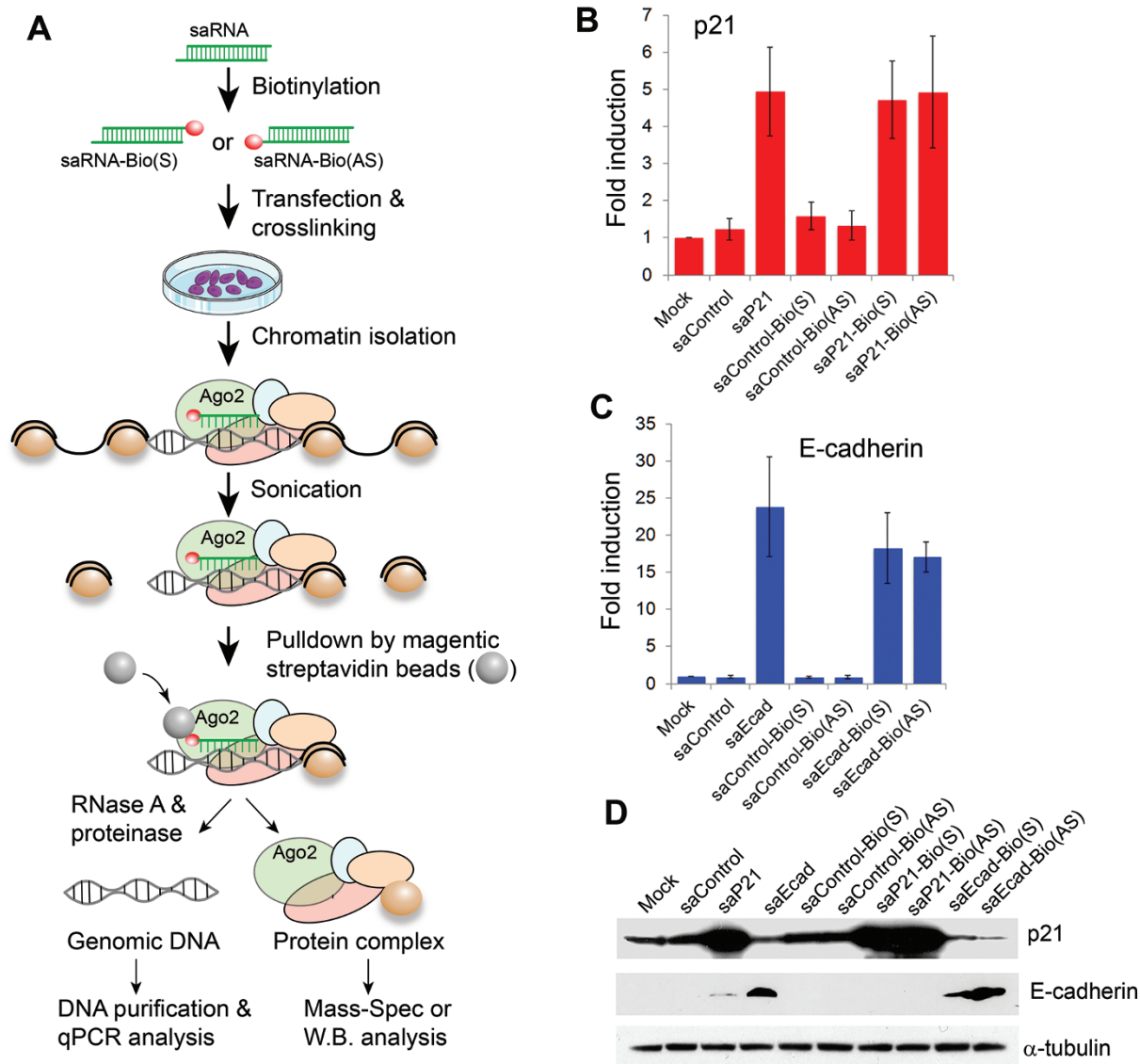


Figure 3 Chromatin isolation by biotinylated RNA pull down (ChlBRP) assay. **(A)** Flowchart of ChlBRP assay. A biotin group was attached to the 3' end of either strand in an saRNA duplex, resulting two duplex versions (Bio(S) and Bio(AS)). The duplexes were individually transfected into cells which were treated, 48 h later, with formaldehyde to crosslink chromatin DNA/protein complexes. Chromatin was purified and associated DNA sheared by sonication. Chromatin complexes associated with the biotinylated saRNA were isolated with magnetic streptavidin beads. DNA and protein fractions were eluted for further analysis. W.B., western blotting. **(B, C)** RT-qPCR quantification of *p21* **(B)** and *E-cadherin* **(C)** mRNA expression. saRNA targeting either the *p21* or *E-cadherin* promoter was biotinylated at the 3' end of either the sense [Bio(S)] or antisense [Bio(AS)] strand of the duplex, and transfected into PC-3 cells for 48 h. Data represent the means \pm SD of at least two independent experiments and are plotted as fold changes relative to mock transfection. **(D)** Immunoblotting analysis of *p21* and *E-cadherin* protein levels in cells transfected as in **B**. Detection of α -tubulin protein levels served as a protein loading control.

nylated saP21 and saEcad (Figure 4A-4D) to address the questions mentioned above. We transfected each saRNA duplex (either sense or antisense labeled) into cells and performed ChlBRP to pull down the biotinylated strand of the duplex and its associated proteins. We then detected Ago2 by immunoblotting in the resulted protein frac-

tion (Figure 4E and 4F). In RNAi, it is known that Ago2 uses the strand with lower thermodynamic stability at its 5' end relative to its 3' end as the guide, and destroys the other (passenger) strand in an siRNA duplex [37]. In support, we found that saP21 and saEcad had different Ago2-loading profiles (Figure 4E and 4F), which can be

