nature chemical biology

ARTICLE

PUBLISHED ONLINE: 27 JUNE 2010 | DOI: 10.1038/NCHEMBIO.400

# Transcriptional regulation by small RNAs at sequences downstream from 3′ gene termini

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**Transcriptome studies reveal many noncoding transcripts overlapping 3′ gene termini. The function of these transcripts is unknown. Here we have characterized transcription at the progesterone receptor (PR) locus and identified noncoding transcripts that overlap the 3′ end of the gene. Small RNAs complementary to sequences beyond the 3′ terminus of PR mRNA modulated expression of PR, recruited argonaute 2 to a 3′ noncoding transcript, altered occupancy of RNA polymerase II, induced chromatin changes at the PR promoter and affected responses to physiological stimuli. We found that the promoter and 3′ terminal regions of the PR locus are in close proximity, providing a potential mechanism for RNA-mediated control of transcription over long genomic distances. These results extend the potential for small RNAs to regulate transcription to target sequences beyond the 3′ termini of mRNA.**



Modulation of gene expression in mammalian cells by small duplex RNAs is typically associated with recognition of mRNA[1]. Reports suggest that duplex RNAs complementary to gene promoters can silence or activate gene expression in mammalian cells[2–6]. Argonaute 2 (AGO2), a key protein involved in RNA interference (RNAi)[7], is required for the action of promoter-targeted RNAs[5,8], and a related protein, AGO1, has also been implicated in the mechanism[9]. The mechanism of promoter-targeted RNAs may involve recognition of noncoding transcripts that overlap gene promoters[10,11]. Over 70% of all genes have noncoding transcripts that overlap their promoters, and these transcripts provide potential target sites for small RNA duplexes[12–17].

Promoter-targeted RNAs are robust modulators of progesterone receptor (PR) transcription in T47D and MCF7 breast cancer cells[4,6,8,11]. We term these small RNAs antigene RNAs (agRNAs) to distinguish them from duplex RNAs that target mRNA. The main difference between activation or inhibition of gene expression by closely related agRNAs is the basal expression of PR. Gene silencing is observed in T47D cells that constitutively express PR at high basal levels, whereas activation of PR expression is observed in MCF7 cells that express PR at low levels[6].

Both activating and inhibitory agRNAs modulate PR expression through binding to complementary target sequences within an antisense transcript that originates from inside the PR gene and is transcribed through the promoter region. agRNAs recruit AGO protein to the antisense transcript, affect levels of RNA polymerase II (RNAP2) at the promoter and alter the mix of regulatory proteins that bind the antisense transcript and the PR promoter[11].

Noncoding RNAs also overlap the 3′ untranslated region (3′ UTR) of many genes[15–17]. The 3′ UTR plays a major role in cellular regulation and disease pathology[18] and is involved in a variety of post-transcriptional processes, including mRNA transport, localization and stability. The function of 3′ noncoding transcripts is unclear, but their proximity to the 3′ UTR suggests that they may affect gene regulation.

There has been little investigation into the potential function of overlapping noncoding transcripts at the 3′ region of genes, and no examination of whether these noncoding transcripts might be targets for modulating gene expression by duplex RNAs. The abundance of transcripts that overlap the 3′ UTR, coupled with the ability of agRNAs to modulate gene expression by targeting overlapping 5′ transcripts, suggested that small RNAs might also influence gene expression by recognizing sequences beyond the 3′ end of genes. Here we investigate the potential for small RNAs to recognize regions beyond the 3′ termini of mRNAs and regulate gene expression.

## RESULTS

### Characterization of the 3′ region of PR mRNA

Working with agRNAs requires accurate identification of mRNA termini. Initially, the PR GenBank sequence was inaccurately labeled, with the 5′ end extended too far upstream and the 3′ terminus prematurely truncated (**Fig. 1a**, top). Northern analysis suggested lengths for PR mRNA variants[19] but lacked a precise length for the largest variant (estimated to be 11.4 kilobase pairs (kb)) (**Fig. 1a**, middle). A GenBank update based on a cluster of expressed sequence tags later extended the 3′ UTR downstream to +13,037 (**Fig. 1a**, bottom).

We performed northern analysis (**Supplementary Methods** and **Supplementary Results**) with probes complementary to the protein-encoding region of PR mRNA (probe 1), the terminus of PR mRNA at +13,037 predicted by the recent GenBank update (probe 2) and a region immediately downstream from the predicted +13,037 terminus (probe 3) (**Supplementary Fig. 1a** and **Supplementary Table 1**). Probe 1 yielded major products at ~5.5 kb and >10 kb, similar to results observed previously[19] (**Supplementary Fig. 1b**). Probe 2 (complementary to the region at the predicted PR terminus) yielded only the >10 kb band (**Supplementary Fig. 1b,c**), consistent with the conclusion that the band is full-length PR mRNA. Probe 3 (complementary to the region immediately downstream of +13,037) did not detect transcript (**Supplementary Fig. 1c**). Quantitative PCR (qPCR) revealed that RNA levels were relatively constant before abruptly dropping after nucleotide +13,037 (**Supplementary Fig. 1d,e** and **Supplementary Table 2**). 5′ and 3′ rapid amplification of cDNA ends (RACE) detected polyadenylation sites near +13,037 (**Supplementary Fig. 2** and **Supplementary Table 3**).

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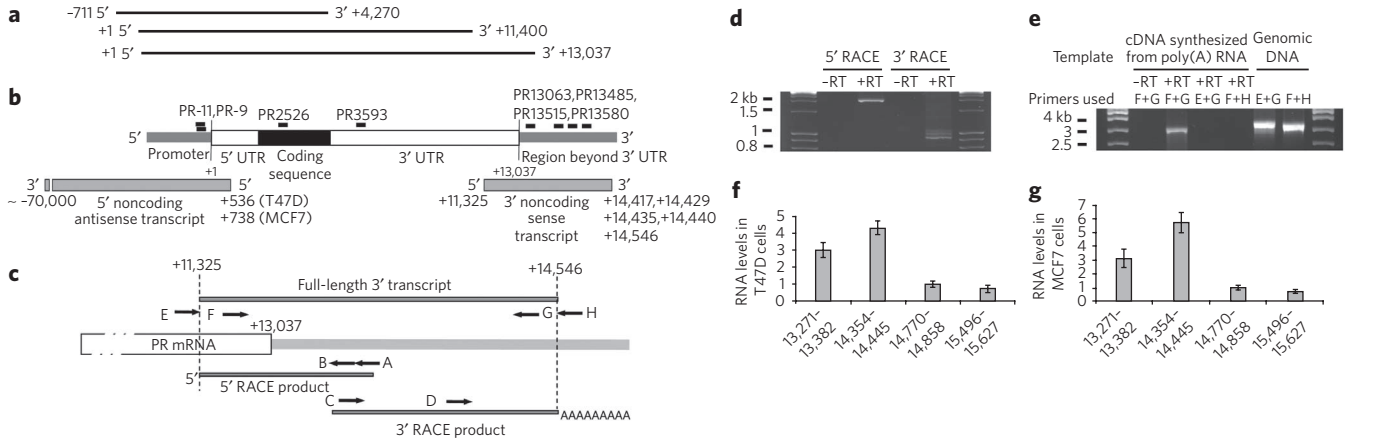


