

An antisense RNA capable of modulating the expression of the tumor suppressor microRNA-34a

Jason Serviss¹, Per Johnsson¹, Miranda Houtman¹, Katja Pokrovskala¹, Dan Grandér^{1*}

¹ Department of Oncology and Pathology, Karolinska Institutet, Stockholm, Sweden

* Correspondence: Dan Grandér, Department of Oncology and Pathology, Karolinska Institutet, Stockholm, Sweden, SE-17177.

dan.grander@ki.se

ABSTRACT

Long non-coding RNAs transcribed in an antisense orientation to sense protein-coding genes have been increasingly shown to play pivotal roles in regulating gene expression in both *cis* and *trans*. Expression of these antisense transcripts has often been shown to be dysregulated in cancer giving rise to an altered protein-coding gene expression. Here we for the first time describe the ability of a human antisense RNA to regulate levels of a microRNA. This microRNA is *miR34a* which is a downstream target of *p53* and mediates critical cellular functions such as apoptosis and senescence. We find that the *miR34a* antisense RNA is critical for *miR34a* expression and mediation of its cellular functions.

INTRODUCTION

In recent years advances in functional genomics has revolutionized our understanding of the human genome. Evidence now points to the fact that approximately 75% of the genome is transcribed but only ~1.2% of this is responsible for encoding proteins [1, 2]. The newly discovered non-coding elements have been categorized dependent on their function, size, localization, and orientation although a strict definition of these categories is an ongoing process. Of these recently identified elements, long non-coding (lnc) RNAs are defined as transcripts exceeding 200bp in length with a lack of a functional open reading frame. LncRNAs tend to exhibit increased tissue specificity, decreased expression levels, and less conservation than protein coding genes [3]. The vast prevalence of transcribed lncRNAs throughout the genome originally led to the speculation that these transcripts were non-functional “transcriptional relics” although further investigation has found lncRNAs to have important regulatory functions in processes such as development, cell fate, and oncogenesis [4-6]. Although many lncRNAs have been identified, the majority still have an unknown

biological role and are yet to be functionally characterized [3].

Some lncRNAs are dually classified as antisense (as) RNAs which are expressed from the same locus as a sense transcript in an antisense orientation. The phenomenon of asRNA transcription has been described in a large variety of eukaryotic organisms and was first discovered long before the advent of modern sequencing technologies [7]. With new high throughput transcriptome sequencing, current estimates indicate that up to 20-40% of the estimated 20,000 protein-coding genes exhibit antisense transcription [8, 9]. asRNAs have been shown to be expressed in both a concordant and discordant fashion with their sense transcript and can arise from independent promoters, bi-directional promoters exhibiting divergent transcription, as well as cryptic promoters [10-13]. Examples of asRNA-mediated gene regulation are becoming increasingly prevalent and are often, but not exclusively, mediated in *cis* resulting in the modulation of sense gene levels. The mechanisms by which they accomplish this are largely diverse stretching from recruitment of chromatin modifying factors [4], acting as microRNA (miRNA) sponges [14], and causing transcriptional interference [15], to mention only a few.

The hypothesis that asRNAs play an important role in oncogenesis was first proposed when studies increasingly found examples of aberrant expression of these transcripts and other lncRNA subgroups in tumor samples [16, 17]. Functional characterization of individual transcripts led to the discovery of asRNA-mediated regulation of several known tumorigenic factors. The asRNA *ANRIL* was found to be up-regulated in leukemia and function by repressing *CDKN2B*, an important regulator of cell cycle G1 progression [5]. Furthermore, the tumor suppressor *PTEN* has been shown to be regulated both transcriptionally and post-transcriptionally by asRNA transcripts [18]. As well, the asRNA *HOTAIR* has been shown to negatively regulate the *HOXD* locus via recruitment of Polycomb Repressive Complex 2 mediating epigenetic silencing [4]. Although studies characterizing the functional importance of asRNAs in cancer are limited to date, it is becoming increasingly apparent that they play critical roles in regulating key cancer initiation and progression pathways (for a review on this topic please refer to [19]).

Apoptosis and senescence are two highly ordered and orchestrated cellular pathways which are commonly dysregulated in cancer. Cellular signaling in response to DNA damage, sustained oncogene expression, and other forms of cellular stress converge on the transcription factor *p53* which regulates transcription of coding and non-coding downstream targets. One non-coding target of *p53* is the tumor suppressor microRNA (miRNA) known as *miR34a* [20]. Upon *p53* activation *miR34a* expression is increased allowing it down-regulate its targets involved in cellular pathways such as DNA damage repair, growth factor signaling, apoptosis, and cellular senescence [21]. *miR34a* is a crucial factor in mediating activated *p53*

response and its expression is therefore often decreased in human cancers [22-26]. Reduced *miR34a* levels have been shown to be mediated via epigenetic regulation in several cancer types such as colorectal-, pancreatic-, and ovarian cancer [27] as well as various types of hematological malignancies [28]. Phase 1 clinical trials are currently underway to evaluate the safety of *miR34a* replacement therapy in hepatocellular carcinoma patients making it the first miRNA replacement therapy to be clinically tested [29].

While the functional outcome of antisense transcription at protein-coding genes has been well established, the presence and functional importance of antisense transcription at miRNA host genes (HG) remains unknown. In this study we identify and characterize a *miR34a* HG antisense transcript, finding that its expression is necessary for an appropriate *miR34a* response to apoptotic stimuli. We also find that modulating the levels of the *miR34a* asRNA is sufficient to increase levels of *miR34a* resulting in G1 arrest in a *p53*-null background.

RESULTS

Many functionally characterized asRNAs have been shown to interact with chromatin modifying factors aiding in the recruitment of these factors to specific loci and mediating transcriptional activation or inhibition [30]. Therefore members of our laboratory* began this study by bioinformatically evaluating evidence for asRNA transcription at the site of 20 miRNAs that are commonly epigenetically dysregulated in various cancer types [27, 31-35]. The bioinformatic analysis identified seven candidates which were further validated by screening a panel of various cell lines (**Supplementary Fig. 1a and 1b**). All screened asRNA candidates showed evidence for expression in at least one cell line although further bioinformatic analysis revealed that several may have coding potential. We filtered out asRNA candidates with protein-coding potential and also removed short unspliced transcripts which are potential relics from spurious transcription. Of the candidates remaining we choose an antisense transcript to the tumor suppressor *miR34a* for further investigation.

Characterization of the *miR34a* asRNA transcript

Bioinformatic analysis of the *miR34a* locus revealed one annotated lncRNA (*RP3-510D11.2*, GENCODE v7) and one spliced expressed sequence tag read (*BP872819*) indicating the presence of a *miR34a* asRNA transcript in a “head-to-head” orientation with approximately 100bp overlap with *miR34a* HG (**Fig. 1a**). We were able to detect co-expression of both *miR34a* HG and *miR34a* AS in a variety of cell lines, including HCT116 human colon cancer cells and 293T human embryonic kidney cells (**Fig. 1b**). We included both *p53*^{+/+} and *p53*^{-/-}

* Kindly note that the results sections concerning characterization and regulation of the *miR34a* asRNA includes previous unpublished laboratory results that were not contributed by the author. Please see the acknowledgments section for more details.

cell lines in the cell line panel due to the fact that *miR34a* is a known downstream target of *p53*. A correlation was observed between both *miR34a* HG and miR34a asRNA expression and *p53* status, with *p53*^{+/+} cell lines tending to have higher expression of both transcripts (**Supplementary Fig. 2a**).

We next sought to determine the 5' transcription start site and 3' termination site for the miR34a asRNA transcript. To this end we first performed 3' rapid amplification of cDNA ends (RACE) using the U2OS osteosarcoma cell line that exhibited high levels of miR34a asRNA expression in the cell panel screening. Sequencing of the resulting cloned cDNA indicated the transcripts 3' transcription termination site to be 125bp upstream of the *RP3-510D11.2* transcript's annotated termination site (**Fig. 1c**). Next we characterized the miR34a asRNA 5' transcription start site by carrying out a primer walk assay. A common reverse primer was placed in exon 2 and forward primers were gradually staggered upstream of the transcripts annotated start site (**Fig. 1d**). Our results indicated that the 5' start site for miR34a asRNA is in fact approximately 200bp upstream of the annotated start site (**Fig. 1e**). We also investigated the propensity of miR34a asRNA to be alternatively spliced, using PCR cloning and sequencing, finding that the transcript is post-transcriptionally spliced to form several different isoforms (**Supplementary Fig. 2b**).