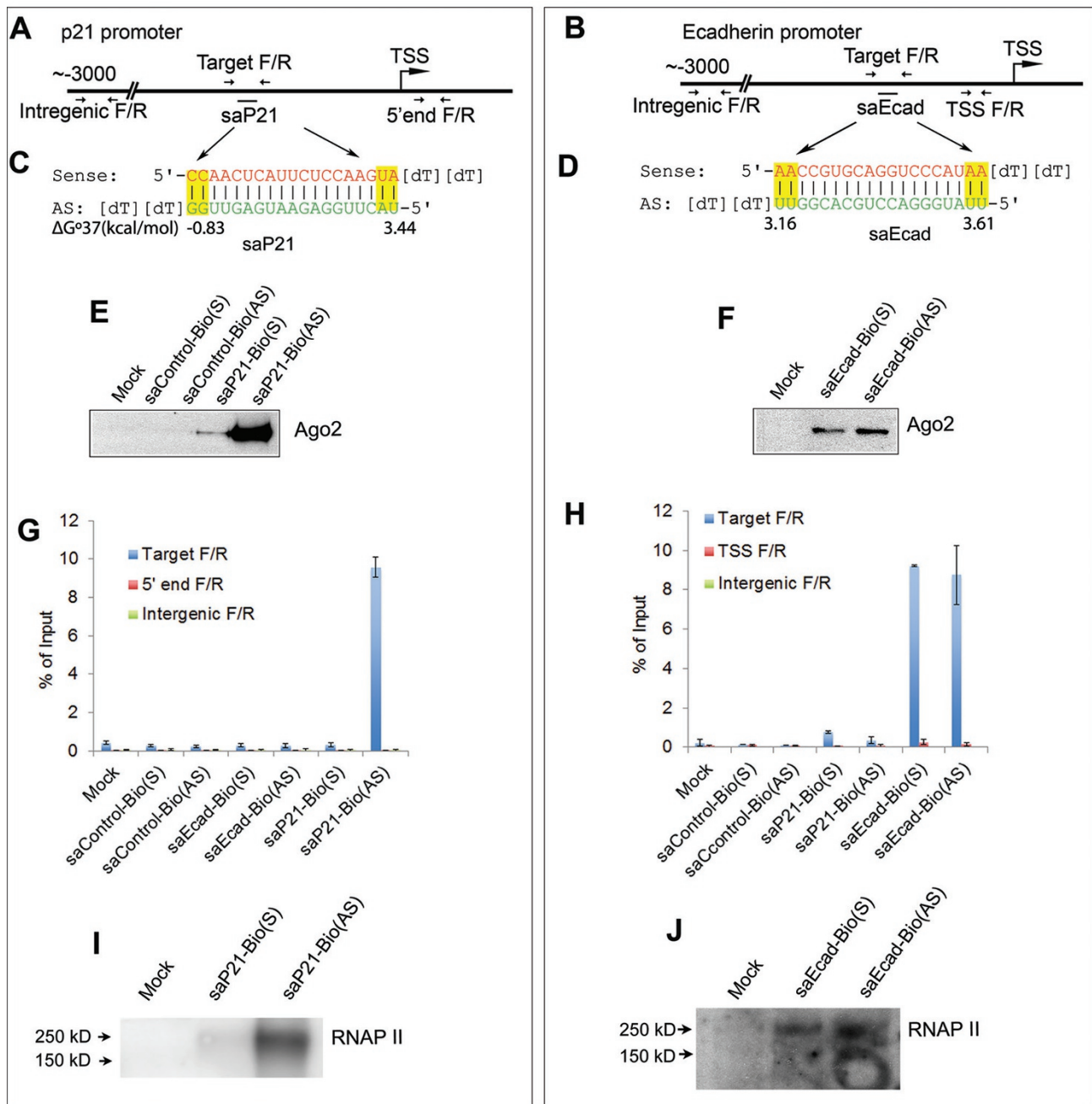


Figure 4 Ago2 loading of saRNA strands and their association with promoter DNA and interaction with RNAP II. **(A, B)** Schematic of the promoter region of the *p21* **(A)** and *E-cadherin* **(B)** promoters studied by Chlbrp. Locations of the saRNA target, primer binding sites and transcription start site (TSS) on the promoter are shown. Three sets of primers were designed to analyze enriched DNA for each promoter, including primers that amplify the saRNA target region (Target F/R), intergenic region ("Intergenic F/R"), and regions close to the TSS ("5' end F/R" for *p21*, and "TSS F/R" for *E-cadherin*). **(C, D)** Duplex saRNA sequence for saP21 **(C)** and saEcad **(D)** with calculated terminal ΔG values at 37 °C shown below the sequences. AS, anti-sense. **(E, F)** Strand-specific association of saP21 **(E)** and saEcad **(F)** with Ago2 detected by Chlbrp immunoblotting. PC-3 cells were transfected with the indicated duplex saRNAs for 48 h. The protein fraction of Chlbrp samples was used to detect Ago2 by immunoblotting. Bio(S) and Bio(AS), a biotin group was added to the sense and antisense strand respectively (see Materials and Methods section). **(G, H)** Strand-specific DNA association of saP21 **(G)** or saEcad **(H)** detected by Chlbrp qPCR. The DNA fraction from cells transfected as in **E** or **F** was used to amplify the DNA target region of the saRNAs using "Target F/R" primers or primers for control regions. Data represent the means \pm SD of at least two independent experiments and are plotted as % of input. **(I, J)** Chlbrp detection of RNAP II protein in association with saP21 **(I)** or saEcad **(J)**. Chlbrp-retrieved proteins were subject to immunoblotting of RNAP II.

explained by the different thermodynamic properties of their termini. For duplex saP21, which has an asymmetric terminal thermodynamic stability with a calculated 5' end ΔG_{37}° (kcal/mol) equal to -0.83 compared with 3.44 for its 3' end (Figure 4C), Ago2 was mainly associated with the antisense strand (Figure 4E). In contrast, saEcad is almost symmetric in its terminal stability (Figure 4D), and, as a result, the two strands showed equal Ago2 association (Figure 4F). Surprisingly, we found no detectable Ago2 association of either strand of saControl (Figure 3E), which does not have any known target in the cells. Because in ChIRP assay the chromatin-enriched fraction is isolated, the lack of Ago2 association for saControl can perhaps be attributed to its lack of targets in the nucleus. In this regard, a previous study has shown that only dsRNAs with a nuclear target could localize in the nuclear compartment [38].