**Figure 1 | Characterization of PR mRNA and PR 3′ noncoding transcript.** (**a**) Differing annotations of PR mRNA. Top, pre-2008 GenBank (NM_000926.3); middle, predicted largest transcript based on northern analysis in published reports; bottom, current GenBank (NM_000926.4). (**b**) Location of target sequences for duplex RNAs relative to PR mRNA. The 5′ and 3′ noncoding transcripts that overlap the transcription start site and polyadenylation site are shown. (**c**) Locations of RACE (A, B, C and D) or RT-PCR (E, F, G and H) primers relative to PR mRNA and the 3′ noncoding transcript. (**d**) Agarose gel analysis of RACE products. Total RNA used in RACE was treated with DNase before reverse transcription. (**e**) Agarose gel analysis of RT-PCR amplification using primers E, F, G and H. Poly(A) RNA was treated with DNase before reverse transcription. Amplification of genomic DNA was included as a positive control for primer function. Complete data including sequencing of amplified products are shown in **Supplementary Figure 2**. (**f**,**g**) qPCR of relative poly(A) RNA levels in T47D (**f**) cells and MCF7 cells (**g**) using primer sets upstream or downstream of the predicted +14,546 terminus of the 3′ noncoding transcript (**Supplementary Fig. 1**). −RT: RNA samples that were not treated with reverse transcriptase (−RT) were used as negative control. +RT: reverse transcriptase added. Data are from triplicate independent experiments.



## Transcripts at the 3′ terminus of PR mRNA

We used 3′ and 5′ RACE to search for noncoding transcripts that overlap the 3′ terminus of PR and identified transcripts that are transcribed in the sense orientation (in other words, synthesized in the same direction) relative to PR mRNA (**Fig. 1b–d**, **Supplementary Figs. 1** and **2**). These 3′ transcripts shared a transcription start site at +11,325 and multiple poly(A) sites 1,400–1,500 bases downstream from the +13,037 terminus of PR mRNA. We did not detect antisense transcripts in this region.

As the 3′ transcript was transcribed in the same direction as PR mRNA, we tested whether it was a long variant of PR mRNA. We performed RT-PCR using a forward primer (primer E) complementary to PR mRNA that was directly upstream of +11,325 (and therefore had no complementarity to the +11,325-to-+14,546 transcript) and a reverse primer (primer G) upstream of +14,546 (**Fig. 1c**). We did not detect amplified product (**Fig. 1e** and **Supplementary Fig. 2c**,**d**). By contrast, RT-PCR using a forward primer (primer F) directly downstream of +11,325 and reverse primer directly upstream of +14,546 (primer G) detected the +11,325-to-+14,546 product (**Fig. 1e**). Sequencing confirmed the identity of the amplified primer F–primer G product as the +11,325-to-+14,546 transcript and revealed that the transcript was unspliced (**Supplementary Fig. 2e**). Beyond the +14,546 terminus of the noncoding transcript, qPCR revealed that RNA levels drop abruptly in both T47D and MCF7 cells (**Fig. 1f**,**g** and **Supplementary Fig. 1d**,**e**). RT-PCR with one primer (primer F) complementary to a sequence shared by PR mRNA and the 3′ noncoding transcript and another primer (primer H) downstream from +14,546 detected no product, suggesting that the mRNA does not extend past +13,037 (**Fig. 1e**).

RNAP2 transcribes genes beyond their poly(A) sites with subsequent cleavage of the transcript to form the mature mRNA. Our PCR did not detect evidence for longer PR mRNA (**Fig. 1e**), but we cannot with certainty exclude its existence or its involvement in the mechanism of 3′ agRNAs. qPCR revealed that the 3′ noncoding transcript was almost entirely in the nuclear fraction of cell lysates whereas PR mRNA was mostly in the cytoplasm (**Supplementary Fig. 3**).

We verified our data using the branched DNA (bDNA) assay (**Supplementary Fig. 4**). The bDNA assay directly detects RNA using strand-specific probes that allow discrimination of sense and antisense transcripts. A probe set complementary to the sense transcript beyond +13,037 detected RNA at ~4% relative to the level of PR mRNA in either T47D or MCF7 cells. Assays with a probe set designed to detect a possible antisense transcript downstream from +13,037 suggested that an antisense transcript either was not present or was present at levels too low to detect.

## Target sequences for duplex RNAs

We chose target sequences within the +11,325/+14,546 region of the 3′ noncoding transcript but downstream from +13,037 terminus of PR mRNA (**Fig. 1b** and **Supplementary Table 4**). We numbered duplex RNAs by the position of the most upstream base relative to the +1 transcription start site for PR mRNA. We also tested duplex RNAs that target the PR promoter or PR mRNA to allow comparisons with other modulatory RNAs. We previously characterized agRNAs that target the promoter region (5′ agRNAs) and modulate PR expression. PR-11 targets the region from −11 to +8 relative to the transcription start site and activates PR transcription in MCF7 and T47D cells[6]. PR-9 targets the region from −9 to +10 and is a robust transcriptional silencing agent in T47D cells[4]. PR3593 and PR2526 target PR mRNA. The nomenclature for mismatch-containing RNAs is based on the parent fully complementary RNA. For example, PR13515_MM3 has three mismatched bases relative to parent RNA PR13515.

## Gene inhibition by targeting beyond the 3′ UTR

We tested several 3′ agRNAs complementary to sequences downstream from the +13,037 terminus of PR mRNA (**Fig. 1b** and **Supplementary Table 4**). agRNAs PR13485 and PR13580 inhibited PR protein expression in T47D cells (**Fig. 2a** and **Supplementary Fig. 5**). PR has two major protein isoforms, PRB and PRA[19], and both isoforms appear as distinct bands during western analysis. The half-maximal inhibitory concentration value ($IC_{50}$) for inhibition of PR13580 was 10.7 nM, similar to the value for inhibition by mRNA-targeting RNA PR2526,

