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Argonaute-1 directs siRNA-mediated transcriptional gene silencing in human cells

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Argonaute proteins are the core components of effector complexes that facilitate RNA interference (RNAi). Small interfering RNAs (siRNAs) targeted to promoter regions mediate transcriptional gene silencing (TGS) in human cells through heterochromatin formation. RNAi effector complexes have yet to be implicated in the mechanism of mammalian TGS. Here we describe the role of the human Argonaute-1 homolog (AGO1) in directing TGS at the promoters for human immunodeficiency virus-1 coreceptor CCR5 and tumor suppressor RASSF1A. AGO1 associates with RNA polymerase II (RNAPII) and is required for histone H3 Lys9 dimethylation and TGS. AGO1, TAR RNA-binding protein-2 (TRBP2) and Polycomb protein EZH2 colocalize to the siRNA-targeted *RASSF1A* promoter, implicating Polycomb silencing in the mechanism of mammalian TGS. These results establish a connection between RNAi components AGO1 and TRBP2, RNAPII transcription and Polycomb-regulated control of gene expression.

Argonaute family proteins are integral effectors of transcriptional and post-transcriptional RNA-silencing pathways[1]. Ago4 directs repressive chromatin modifications in plants, and a mutation in Ago4 correlates with the loss of histone H3 Lys9 dimethylation (H3K9me2) and TGS[2]. Ago1 mutant strains in fission yeast also show a loss of H3K9me2 and heterochromatin formation[3]. The mechanism of small RNA–directed TGS in fission yeast involves the Ago1-containing RNA-induced transcriptional silencing (RITS) complex[4]. Targeting promoters with sequence-specific siRNAs leads to TGS in human cells[5] by inducing H3K9me2 and histone H3 Lys27 trimethylation (H3K27me3)[6], which are characteristic of facultative heterochromatin[7]. To identify the role of RNAi components in siRNA-mediated TGS in human cells, we investigated the silencing potential of the therapeutically relevant human immunodeficiency virus-1 (HIV-1) coreceptor *CCR5* promoter[8] and the *RASSF1A* promoter, which is epigenetically silenced in cancers[9].

RESULTS

TGS induces heterochromatin formation

We generated a stable cell line expressing green fluorescent protein (GFP) driven by the *CCR5* promoter in HEK293T cells (293T CCR5-GFP) to assess the levels of siRNA-mediated TGS at the *CCR5* promoter. Four candidate siRNAs with sequence homology to the *CCR5-GFP* promoter were screened for knockdown of GFP expression at 48 h after transfection, and two siRNAs (R61 and R149) induced ~50% reduction of protein abundance (**Fig. 1a**). *GFP* mRNA abundance was assessed at 24 h after transfection using real-time quantitative reverse-transcription PCR (qRT-PCR). Cells transfected with promoter-specific R61 siRNA showed ~70% knockdown of *GFP* mRNA transcript abundance compared to cells transfected with control siRNA specific for *CCR5* mRNA (**Fig. 1b**), similar to previous observations with siRNAs targeted to RNAPII promoters in human cells[5,6,10]. We also examined siRNA-mediated TGS at the endogenous *RASSF1A* promoter using HeLa stable cell lines expressing either a short hairpin RNA (shRNA) that is processed *in vivo* into an siRNA targeting the *RASSF1A* promoter or a control vector without shRNA expression[11]. *RASSF1A* mRNA transcript abundance also showed ~70% knockdown through siRNA-mediated TGS of this endogenous promoter (**Fig. 1b**).

