

A piRNA utilizes HILI and HIWI2 mediated pathway to down-regulate ferritin heavy chain 1 mRNA in human somatic cells

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Received January 04, 2018; Revised July 27, 2018; Editorial Decision July 30, 2018; Accepted July 31, 2018

ABSTRACT

The piwi interacting RNAs (piRNAs) are small non-coding RNAs that specifically bind to the PIWI proteins, a functional requirement. The piRNAs regulate germline development, transposons control, and gene expression. However, piRNA-mediated post-transcriptional gene regulation in human somatic cells is not well understood. We discovered a human piRNA (piR-FTH1) which has a complementary sequence in the ferritin heavy chain 1 (*Fth1*) mRNA. We demonstrated that expression of piR-FTH1 and *Fth1* are inversely correlated in the tested tumor cell lines. We found that piR-FTH1 negatively regulates the *Fth1* expression at post-transcriptional level in triple negative breast cancer (TNBC) cells. Additionally, we confirmed that transfected piR-FTH1 knocks down the *Fth1* mRNA via the HIWI2 and HILI mediated mechanism. piR-FTH1 mediated *Fth1* repression also increased doxorubicin sensitivity by a remarkable 20-fold in TNBC cells. Since the current piRNA-mediated knockdowns of target mRNA are mostly reported in germ line cells, piRNA-mediated post-transcriptional gene regulation in somatic cells is rather unique in its application and mechanistically uses an alternative pathway to siRNA and miRNA. This work begins to lay the groundwork with a broader impact on treatment of various diseases that are linked to elevated levels of specific mRNAs which have a piRNA target.

INTRODUCTION

Numerous non-coding RNAs (ncRNAs) have been identified in the past few years and are mainly involved in regulation of gene expression (1). Small and long ncRNAs are the two major classes of ncRNAs. Among the small ncRNAs, there are three types of RNAs in eukaryotes: microRNAs (miRNA), PIWI interacting RNAs (piRNAs) and small interfering RNAs (siRNA) (1,2). PIWI-interacting RNAs

(piRNAs) are small RNAs which are defined by their ability to specifically bind to the PIWI proteins (3–5). The piRNAs are between 24 and 32 nucleotides long, prefer a 5'-uracil and contain a 3'-end ribose sugar that is 2'-*O*-methylated (6–8). They are generated from single-stranded precursor in a manner independent of RNase III enzymes (6,9).

The piRNAs act as sequence-specific guides for PIWI proteins to regulate the expression of transposons at both transcriptional and post-transcriptional levels in germline cells (4,5,10). piRNA-mediated transcriptional gene silencing had been studied in recent years and was found to be independent of PIWI slicing activity (6,11–15). Instead it leads to turning off the target gene expression through chromatin modification, such as, repressive histone marks and DNA methylations (16–19). In contrast, piRNA directed post-transcriptional gene silencing depends on sequence specificity and RNase H-like activity of PIWI proteins (12,15,20,21).

Post-transcriptional gene silencing by piRNA is mediated by two different mechanisms: (i) piRNAs target the transposon sequences found in the 3'-UTRs or 5'-UTRs of mRNAs (8,22–25) and (ii) if the piRNAs are derived from pseudogenes and antisense transcripts, transcribed from the opposite strand of the endogenous genes then that piRNA target the mRNA of the corresponding endogenous gene (10). The ping-pong cycle utilizes post-transcriptional gene silencing mechanisms to eliminate transposon mRNAs (6,13). In recent years, an increasing number of post-transcriptional gene silencing mechanisms for piRNAs beyond transposon silencing have been identified (10). It has been reported that piRNA mediates the decay of maternal *nanos* mRNA in *Drosophila* embryos through deadenylation (26). In the fly testis, *Su (ste)* pseudogene produces piRNAs which target *Stellate* mRNA for degradation (27–29). A unique one to one ping-pong piRNA system (*fem* piRNA-*Mas* mRNA) determines the sex in silkworms through post-transcriptional regulation (30).