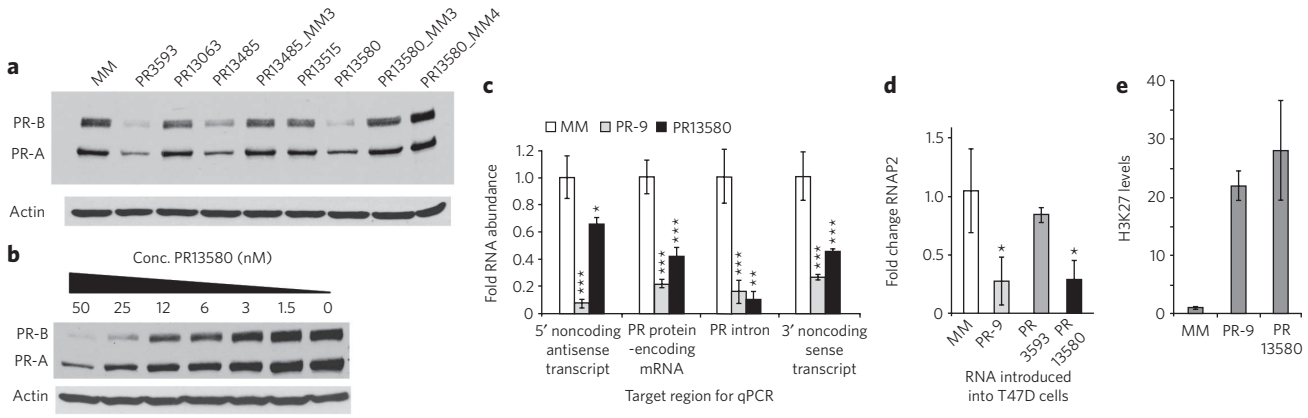


**Figure 2 | Inhibition of PR expression in T47D cells by agRNAs complementary to sequences downstream from the terminus of PR mRNA.** (**a**) Western analysis showing inhibition of protein expression by duplex RNAs (50 nM). (**b**) Dose response for PR13580. (**c**) qPCR showing reduction of PR mRNA levels by duplex RNAs (25 nM). Four different primer sets were used, each complementary to different regions of the PR gene. Values are relative to mismatch-treated cells. (**d**) Presence of RNAP2 at the PR transcription start site (25 nM duplex RNA) evaluated by ChIP relative to mismatch-treated cells. (**e**) ChIP for the H3K27 trimethylation (H3K27me3) marker within the PR gene locus in T47D cells. Values are relative to mismatch-treated cells. ***$P < 0.005$, **$P < 0.01$ and *$P < 0.05$ as compared to cells treated with a mismatch RNA. *P* values were calculated using the two-tailed unpaired Student's *t*-test with equal variances. All error bars represent s.d. Data in parts **c** and **d** are the result of triplicate independent experiments. Data in parts **a** and **b** are representative of duplicate experiments.



11.5 nM (**Fig. 2b** and **Supplementary Fig. 6**). Mismatch-containing duplex RNAs did not inhibit gene expression. Some of the control RNAs maintained the potential for seed sequence[20] recognition of the duplex (PR13485_MM3, PR13580_MM3), demonstrating that seed sequence complementarity is not sufficient to induce the observed silencing (**Fig. 2a**).

Small RNAs can induce off-target effects through induction of the interferon response[21]. Inhibitory 3′ agRNA PR13580 did not significantly enhance expression of interferon-responsive genes (**Supplementary Fig. 7a**) and was chosen for detailed investigation. Addition of poly(I:C), a potent inducer of the interferon response, did not alter PR gene expression (**Supplementary Fig. 7b**).

We used qPCR to investigate how addition of inhibitory agRNA PR13580 affected RNA levels throughout the PR locus (**Fig. 2c** and **Supplementary Table 5**). Our strategy was to use different primer sets, each designed to amplify a different RNA species including (i) the 5′ antisense transcript at the PR promoter previously implicated in agRNA-mediated control of transcription[11], (ii) the protein encoding region for PR mRNA, (iii) intron 7 within PR pre-mRNA, an indicator of whether gene modulation occurs before or after splicing and (iv) the +11,325/+14,546 noncoding transcript overlapping the terminus of PR mRNA (**Fig. 1b**).

Addition of agRNA PR13580 to T47D cells reduced levels of PR mRNA (**Fig. 2c**). We also observed reduced levels of PR pre-mRNA, suggesting that modulation of RNA occurs before splicing. qPCR revealed reduced levels of the noncoding transcript downstream from the terminus of the 3′ UTR and 5′ noncoding transcript overlapping the transcription start site of PR. Negative control duplex RNA PR13063 did not reduce levels of PR mRNA (**Supplementary Fig. 8**). The bDNA assay yielded similar results (**Supplementary Fig. 9**).

To test whether agRNAs targeted to sequences beyond the 3′ UTR altered gene transcription, we measured RNAP2 occupancy at the PR promoter (**Supplementary Table 6**). Chromatin immunoprecipitation (ChIP) revealed that duplex agRNA PR13580 reduced occupancy of RNAP2 at the promoter (**Fig. 2d**). We also measured levels of the histone modification H3K27me3 (trimethylation of histone H3 at position Lys27), which serves as a chromatin-level marker for gene silencing[22]. Addition of PR13580 to T47D cells led to a 27-fold increase in H3K27me3 levels within the PR gene, relative to cells treated with mismatched control duplex (**Fig. 2e**). Addition of promoter-targeted agRNA PR-9 also caused changes in RNAP2 occupancy and levels of H3K27me3. RNA PR3593, which targets PR mRNA, did not decrease RNAP2 occupancy (**Fig. 2d**), emphasizing a basic difference in mechanism between agRNAs (inhibition of transcription) and duplex RNAs that function through post transcriptional gene silencing (PTGS) (inhibition of translation).

## Gene activation by targeting beyond the 3′ UTR

We hypothesized that agRNAs complementary to sequences beyond the 3′ terminus of PR might also yield enhanced gene expression in MCF7 cells, a cell line in which PR expression is low and increased expression is easily detectable. agRNA PR13515 increased levels of PR protein and mRNA (**Fig. 3a–c**). Levels of PR pre-mRNA increased (**Fig. 3c**), consistent with the suggestion that RNA levels increase before splicing. For comparison, we also measured RNA levels after addition of agRNA PR-11 that was complementary to the transcript of the PR promoter and 5′ noncoding RNA[11] and observed similar effects with all primer sets (**Fig. 3c**). The bDNA assay also revealed increased levels of PR mRNA and the 3′ noncoding transcript upon addition of PR13515 or PR-11 (**Supplementary Fig. 9c**).

Addition of either PR-11 or PR13515 enhanced RNAP2 recruitment at the PR transcription start site (**Fig. 3d**) and caused a decrease in the silencing marker H3K27me3 (**Fig. 3e**). The similarity of gene activation between PR-11 and PR13515 reinforced the idea that modulation of gene transcription by agRNAs proceeds through similar mechanisms regardless of whether the target regions are located beyond the 5′ or 3′ boundaries of mRNA.

We tested whether increased stability of PR mRNA contributed to its increased cellular levels after treatment with PR13515. Actinomycin D, a small molecule that inhibits transcription[23], was added to cells that had been treated previously with PR13515 or PR-11. The half-life of PR in MCF7 cells is 7–10 h[24]. In MCF7 cells treated with either PR13515 or PR-11, addition of actinomycin D reversed the agRNA-mediated increase in PR mRNA. PR mRNA levels decreased by 8–10-fold within 30 h. (**Fig. 3f**). These data suggested that increased levels of PR mRNA were due to enhanced transcription.