Previous work has shown that the facultative heterochromatin marks H3K9me2 and H3K27me3 are associated with siRNA-targeted promoters in human cells[6,10]. To determine whether the *CCR5-GFP* promoter–targeting R61 siRNA could induce these repressive histone modifications, we screened the *CCR5-GFP* promoter for H3K9me2 at regions overlapping the R61 siRNA target site, using chromatin immunoprecipitation (ChIP). A ~14-fold enrichment of H3K9me2 was observed 24 h after transfection of R61 siRNA, relative to cells transfected with the control siRNA (**Fig. 2a**). We also performed ChIP experiments using primers spanning a region 100–300 base pairs downstream of the R61 siRNA target site (3′ site) and observed an approximately seven-fold enrichment of H3K9me2 (**Fig. 2a**). Time-course ChIP experiments showed an increase in H3K9me2 enrichment at the targeted *CCR5-GFP* promoter between 12 h and 24 h after transfection of R61 siRNA (**Fig. 2b**). However, negligible amounts of DNA methylation were observed at the

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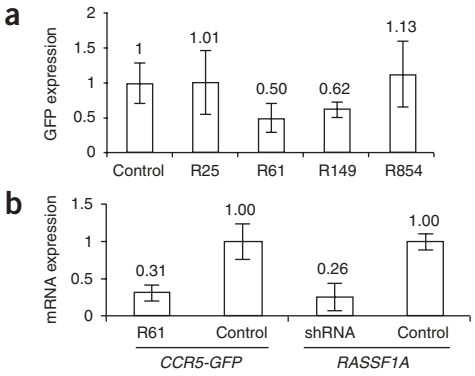

Figure 1 Transcriptional gene silencing of *CCR5-GFP* and *RASSF1A*. (**a**) GFP expression in 293T CCR5-GFP cells 48 h after transfection with *CCR5-GFP* promoter–specific or control siRNA, as determined by FACS analysis. Error bars show s.d. from three independent experiments. (**b**) *GFP* mRNA expression in 293T CCR5-GFP cells 24 h after transfection with promoter-specific R61 or control siRNA, and *RASSF1A* mRNA expression in HeLa stable cells expressing promoter-specific shRNA or control vector, as determined by qRT-PCR and normalized to *GAPDH* abundance. Error bars show s.e.m. for four (*GFP*) and three (*RASSF1A*) independent samples.

siRNA-targeted *CCR5-GFP* and *RASSF1A* promoters using a DNA methylation assay based on methylation-sensitive restriction enzyme digestions (**Fig. 2c**).

AGO1 directs TGS

We next investigated whether human AGO1 or AGO2 (also called EIF2C1 and EIF2C2) might be involved in siRNA-mediated TGS in human cells, as studies using model organisms have demonstrated the involvement of Argonaute proteins in TGS pathways[2–4]. ChIP experiments in 293T CCR5-GFP cells transfected with R61 siRNA showed ~18-fold enrichment of AGO1 at the *CCR5-GFP* promoter, relative to cells transfected with control siRNA, and ~14-fold enrichment of AGO1 at the 3′ site (**Fig. 3a**). We did not observe enrichment for AGO2 in our ChIP experiments on the targeted *CCR5-GFP* promoter (**Fig. 3b**), but we cannot rule out the possibility that AGO2 may be involved in other RNA-silencing pathways in the nucleus[12]. AGO1 was also enriched ~21-fold at the siRNA-targeted *RASSF1A* promoter (**Fig. 3a**). To determine the temporal nature of AGO1 association with the siRNA-targeted promoter, time-course ChIP experiments were conducted at 6-h intervals from 12–30 h after transfection of R61 siRNA into 293T CCR5-GFP cells. The time-course data showed an initial peak and subsequent decrease in AGO1 recruitment to the targeted promoter (**Fig. 3c**), correlating with the increase in H3K9me2 mentioned previously (**Fig. 2b**). These data suggest that AGO1 directs siRNA-mediated TGS by recruiting histone methyltransferase activity for H3K9me2.

We next used RNAi-mediated knockdown of AGO1 to investigate the requirement of AGO1 in directing H3K9me2 and silencing gene expression. 293T CCR5-GFP cells were transfected with *AGO1* mRNA–specific siRNA[12], and knockdown of AGO1 was observed at 48 h after transfection (**Fig. 4a**). Cells transfected with *AGO1* siRNA (*AGO1*$^{-}$) or control siRNA (*AGO1*$^{+}$) were transfected 24 h later with R61 siRNA. *GFP* transcript abundance was much greater in *AGO1*$^{-}$ than in *AGO1*$^{+}$ cells at 24 h after transfection of R61 siRNA (**Fig. 4b**). Knockdown of AGO1 resulted in the loss of both AGO1 and H3K9me2 enrichment at the *CCR5-GFP* promoter in *AGO1*$^{-}$ cells transfected with R61 siRNA (**Fig. 4c**). These data indicate that AGO1 recruitment to the targeted promoter is required for H3K9me2 and siRNA-mediated TGS in human cells.