By mutation assay, we have shown that an intact saP21 target in *p21* promoter is required for RNAa activity (Figure 1D-1F). To further assess whether each Ago2-loaded guide saRNA strand binds to its intended promoter target sequence, we transfected cells with biotinylated duplex saRNAs and probed for DNA in the ChIRP pull-down DNA fraction by qPCR using primer pairs designed to amplify the target region ("Target F/R") of each saRNA and two genomic control regions, including regions close to the TSS ("TSS F/R" or "5' end F/R") and upstream intergenic regions ($\sim 3\ 000$ bp, "Intergenic F/R"; Figure 4A). We observed a 24.7-fold enrichment in the amount of target DNA retrieved by the antisense strand of saP21 (saP21-Bio(AS)) relative to the intergenic control region, suggesting strong association of the antisense strand with target DNA (Figure 4G). However, DNA retrieved by saP21-Bio(S) was not enriched in any PCR amplicon region (Figure 4G), suggesting that no binding event for saP21-Bio(S) had occurred. In contrast, for saEcad, whose two strands have equal Ago2 loading (Figure 4F), we detected 16.8- and 14.8-fold enrichment in the DNA retrieved by the saEcad-Bio(S) and saEcad-Bio(AS) strands, respectively, at their target locations (Figure 4H). In addition, we used the two versions of saP21 and saEcad as cross controls for each promoter, and did not detect any binding at the saP21 target site by saEcad (saEcad-Bio(S) and saEcad-Bio(AS); Figure 4G) nor at the saEcad target site by saP21 (saP21-Bio(S) and saP21-Bio(AS); Figure 4H). The observed significant enrichment of target promoter DNA retrieved by Ago2-loaded saRNA guide strands strongly suggests that saRNAs bind to their intended targets at gene promoters in a sequence-specific manner. Consistent with their Ago2-loading patterns, only saRNA strands loaded by Ago2 were able to associate with target DNA (compare

Figure 4E and 4G with Figure 4F and 4H). This points to the possibility that most, if not any, chromosome-targeted dsRNAs could guide Ago2 to its target site as long as it can program Ago2, supporting the view that Ago2 is a RNA-programmable homology search and binding protein [19]. This finding also raises an intriguing question: which saRNA strand provides RNAa activity if both strands can be loaded by Ago2 and bind to the promoter target as in the case of saEcad? Given that our previous study showed by sequence mutation and Ago2 blocking analysis that the antisense strand of saEcad is likely the activator strand [13, 22], the two strands of saEcad may not have equal RNAa activity even though both are capable of guiding Ago2 to the promoter target and interact with RNAP II (Figure 4H).

As our results showed an increased rate of transcription upon saRNA treatment (Figure 1B and 1C), we further investigated the potential interaction of the saRNA-Ago2 complex with the transcription machinery upon binding of the latter to promoters. We addressed this question by detecting RNAP II in the protein fraction of ChIRP samples from cells transfected with biotinylated duplex saRNAs. As shown in Figure 4I and 4J, the AS strand in saP21 (Figure 4I) and both the S and AS strands in saEcad (Figure 4J) were able to associate with RNAP II, exhibiting a similar strand specificity found for Ago2 loading. Together, these findings clearly indicate that saRNAs predictably bind to their promoter targets in an Ago2-dependent manner and interact with the transcription machinery.

Identification of protein components of RNAa effector complexes

Having identified that the Ago2-loaded antisense strand of saP21 guides Ago2 to its promoter target where the saRNA-Ago2 complex interacts with RNAP II, we next examined what additional proteins are recruited by the saRNA-Ago2 complex to facilitate the Ago2-RNAP II interaction and transcriptional activation. To this end, we transfected cells with biotinylated saP21 for 48 h and applied the ChIRP assay to the nuclear fraction pulled down with either saP21-Bio(S) or saP21-Bio(AS) and subjected the associated proteins to mass spectrometry (MS) analysis after protein separation by SDS-PAGE. Proteins pulled down by saP21-Bio(S), the passenger strand, served as a control for background proteins. Immunoblotting analysis of the prepared nuclear fraction revealed it to be free of cytoplasmic protein contamination (Supplementary information, Figure S3). MS analysis identified 42 proteins to be associated with saP21-Bio(AS) and only 15 with saP21-Bio(S) (Supplementary information, Figure S4, Table S2). Of the 42 proteins, 27

were uniquely associated with the guide strand saP21-Bio(AS) and the other 15 were shared by both strands (Supplementary information, Figure S4). Although Ago2 was recovered by both saP21-Bio(S) and saP21-Bio(AS), more Ago2 peptides were identified in the saP21-Bio(AS) sample ($n = 116$) compared with saP21-Bio(S) sample ($n = 30$). This finding is consistent with the ChIP-RBP results for the Ago2-loading pattern (Figure 3E) and further supports that the antisense strand of saP21 is preferentially loaded by Ago2 and serves as the functional (guide) strand. Among the 42 proteins associated with saP21-Bio(AS), there are additional dsRNA-binding proteins such as Ago3, Stau1 and ILF3, and proteins involved in RNA binding and splicing, including heterogeneous nuclear ribonucleoproteins (hnRNPs; Table 1). Of great interest are several of the proteins uniquely associated with the saP21 guide strand and known to be involved in RNA/DNA binding, transcriptional regulation and chromatin modification, including CTR9, RHA (also known as DHX9), ubiquitin B (UBB), histone H2A type 1-B/E (HIST1H2AB/E), poly(rC) binding protein 2 (PCBP2) and splicing factor proline/glutamine-rich (Table 1 and Supplementary information, Table S2). In addition, we identified translational, metabolic, ribosomal, cytoskeletal and chaperone proteins associated with saP21-Bio(AS) or both strands (Table 1). Surprisingly, RNAi-related proteins known as RISC-loading factors were absent from our list, including Dicer, TRBP and members of the TNRC6 family which are known to be almost invariably associated with Agos in human cells [39-41]. Of the identified proteins with a potential role in RNAa, we chose CTR9 and RHA as candidates for further validation since both proteins are known transcriptional activators with DNA/RNA-unwinding/binding activity and are potential interactors with RNAP II [42-45].