AsRNAs are known to be capable of exerting regulatory functions on both a transcriptional and post-transcriptional level and therefore, the cellular localization of the asRNA can be informative concerning the method of regulation. Semi-quantitative PCR revealed that the miR34a asRNA transcript localizes to both the nucleus and cytoplasm in both spliced and unspliced forms (**Fig. 1f**). Polyadenylation status was also evaluated indicating that the miR34a asRNA is polyadenylated although the unspliced form seems to only be in the polyA negative state (**Fig. 1g**).

Next we utilized a bioinformatic approach to evaluate the coding potential of the miR34a asRNA transcript. The Coding-potential assessment tool uses a linear regression model to evaluate coding-potential by examining ORF length, ORF coverage, Fickett score [36] and hexamer score [37]. Results indicated that miR34a asRNA has a similar lack of coding capacity to the known non-coding transcripts *HOTAIR* and *XIST* and differs greatly when examining these parameters to the known coding transcripts beta-actin, tubulin, and *MYC* (**Fig. 1h**). We further confirmed these results using the Coding-potential Calculator which utilizes a support based machine-based classifier and accesses an alternate set of discriminatory features (**Supplementary Fig. 2c**). However, to fully evaluate coding potential methods such as mass spectrometry or ribosome profiling must be used [38]. Despite this, bioinformatic tools are increasingly reliable in assessment of coding potential. In summary, the miR34a asRNA presents little evidence of coding potential as evaluated by these two

bioinformatic approaches.

Regulation of miR34a asRNA expression

miR34a is a known downstream target of *p53* and has been previously shown to exhibit increased expression with cellular apoptotic signaling [20]. We hypothesized that miR34a asRNA may be regulated in a similar fashion whereby transcription is stimulated by activation of *p53*. To test this we treated *p53^{+/+}* and *p53^{-/-}* HCT116 cells with increasing concentrations of the DNA damaging agent doxorubicin and monitored miR34a asRNA expression. We observed increasing *miR34a* HG and asRNA expression with increasing doses of doxorubicin indicating that these two transcripts are co-regulated (**Fig. 2a and 2b**). Although *p53^{+/+}* cells showed a 12-fold increase in miR34a AS expression at the highest tested doxorubicin concentration, this effect was largely abrogated in *p53^{-/-}* cells, indicating that *p53* is a major regulator of miR34a asRNA expression. Further examination of the effects of DNA damage on miR34a asRNA expression in three independent experiments confirmed these observations (**Fig. 2c**).

It is likely, due to the head-to head orientation of *miR34a* HG and asRNA, that transcription initiation may be activated from a single promoter in a bi-directional manner. To investigate whether miR34a HG and asRNA are transcribed from the same promoter as convergent transcripts, we cloned the *miR34a* HG promoter, including the *p53* binding site, into a luciferase/renilla dual reporter vector which we hereafter refer to as p1 (**Fig. 2d and 2e**). Upon transfection of this construct in two separate cell lines we observed increases in both luciferase and renilla indicating that *miR34a* HG and asRNA expression is regulated by a single promoter contained within the p1 construct (**Fig. 2f and 2g**).

The function of miR34a asRNA

Functional characterization of individual antisense transcripts has previously shown their capability to regulate their sense gene [5, 30]. We therefore investigated the possibility that miR34a asRNA may regulate *miR34a* HG levels. We hypothesized that the overlapping regions of the sense and antisense transcripts may have a crucial role in miR34a asRNAs ability to regulate *miR34a* HG via RNA:DNA or RNA:RNA interaction. Accordingly, we first co-transfected the p1 construct, containing the overlapping region of the two transcripts, and an short hairpin (sh) RNA targeting renilla into HEK293T cells (**Fig. 2d and 2e**). Analysis of luciferase and renilla expression revealed that by decreasing levels of the renilla transcript (corresponding to miR34a asRNA) luciferase (corresponding to *miR34a* HG) levels were concomitantly decreased (**Fig. 3a**). These results indicate that miR34a asRNA is capable of regulating the *miR34a* HG in a concordant manner and that the overlapping region of the miR34a asRNA may be sufficient to mediate this regulation.

Our previous results showed that *miR34a* HG and asRNA are simultaneously up-regulated in response to doxorubicin treatment leading us to consider the possibility that miR34a asRNA may be necessary for an appropriate miR34a response to apoptotic signaling. To explore this possibility we again utilized the p1 construct, co-transfected it with an shRNA targeting renilla, and treating HCT116 cells with increasing levels of doxorubicin. Levels of luciferase were abrogated in the shRenilla-transfected cells compared to control shRNA-transfected cells (**Fig. 3b**). Collectively, these results indicate that miR34a asRNA positively regulates levels of *miR34a* HG and is crucial for a proper *miR34a* response to apoptotic stimuli.

Despite the fact that *p53* regulates *miR34a* HG and asRNA expression we hypothesized that other factors may also be able to regulate this locus. In fact, transcription factor ChIP-sequencing from ENCODE shows evidence for a plethora of transcription factors capable of binding to the *miR34a* locus (**Supplementary Fig. 2d**). Preliminary experiments showed that transient transfection of miR34a asRNA into the *p53*-null PC3 prostate cancer cell line caused an increase in *miR34a* HG expression (**Fig. 3c**). By utilizing a lentiviral system, we stably overexpressed the miR34a asRNA transcript (miR34a AS ref., **Supplementary Fig. 2b**) in PC3 cells (**Supplementary Fig. 2e and Fig. 3d**). We analyzed the levels of miR34a asRNA in these stably overexpressing PC3 cells, compared to HEK293T cells, which have high endogenous levels of miR34a asRNA, finding that the overexpression in the PC3 cells was within physiologically relevant levels (**Supplementary Fig. 2f**). Analysis of *miR34a* levels in the PC3 miR34a asRNA overexpressing cell lines showed increased expression of the miRNA (**Fig. 3e**). These results indicate that miR34a expression may be rescued in *p53*-null PC3 cells by overexpressing miR34a asRNA.

miR34a has been previously shown to regulate cell cycle progression, with increasing levels of *miR34a* causing G1 arrest. This is thought to be mediated by *miR34a*'s ability to target cell cycle regulators such as cyclin D1 and *CDK6* [39]. We therefore sought to determine if the miR34a asRNA overexpressing PC3 cell lines were able to mimic these known phenotypes. Cell cycle analysis via determination of DNA content showed a significant increase in G1 phase cells in miR34a asRNA overexpressing PC3 cells (**Fig. 3f**) indicative of G1 arrest. We also performed western blot analysis for the cell cycle regulator cyclin D1 finding that its levels were reduced ~17% in the miR34a asRNA overexpressing cell line (**Fig. 3g and 3h**). In summary, we find that overexpression of miR34a asRNA is sufficient to increase *miR34a* expression and give rise to known phenotypes observed with increased *miR34a* expression.

DISCUSSION

The expression levels of asRNAs are crucial for the appropriate regulation of cancer-associated protein-coding genes. Here we show for the first time that asRNAs are also capable of regulating cancer-associated miRNAs (**Fig. 4**). We find that, in the presence of *p53* activation, miR34a asRNA provides an additional regulatory level by increasing *miR34a* expression. In addition, co-expression of miR34a asRNA is necessary to induce an appropriate *miR34a* response leading to cell cycle arrest.

The ability of miR34a asRNA to increase levels of *miR34a* in *p53*-null PC3 cells was also demonstrated in this study. These results indicate that *p53* is not necessary for activation of *miR34a* expression in the PC3 cell line. These results are in conflict with the results that we observed in HCT116 *p53*^{-/-} cells, which were not capable of an appropriate up-regulation of *miR34a* in the absence of *p53*. We hypothesize that miR34a asRNA may interact with one or several co-factors which, independently or in concert with *p53*, increase the transcriptional output of the *miR34a* locus. We further hypothesize that the differences observed between PC3 and HCT116 *p53*^{-/-} cell lines may be accounted for due to the presence of these co-factors in the respective cell lines. As of yet, we are not aware of the interacting partners of miR34a asRNA, and an ongoing investigation of this is underway.