TRBP2 and RNAPII are involved in TGS

As the double-stranded RNA–binding protein TRBP2 (ref. 13) associates with AGO2 and contributes to the formation of the RNA-induced silencing complex (RISC) in human cells[14], we sought to determine whether TRBP2 might also function in AGO1-directed TGS. ChIP experiments indicated ~16-fold enrichment of TRBP2 at the siRNA-targeted *RASSF1A* promoter (**Fig. 5a**). To assess the requirement for TRBP2 in TGS, 293T CCR5-GFP cells were transfected with *TRBP2* mRNA–specific siRNA[14], and knockdown of *TRBP2* mRNA

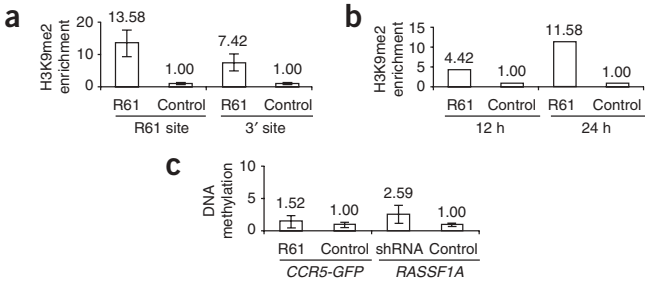

Figure 2 H3K9me2 and DNA methylation at targeted promoters. (**a**) ChIP of the *CCR5-GFP* promoter using anti-H3K9me2 in 293T CCR5-GFP cells 24 h after transfection with R61 or control siRNA. Error bars show s.e.m. for three independent experiments. (**b**) Time-course ChIP of the *CCR5-GFP* promoter using anti-H3K9me2 in 293T CCR5-GFP cells 12 and 24 h after transfection with R61 or control siRNA. (**c**) DNA methylation levels at the R61- or control siRNA–targeted *CCR5-GFP* promoter in 293T CCR5-GFP cells and at the endogenous *RASSF1A* promoter in HeLa stable cells expressing promoter-specific shRNA or control vector, measured using AvaI- and ApaI-based DNA methylation assays for the *CCR5-GFP* and *RASSF1A* promoters, respectively. Error bars show s.e.m. for three independent samples.

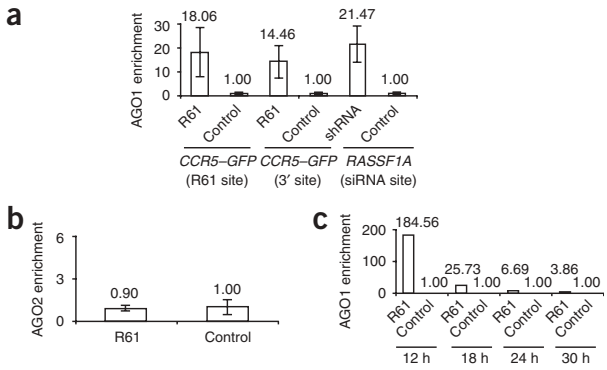

Figure 3 AGO1 recruitment to siRNA-targeted promoters. (**a**) ChIP of the *CCR5-GFP* promoter using anti-AGO1 in 293T CCR5-GFP cells 18 h after transfection with R61 or control siRNA, and ChIP of the *RASSF1A* promoter from HeLa stable cells expressing shRNA or control vector. Error bars show s.e.m. for three independent experiments. (**b**) ChIP of the *CCR5-GFP* promoter using anti-AGO2 in 293T CCR5-GFP cells 18 h after transfection with R61 or control siRNA. Error bars show s.e.m. for three independent experiments. (**c**) Time-course ChIP of the *CCR5-GFP* promoter from 293T CCR5-GFP cells, performed at 6-h intervals after R61 or control siRNA transfection.