All of the previous studies regarding the piRNA-mediated post-transcriptional gene silencing have been reported in germ line cells and adult testis (10). There is little known about target mRNA degradation by piRNAs in hu-

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man somatic cells. A few recent studies indicated the presence of PIWIs and their piRNA partners in somatic cells from lower eukaryotes to human (19,20,31,32). The PIWI–piRNA pathway plays diverse roles in soma including epigenetic regulation, transposons silencing, genome rearrangement and developmental regulations (19,32). The elevated expression of HIWI family (human PIWI homolog) proteins were detected in many human cancers (33,34). For example, it has been reported that HIWI2 (human PIWIL4) protein associates with the genomic tRNA cluster derived piRNAs in the MDA-MB-231 cells, a human Triple Negative Breast Cancer (TNBC) cell line (35). These previous reports indicated that an active PIWI–piRNA pathway is present in human somatic cells. Here, we report the identification of post-transcriptional regulation of *Fth1* mRNA by naturally occurring piRNA (piR-FTH1) in MDA-MB-231 cell lines, which is mediated by HIWI2 and HILI proteins. These findings indicated that piRNA can be involved at the level of post-transcriptional regulation that extends beyond the germ line cells, and this pathway can be harnessed to silence the expression of targeted genes.

MATERIALS AND METHODS

Preparation of oligonucleotide sequences

The 3'-end 2'-*O*-methylated RNA sequences (piR-FTH1 and scramble piR-FTH1) were purchased from Dharmacon, Inc. The non-methylated RNA sequences (piR-FTH1(NM) and scramble piR-FTH1(NM)) sequences were *in vitro* transcribed (36). All DNA oligonucleotides (Anti-piR-FTH1 and scramble Anti-piR-FTH1) were purchased from Integrated DNA Technologies (IDT). All RNA and DNA oligonucleotides sequence information is reported in supplementary information Table S1. Details of purification of the oligonucleotides is described in the supplementary methods section.

5'-end radiolabeling of RNA oligonucleotides

The 5'-terminal phosphates of transcribed RNAs were removed by treating with Calf intestinal alkaline phosphatase (New England Biolabs) for 45 min at 37°C. Then proteins were extracted by adding phenol chloroform, and RNAs were isolated and purified by ethanol precipitation. The RNAs purchased from company do not require removal of terminal phosphate group. The RNAs were 5'-end labeled using standard T4 Kinase labeling reaction. For details, see supplementary methods section.

Cell culture and transfection

MDA-MB-231 cells were grown in 96-well plates (for MTS assay) or six-well plates (for RT-qPCR and western blotting) in Dulbecco's modified Eagle's medium (DMEM) with high glucose supplemented with 10% fetal bovine serum and 1% antibiotics (streptomycin and penicillin) at 37°C in 5% CO₂ in a humidified incubator for 24 hrs. MCF-7, HEK293, A549, A2008, PC3, RF3 and Hela cell lines were maintained according to ATCC's recommendations in six-well plates.

All the oligonucleotides (RNA and DNA) were transfected with 0.5 μl of jetPRIME™ transfection reagent (Polyplus) for cells grown in 200 μl of medium (96 well plate) and 5 μl for cells grown in 2 ml of medium (six-well plate). For knockdown studies, siRNAs for HIWI (sc-40677), HILI (sc-62456), HIWI2 (sc-62458) and AGO2 (sc-44409) were purchased from Santa Cruz. The siRNAs were transfected with 5 μl of jetPRIME™ transfection reagent (Polyplus) for cells grown in six-well plates. The cells transfected with control siRNA (sc-44232) served as a control. The sequence information for siRNAs are included in supplementary information Table S2.

RT-qPCR assay

Total RNAs were isolated using E.Z.N.A.® MicroElute Total RNA Kit (Omega), and DNA was removed by on-membrane DNase I (Omega) digestion according to the manufacturer's protocol. Then 1 μg of RNA was used for cDNA synthesis. The cDNA was synthesized using qScript cDNA SuperMix (Quanta Biosciences) and oligo d(T) primer according to the manufacturer's protocol. The details of the cDNA synthesis and PCR reactions are provided in the supplementary methods section. The primers used are shown in the supplementary information Table S3. The relative fold change in expression was measured using the Lival method (37).

TaqMan small RNA assay

Total RNAs were isolated using E.Z.N.A.® MicroElute Total RNA Kit (Omega) according to the manufacturer's protocol. To quantify piRNAs (piR-FTH1 and piR-58320) and miRNA (miR-382) expression, the custom designed hydrolysis probes (for TaqMan™ small RNA assays) were purchased from Applied Biosystem, and RT-qPCR reactions were performed according to the manufacturer's protocol using U6 snoRNA as a reference. We followed their protocol with the minor modifications described in previous studies (38). Further details of the assay are provided in the supplementary method section.