Altered expression patterns of cancer-associated miRNAs often takes place via epigenetically mediated mechanisms. *miR34a* is no exception to this and its promoter has been found to be aberrantly methylated and de-acetylated in various cancer types [27, 28, 40]. We hypothesize that miR34a asRNA may be instrumental in relieving these inhibitory chromatin marks allowing for increased expression of the *miR34a* locus. Whether transient expression of miR34a asRNA may be able to reinstate an epigenetically stable *miR34a* expression independent of *p53* status is yet to be seen, but indicates novel therapeutic prospects. *MiR34a* expression in a *p53*-null background has previously been shown to be sufficient to up-regulate *p21*, via *miR34a* targeting of *HDAC1*, resulting in reduced cancer cell proliferation [41]. Although currently, efficient methods are not in place allowing the transfer of long oligonucleotides to *in vivo* tumor cells, we hypothesize that therapies mediating stable epigenetic changes causing decreased cell proliferation via G1 arrest and/or apoptosis in *p53* mutated cancer types may be a largely beneficial therapeutic prospect.

In conclusion, we have found miR34a asRNA to be a critical component of the *miR34a* response. Ongoing studies of the mechanisms underlying this interaction are currently underway and will hopefully shed light on the complexities of dysregulated apoptosis and senescence which mediate therapy resistance during tumorigenesis.

EXPERIMENTAL PROCEDURES

Cell culture

All cell lines were cultured at 5% CO₂ and 37° C with HEK293T cells cultured in DMEM high glucose (Hyclone), HCT116 cells in McCoy's 5a (Life Technologies), and PC3 cells in RPMI (Hyclone) and 2 mM L-glutamine. All growth mediums were supplemented with 10% heat-inactivated FBS and 50 µg/ml of streptomycin and 50 µg/ml of penicillin.

Bioinformatics

The USCS genome browser [42] was utilized for the bioinformatic evaluation of antisense transcription utilizing the Human EST and ENCODE RNAseq tracks.

Coding Potential

Protein-coding capacity was evaluated using the Coding-potential Assessment Tool [43] and Coding-potential Calculator [44] with default settings. Transcript sequences for use with Coding-potential Assessment Tool were downloaded from the UCSC genome browser using the following IDs: *HOTAIR* (ENST00000455246.1), *XIST* (ENST00000429829.1), β-actin (ENST00000331789.5), Tubulin (ENST00000427480.1), and *MYC* (ENST00000377970). Transcript sequences for use with Coding-potential Calculator were downloaded from the UCSC genome browser using the following IDs: *HOTAIR* (uc031qho.1), β-actin (uc003soq.4).

sh-RNAs.

shRNA-expressing constructs were cloned into the U6M2 construct by using the BglII and KpnI restriction sites as previously described [45]. ShRNA constructs were transfected by using Lipofectamine 2000 (Life Technologies). The sequence targeting renilla is as follows: AAT ACA CCG CGC TAC TGG C

Lentiviral particle production, infection, and selection.

Lentivirus production was performed as previously described in [46]. Briefly, HEK293T cells were transfected with viral and expression constructs using Lipofectamine 2000 (Life Technologies), after which viral supernatants were harvested 48 and 72 hours post-transfection. Viral particles were concentrated using PEG-IT solution (Systems Biosciences) according to the manufacturer's recommendations. HEK293T cells were used for virus titration and GFP expression was evaluated 72hrs post-infection via flow cytometry after which TU/ml was calculated.

Western Blotting.

Samples were lysed in 50 mM Tris-HCl, pH 7.4, 1% NP-40, 150 mM NaCl, 1 mM EDTA, 1% glycerol, 100 µM vanadate, protease inhibitor cocktail and PhosSTOP (Roche Diagnostics GmbH). Lysates were subjected to SDS-PAGE and transferred to PVDF membranes. The proteins were detected by western blot analysis by using an enhanced chemiluminescence system (Western Lightning-ECL, PerkinElmer). Antibodies used were specific for *CCND1* (Cell Signaling, cat. no. 2926, 1:1000), and β-actin (Sigma-Aldrich, cat. no. A5441, 1:5,000). All western blot quantifications were performed using ImageJ [47].

Generation of U6-expressed miR34a AS lentiviral constructs.

The U6 promoter was amplified from the U6M2 cloning plasmid [45] and ligated into the Not1 restriction site of the pHIV7-IMPDH2 vector [46]. miR43a asRNA was PCR amplified and subsequently cloned into the Nhe1 and Pac1 restriction sites in the pHIV7-IMPDH2-U6 plasmid.

Promoter activity.

Cells were cotransfected with the renilla/firefly bidirectional promoter construct [48] and GFP by using Lipofectamine 2000 (Life Technologies). The expression of GFP and luminescence was measured 24 h post transfection by using the Dual-Glo Luciferase Assay System (Promega) and detected by the GloMax-Multi+ Detection System (Promega). The expression of luminescence was normalized to GFP.

Subcellular Fractionation.

Fractionation was performed using the PARIS kit according to the manufacturer's recommendations (Life Technologies).

Flow Cytometry.

Cells were harvested, centrifuged and, either re-suspended in PBS, 5% FBS and analyzed for GFP expression using the LSRII machine (BD Biosciences).

RNA extraction and cDNA synthesis.

For downstream SYBR green applications, RNA was extracted using the RNeasy mini kit (Qiagen) and subsequently treated with DNase (Ampion Turbo DNA-free, Life Technologies). 500ng RNA was used for cDNA synthesis using MuMLV (Life Technologies) and a 1:1 mix of oligo(dT) and random nanomers.

For analysis of miRNA expression with Taqman, samples were isolated with trizol (Life Technologies) and further processed with the miRNeasy kit (Qiagen). cDNA synthesis was performed using the TaqMan MicroRNA Reverse Transcription Kit (Life Technologies) using the corresponding oligos according to the manufacturer's recommendations.

QPCR and PCR.

PCR was performed using the KAPA2G fast mix (Kapa Biosystems) with corresponding primers. QPCR was carried out using KAPA 2G SYBRGreen (Kapa Biosystems) using the Applied Biosystems 7900HT machine with the cycling conditions: 95 °C for 3 min, 95 °C for 3 s, 60 °C for 30 s.

QPCR for miRNA expression analysis was performed according to the protocol for the TaqMan microRNA Assay kit (Life Technologies) with the same cycling scheme as above.

Bi-directional promoter.

The overlapping region (p1) corresponds with the sequence previously published as the p53 binding site in [20] which we cloned into the pLucRluc construct [48].

Cell-cycle distribution.

Cells were washed in PBS and fixed in 4% PFA at room temperature overnight. PFA was removed, and cells were resuspended in 95% EtOH. The samples were then rehydrated in distilled water, stained with DAPI and analyzed by flow cytometry on a LSRII (BD Biosciences) machine. Resulting data was further processed using Flowjo software.

3'-RACE

3'-RACE was performed as described as previously in [18]. Briefly, U2OS cell RNA was polyA-tailed using yeast polyA polymerase after which cDNA was synthesized using oligo(dT) primers. Nested-PCR was performed first using a forward primer in miR34a asRNA exon 1 and a tailed oligo(dT) primer followed by a second PCR using an alternate miR34a asRNA exon 1 primer and a reverse primer binding to the tail of the previously used oligo(dT) primer. PCR products were gel purified and cloned the Strata Clone Kit (Agilent Technologies), and sequenced.

ACKNOWLEDGMENTS

I would like to kindly acknowledge Miranda Houtman for contributing unpublished data used to create Figures 1B-C, Figures 2A-C and 2F-G. I would also like to thank Professor Dan Grandér and Per Johnsson for their theoretical guidance and input.