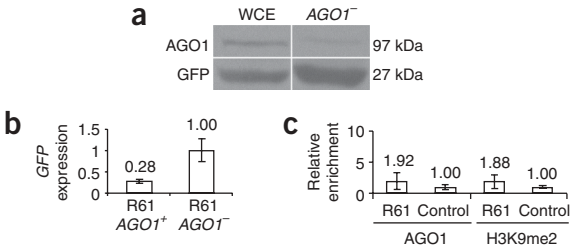

Figure 4 AGO1 is required for transcriptional gene silencing. (**a**) Whole-cell extracts (WCE) from 293T CCR5-GFP cells transfected with control siRNA and extracts from 293T CCR5-GFP cells transfected with AGO1 mRNA-specific siRNA (AGO1⁻), at 48 h after transfection, analyzed by western blotting using anti-AGO1 or anti-GFP (loading control). (**b**) *GFP* mRNA expression in *AGO1*⁺ or *AGO1*⁻ 293T CCR5-GFP cells 24 h after transfection with R61 siRNA, as determined by qRT-PCR and normalized to *GAPDH* abundance. Error bars show s.e.m. for three independent samples. (**c**) ChIP of the *CCR5-GFP* promoter using anti-AGO1 or anti-H3K9me2 in *AGO1*⁻ 293T CCR5-GFP cells 24 h after transfection with R61 or control siRNA. Error bars show s.e.m. for three independent experiments.

expression was observed at 48 h after transfection (**Fig. 5b**). Cells transfected with *TRBP2* siRNA (*TRBP2*⁻) or control siRNA (*TRBP2*⁺) were transfected 24 h later with R61 siRNA. As in AGO1 knockdown experiments, greater *GFP* transcript abundance was observed at 24 h after transfection of R61 siRNA in *TRBP2*⁻ cells than in *TRBP2*⁺ cells (**Fig. 5c**), indicating that TRBP2 is also required along with AGO1 in the mechanism of TGS.

Recent studies in fission yeast[15] and human cells[6] have also shown that RNAPII is required for siRNA-mediated TGS. To examine whether RNAPII associates with AGO1, we performed coimmunoprecipitation experiments in 293T CCR5-GFP cells and observed that AGO1 coimmunoprecipitated with RNAPII having an unphosphorylated C-terminal domain (CTD) (**Fig. 6a**). To test whether AGO1 associates with RNAPII via protein-protein interactions or whether a single-stranded RNA species tethers AGO1 to RNAPII, 293T CCR5-GFP cells were treated with RNase A. *GADPH* mRNA expression in 293T CCR5-GFP cells was measured by qRT-PCR to assess the extent of RNA degradation by RNase A (**Fig. 6b**). RNase A treatment did not affect the coimmunoprecipitation of RNAPII and AGO1 (**Fig. 6a**), suggesting that their association involves protein-protein interactions.

TGS is linked with EZH2 and Polycomb silencing

We next examined whether AGO1-directed TGS is coupled to the Polycomb silencing machinery[16]. ChIP experiments showed ~14-fold enrichment of a Polycomb component, the histone methyltransferase EZH2 (ref. 17), at the siRNA-targeted *RASSF1A* promoter in HeLa cells (**Fig. 7a**). EZH2 has been shown to have histone methyltransferase activity for H3K9 and H3K27 (ref. 18), and we also observed approximately nine-fold enrichment of H3K27me3 at the siRNA-targeted *RASSF1A* promoter in ChIP experiments (**Fig. 7a**). The recruitment of Polycomb component EZH2 to the siRNA-targeted *RASSF1A* promoter suggested that RNAi components might be involved in the endogenous mechanism of Polycomb silencing. We therefore examined a Polycomb target, the *MYT1* promoter[19], by ChIP experiments in HeLa cells and observed enrichment for EZH2, H3K27me3 and, notably, AGO1 at this endogenously silenced promoter (**Fig. 7b**). The *CCR5* promoter has also been characterized as a Polycomb target in human cells[20], and we tested whether RNAi components also localized to the endogenously silenced *CCR5* promoter in HeLa cells. AGO1 and TRBP2, along with EZH2 and RNAPII with unphosphorylated CTD, were all enriched at the *CCR5* promoter (**Fig. 7c**), further implicating RNAi in Polycomb silencing pathways in human cells.