To validate the MS results and confirm physical interactions between each of the two candidate proteins (RHA and CTR9) and the two proteins (Ago2 and RNAP II) we already determined from earlier experiments to be either essential for RNAa (Ago2; Figure 3E and refs [13, 22]) or associated with the antisense strand of saP21 (RNAP II; Figure 4I), we first performed immunofluorescence staining of Ago2, RHA and CTR9. As expected, Ago2 is preferentially distributed in the cytoplasm, with scattered and punctate staining in the nucleus, whereas CTR9 and RHA almost exclusively reside in the nucleus (Supplementary information, Figure S5). We then conducted reciprocal co-immunoprecipitation assays. First, from the nuclear fraction (Supplementary information, Figure S3) of cells transfected with either saControl or saP21, we immunoprecipitated CTR9 or RHA using their specific

antibodies. Ago2 and RNAP II were then detected by immunoblotting in the immunoprecipitated complexes. As shown in Figure 5A, CTR9 co-immunoprecipitated both Ago2 and RNAP II in cells transfected with saP21, but not saControl. Similarly, RHA co-immunoprecipitated both Ago2 and RNAP II in saP21 transfected cells; whereas in saControl transfected cells, minimal association of Ago2 and RHA was observed (Figure 5A). These results thus validated our MS findings and suggest a strong association between the saRNA-Ago2 complex and RHA, CTR9 and RNAP II. Since CTR9 is a component of a bigger complex, the PAF1 complex (PAF1C) known to interact with histone-modifying enzymes and RNAP II to regulate transcription, we assessed whether PAF1 protein was also present in the complex with CTR9 by detecting PAF1 in immunoprecipitates of CTR9 or RHA. As shown in Figure 5B, PAF1 was associated with CTR9, but not with RHA, in both saControl- and saP21-treated cells (Figure 5B), suggesting that CTR9 and PAF1 form the known PAF1C. Further, we immunoprecipitated Ago2 in similarly treated cells and then probed for RHA, CTR9 and RNAP II. We found that RHA and CTR9 was uniquely co-immunoprecipitated with Ago2 in saP21- but not in saControl-transfected cells (Figure 5C). We also detected, in Ago2 immunoprecipitates, PAF1, RNAP II and PCBP2 (hnRNP E2), one of the major cellular poly(rC)-binding proteins involved in RNA binding (Table 1), and found that they were associated with Ago2 mainly in saP21-treated cells (Figure 5C).

To further test whether CTR9 is recruited to saRNA target site on promoters, we performed CTR9 ChIP in cells transfected with saP21 or saControl. No obvious enrichment of CTR9 was detected on *p21* promoter in saControl-transfected cells, whereas in saP21-transfected cells, a significantly increased enrichment of CTR9 occurred surrounding the saP21 target site on *p21* promoter and extended into the TSS region (Figure 5D). Together with the data shown in Figure 2B and Figure 5A-5C, this result suggests that CTR9 was recruited by the saRNA-Ago2 complex to the saRNA's promoter target.

To confirm functional involvement of proteins identified by MS in RNAa, we examined the requirement of select candidate proteins for *p21* activation. Specifically, we knocked down *CTR9* and *RHA* by RNAi and determined whether *p21* could still be activated by saP21. Knockdown of *CTR9* (20% of mock; Figure 5E) and *RHA* (3% of mock; Figure 5F) significantly impaired the RNAa activity of saP21 as assessed at both the mRNA (Figure 5G and 5H) and protein (Figure 5I and 5J) levels of *p21* expression. We also examined the requirement of CTR9 in the activation of *KLF4* and *E-cadherin* by their saRNAs. As shown in Supplementary informa-

Table 1 Proteins associated with saP21 identified by MS following ChIbRP[§]

Accession#	Gene Symbol	Protein Name	saP21- Bio(S)*	saP21- Bio(AS)*	Unique to Bio(AS)
dsRNA binding					
O95793	STAU1	dsRNA-binding protein Staufen homolog 1	2	4	
Q12906	ILF3	Interleukin enhancer-binding factor 3	4	18	
Q5TA56	EIF2C3	Eukaryotic translation initiation factor 2C, 3		2	Yes
Q9UKV8	EIF2C2	Protein argonaute-2	18	44	
Nucleic acids binding/ Splicing/ Chromatin/ Transcription					
P11940	PABPC1	Polyadenylate-binding protein 1	2	5	
P22626	HNRNPA2B1	Heterogeneous nuclear ribonucleoproteins A2/B1		2	Yes
P31943	HNRNPH1	Heterogeneous nuclear ribonucleoprotein H		1	Yes
P52272	HNRNPM	Heterogeneous nuclear ribonucleoprotein M		2	Yes
Q15366	PCBP2	Poly(rC)-binding protein 2		2	Yes
P23246	SFPQ	Splicing factor, proline- and glutamine-rich		1	Yes
Q6PD62	CTR9	RNA polymerase-associated protein CTR9 homolog		1	Yes
Q08211	RHA	ATP-dependent RNA helicase A		2	Yes
P04908	HIST1H2AB/E	Histone H2A type 1-B/E		1	Yes
P0CG47	UBB	Polyubiquitin-B		1	Yes
P06748	NPM1	Nucleophosmin	2	6	
Translation					
P60842	EIF4A1	Eukaryotic initiation factor 4A-I		1	Yes
P68104	EEF1A1	Elongation factor 1-alpha 1	3	2	
Metabolic proteins					
O00763	ACACB	Acetyl-CoA carboxylase 2		1	Yes
P06733	ENO1	Alpha-enolase		1	Yes
Q13085	ACACA	Acetyl-CoA carboxylase 1	2	14	
P04075	ALDOA	Fructose-bisphosphate aldolase A		5	Yes
P14618	PKM	Pyruvate kinase isozymes M1/M2	2	8	
P00558	PGK1	Phosphoglycerate kinase 1		4	Yes
Chaperones					
P07900	HSP90AA1	Heat shock protein HSP 90-alpha		5	Yes
P08107	HSPA1A/B	Heat shock 70 kDa protein 1A/1B		1	Yes
P11142	HSPA8	Heat shock cognate 71 kDa protein	6	9	
Cytoskeleton proteins					
A6NMY6	ANXA2P2	Putative annexin A2-like protein		2	Yes
P07355	ANXA2	Annexin A2	1	4	
P04350	TUBB4A	Tubulin beta-4 chain		1	Yes
P07437	TUBB	Tubulin beta chain	2	3	
P18206	VCL	Vinculin		1	Yes
P60709	ACTB	Actin, cytoplasmic 1	12	17	
Q15149	PLEC	Plectin		7	Yes
Q5D862	FLG2	Filaggrin-2		2	Yes
P21333	FLNA	Filamin-A		1	Yes
P68363	TUBA1B	Tubulin alpha-1B chain	2	4	
Ribosomal proteins					
P08865	RPSA	40S ribosomal protein SA	2	4	
P15880	RPS2	40S ribosomal protein S2		1	Yes
P18124	RPL7	60S ribosomal protein L7		1	Yes
Miscellaneous					
P01833	PIGR	Polymeric immunoglobulin receptor		2	Yes
Q8IVZ3	OSBPL1A	OSBPL1A protein	1	1	
P02808	STATH	Statherin		1	Yes
Total non-redundant proteins			15	42	

[§]For detailed listing of proteins recovered by MS, see Supplementary information, Table S2.