REFERENCES

1. Djebali, S., et al., *Landscape of transcription in human cells*. Nature, 2012. **489**(7414): p. 101-8.
2. International Human Genome Sequencing, C., *Finishing the euchromatic sequence of the human genome*. Nature, 2004. **431**(7011): p. 931-45.
3. Derrien, T., et al., *The GENCODE v7 catalog of human long noncoding RNAs: analysis of their gene structure, evolution, and expression*. Genome Res, 2012. **22**(9): p. 1775-89.
4. Rinn, J.L., et al., *Functional demarcation of active and silent chromatin domains in human HOX loci by noncoding RNAs*. Cell, 2007. **129**(7): p. 1311-23.
5. Yap, K.L., et al., *Molecular interplay of the noncoding RNA ANRIL and methylated histone H3 lysine 27 by polycomb CBX7 in transcriptional silencing of INK4a*. Mol Cell, 2010. **38**(5): p. 662-74.
6. Struhl, K., *Transcriptional noise and the fidelity of initiation by RNA polymerase II*. Nat Struct Mol Biol, 2007. **14**(2): p. 103-5.
7. Vanhee-Brossollet, C. and C. Vaquero, *Do natural antisense transcripts make sense in eukaryotes?* Gene, 1998. **211**(1): p. 1-9.
8. Chen, J., et al., *Over 20% of human transcripts might form sense-antisense pairs*. Nucleic Acids Res, 2004. **32**(16): p. 4812-20.
9. Katayama, S., et al., *Antisense transcription in the mammalian transcriptome*. Science, 2005. **309**(5740): p. 1564-6.
10. Sigova, A.A., et al., *Divergent transcription of long noncoding RNA/mRNA gene pairs in embryonic stem cells*. Proc Natl Acad Sci U S A, 2013. **110**(8): p. 2876-81.
11. Neil, H., et al., *Widespread bidirectional promoters are the major source of cryptic transcripts in yeast*. Nature, 2009. **457**(7232): p. 1038-42.
12. Seila, A.C., et al., *Divergent transcription from active promoters*. Science, 2008. **322**(5909): p. 1849-51.
13. Core, L.J., J.J. Waterfall, and J.T. Lis, *Nascent RNA sequencing reveals widespread pausing and divergent initiation at human promoters*. Science, 2008. **322**(5909): p. 1845-8.
14. Memczak, S., et al., *Circular RNAs are a large class of animal RNAs with regulatory potency*. Nature, 2013. **495**(7441): p. 333-8.
15. Conley, A.B. and I.K. Jordan, *Epigenetic regulation of human cis-natural antisense transcripts*. Nucleic Acids Res, 2012. **40**(4): p. 1438-45.
16. Brunner, A.L., et al., *Transcriptional profiling of long non-coding RNAs and novel transcribed regions across a diverse panel of archived human cancers*. Genome Biol, 2012. **13**(8): p. R75.
17. Prensner, J.R., et al., *Transcriptome sequencing across a prostate cancer cohort identifies PCAT-1, an unannotated lincRNA implicated in disease progression*. Nat Biotechnol, 2011. **29**(8): p. 742-9.
18. Johnsson, P., et al., *A pseudogene long-noncoding-RNA network regulates PTEN transcription and translation in human cells*. Nat Struct Mol Biol, 2013. **20**(4): p. 440-6.
19. Spizzo, R., et al., *Long non-coding RNAs and cancer: a new frontier of translational research?* Oncogene, 2012. **31**(43): p. 4577-87.
20. Raver-Shapira, N., et al., *Transcriptional activation of miR-34a contributes to p53-mediated apoptosis*. Mol Cell, 2007. **26**(5): p. 731-43.
21. Lal, A., et al., *Capture of microRNA-bound mRNAs identifies the tumor suppressor miR-34a as a regulator of growth factor signaling*. PLoS Genet, 2011. **7**(11): p. e1002363.
22. Cole, K.A., et al., *A functional screen identifies miR-34a as a candidate neuroblastoma tumor suppressor gene*. Mol Cancer Res, 2008. **6**(5): p. 735-42.
23. Gallardo, E., et al., *miR-34a as a prognostic marker of relapse in surgically resected non-small-cell lung cancer*. Carcinogenesis, 2009. **30**(11): p. 1903-9.

24. Zenz, T., et al., *miR-34a as part of the resistance network in chronic lymphocytic leukemia*. Blood, 2009. **113**(16): p. 3801-8.
25. Cheng, J., et al., *The impact of miR-34a on protein output in hepatocellular carcinoma HepG2 cells*. Proteomics, 2010. **10**(8): p. 1557-72.
26. Liu, C., et al., *The microRNA miR-34a inhibits prostate cancer stem cells and metastasis by directly repressing CD44*. Nat Med, 2011. **17**(2): p. 211-5.
27. Vogt, M., et al., *Frequent concomitant inactivation of miR-34a and miR-34b/c by CpG methylation in colorectal, pancreatic, mammary, ovarian, urothelial, and renal cell carcinomas and soft tissue sarcomas*. Virchows Arch, 2011. **458**(3): p. 313-22.
28. Chim, C.S., et al., *Epigenetic inactivation of the miR-34a in hematological malignancies*. Carcinogenesis, 2010. **31**(4): p. 745-50.
29. Mirna Therapeutics, I. *A Multicenter Phase I Study of MRX34, MicroRNA miR-RX34 Liposome Injectable Suspension*. 2014 [cited 2014 2014-05-14].
30. Pelechano, V. and L.M. Steinmetz, *Gene regulation by antisense transcription*. Nat Rev Genet, 2013. **14**(12): p. 880-93.
31. Croce, C.M., *Causes and consequences of microRNA dysregulation in cancer*. Nat Rev Genet, 2009. **10**(10): p. 704-14.
32. Toyota, M., et al., *Epigenetic silencing of microRNA-34b/c and B-cell translocation gene 4 is associated with CpG island methylation in colorectal cancer*. Cancer Res, 2008. **68**(11): p. 4123-32.
33. Ferraro, A., et al., *Epigenetic regulation of miR-21 in colorectal cancer: ITGB4 as a novel miR-21 target and a three-gene network (miR-21-ITGBeta4-PDCD4) as predictor of metastatic tumor potential*. Epigenetics, 2014. **9**(1): p. 129-41.
34. Skarn, M., et al., *Epigenetic regulation and functional characterization of microRNA-142 in mesenchymal cells*. PLoS One, 2013. **8**(11): p. e79231.
35. Chang, S. and S.K. Sharan, *Epigenetic control of an oncogenic microRNA, miR-155, by BRCA1*. Oncotarget, 2012. **3**(1): p. 5-6.
36. Fickett, J.W., *Recognition of protein coding regions in DNA sequences*. Nucleic Acids Res, 1982. **10**(17): p. 5303-18.
37. Fickett, J.W. and C.S. Tung, *Assessment of protein coding measures*. Nucleic Acids Res, 1992. **20**(24): p. 6441-50.
38. Kuersten, S., et al., *Translation regulation gets its 'omics' moment*. Wiley Interdiscip Rev RNA, 2013. **4**(6): p. 617-30.
39. Sun, F., et al., *Downregulation of CCND1 and CDK6 by miR-34a induces cell cycle arrest*. FEBS Lett, 2008. **582**(10): p. 1564-8.
40. Nalls, D., et al., *Targeting epigenetic regulation of miR-34a for treatment of pancreatic cancer by inhibition of pancreatic cancer stem cells*. PLoS One, 2011. **6**(8): p. e24099.
41. Zhao, J., et al., *TP53-independent function of miR-34a via HDAC1 and p21(CIP1/WAF1)*. Mol Ther, 2013. **21**(9): p. 1678-86.
42. Kent, W.J., et al., *The human genome browser at UCSC*. Genome Res, 2002. **12**(6): p. 996-1006.
43. Wang, L., et al., *CPAT: Coding-Potential Assessment Tool using an alignment-free logistic regression model*. Nucleic Acids Res, 2013. **41**(6): p. e74.
44. Kong, L., et al., *CPC: assess the protein-coding potential of transcripts using sequence features and support vector machine*. Nucleic Acids Res, 2007. **35**(Web Server issue): p. W345-9.
45. Amarzguioui, M., J.J. Rossi, and D. Kim, *Approaches for chemically synthesized siRNA and vector-mediated RNAi*. FEBS Lett, 2005. **579**(26): p. 5974-81.
46. Turner, A.M., et al., *Characterization of an HIV-targeted transcriptional gene-silencing RNA in primary cells*. Hum Gene Ther, 2012. **23**(5): p. 473-83.
47. Schneider, C.A., W.S. Rasband, and K.W. Eliceiri, *NIH Image to ImageJ: 25 years of image analysis*. Nat Methods, 2012. **9**(7): p. 671-5.