We tested several control RNAs to evaluate specificity. Control RNA PR13515_MM4 contained mismatched bases substituted throughout the sequence of PR13515. Control RNAs

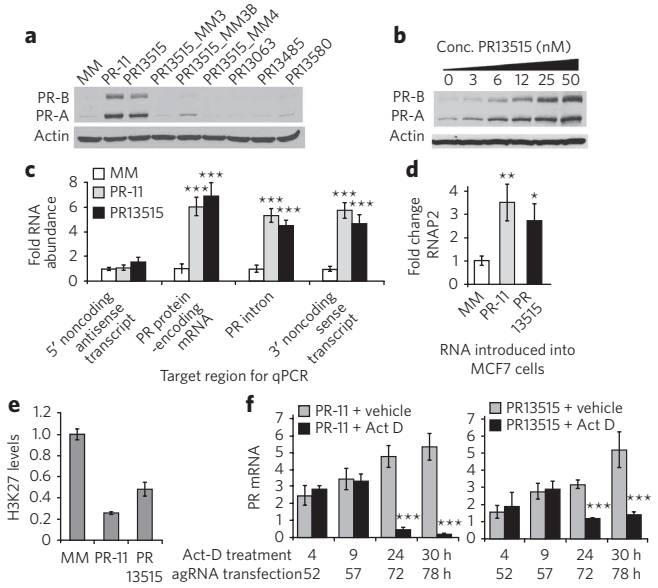


**Figure 3 | Enhanced PR expression in MCF7 cells by an RNA complementary to a sequence downstream from the terminus of the PR 3′ UTR.** (**a**) Western analysis showing activation of protein expression by duplex RNAs. (**b**) Dose response for RNA PR13515. (**c**) qPCR showing effect on RNA levels relative to cells treated with mismatched RNA (MM). Four different primer sets were used, each complementary to different regions near the PR gene. (**d**) Recruitment of RNAP2 to the PR promoter upon addition of PR13515 or PR-11 evaluated by ChIP relative to cells treated with RNA MM. (**e**) ChIP for the H3K27 trimethylation (H3K27me3) marker within the PR gene locus relative to RNA MM. (**f**) Cells were transfected with either PR-11 or PR13515. After 2 d, actinomycin D (act D, 1 μg ml$^{-1}$) or vehicle was added to the medium. Cells were harvested at the indicated time points (hours after addition of actinomycin or vehicle or transfection with agRNA). Data were normalized to levels of 18S rRNA that did not significantly change. ***$P < 0.005$, **$P < 0.01$ and *$P < 0.05$ as compared to cells treated with RNA MM. $P$ values were calculated using the two-tailed unpaired Student's $t$-test with equal variances. Error bars represent s.d. Duplex RNAs were added to cells at 25 nM unless otherwise noted. Data in **a**–**d** are the results of triplicate experiments; data in **f** are the result of four to six independent experiments.

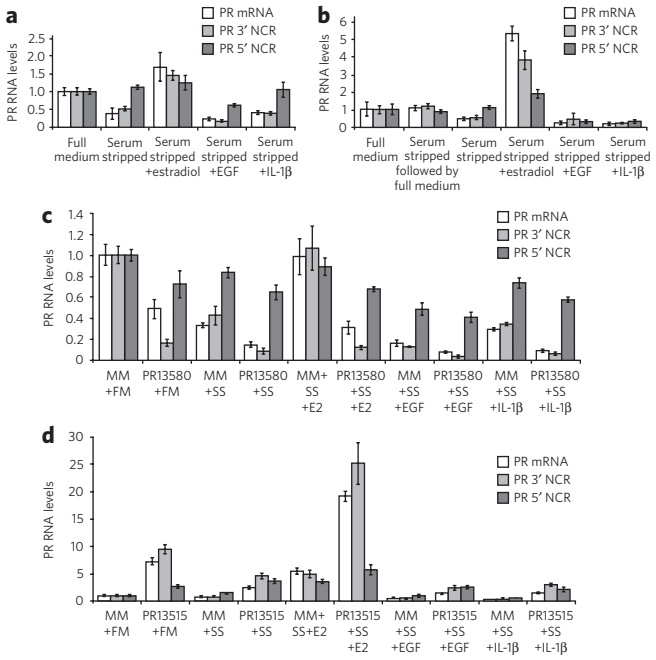


**Figure 4 | Effect of combining physiological stimuli with agRNAs.** (**a**,**b**) qPCR analysis showing the effect of physiological stimuli on transcript expression in T47D cells (**a**) and MCF7 cells (**b**). Values are relative to cells grown in full medium. For the sample labeled "serum stripped followed by full medium," cells were grown in serum-stripped medium. (**c**,**d**) The medium was replaced by full medium for 1 d before harvesting. qPCR analysis of the effect of physiological stimuli and agRNA (25 nM) addition on transcript expression in T47D cells (**c**) and MCF7 cells (**d**). PR 3′ NCR, 3′ noncoding PR RNA; PR 5′ NCR, 5′ noncoding PR RNA; SS, serum-stripped medium; FM, full medium; E2, 17β estradiol treatment (100 nM); IL-1 β, interleukin 1β treatment (10 ng ml$^{-1}$); EGF, epidermal growth factor treatment (100 ng ml$^{-1}$). Results are from three to six independent replicates.

PR13515_MM3 and PR13515_MM3B had mismatched bases clustered to preserve the potential for off-target effects that might arise from seed sequence complementarity[21]. These control RNAs did not enhance protein expression (**Fig. 3a**). PR13515 did not increase expression of interferon-responsive genes, and activation of the interferon response did not increase PR expression (**Supplementary Fig. 7c**,**d**). Off-target effects are a concern whenever duplex RNAs are introduced into cells. Our 3′ and 5′ agRNAs have partial complementarity to other genes, but these matches do not suggest any source for off-target regulation of PR.

## Combining physiological stimuli and agRNAs

17β-Estradiol is a potent and well-characterized activator of PR gene expression in breast cancer cells[24]. Conversely, expression of PR decreases in cells grown in medium containing charcoal-stripped serum and supplemented with either interleukin-1β (IL-1β) or epidermal growth factor (EGF)[25–27].

We investigated how these physiological stimuli would affect expression of the 3′ and 5′ noncoding transcripts (**Fig. 4a**,**b** and **Supplementary Table 7**). Growth of T47D or MCF7 cells in charcoal-stripped serum reduced PR mRNA levels. Addition of EGF or IL-1β further reduced levels of PR mRNA, whereas addition of 17β-estradiol increased PR mRNA expression. In all cases, levels of the 3′ noncoding transcript varied proportionally with PR mRNA (**Fig. 4a**,**b**). By contrast, expression of the 5′ noncoding transcript did not change significantly in T47D or MCF7 cells grown in charcoal-stripped serum or in medium supplemented with 17β-estradiol. Addition of EGF or IL-1β decreased expression of the 5′ transcript in MCF7 cells.

We then tested whether the addition of agRNAs would affect regulation of PR expression by physiologically relevant stimuli and, conversely, whether physiological stimuli could block the action of agRNAs (**Fig. 4c**,**d** and **Supplementary Fig. 10**). Addition of inhibitory 3′ agRNA PR13580 to T47D cells cultured in charcoal-stripped serum supplemented with IL-1β or EGF led to a proportionate reduction in expression of PR mRNA and the 3′ noncoding transcript to levels lower than those achieved by the physiological treatments alone. Treating cells with 17β-estradiol increases PR mRNA expression (**Fig. 4a**,**c**). Inhibitory agRNA PR13580 reversed this effect, leading to low expression of PR (**Fig. 4c**) and almost-unchanged expression of the 5′ noncoding transcript regardless of physiological treatment.