*Number of peptides for each unique protein associated with either saP21-Bio(S) or saP21-Bio(AS).

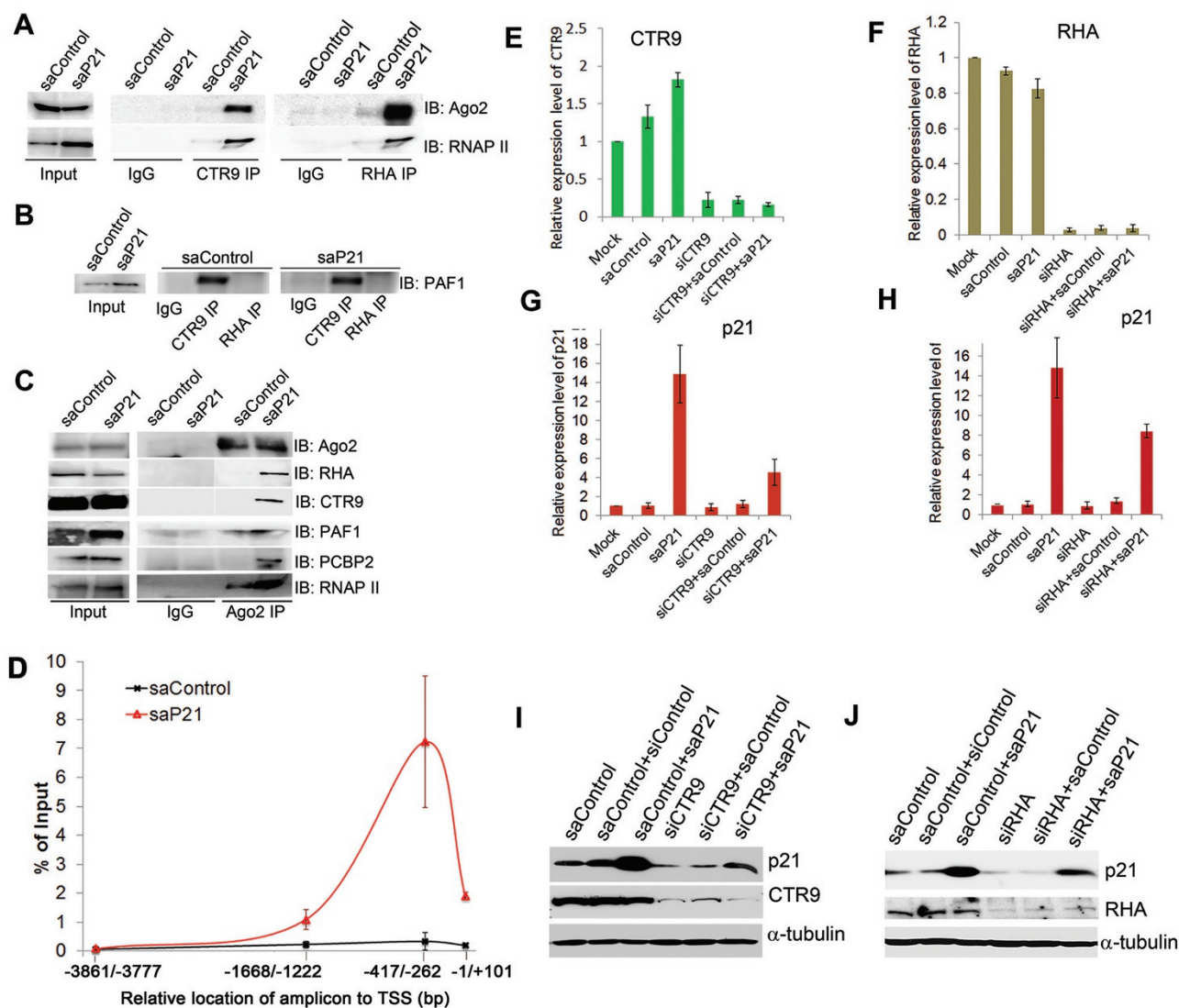


Figure 5 Characterization of the RNAa effector complex. **(A)** Association of Ago2 and RNAP II with CTR9 or RHA. PC-3 cells were transfected with the indicated saRNA for 48 h. Total protein extract from each treatment was immunoprecipitated with either anti-CTR9 or anti-RHA antibody. Effector proteins Ago2 and RNAP II were detected in the eluates by immunoblotting. **(B)** Association of PAF1 with CTR9. Proteins were immunoprecipitated using CTR9 or RHA antibodies as in **A** and probed for PAF1 by immunoblotting. **(C)** Association of Ago2 with RHA, PAF1, CTR9, PCBP2 and RNAP II. Reciprocal immunoprecipitation with anti-Ago2 antibody followed by immunoblotting detection of associated effector proteins. **(D)** Enrichment of CTR9 at saP21 target site. CTR9 ChIP was performed in PC-3 cells transfected for 48 h with saP21 or saControl. The immunoprecipitated DNA was amplified by qPCR using 4 sets of primers located at p21 promoter as indicated. The primer set at -3 861/-3 777 served as negative control for CTR9 binding. Results represent % of input in each experiment and are means \pm SD of at 3 independent experiments. **(E-J)** Knockdown of CTR9 and RHA impairs RNAa of p21. PC-3 cells were transfected with siRNA targeting CTR9 or RHA or control siRNA for 24 h, and the next day the cells were further transfected with saRNA for an additional 48 h. The transfected cells were then assessed by RT-qPCR for mRNA expression of CTR9 (**E**), RHA (**F**) and p21 (**G**, **H**), and by immunoblotting for protein expression of CTR9 (**I**), RHA (**J**) and p21 (**I**, **J**).

tion, Figure S6A-S6F, the knockdown of *CTR9* attenuated saRNA-induced activation of both *KLF4* and *E-cadherin*. The knockdown of unrelated proteins such as XBP and CID1 by their siRNAs (siXBP and siCID1) had no

significant effect on *p21* activation by saP21 (Supplementary information, Figure S6G). These results indicate that CTR9 is a component in RITA essential for RNAa.