48. Polson, A., E. Durrett, and D. Reisman, *A bidirectional promoter reporter vector for the analysis of the p53/WDR79 dual regulatory element*. Plasmid, 2011. **66**(3): p. 169-79.

FIGURES

Figure 1

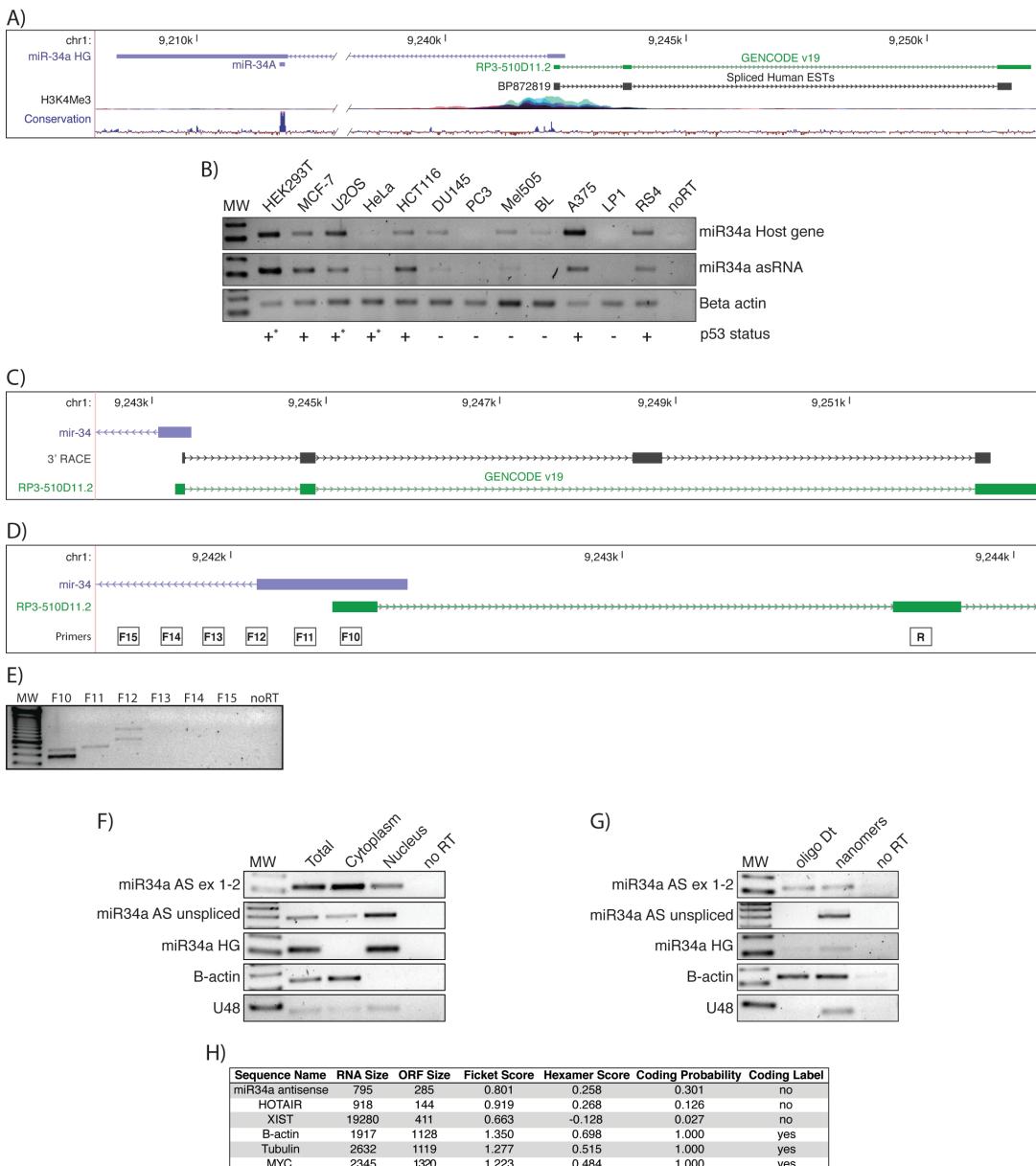


Figure 1: Characterization of the miR34a asRNA transcript. **A)** A schematic picture of the *miR34a* locus from UCSC genome browser including *miR34a* HG and mature *miR34a* (purple) as well as bioinformatic evidence for antisense transcription from GENCODE v19 (green) and the spliced human EST track (black). H3K4me3 ChIP-seq data from ENCODE indicating the promoter region and conservation over the locus is also shown. **B)** Semi-quantitative PCR data from the screening of a panel of cell lines. * Indicates that, although the cell line is *p53wt*, other mechanisms are present which inhibit *p53* function. **C)** 3'-RACE sequencing results displayed in the UCSC genome browser together with the annotated miR34a asRNA transcript from GENCODE. **D)** A schematic picture of the miR34a asRNA locus including the location of the primers used in the primer walk assay. **E)** Semi-quantitative PCR results from the primer walk assay performed using HEK293T cells. **F)** Semi-quantitative PCR results from fractionated HEK293T cells investigating the cellular localization of the miR34a asRNA transcript. **G)** Polyadenylation status of the spliced and unspliced variants of miR34a asRNA in HEK293t cells. **H)** Coding potential analysis results from the Coding-potential Assessment Tool including miR34a asRNA and 2 characterized non-coding transcripts (*HOTAIR* and *XIST*) and 2 known coding transcripts (β -actin, tubulin, and *MYC*).