In MCF7 cells, addition of activating agRNA PR13515 in combination with 17β-estradiol yielded enhanced activation of PR gene expression to levels substantially above those achieved by addition of 17β-estradiol or PR13515 alone (**Fig. 4d**). PR13515 reversed the repressive effects of growth in charcoal-stripped serum, EGF addition and IL-1β addition on PR expression (**Fig. 4d**). These data suggested

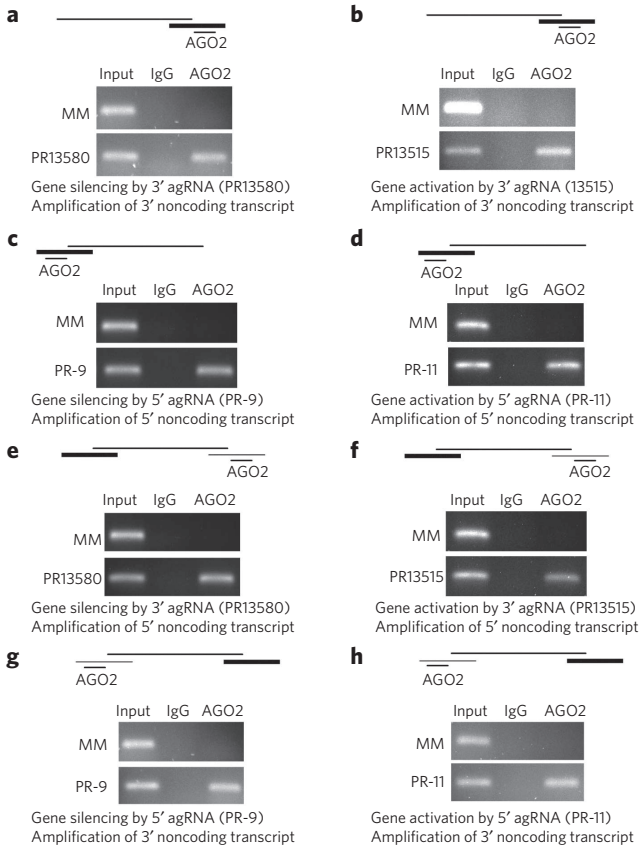


**Figure 5 | Effect of 3′ or 5′ agRNAs on recruitment of AGO2 protein to the 3′ or 5′ noncoding transcripts at the PR locus.** (**a**,**b**) RNA immunoprecipitation (RIP) of 3′ noncoding RNA using an anti-AGO2 antibody after treatment with inhibitory RNA PR13580 in T47D cells (**a**) or activating RNA PR13515 in MCF7 cells (**b**) on recruitment of AGO2 protein to the 3′ noncoding transcript. (**c**,**d**) Effect of adding inhibitory RNA PR-9 to T47D cells (**c**) or activating RNA PR-11 to MCF7 cells (**d**) on recruitment of AGO2 protein to the 5′ noncoding transcript. (**e**,**f**) Effect of adding inhibitory RNA PR13580 to T47D cells (**e**) or activating RNA PR13515 to MCF7 cells (**f**) on co-immunoprecipitation of AGO2 protein with the 5′ noncoding transcript. (**g**,**h**) Effect of adding inhibitory RNA PR-9 to T47D cells (**g**) or activating RNA PR-11 to MCF7 cells (**h**) on co-immunoprecipitation of AGO2 protein with the 3′ noncoding transcript. The scheme above each gel depicts PR mRNA, the 3′ and/or 5′ noncoding transcripts and AGO2-bound agRNA. The heaviest line represents the transcript being amplified. Duplex RNAs were added to cells at 25 nM. Experiments are representative of two independent determinations.

that activating and inhibitory agRNAs can counteract or supplement the effects of physiological regulators on PR gene expression.

## Recruitment of argonaute to noncoding RNAs

Duplex agRNAs PR-9 and PR-11 are complementary to sequences within the PR promoter and recruit AGO protein to a noncoding transcript that overlaps the PR gene promoter[11]. The sense noncoding transcript overlapping the 3′ terminus of the PR gene (**Fig. 1b**) contains complementary target sites for inhibitory or activating agRNAs PR13580 and PR13515 and was a candidate for involvement in RNA-mediated gene modulation.

We used RNA immunoprecipitation (RIP)[28] from isolated nuclei with an anti-AGO2 antibody (**Fig. 5**, **Supplementary Table 8** and **Supplementary Fig. 11**) to examine recruitment of AGO2 protein to the 3′ noncoding transcript during modulation of gene expression by agRNAs. When we added silencing agRNA PR13580 to T47D cells (**Fig. 5a**) or activating agRNA PR13515 to MCF7 cells (**Fig. 5b**), we observed association between AGO2 and the 3′ noncoding transcript. 5′ agRNAs targeting a noncoding RNA at the PR promoter showed similar recruitment of AGO2 to the 5′ noncoding transcript (**Fig. 5c**,**d**). Sequencing confirmed the identity of the RIP products (**Supplementary Fig. 11**). RIP with an anti-AGO1 antibody did not detect association of AGO1 (**Supplementary Fig. 12**). Inhibition of AGO2 expression with an anti-AGO2 siRNA reversed gene silencing by PR13580. Inhibition of AGO1, AGO3 or AGO4 expression did not reverse gene silencing (**Supplementary Fig. 13**). These data are consistent with a primary role for AGO2.

We observed the same results when using a well-characterized antibody that recognizes all four AGO proteins[29] (**Supplementary Fig. 14a–d**). Identical RIP results using two different anti-AGO antibodies support the specificity of AGO involvement in the mechanism of 3′ agRNAs. No product was observed in the absence of reverse transcriptase (**Supplementary Fig. 15**) or after transfection of cells with mismatch-containing duplex RNAs. Cytoplasmic proteins GAPDH and tubulin were not detected in the nuclei, indicating that we are not detecting interactions in the cytoplasm.

## Cleavage of the 3′ noncoding transcript is not detected

Small RNAs that are complementary to mRNA can induce cleavage of their target transcripts[1]. An important question of mechanism is whether 3′ agRNAs act by promoting cleavage of the 3′ noncoding transcript. We readily detected cleavage of PR mRNA by PR2562, a duplex RNA that is complementary to the coding region of PR mRNA (**Supplementary Fig. 16**).

We did not detect cleavage of the 3′ noncoding RNA using silencing agRNA PR13580 in T47D cells or activating agRNA PR13515 in MCF7 cells (**Supplementary Fig. 16**). The 3′ noncoding transcript was expressed at relatively low levels, and it is possible that cleavage might occur without being detectable by 5′ RACE. Failure to detect any cleavage, however, was consistent with our observation that addition of PR13515 enhances levels of the 3′ noncoding transcript (**Fig. 3c**) rather than reducing those levels as would be predicted if transcript cleavage were occurring. It was also consistent with previous results showing that 5′ agRNA PR-11 does not alter levels of the 5′ noncoding transcript and does not cause detectable cleavage of the transcript[6] and with RIP data showing association of AGO2, because the transcript must be intact if it is to be detected during RIP.