Together, we showed that Ago2 biochemically as-

sociates in the nucleus with CTR9 and RHA, both of which are functionally essential for RNAa and form the minimal core RITA complex with an RNA-programmed Ago2.

saRNA targeting causes monoubiquitination of histone 2B

Histone 2B (H2B) ubiquitination is a histone modification that stimulates methylation of H3K4, leading to active transcription [46]. Since UBB polypeptide exists in the complex associated with saP21-Bio(AS) (Table 1), and PAF1C has been shown to induce monoubiquitination of histone 2B (H2Bub1), we decided to investigate the presence of H2Bub1 on *p21* gene by ChIP using an antibody recognizing H2Bub1 in cells transfected for 48 h with saP21 (Figure 6A). Neither saP21 nor control treatments affected global H2Bub1 levels (Figure 6B). However, on *p21* gene, we observed increased levels of H2Bub1 (compared with control treatments) starting from position -141 and continuing into the entire *p21* transcribed region, with the enrichment ranging from 2.3- to 9.1-fold (Figure 6A). These data suggest that H2B ubiquitination could be an early event in the chain of transcriptional/epigenetic events that eventually lead to

transcriptional activation of *p21*.

Discussion

Using the ChIP assay, which allows us to isolate and identify target-specific nucleic acids and proteins, we were able to address critical questions concerning RNAa. In the present work, we show that saRNAs are loaded by Ago2 and bind to their intended promoter targets, where Ago2 recruits RHA and CTR9 to interact with RNAP II, leading to ubiquitination of H2B and productive transcription elongation.

Central role of Ago2 in the RITA complex

Since the discovery of the essential role of Ago proteins in mediating posttranscriptional RNAi, surprising additional functions of Ago proteins, including roles in transcriptional regulation [47] and alternative splicing [41], have been uncovered. In these mechanisms, Ago proteins serve as an RNA-programmable recruitment platform which can be targeted by small RNAs to their cognate target molecules in both cytoplasmic and nuclear compartments, where Ago proteins then recruit diverse arrays of proteins of particular functions to influence dif-

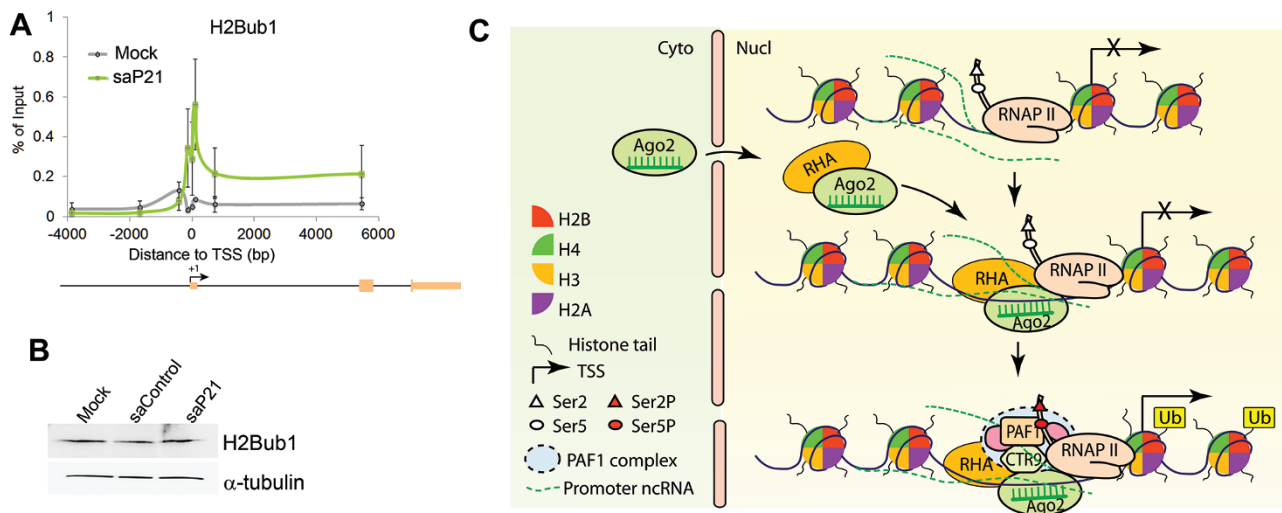


Figure 6 saRNA targeting induces H2Bub1 in the *p21* gene and a working model for RNAa. **(A)** Increased enrichment of H2Bub1 in the *p21* gene in response to saP21 treatment. H2Bub1 ChIP was performed in PC-3 cells transfected with saP21. The diagram beneath the graph shows the gene structure in reference to the graphical data. Results represent % of input in each experiment and are means \pm SD of at least two independent experiments. **(B)** No change in global H2Bub1 levels in response to saRNA transfection. Immunoblotting analysis of H2Bub1 protein levels in PC-3 cells transfected with saP21, saControl or mock-transfected. **(C)** A working model for RNAa. A promoter-targeted saRNA is loaded by an Ago2 protein which uses one of the duplex strands as the guide to form an active saRNA-Ago2 complex. The saRNA-loaded Ago2 in partnership with RHA binds to the cognate sequence (DNA or non-coding transcripts (dotted green lines)) of the saRNA at the target promoter, on which RNAP II transcription is paused. Recruitment by Ago2 of PAF1C to the target site results in the formation of a RITA complex which phosphorylates RNAP II CTD on Ser2 and monoubiquitinates H2B, leading to increased transcription initiation and productive elongation. Ub, ubiquitination.

ferent aspects of gene regulation.