Western blotting

Proteins were extracted from the cells with RIPA buffer (Santa Cruz) 24 hrs after transfection and 50 μg of protein lysate was separated by 15% SDS–PAGE. The proteins were detected by mouse monoclonal FTH1 antibody (B-12, sc-376594), rabbit polyclonal HIWI antibody (ab 12337), rabbit polyclonal HILI antibody (ab 181340), rabbit polyclonal HIWI2 antibody (ab180867), rabbit polyclonal AGO2 antibody (ab 226943) at 1:200 dilution and GAPDH (G-9, sc-365062) antibody at 1:5000 dilution respectively. Horseradish peroxidase-conjugated goat anti-mouse IgG (sc-2005) was used as the secondary antibody at 1:1000 dilutions for GAPDH and FTH1. Horseradish peroxidase-conjugated goat anti-rabbit IgG (STAR124P) was used as secondary antibody at 1:1000 dilutions for HIWI, HILI, HIWI2 and AGO2 detection. Proteins were visualized by Western Blotting Luminol Reagent (sc-2048) in ChemiDoc-ItTS2 Imager (UVP, LLC).

Periodate oxidation and alkaline β -elimination

Total small RNAs were isolated using mirVana™ miRNA Isolation Kit (Fisher) according to the manufacturer's protocol. Then two portions, each containing 50 μ g of RNA were dissolved in 87.5 μ l 0.06 M borate buffer (pH 8.6). Then 12.5 μ l of nuclease-free water (to control group) or 200 mM sodium periodate (to treatment group) were added into the reaction. Sodium periodate was freshly prepared before every treatment. Samples were incubated in the dark at room temperature for 1 hr. After the incubation, 10 μ l of glycerol was added and incubated for another 30 min to stop the reaction. For the control group, 12.5 μ l sodium periodate was incubated with glycerol for 60 min prior to adding to the samples to maintain the same ion strength between the control and treatment. Then RNA was precipitated by ethanol precipitation method. Precipitated RNA was dissolved in 100 μ l of 0.055 M borate buffer (pH 9.5) and incubated for 60 min at 45°C. RNA was then precipitated, washed, and used for TaqMan small RNA assay.

Immunoprecipitation

HEK-293 cells were used for checking the endogenous loading of piR-FTH1 into the HIWI protein. To check the association of synthetic piR-FTH1 with HIWI proteins, MDA-MB-231 cells were transfected with radiolabeled piR-FTH1, scramble piR-FTH1 and piR-FTH1(NM). After 24 hrs transfection, cells were harvested by trypsinization and resuspend in $1 \times$ PBS. Then 1 ml (5 mg) of cell extract was pre-cleared by incubation with 30 μ l of Protein A-agarose (Santa Cruz) at 4°C for 1 hr. Then HIWI2 and HILI complexes were immunopurified from the pre-cleared mixture using HIWI2 and HILI antibodies. The pre-cleared mixture incubated with mouse IgG and rabbit IgG acted as a control for immunoprecipitation. Reaction mixtures were rocked for overnight at 4°C. Then samples were incubated with protein A-agarose for 2 hrs at 4°C. Then the beads washed extensively with a washing buffer (150 mM KCl, 25 mM Tris pH 7.4, 5 mM EDTA, 0.5 mM DTT, 0.5% NP40, 100 U/ml RNAase inhibitor SUPERase•in™ and Protease inhibitor cocktail). RNAs were isolated from the immunoprecipitates (IPs) by resuspending the beads in TRIzol RNA extraction reagent according to manufacturer's instructions. The eluted RNA from HEK-293 cells IPs were subjected to TaqMan small RNA assay. The RNA eluted from MDA-MB-231 cells were separated on a 17% denaturing polyacrylamide gel, dried on Whatman paper, and exposed to a phosphorimager screen overnight. The gel images were obtained by scanning the screen on a Phosphorimager Typhoon FLA9500 (GE).