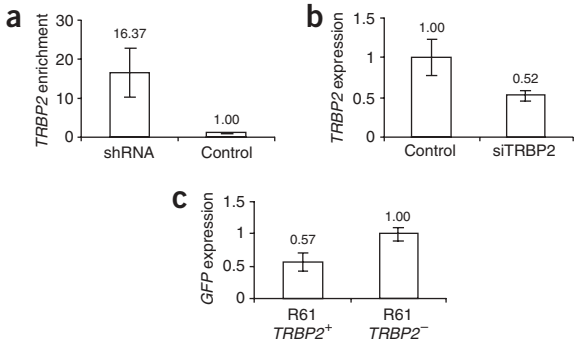

Figure 5 TRBP2 is required for transcriptional gene silencing. (**a**) ChIP of the *RASSF1A* promoter using anti-TRBP2 from HeLa stable cells expressing shRNA or control vector. Error bars show s.e.m. for three independent experiments. (**b**) *TRBP2* mRNA expression in 293T CCR5-GFP cells 48 h after transfection with control siRNA or *TRBP2* mRNA–specific siRNA (siTRBP2), as determined by qRT-PCR and normalized to *GAPDH* abundance. Error bars show s.e.m. for three independent samples. (**c**) *GFP* mRNA expression in *TRBP2*⁺ or *TRBP2*⁻ 293T CCR5-GFP cells 24 h after transfection with R61 siRNA, as determined by qRT-PCR and normalized to *GAPDH* abundance. Error bars show s.e.m. for three independent samples.

DISCUSSION

Data presented here show that AGO1 directs siRNA-mediated TGS in human cells by associating with targeted promoters. AGO1 may then recruit EZH2, which induces H3K27me3 (ref. 17), possibly H3K9me2 (ref. 18) and facultative heterochromatin assembly. AGO1 association with RNAPII having an unphosphorylated CTD may prevent the formation of an elongation-competent RNAPII complex at the siRNA-targeted promoter through Polycomb silencing mechanisms[21]. The findings presented here suggest a model for siRNA-mediated TGS in human cells involving AGO1, TRBP2, RNAPII and EZH2. Although the role of TRBP2 in mammalian TGS is presently unclear, TRBP2 may couple the initiation and effector steps of AGO1-directed TGS by binding promoter-specific double-stranded siRNA[14] and facilitating the loading of the siRNA antisense strand[6] into AGO1. The involvement of AGO1 in TGS also suggests that the siRNA antisense strand may recognize an RNA target that RNAPII with unphosphorylated CTD transcribes through promoter regions as a noncoding RNA in the sense orientation. Targeting of nascent RNA transcripts by RNAi components in the mechanism of TGS is supported by recent observations in fission yeast[22]. An alternative role for TRBP2 in mammalian TGS may be to stabilize an RNA-RNA

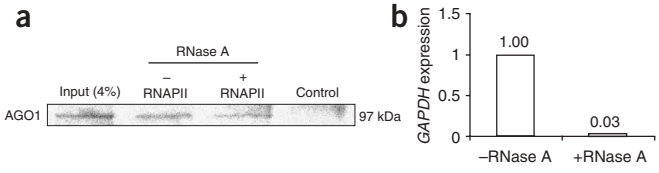

Figure 6 RNAPII with unphosphorylated CTD associates with AGO1. (**a**) Anti-RNAPII (unphosphorylated CTD) immunoprecipitates from 293T CCR5-GFP cell extracts treated (+) or untreated (–) with RNase A, analyzed by western blotting using anti-AGO1. (**b**) *GAPDH* mRNA expression in –RNase A or +RNase A 293T CCR5-GFP cells, as determined by qRT-PCR.