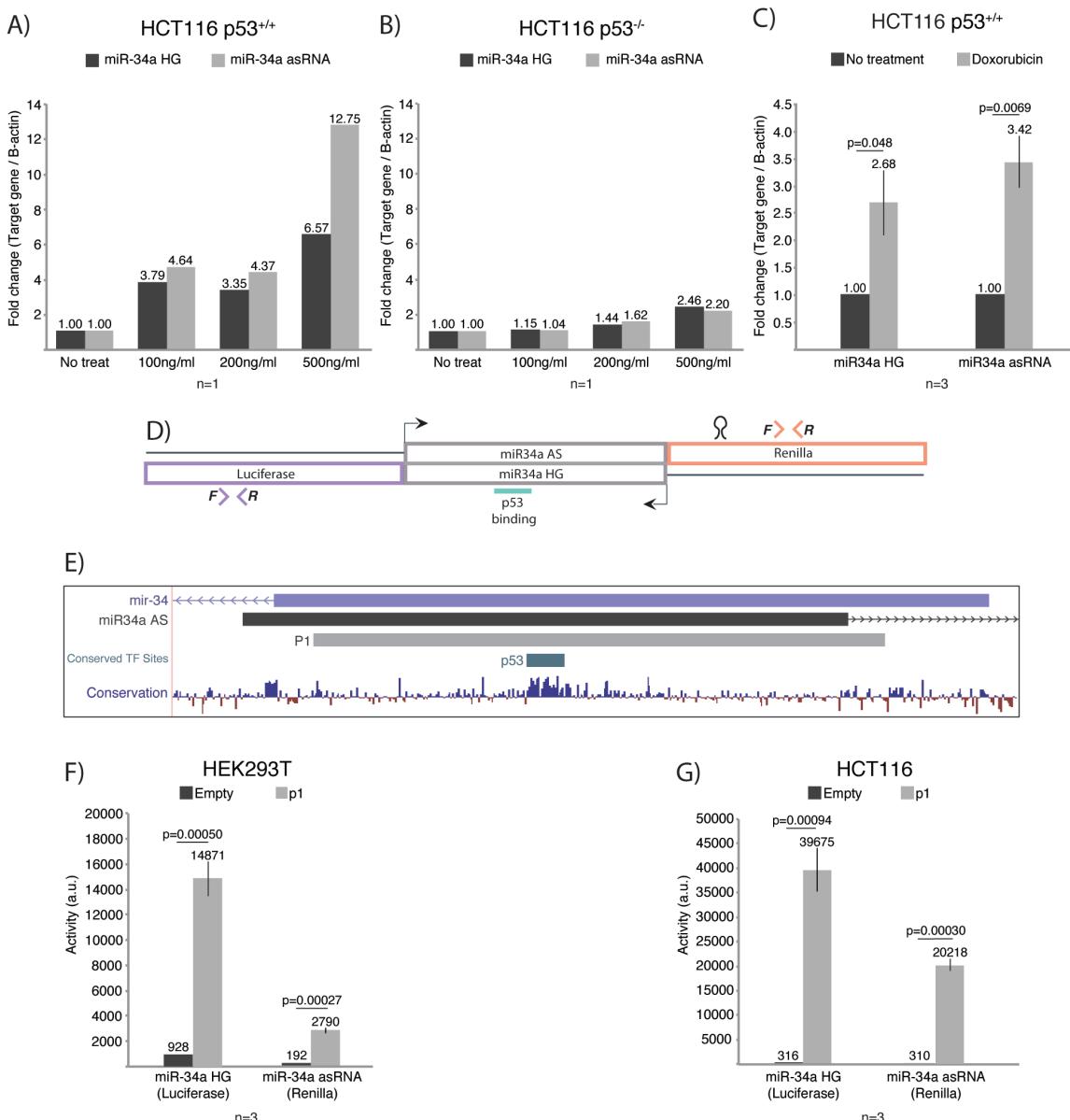
Figure 2

Figure 2: Regulation of the miR34a locus. Monitoring the effects of 25 hours doxorubicin treatment on miR34a asRNA and HG in P53^{+/+} (**A**) and p53^{-/-} (**B**) HCT116 cells. **C**) Three independent experiments evaluating the effects of 24 hours of treatment with 200 ng/ml doxorubicin on *miR34a* asRNA and HG. Error bars represent +/- s.e.m. **D**) A representative picture of the p1 construct including forward (F) and reverse (R) primer locations and the renilla shRNA targeting site. **E**) A UCSC genome browser illustration indicating the location of the promoter region cloned into the p1 construct including the conserved p53-binding site. **F**) Quantification of luciferase and renilla levels after transfection of HEK293T cells with the p1 construct. Error bars represent +/- s.e.m. **G**) Quantification of luciferase and renilla levels after transfection of HCT116 cells with the p1 construct. Error bars represent +/- s.e.m.

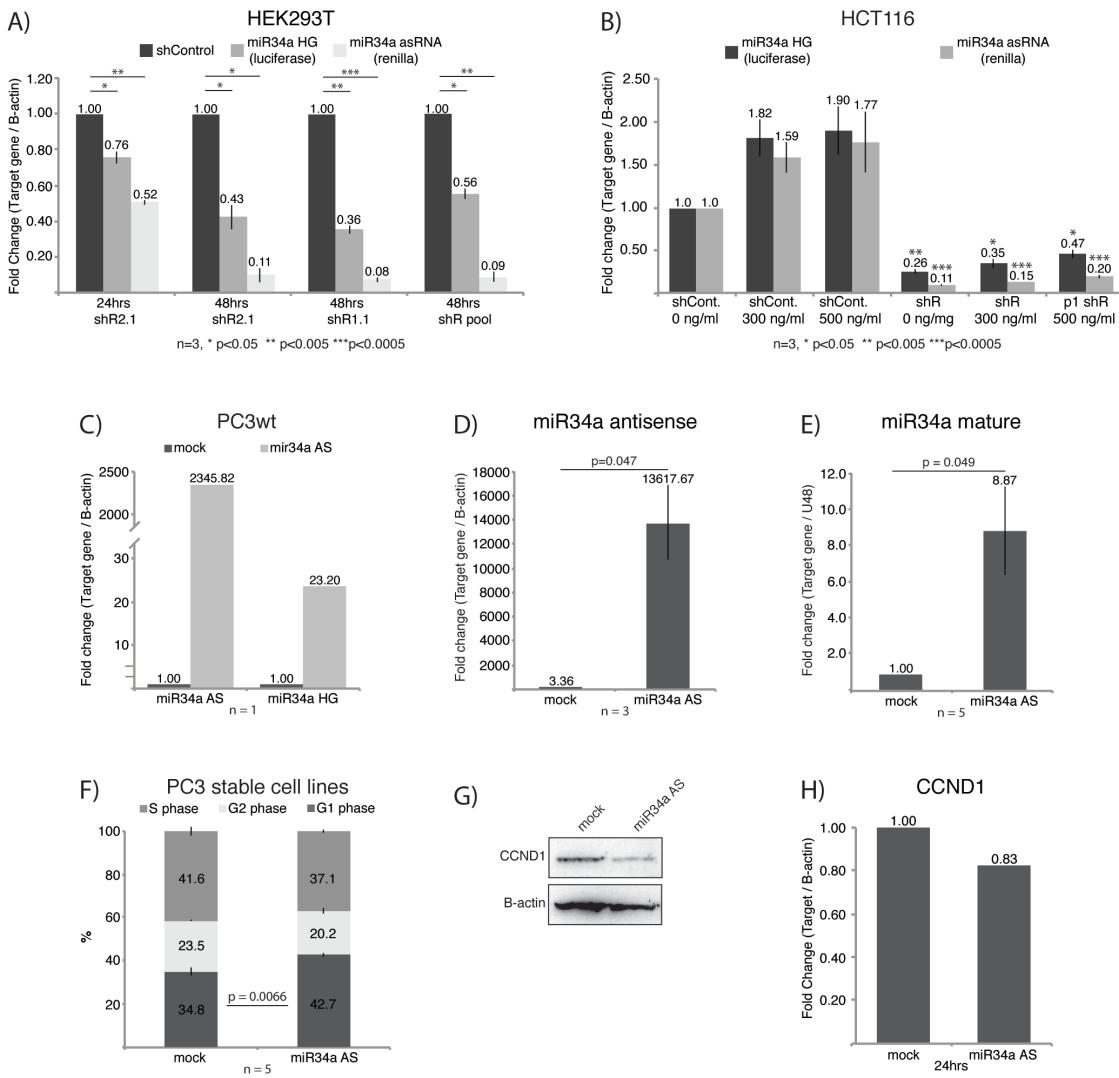
Figure 3

Figure 3: The function of miR34a asRNA. **A)** HEK293T cells which were co-transfected with the p1 construct and two different shRNAs targeting renilla and a combination (pool) of both. Renilla and luciferase levels were quantified with QPCR at 24 and 48 hour time points. Error bars represent +/- s.e.m. for three independent experiments. **B)** HCT116 cells were co-transfected with the p1 construct and shRNA 2.1 (shR) targeting renilla or an shRNA control (shCont.). 24 hours post-transfection doxorubicin treatment was initiated. After an additional 24hours, cells were harvested and renilla and luciferase levels were measured using QPCR. All statistical testing compared the shRNA control with shRNA renilla transfected cells using the respective treatment concentration. Error bars represent +/- s.e.m. for three independent experiments. **C)** Transient transfection of miR34a asRNA in *p53* null PC3 cells followed by QPCR detecting both *miR34a* HG and asRNA. **D)** Quantification of miR34a asRNA expression in stable PC3 cells. Error bars represent +/- s.e.m. for three independent experiments. **E)** Quantification of miR34a expression in stable PC3 cells. Error bars represent +/- s.e.m. for three independent experiments. **F)** Cell cycle analysis comparing stably overexpressing miR34a asRNA PC3 cells to mock expressing PC3 cells. Error bars represent +/- s.e.m. **G)** Western blot analysis of *CCND1*, a validated target of *miR34a*, in miR34a asRNA overexpressing PC3 stable cell lines. **H)** Quantification of the *CCND1* western blot.