Several previous proteomic approaches have characterized proteins that form complexes with Ago proteins immunoprecipitated from either cytoplasmic or nuclear fractions [39-41, 48]. Commonly recovered proteins include components of the RISC-loading complex (e.g., Dicer, TRBP and TNRC6 family members), RNA helicases such as RHA, hnRNPs, splicing factors and histones. We used our ChIRP approach to pull down proteins associated with Ago2-loaded saRNA, and recovered categories of proteins common to those identified by Ago immunoprecipitation, thus validating our method as an effective alternative to Ago immunoprecipitation. More importantly, ChIRP has the advantage of recovering not only general proteins associated with Agos, but also target-specific proteins associated only with the saRNA-Ago2 complex. Surprisingly, members of the RISC-loading complex are absent from the RITA complex, whereas proteins involved in transcriptional regulation such as CTR9 were identified for the first time in our analysis. Our findings suggest that Ago2, guided by a promoter-targeted saRNA, serves as a promoter-specific platform to recruit a unique set of proteins distinct from the RISC complex to form a RITA complex.

The RITA complex

Previous work using a candidate approach [49] or MS [34] identified several hnRNPs in association with saRNAs. By using ChIRP, followed by MS analysis, we were able to identify hnRNPs (e.g., PCBP2) as well as additional factors such as RHA and CTR9 that form the RITA complex on the target promoter.

RHA, also known as nuclear DNA helicase II, is a member of the DEAH family of helicase superfamily 2 and was first isolated from nuclei of calf thymus using a DNA-unwinding assay [50]. It contains two dsRNA binding domains, a helicase domain, and an RCG-rich region which possesses both RNA and DNA helicase activities [44, 51]. RHA directly binds to DNA or interacts with other transcription factors to mediate transcriptional activation by recruiting basal transcription machinery to promoter DNA and/or modifying chromatin structure [42, 43, 52-57]. RHA can also act as a bridging factor to recruit RNAP II into pre-initiation complexes [51, 55, 58]. Of particular interest, RHA can unwind triple-helical DNA structures in which the third strand contains a 3' single-stranded overhang [42], a scenario similar to saRNA binding to dsDNA of a promoter. Although RHA has been shown to be a component of the RISC complex [59], it may play a critical role in RNAa given its nuclear localization and established functions in DNA unwinding and transcriptional activation. It is possible that RHA is

both a general Ago2-interacting protein and an RNAa facilitator. Another interesting aspect of RHA is that the *Drosophila* homolog of RHA, known as maleless, is a component of the dosage compensation complex which is directed also by ncRNA to the male X chromosome to maintain upregulation of all genes on the chromosome [60, 61].

CTR9 is a component of PAF1C, which, in addition to PAF1, contains at least 4 other proteins (LEO1, CDC73, RTF1 and SKI8) [62, 63], and is involved in both transcription initiation and elongation [45, 64-67]. PAF1C is required to establish several histone modifications associated with active genes by recruiting histone-modifying factors such as E2/E3 ubiquitin ligase to the RNAP II complex to cause H2B ubiquitination and downstream H3K4 and H3K79 methylation [45, 62, 68]. PAF1C is also required for efficient phosphorylation of Ser2 of RNAP II CTD, suggesting it has a role in the transition between early and productive transcription elongation [67]. Similar to RHA, CTR9 has also been shown to bind a triple helix structure [69, 70]. The observed association of these two components of PAF1C (CTR9 and PAF1) with Ago2 suggests that components of PAF1C especially CTR9 may participate in RNAa.

The involvement of two proteins (RHA and CTR9) with DNA/RNA unwinding or binding activity is intriguing. Perhaps, such activity is needed for opening up the strands of the DNA duplex at the target site. The physical association of RHA and CTR9 with the guide strand and Ago2, and their function in DNA binding and unwinding and in transcriptional activation, strongly suggest that they are bona fide components of the RITA complex.

Transcriptional and epigenetic activation by RNAa

A fundamental question regarding RNAa is how saRNAs stimulate transcription. saRNA and promoter-targeted miRNA have been shown to cause an enrichment of RNAP II at target promoters, as evidenced by CHIP analysis [25, 32, 33], suggesting potential recruitment of RNAP II by Ago proteins. However, biochemical evidence is missing. We showed a direct interaction of Ago2-loaded saRNAs with RNAP II, and the accumulation of initiating RNAP II at the *p21* promoter and RNAP II Ser2P in gene body upon saRNA treatment. We thus conclude that RNAa of *p21* causes an increase of both transcription initiation and transition to productive elongation.

Previous studies have shown that different epigenetic changes such as demethylation of H3K9 and dimethylation and trimethylation of H3K4 [13, 14, 24, 34] are associated with gene activation induced by saRNA. However, it is unclear whether such changes are the cause or

consequence of transcriptional activation. The presence of both histones (H2A) and the factors facilitating H2B ubiquitination prompted us to examine for the first time H2B ubiquitination, which is regarded as a prerequisite for H3K4 methylation and acetylation and is at the top of histone modification cascades [71]. It is also known that upon transcription initiation, phosphorylation of Ser5 on RNAP II CTD serves as a signal to trigger a series of events through PAF1C, by which the E2/E3 ligase complex containing Rad6 and Bre1 is recruited to monoubiquitinate histone H2B at lysine 123 in yeast and lysine 120 in humans [72, 73]. The observed massive increase in H2Bub1 starting from the saRNA target site and its propagation into gene body in response to saRNA treatment clearly suggest that H2Bub1 is an important and early epigenetic event, potentially facilitated by PAF1C, leading to *p21* activation. In support, a previous study showed that, in p53-induced *p21* activation, H2Bub1 of the *p21* gene occurs at the same time when RNAP II accumulates at the *p21* promoter, but prior to any increase in *p21* mRNA level, and subsequently leads to H3K4me2 and productive elongation [74]. Further study is needed to establish a general causal role for H2Bub1 in RNAa and to identify the ubiquitin ligase(s) responsible for H2B ubiquitination.