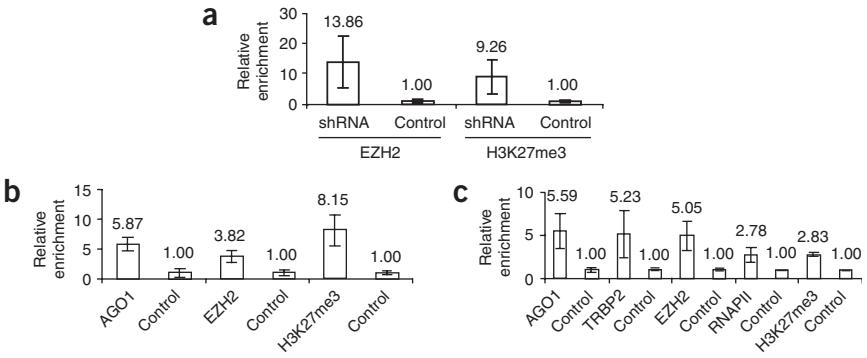

Figure 7 Polycomb connection to transcriptional gene silencing. (**a**) ChIP of the *RASSF1A* promoter using anti-EZH2 or anti-H3K27me3 from HeLa stable cells expressing shRNA or control vector. Error bars show s.e.m. for three independent experiments. (**b**) ChIP of the Polycomb target *MYT1* promoter using anti-AGO1, anti-EZH2, anti-H3K27me3 or no antibody (controls) in HeLa cells. Error bars show s.e.m. for three independent experiments. (**c**) ChIP of the Polycomb target CCR5 promoter using indicated antibodies or no antibody (controls) in HeLa cells. Error bars show s.e.m. for three independent experiments.

interaction between the siRNA antisense strand and the nascent promoter RNA transcript using a double-stranded RNA–binding domain[23]. RNAi components have been linked to Polycomb silencing in flies[24], and the association of AGO1 and TRBP2 with endogenous Polycomb target promoters in human cells indicates a potential role for small noncoding RNAs and the RNAi machinery in directing mammalian Polycomb silencing pathways.

METHODS

Cell culture. HEK293T cells (4.0×10^6) were transfected with 5 μg of pR5-GFPsg143 vector (~3 kilobases of *CCR5* promoter and exons 1 and 2, driving expression of red-shifted GFP) using Lipofectamine 2,000 (Invitrogen) and selected with neomycin (800 μg ml^{-1}) to generate a stable cell population (293T CCR5-GFP). HeLa stable cells expressing *RASSF1A* promoter–specific shRNA or control vector alone were generated as described[11].

Screening of siRNAs. 293T CCR5-GFP cells were plated (9.4×10^5 per well) in 12-well plates and transfected 24 h later with 10 nM *CCR5-GFP* promoter–specific siRNA or control siRNA specific for *CCR5* mRNA, using MPG at a 10:1 charge ratio (MPG/siRNA) as described[5]. *CCR5-GFP* promoter–specific siRNAs were constructed from oligonucleotides following established methodologies for T7-expressed siRNA synthesis (Ambion Silencer). Forty-eight hours after transfection, cultures were collected for FACS analysis of GFP expression. Sequences of *CCR5-GFP* promoter–specific siRNAs are as follows: control, 5′-AAUUCUUUGGCCUGAAUAAAA-3′; R25, 5′-GCCAAAGCUUUUUAUUCUAAA-3′; R61, 5′-GCCCAGAGGGCAUCUUGUGAA-3′; R149, 5′-CCGCCAAGAGAGCUUGAUAAA-3′; R854, 5′-GCCCGUAAAUAAACUUUCAAA-3′.