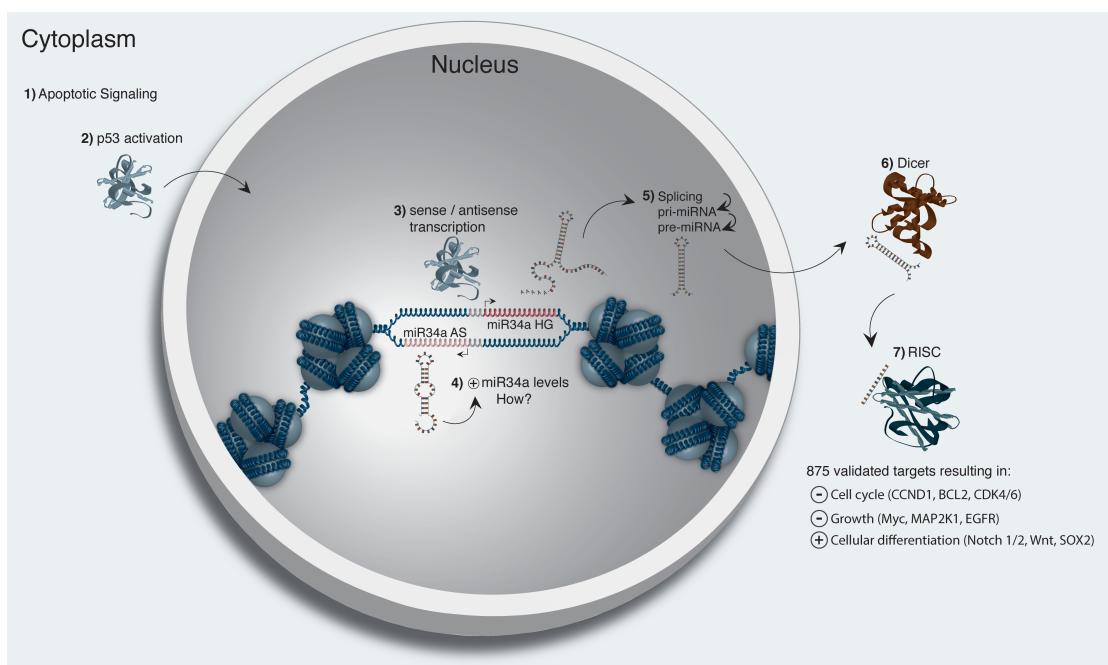
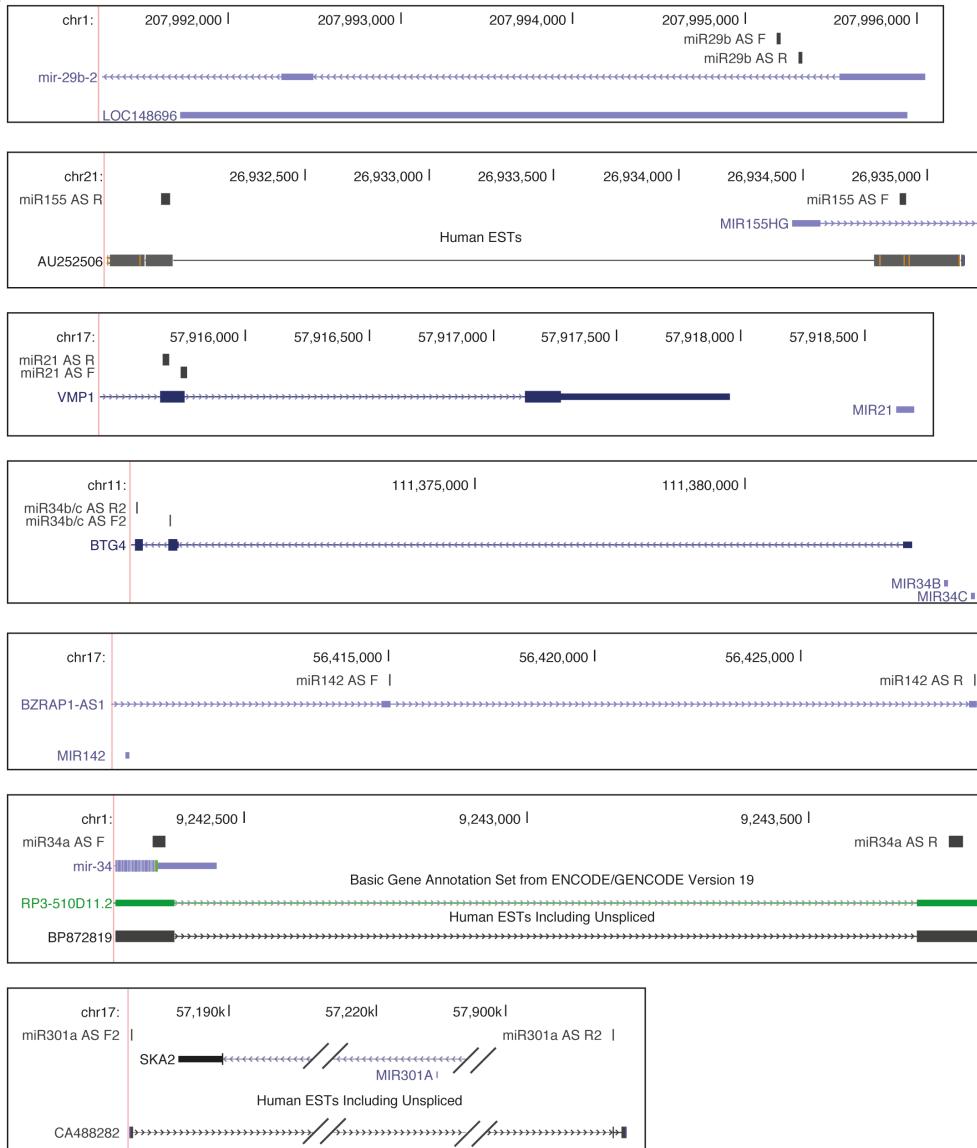
Figure 4

Figure 4: A graphical summary of the proposed miR34a asRNA function. Apoptotic signaling (1) leading to activation of *p53* (2) causes *p53* to migrate to the nucleus. *P53* is then capable of activating *miR34a* HG and asRNA transcription (3). *miR34a* asRNA is then capable of increasing *miR34a* HG levels (4) although whether this regulation is transcriptional or post-transcriptional is unclear. *miR34a* HG is then spliced, processed by the RNase III enzyme Drosophila, and exported to the cytoplasm (5). The *miR34a* pre-miRNA then binds to Dicer where the hair-pin loop is cleaved and mature *miR34a* is formed (6). Binding of the mature *miR34a* miRNA to the RISC complex then allows it to bind and repress its targets (7).

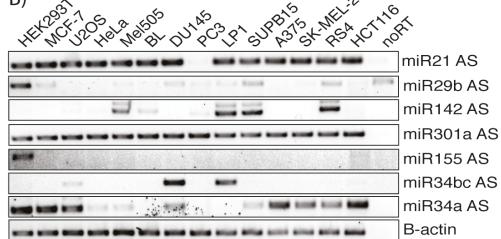
SUPPLEMENTAL FIGURES

Supplemental Figure 1

A)