Chromatin targeting by the saRNA-Ago2 complex

The ability to precisely target any region of the genome has enormous potential for genome editing and gene expression control, and has been realized with varying degree of success by using both protein- (e.g., zinc finger nuclease and TALENS [transcription activator-like effector nucleases] and nucleic acid- (e.g., TFO and CRISPR) [75] based methods. However, these methods are not perfect, as they suffer from off-target effects and/or require sophisticated designs or an exogenous protein partner [75]. We demonstrate here that promoter-targeted dsRNAs bind to their intended targets on promoters, revealing another sequence homology-driven genome-targeting mechanism. Using the ChIRP assay, we found that saRNA can strongly associate with its target DNA on promoters with a predictable strand specificity, and that this interaction depends on Ago2 loading of the saRNA, which in turn is determined by the thermodynamics of the saRNA termini. However, there is still possibility that non-coding transcripts that overlap saRNA targets could serve as the actually binding molecules of saRNAs as have been shown in several other example genes [25, 76, 77].

In summary, we demonstrate that RNAa is a transcriptional mechanism of gene regulation in which saRNAs in complex with Ago2 bind to their intended targets on

gene promoters. Characterization of transcriptional and epigenetic events during RNAa and identification of the RITA complex allow us to build a working model (Figure 6C) in which RNA-programmed and RHA-associated Ago2 serves as a recruitment platform on which components of PAF1C are assembled to stimulate transcription initiation and license RNAP II for productive elongation.

Materials and Methods

Cell culture and transfection

Human prostate cancer cell line PC-3 was cultured in RPMI-1640 medium. Transfection of duplex RNA was done using Lipofectamine RNAiMAX (Invitrogen). Detailed procedures and the duplex RNAs used are provided in the extended experimental procedures in Supplementary information, Data S2.

RNA purification and mRNA expression analysis

Total RNA was purified using either RNeasy Mini kit (Qiagen), MasterPure Complete DNA and RNA Purification kit (Epicentre) or GenElute Mammalian Total RNA Purification Mini kit (Sigma). Total RNA was reverse transcribed and the resulting cDNA was amplified by quantitative or regular RT-PCR. Detailed procedures are provided in Supplementary information, Data S2.

Immunoblotting

Proteins were purified using the M-PER mammalian protein extraction reagent (Thermo Scientific), separated by SDS-PAGE gel and transferred to a PVDF membrane. The resulting membrane was blocked and blotted with a primary antibody against Ago2 CTR9, RHA, RNAP II, *p21*, α -tubulin, *p21*, H2Bub1 or topoisomerase I at dilutions suggested in manufacturer's manuals. Detailed procedures are provided in Supplementary information, Data S2.

Subcellular fractionation

Cytoplasmic and nuclear fractions were prepared using NE-PER (Pierce) according to the manufacturer's manual. Immunodetection of either β -tubulin or topoisomerase I was used as cytoplasmic and nuclear marker, respectively.

ChIP assay

ChIP assay was performed as previously described [32]. Briefly, chromatin was prepared from PC-3 cells following crosslinking with formaldehyde. DNA was sheared to an average size of ~500 bp using a Bioruptor sonicator (Diagenode). Chromatin was immunoprecipitated overnight at 4 °C using 5 μ g of the following antibodies: anti-RNAP II (Millipore, cat #05-623), anti-RNAP II Ser2P (Abcam, cat #ab5095), anti-RNAP II Ser5P (Abcam, cat #ab5131). The following day, the samples were incubated with 25 μ l Protein G Dynabeads (Invitrogen) for 2 h at 4 °C. Immunoprecipitates were sequentially washed with low salt, high salt, and TE buffer. Eluates were collected and reverse crosslinked at 65 °C overnight. ChIP DNA was treated with Proteinase K, purified with phenol/chloroform, treated with RNase A, and purified using the Qiaquick PCR purification kit (Qiagen). Target amplification and detection was performed on a 7500 Fast Real-Time System (Applied Biosystems). Enrichment was determined by using the $2^{-\Delta\Delta Ct}$ method relative to input DNA or IgG control.

Chromatin Isolation by biotinylated RNA Pulldown

ChlBRP protocol was based on standard ChIP assay [32] and ChIRP protocol [78] with modifications. Briefly, ChlBRP was performed on cells transfected with biotinylated saRNA by following the standard ChIP procedures as described above. After the last wash and completely removal of buffer, beads were taken to different elution protocols including elution of protein and DNA. Detailed procedures are provided in Supplementary information, Data S2.

NRO assay

Isolation of nuclei from cells was carried out as previously described [79] with several modifications. Briefly, PC-3 cells were swelled in ice cold swelling buffer and pelleted. The resulting cell pellet was resuspended and nuclei were pelleted again and washed and pelleted once more in 10 ml of NP-40 lysis buffer and then resuspended in 1 ml nuclei freezing buffer. Nuclei were then mixed with 5× NRO reaction buffer in the presence of RNase inhibitor. The reaction was resumed for 30 min at 37 °C, followed by RNA purification using RNeasy Mini kit (Qiagen) including on-column DNase I digestion (Qiagen). BrdU RNA was captured by adding protein G agarose beads coupled with an anti-BrdU antibody and the BrdU RNA was then purified and analyzed by RT-qPCR. Detailed procedures are provided in Supplementary information, Data S2.

Immunoprecipitation

Total protein extract from cells was incubated overnight with a particular antibody in immunoprecipitation (IP) buffer 2 (25 mM Tris pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 5% Glycerol). Next day, Protein G magnetic beads (Invitrogen) were added to the lysates and incubated with IPs for 2 h at 4 °C, then the beads were washed 3 times in IP buffer 2, once in IP buffer 2 plus 500 mM NaCl and eluted in 2× protein loading buffer by boiling the samples for 5 min. Proteins were next resolved on a protein gel and analyzed by immunoblotting.

Immunofluorescence (IF) and confocal microscopy

Cells on coverslips were fixed, permeabilized and stained with a primary antibody against Ago2, RHA or CTR9. The cells were then incubated with a fluorochrome-conjugated secondary antibody and analyzed by confocal microscopy. Detailed procedures are provided in Supplementary information, Data S2.

Detailed experimental procedures can be found in the Supplementary information, Data S2.

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Author Contributions

LCL, VP, HL and AP designed the study, interpreted the data and wrote the manuscript; VP, SHSL, KHL and ZHH performed the experiments and analyzed the data.

Competing Financial Interests

LCL is a named inventor on patents related to RNAa which have been licensed to MiNA Therapeutics, Ltd.

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