Chromatin immunoprecipitation. ChIP assays were performed as described[6]. 293T CCR5-GFP cells (4.0×10^6) were transfected with 30 nM R61 or control siRNA (IDT) using Lipofectamine 2,000 (Invitrogen). Cells were cross-linked with 1% (v/v) formaldehyde for 10 min. Antibodies used were anti-H3K9me2 (Upstate 07-441), anti-AGO1 (Upstate 07-599), anti-RNAPII (Abcam ab817), anti-H3K27me3 (Upstate 07-449), anti-EZH2 (Upstate 07-400), anti-AGO2 (Upstate 07-590) and anti-TRBP. Immunoprecipitated DNA was analyzed using real-time PCR with primers 5′R5-2 (5′-GGGGTCTCATTTGCCTTCTTAGAGATCACA-3′) and 3′R5-3 (5′-TAAGTATATGGTCAAGTTCAGGTTC-3′), spanning the R61 siRNA target site, or primers 5′R5W-1 (5′-GTCTTCTCAGCTCTGCTGACAATACT-3′) and 3′R5W-2 (5′-GGATTTTCACTCTGTTCACTATTTTGTTGC-3′), spanning a region 100–300 base pairs downstream of the R61 siRNA target site (3′ site), standardized to plasmid pR5-GFPsg143 and normalized to input values. Primers used for the *RASSF1A* promoter were 5′RAS-1 (5′-GAAGGAAGGGCAAGGCGGGGGGGGCTCTGC-3′) and 3′RAS-1 (5′-GGCCCGGTTGGGCCCGTGCTTCGCT-3′), and primers used for the *MYT1* promoter were as described[19].

Quantitative RT-PCR. The SuperScript III Platinum SYBR Green One-Step qRT-PCR kit (Invitrogen) was used to amplify *GFP*, *RASSF1A*, *TRBP2* and *GAPDH* mRNAs from total RNA isolated with RNA STAT-60 (Tel-Test), using primers 5′GFP-1 (5′-CACCCAGTCCGCCCTGAGCAAAGAC-3′) and 3′GFP-1 (5′-TTACTTGTACAGCTCGTCCATGCCG-3′) for *GFP*, primers 5′GAP-1 (5′-CGCTCTCTGCTCCTCCTGTT-3′) and 3′GAP-1 (5′-CCATGGTGTCTGAGCGATGT-3′) for *GAPDH* and primers for *RASSF1A* (ref. 11) and *TRBP2* (ref. 14) as previously described.

Western blotting and RNase A treatment. Total protein from *AGO1*$^+$ or *AGO1*$^-$ 293T CCR5-GFP extracts and control anti-FLAG (Sigma) or anti-RNAPII (Abcam) immunoprecipitates from 293T CCR5-GFP extracts were treated or untreated with 50 μg ml^{-1} RNase A (Sigma) for 30 min at 25 °C. The extracts were then heated for 5 min at 95 °C, separated by electrophoresis in 4%–12% SDS polyacrylamide, transferred to PVDF membranes, probed with anti-AGO1 (Upstate) or anti-GFP (Abcam) and developed with horseradish peroxidase–conjugated anti-rabbit IgG (Amersham Biosciences) and Luminol detection reagent (Fisher).

Promoter methylation analysis. Genomic DNA from 293T CCR5-GFP cells transfected with R61 or control siRNA, or from HeLa stable cells expressing *RASSF1A* shRNA or control vector, was digested for 1 h with AvaI or ApaI (NEB) at 37 °C and amplified using real-time PCR with ChIP primers for the *CCR5-GFP* and *RASSF1A* promoters. PCR amplification indicated that the AvaI or ApaI site(s) in the targeted promoter sequences are methylated and protected from restriction-enzyme digestion. All measurements were normalized to equivalent amounts of undigested genomic DNA samples incubated in NEBuffer no. 4 (NEB) alone.

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AUTHOR CONTRIBUTIONS

D.H.K., K.V.M. and J.J.R. designed the study, D.H.K. carried out most of the experiments, K.V.M. and L.M.V. generated 293T CCR5-GFP cells and performed siRNA screening and D.H.K, K.V.M. and J.J.R. interpreted data and wrote the manuscript.

COMPETING INTERESTS STATEMENT

The authors declare they have no competing financial interests.

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