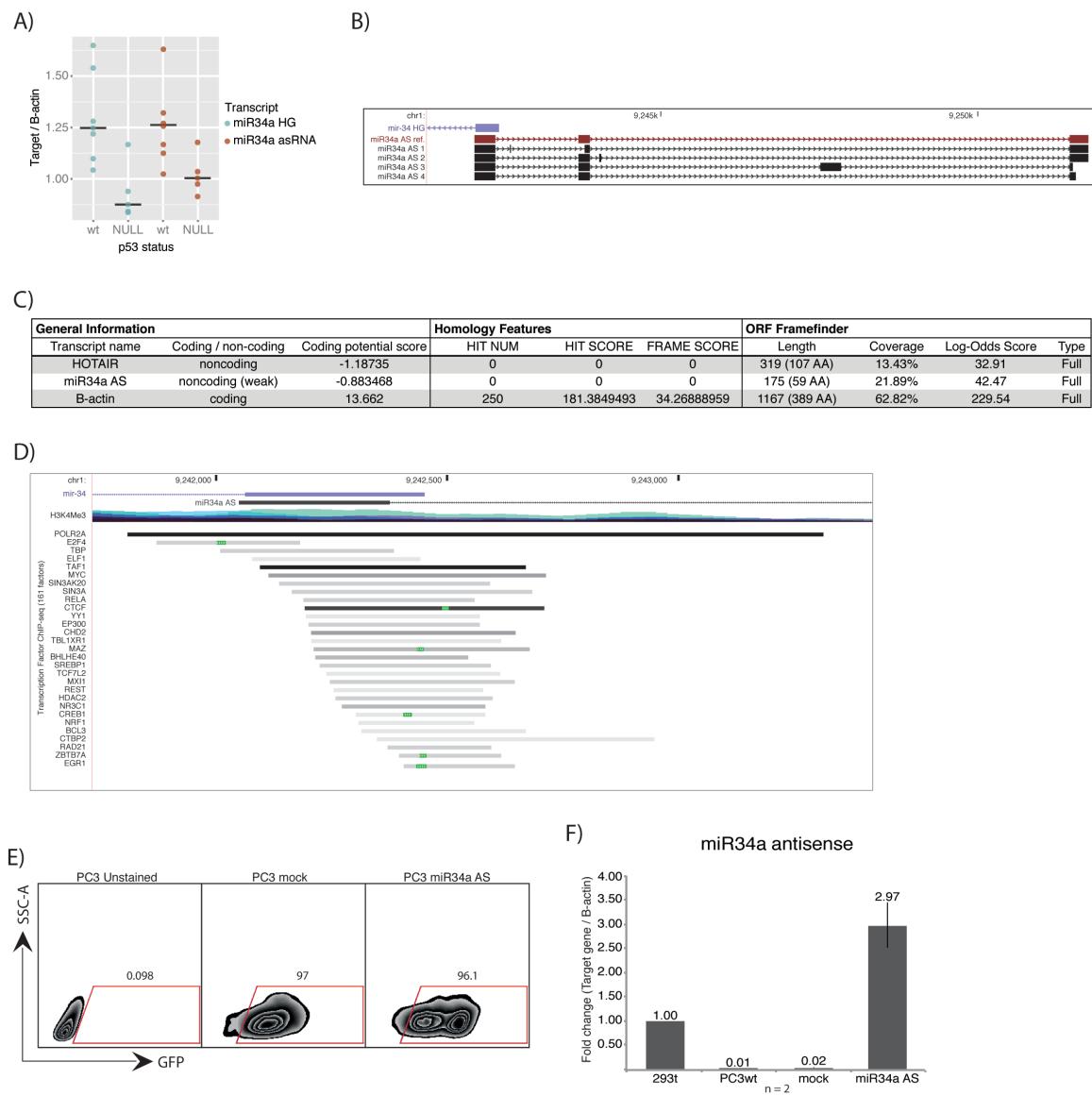


B)



Supplementary Figure 1: **A)** Bioinformatic analysis of asRNA transcription at the locus of the 7 miRNA candidates using the UCSC genome browser. The miRNA or miRNA HG, primers used in the subsequent PCR screening, and the amplified transcript (either UCSC/Gencode annotated or Human EST reads) are shown. **B)** Semi-quantitative PCR results from the screening of a panel of cell lines to directly detect candidate asRNA transcripts.

Supplemental Figure 2



Supplementary Figure 2: **A)** Quantification results from the cell line screening (figure 1b) showing the expression levels of *miR34a* HG and asRNA in *p53**wt* and *p53**-null* cell lines **B)** Sequencing results from the analysis of *miR34a* asRNA isoforms in U2OS cells. *miR34a* AS ref. refers to the full length transcript as defined by the 3'-RACE and primer walk assay. **C)** Analysis of coding potential of the *miR34a* asRNA transcript using the Coding-potential Calculator. **D)** Transcription factor binding at the *miR34a* promoter from the ENCODE project ChIP-seq data. The illustration was downloaded from the UCSC genome browser track Txn Factor ChIP. Histone ChIP-seq data for H3K4me3 is also shown to highlight the promoter region. **E)** FACS analysis of GFP expression in post-selection PC3 stable cell lines. **F)** Comparison of *miR34a* asRNA expression in HEK293T cells (high endogenous *miR34a* asRNA), PC3wt cells (low endogenous *miR34a* asRNA expression), and the stable PC3 mock and *miR34a* asRNA overexpressing cell lines.

Supplemental Table 1

transcript	primer name	sequence/cst.number	Fig. 1B	Fig. 1C	Fig. 1E	Fig. 1F	Fig. 1G	Fig. 2A	Fig. 2B	Fig. 2C	Fig. 3A	Fig. 3B	Fig. 3C	Fig. 3D	Fig. 3E	Supp. Fig.	Supp. Fig.
Other antisense primers	>miR155_asF	AAAC CTG GTC CAG GGC CAT TAT ACA															
	>miR155_asR	TGA CCT CAT GAT CCA GTC ACC TCA															
	>miR301a_asF2	CAA CGG CAG AAG CCT CAC CAC AAA															
	>miR301a_asR2	CGC TGC TGG ATT CCT TTG GCA TAA															
	>miR24bc_asF2	ACA GAA GTC ACT GGC ACT CTG A															
	>miR24bc_asR2	ACC CAT ATG GTC ATC TCC TTC GGA															
	>miR21_asF	AGT CCT ACC TGT GGT GTG CCC ATT															
	>miR21_asR	AGG TCC ATC TCT GCA GAA GCC ATT															
	>miR29b_asF	TGT AAT AAG AAA GGC GGT GCT GGT															
	>miR29b_asR	AAC CTG TCT TTG TCG CAG GTC TCA															
	>miR142_asRNA	TTG ATC GTC ATG CTG ACC CAG TGA															
	>miR142_asRNA	GGG CGG TCT TAC ATG CCT TTG ATT															
miR34a asRNA primers	>miR34a_asF10	ACG CGT CTG TCC ATG CGG CGG GGA T															
	>miR34a_asF10	ACG CGT CTG TCC ATG CGG CGG GGA T															
	>miR34a_asF11	ATC TGC CTG GTC ACC GAA AGG CA															
	>miR34a_asF12	AAA CAC AAG CGT TTA CCT GGG TGC															
	>miR34a_asF13	ACG GAG GCT ACA CAA TTG AAC AGG															
	>miR34a_asF14	AGG GAA GAA AGA ACT ATG CGA GCA															
	>miR34a_asF15	CAT TTG CTG CAA TAT CAC CGT GGC															
	>miR34a_as_int1R1	TGC CGA AAC TAG CGG CTGCT T															
	>miR34a_asF2	AAA CGA AAG GGG GGA TTG TCC TTG															
	>miR34a_asR2	ATA GGT TCA TTG CGG CGA TTG CGC															
	>miR34a_asF3	GGC ACT EGG AGA AGA CGA TTG TTT															
	>miR34a_asR3	CCA CAG CTG TTG CCT CTG AAT GCT															
	>miR34a_asEx3_R1	GTT GGC TTC TTC GCA ACT CCT TGT															
	>miR34a_asF1	AGC GGC ATC TCC ACC ATG TGA AA															
	>miR34a_asR1	TTG CCT CTG GAC TCC AAG GAG ATT															
miR34a HG primers	>miR34a_HG_F	TCT GCT CCA GTG GCT GAT GAG AAA															
	>miR34a_HG_R	GTT CAG CTG CCT CAA AGT TGG CAT															
	>miR34a_HG_F1	GGC AGT GTC TTA GCT GGT TGT TGT															
	>miR34a_HG_R1	TCT GGG CAT CTC TCG CTT CAT CTT															
miR34a mature primers	>miR34a_mature_F	Life Technologies Cat. # 4426961															
	>miR34a_mature_R	Life Technologies Cat. # 4426961															
Other primers	>B_gatII_F	AGG TCA TCA CCA TTG GCA ATG AG															
	>B_gatII_R	CTT TGC CGG TTG CCA CGT CA															
	>T7_leng_reverse	CAG TGA ATT GTA ATA CGA CTC ACT ATA GGG ACA TCC GTC GCT CGT CCA GGA CCC TTT TTT TTT TTT VN															
	>5AM_primer	CCG TAG CTG GTC CAG GAC CC															
	>U48_F	AGT GAT GAT GAC CGG AGG TA															
	>U48_R	GGT CAG AGC GCT GGG GTG AT															
	>Renilla_pBDir_F1	TAA CGC CGC CTC TTG TTA TT															
	>Renilla_pBDir_R1	GAT TTG CCT GAT TTG CCC ATA															
	>Luc_set_I_F	AAG ATT CAA AGT CGG CTG CTG															
	>Luc_set_I_R	TTG CCT GAT ACC TTG CAG ATG															

Supplementary Table 1: Primers used in this study.