## Overexpression of 3′ RNA does not affect PR mRNA levels

It is possible that the 3′ noncoding transcript recruits AGO2 protein before its release from chromosomal DNA. Alternatively, the 3′ noncoding transcript might be released from the chromosome and subsequently return to act at the PR locus. This latter mechanism would be similar to the mechanisms of protein transcription factors that are synthesized in the cytoplasm and return to act on their target promoters in the nucleus.

To examine the effect of altered cellular levels of the 3′ noncoding transcript and PR mRNA, we cloned and overexpressed the 3′ noncoding transcript in three breast cancer cell lines with varying basal PR expression levels (T47D (high), MCF7 (low) and MDA-MB231 (undetectable)). Overexpression of the 3′ noncoding transcript by as much as 100-fold above endogenous levels did not yield a significant change in PR mRNA levels in any of these three cell lines (**Supplementary Fig. 17**). These results suggest that the cellular concentration of 3′ noncoding RNA does not affect PR expression.

## Gene looping brings 5′ and 3′ sequences into proximity

Modulation of gene transcription by small RNAs complementary to sequences beyond the PR 3′ UTR suggests that recognition of downstream sequences influences activity at gene promoters. This influence

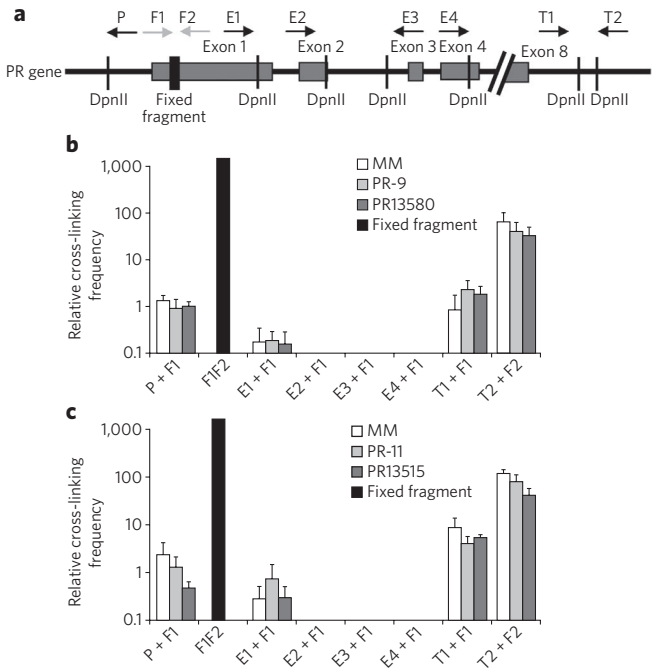


**Figure 6 | 3C analysis of the PR locus.** (**a**) Schematic of the PR gene showing DpnII cleavage sites, exon boundaries and locations of primers used for 3C analysis. The primer pairs used for 3C amplification are shown on the *x* axes of **b** and **c**. (**b**) qPCR showing the relative levels of detection of cross-linked product after treatment with a mismatch-containing RNA duplex or inhibitory duplexes PR-9 or PR13580. (**c**) qPCR showing the relative levels of cross-linked product after treatment with a mismatch-containing RNA duplex or activating duplexes PR-11 or PR13515. Primers E1, E2, E3 and E4 amplify sequences within PR exons 1–4. Primers T1 and T2 amplify sequences beyond the terminus of PR mRNA. Primer set F1F2 yields a fixed fragment, a normalization control derived from genomic DNA by primers complementary to sequences within exon 1 (ref. 35). The bar represents performance of the normalization control, not its absolute value. Values in **b** and **c** are relative to amplification of sequence at the PR promoter using primer P. Duplex RNAs were added to cells at 25 nM. The positive control shows amplification of a synthetic DNA. Data are from three independent experiments.

must be exerted over a long distance because the genomic locations of the PR promoter and the agRNA target sites are ~100 kb apart. Previous reports suggest that gene promoters and termini can be held in close proximity to one another[30–32]. Such proximity might facilitate the modulation of gene expression between otherwise distant 3′ and 5′ regions.

We investigated whether the promoter and terminal regions of PR might also be in proximity using chromosome conformation capture (3C) analysis[31,32], a technique that examines the proximity of sequences within chromosomal DNA. In this technique, chromosomal DNA is cross-linked, digested with restriction enzyme and treated with DNA ligase to join DNA ends that are in close proximity. After reversal of cross-links, the DNA is amplified and sequenced to evaluate the proximity of target regions. We examined amplification of 3C products using multiple primer sets that vary in distance from the 5′ promoter and 3′ terminal regions of PR (**Fig. 6a** and **Supplementary Table 9**). For example, amplification by primer T2 (complementary to a sequence beyond the PR 3′ UTR termini) and primer F2 (complementary to sequences within PR exon 1) would only be predicted to occur if the 5′ and 3′ ends of the PR locus are held in proximity by gene looping.

We observed ligation of the 5′ promoter region of the PR gene to the 3′ terminal regions in T47D (**Fig. 6b**) and MCF7 (**Fig. 6c**) cells. Even though expression of PR was much higher in T47D than MCF7 cells, we did not detect a difference in the relative amount of looping (**Supplementary Figs. 18** and **19**). Addition of inhibitory agRNAs PR13580 or PR-9 to T47D cells or activating agRNAs PR13515 or PR-11 to MCF7 cells did not affect the relative amount of looping at the PR locus (**Fig. 6b**,**c**, **Supplementary Figs. 20** and **21**).

We also examined the effects of physiological stimuli on looping. We have previously described the effects on PR expression of adding estrogen, growing cells in serum-stripped medium, treating cells with epidermal growth factor (EGF) or adding IL-1β (**Fig. 4**). These treatments did not change the relative amounts of gene looping (**Supplementary Fig. 22**). Our data suggest that gene looping at the PR locus remains constant under a range of different cell types, environmental stimuli and agRNA treatments.

It is most likely that the looping is intrachromosomal between the termini of a single PR gene. We are unaware of evidence for interchromosomal contacts between different alleles of the same gene on different chromosomes, but we note that such contacts may be possible.

## Proximity of 5′ and 3′ noncoding transcripts

We investigated the possibility that gene looping might allow the 5′ and 3′ noncoding transcripts to form long-distance associations. We used RIP with anti-AGO2 antibodies to examine the proximity of the noncoding transcript at the 3′ UTR to the noncoding transcript at the PR promoter. Addition of inhibitory 3′ agRNA PR13580 (**Fig. 5e**) or activating 3′ agRNA PR13515 (**Fig. 5f**) followed by RIP led to detection of the 5′ noncoding transcript. Similarly, addition of duplex agRNAs complementary to the PR promoter, inhibitory agRNA PR-9 (**Fig. 5g**) and activating agRNA PR-11 (**Fig. 5h**) led to recovery of the 3′ noncoding transcript.

RIP uses a chemical cross-linking step that allows detection of factors within a complex. Therefore, it is not necessary for association between the 3′ and the 5′ noncoding transcripts to be direct. A more likely explanation is that the association between these noncoding RNAs is indirect. There may be a ribonucleoprotein complex containing AGO and the 5′ noncoding and 3′ noncoding transcripts. We observed the same results when using a second anti-AGO antibody[29] (**Supplementary Fig. 14e–h**).

## Inhibition of *BRCA1* expression by 3′ agRNAs

To test whether gene modulation by 3′ agRNAs might apply to other genes, we targeted sequences beyond the 3′ UTR of the tumor suppressor breast cancer–associated gene 1 (*BRCA1*). We chose *BRCA1* because its 3′ termini had been well characterized[33], its expression is lowered in a significant percentage of human cancers[34], it is expressed in T47D breast cancer cells and the chromosomal loci juxtapose its promoter and termination regions[31]. 3′ RACE and qPCR confirmed the previously reported[35] termination site for *BRCA1* mRNA (**Supplementary Fig. 23** and **Supplementary Tables 10–12**). 3C analysis confirmed that the *BRCA1* promoter and 3′ terminal regions are in proximity (**Supplementary Fig. 24**).

3′ agRNAs inhibited expression of BRCA1 protein, mRNA and pre-mRNA (**Supplementary Fig. 25** and **Supplementary Table 4**). BRCA7851 reduced levels of RNAP2 at the *BRCA1* promoter. An siBRCA[35] complementary to *BRCA1* mRNA did not decrease recruitment of RNAP2 to the *BRCA1* promoter. The 3′ agRNAs did not activate interferon responsive genes (**Supplementary Fig. 6e**,**f**). These data suggested that 3′ agRNAs can also regulate expression of *BRCA1*.

## DISCUSSION

Our data show that duplex RNAs can modulate transcription by targeting sequences beyond the 3′ UTR. These results (i) expand the pool of RNA transcripts that can be targeted by small RNAs; (ii) suggest that interactions encoded beyond the 3′ UTR can be important; (iii) show that RNA-mediated recognition can control

transcription over a 100,000 base distance; (iv) are consistent with gene looping as an explanation for the long-distance control of transcription by RNA; (v) demonstrate that agRNAs can counteract or supplement the effects of physiological stimuli; and (vi) uncover additional layers of regulation and gene structure at the PR locus. agRNAs that target the 5′ promoter region have similar effects on gene expression and involve the essential RNA-binding protein AGO, suggesting that the mechanisms of 3′ agRNAs and 5′ agRNAs are related.

Overexpression of the 3′ noncoding RNA did not affect expression of PR (**Supplementary Fig. 17**), consistent with the suggestion that the 3′ noncoding RNA species becomes involved in the complex before dissociation from chromosomal DNA. Proximity of the newly synthesized RNA to the proteins controlling gene transcription would simplify the challenge of initiating gene-specific inhibition of transcription by increasing the effective concentration of the RNA relative to genomic DNA. Once noncoding RNA leaves the target chromosomal locus, it would face greater obstacles returning and forming sequence-selective interactions upon addition of complementary agRNA.

Our RNA immunoprecipitation data suggested that noncoding RNAs are the direct molecular targets of agRNAs, rather than sequences within chromosomal DNA. AGO2 appeared to play a critical role in the mechanism by promoting binding of the agRNA to the noncoding transcript. After being recruited to the noncoding transcript, AGO2 may form interactions with other proteins or disrupt existing interactions. We have previously shown that agRNAs modulate recruitment or displacement of other proteins such as HP1γ and hnRNPk and also alter histone modifications[11]. A scheme showing a potential orientation of noncoding transcripts, PR genomic DNA, AGO2 and 3′ agRNA is presented in **Figure 7**. Although AGO2 is the best candidate for involvement and we did not observe cleavage of the target transcript, additional experiments will be needed to fully investigate involvement of AGO1, AGO3 or AGO4, as well as whether or not the target transcript is cleaved.

How can transcription be affected by an RNA-mediated binding event across 100,000 bases (**Fig. 7a**)? Three lines of evidence support the conclusion that the PR gene 'loops' (juxtaposes its 3′ and 5′ termini) (**Fig. 7b**): (i) striking similarity in the properties of 3′ and 5′ agRNAs, (ii) 3C analysis demonstrating proximity of 5′ promoter and 3′ terminus regions and (iii) RNA immunoprecipitation showing association of the 3′ and 5′ noncoding transcripts. Gene looping has been observed at the X-inactivation center[36]. The X-inactivation center controls transcriptional inactivation of the X chromosome in females and is regulated by noncoding RNAs *Xist*, *Tsix* and *Xite*. 3C analysis indicates that the loci for these noncoding RNAs are positioned close enough for interactions during the inactivation process, and the molecular mechanism may be related to the mechanism of agRNA-mediated gene regulation that we observe.

Although the molecular basis for the action of nuclear hormone receptors and other protein regulators is still not completely understood after many years of study, insights into how proteins control transcription provide a framework for broadly understanding the mechanism of agRNAs. Like our agRNAs, nuclear hormone receptors recognize specific sequences. For nuclear hormone receptors, ligand binding controls recognition of a coactivator or corepressor protein, whereas agRNAs recruit AGO2. Endogenous RNA coactivators and transcriptional activators have already been identified[37–39], and it is possible that agRNAs and the agRNA–AGO2 complex may make similar interactions at gene promoters. More generally, many transcription factors have domains that contain RNA-binding motifs[39,40]. The HIV-1 Tat protein is an especially compelling example because it binds a viral transcript and interacts with other proteins to control transcription[41,42].

Both 3′ and 5′ agRNAs regulate the levels of PR transcripts. Why do we observe activation under some conditions and inhibition under others? Expression of PR is poised to respond to *in vivo* signals

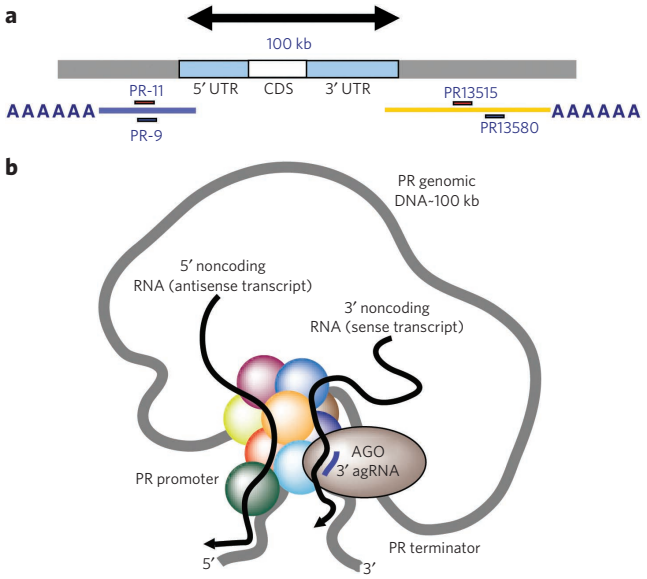


**Figure 7 | Model for modulation of transcription by 3′ agRNAs.** (**a**) 100,000 bases separate the genomic locations of the promoter and 3′ terminal regions of the PR gene. (**b**) Gene looping juxtaposes the 5′ promoter and 3′ terminator, bringing DNA sequences into close proximity. Addition of the 3′ agRNA recruits AGO2 to the 3′ noncoding transcript. The arrival of AGO2 may affect other proteins (here shown as unlabeled spheres) at the gene promoter and alter regulation of transcription. The proximity of 3′ and 5′ noncoding transcripts allows them to co-immunoprecipitate during RIP with anti-AGO antibodies. CDS, coding sequence.

such as hormones, cytokines and growth factors[24,25]. The primary difference between agRNA-mediated activation and repression is that activation is more easily observed in cells that express low basal levels of PR (cells poised to increase expression) whereas inhibition is observed in cells that express relatively high amounts of PR (cells poised to decrease expression). We note that relatively greater PR activation in cells with low basal expression is not restricted to agRNAs. 17β-estradiol also activates PR expression much more robustly in low-PR expressing MCF7 cells than in higher-PR expressing T47D cells.

There are many examples of proteins acting as either activators or repressors depending on context[43–45], on binding to accessory proteins[46,47] or on antagonist and agonist binding[48]. Noncoding RNAs can also either activate or repress transcription through interactions with proteins at promoters, possibly through regulating transcriptional coactivator and corepressor complexes[49].

The transcriptional control machinery can adapt to different stimuli to produce different effects, and this versatility probably underlies the observed mechanism of agRNA action. Conversely, the ability of 3′ agRNAs to modulate gene expression over seemingly large genomic distances emphasizes the remarkable diversity of macromolecules and mechanisms affecting transcription and the power of RNA as a genetic regulator.

## METHODS

**Cell culture.** T47D, MCF7 and MDA-MB-231 cells were cultured at 5% (v/v) $CO_2$ in RPMI medium (Sigma) supplemented with 10% (v/v) fetal bovine serum (FBS) (Atlanta Biologicals), 10 mM HEPES, 0.5% (v/v) non-essential amino acids, 10 μg $ml^{-1}$ insulin and 1 mM sodium pyruvate (Sigma). For some experiments, T47D or MCF7 cells were cultured in medium containing 5% (v/v) dextran and charcoal-stripped FBS (Atlanta Biologicals) 3 d before treatment with either 17β-estradiol (E2) (Steraloids), epidermal growth factor (EGF) (Sigma) or interleukin-1β (Sigma).

**Cellular delivery of duplex RNAs and expression assays.** We used RNAi-max (Invitrogen) to deliver duplex RNAs into cells. Primary antibodies (Ab) included

PR-Ab (6A1, Cell Signaling) and BRCA1-Ab (MS110, Calbiochem). β-actin-Ab (Sigma) was used as an internal control and for quantification. Protein was visualized using horseradish peroxidase (HRP)-tagged secondary anti-mouse antibody (Jackson Immunolabs) and Super Signal developing solution (Pierce).

**Chromatin immunoprecipitation (ChIP) and RNA immunoprecipitation (RIP).** ChIP was performed using monoclonal anti-RNAP2 antibody (Millipore 05-623) or mouse IgG negative control antibody (Millipore 12-371). RIP was performed using the general anti-AGO antibody provided by Z. Mourelatos (Univ. Pennsylvania), anti-AGO1 antibody (Millipore, 07-599) or anti-AGO2 antibody (Millipore 07-590). Samples were treated with DNase I, reverse transcribed and amplified using primers complementary to 5′ or 3′ noncoding transcripts (**Supplementary Table 6**).

**Rapid amplification of cDNA ends (RACE).** 5′ RACE and 3′ RACE were performed according to the manufacturer's protocol using the GeneRacer kit (Invitrogen). This kit includes enzymatic treatments that select for full-length RNA with intact 5′ caps rather than truncated products. For 5′ RACE, RNA was treated with phosphatase before removal of the cap to prevent cloning of truncated transcripts. For 3′ RACE, cDNA was made using oligo dT primers to allow cloning of the polyadenylated 3′ ends. Multiple primer sets (**Supplementary Table 3**) were used to maximize detection of transcripts and reduce the likelihood of bias from any one primer set. We used the Platinum Taq High Fidelity kit (Invitrogen) to produce product for cloning. We sequenced multiple clones from at least two independent experiments to confirm results. Both 3′ and 5′ determinations used techniques optimized for identification of full-length transcripts rather than truncated products.

**Chromatin conformation capture (3C).** Twenty million cells were grown and cross-linked in 1% formaldehyde. Cells were recovered by scraping (5 μg genomic DNA). Nuclei were purified using hypotonic lysis and distributed into 1 million nuclei aliquots. Aliquots were stored at −80 °C. An additional 10 million nuclei were recovered without the use of formaldehyde for a no-cross-link control.

Aliquots were removed from −80 °C storage and resuspended in 500 μl of 1× restriction buffer (RB) and 3% (w/v) SDS and incubated for 1.5 h at 37 °C with shaking at 1,000 rpm to loosen chromatin. Then Triton-X was added up to 1.8% (v/v) and samples were incubated 1 h more at 37 °C with shaking to sequester SDS. Three hundred units of restriction enzyme DpnII were added and incubated overnight at 37 °C with shaking. The next day, SDS was added to 1.6% (w/v), and samples were incubated at 65 °C for 30 min to inactivate restriction enzyme. 150 μl of sample was saved to check restriction enzyme efficiency.

Samples were diluted to 2 ml volume with 1.2× final concentration of ligase buffer and 1% (v/v) final concentration of Triton-X. Samples were incubated at 37 °C for 1 h. Samples were placed on ice, and 40 units of T4 ligase were added and incubated overnight at 16 °C. The next day samples were incubated for 30 min at room temp. Cross-links were reversed by adding NaCl to 200 mM and incubating at 65 °C for 2 h with proteinase K. Samples were then incubated with RNase A for 45 min at 41 °C. Finally DNA was purified by phenol-chloroform extraction and ethanol–sodium acetate precipitation. DNA was resuspended in 50 μl of nuclease-free water and 1 μl was used in each PCR reaction.

Primers were designed to span several DpnII cut sites at the 5′ end, 3′ end and internal sites (**Supplementary Table 9**). Positive control templates for primers were synthesized as single-strand DNA oligonucleotides (Sigma). Product was cloned and sequenced to ensure product was specific. Sequenced products aligned with their respective sites in the genome with the GATC consensus sequence for DpnII between them.

**Statistical analysis.** Data are presented as means ± s.d. of three or more independent results. Statistical significance was assessed using a two-tailed unpaired Student's *t*-test.

Received 29 April 2010; accepted 25 May 2010; published online 27 June 2010

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## Acknowledgments

This work was supported by grants from the US National Institutes of Health (NIGMS GM77253 to D.R.C., GM85080 to B.A.J. and NIBIB EB 05556 to J.C.S.) and the Robert A. Welch Foundation (I-1244 to D.R.C.). B.A.J. was also supported by the High Impact/High Risk Grants Program, UT Southwestern Medical Center, and an Institutional Research Grant from the American Cancer Society (IRG-02-196-04). We gratefully acknowledge Z. Mourelatos (Univ. Pennsylvania) for providing anti-argonaute antibody. We thank A. Bhat for technical assistance and J. Watts for comments on the manuscript.

## Author contributions

All authors designed experiments and analyzed data. All authors except D.R.C. performed experiments. D.R.C. wrote the manuscript. The authors recognize that the contributions of J.C.S. and Y.C. would have been sufficient to merit separate publications.

## Competing financial interests

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturechemicalbiology/.

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