Cell viability assay

For drug sensitivity assay, MDA-MB-231 cells were plated at a density of 10000 cells per well in 96-wells plates and were transfected twice with 50 nM of piR-FTH1 and 50 nM scramble piR-FTH1. Cells were cultured for 24 hrs after the second piRNA treatment and were treated with doxorubicin at concentrations of 0, 10 nM, 25 nM, 50 nM, 100 nM, 250 nM, 500 nM, 1 μ M, 2.5 μ M, 5 μ M, 10 μ M and 20

μ M and incubated for 72 hrs. CellTiter 96 Aqueous Non-Radioactive Cell Assay (Promega) was used to assess the cell viability by measuring the absorbance at 490 nm, as per manufacturer's protocol. The cell viability percentages were calculated by normalizing to the untreated control cells. The IC₅₀ (inhibitory concentration to produce 50% cell death) values were determined by fitting the data in dose-response curves in Origin 8.

Immunocytochemistry

Coverslips were coated with 0.01% of ploy-L-lysine for 15 min at 37°C. Then coverslips were washed well with PBS buffer for three times and then washed with DMEM. The coverslips were placed in 6 well plates and MDA-MB-231 cells were grown on the coverslips. For FTH1 knockdown studies, cells were treated with piR-FTH1 and scramble piR-FTH1 and incubated for 24 hrs. But in case of the HIWI pathway determining experiments, cells were first treated with HIWI2 siRNAs and control siRNAs and then incubated for 24 hrs. The HIWI2 depleted cells and control cells were then treated with piR-FTH1 and scramble piR-FTH1. After 24 hrs of incubations, cells were fixed by incubating them with 4% paraformaldehyde at room temperature for 10 min. Then the cells were incubated with 0.1% Triton X-100 at room temperature for 15 min to increase the permeability. Next the cells were incubated with the staining buffer (1% FBS + 0.1% Tween 20 in PBS) for 1 hr at room temperature and then cells were incubated with either FTH1 primary antibody or both primary antibodies (anti mouse FTH1 antibody from Santa Cruz and anti-rabbit HIWI2 antibody from abcam) for overnight at 4°C. Then the cells were washed three times with the staining buffer and incubated with the secondary antibodies (anti-rabbit Alexa fluor 555 secondary antibody (ab 150074) and anti-mouse Alexa fluor 488 secondary antibody (ab 150117) at 1:500 dilutions for 1 hr at room temperature in the dark. The cells were washed with staining buffer for three times in the dark. Then coverslips were incubated with 0.1 μ g/ml DAPI for 1 min and coverslips were placed under Vectashield mounting media, sealed with clear nail polish, and kept refrigerated until microscopically imaged. Images were acquired using an Olympus 1 \times 81 confocal microscope.

Enzymatic stability of piRNAs

The radiolabeled piR-FTH1 and piR-FTH1(NM) were incubated in serum (Fetal Bovine Serum (FBS)) at 37°C. Then an aliquot of each sample was removed at different time points (0, 1, 2, 4, 8, 15, 30 min, 1, 2, 4, 8 and 24 hrs) and the reaction was stopped by the addition of an equal volume of stop buffer (7 M urea, 10 mM Tris-HCl, and 0.1 mM EDTA (pH 7.5)). Samples were electrophoresed on a 17% denaturing PAGE, dried on Whatman paper, and exposed to a phosphorimager screen overnight. The gel images were obtained by scanning the screen on a Phosphorimager Typhoon FLA9500 (GE).

Statistics

Each experiment was repeated at least three times and triplicates of each sample were tested. The results were indi-

cated as mean \pm standard error of the mean (SEM). Statistical mean differences were calculated by using two-tail *t*-test and *P* values were represented in figure legends.

RESULTS

piR-FTH1 level is inversely correlated to the *Fth1* mRNA level in human cancer cells

piRNAs are known to regulate the mRNA expression post transcriptionally in germline cells, adult testis and ovarian cells (10). Bioinformatics analysis has shown that approximately 28.5% of human mRNA sequences contain at least one retrotransposon sequence in their 3'-UTRs and those mRNAs can be regulated by piRNAs post-transcriptionally. In addition, some previous reports have indicated that piRNAs derived from pseudogenes and antisense transcripts of endogenous gene could regulate mRNA expression (10,22). Generally, a piRNA should have a minimum of 16–22 nt perfect match from the 5'-end of an mRNA which might be enough for PIWI/piRNA-mediated degradation of the mRNA targets in the cells (39). We have identified a naturally occurring piRNA (piR-FTH1) which has a base-pair complementarity (30 nt) in the coding region (CDS) of the *Fth1* mRNA (Figure 1A). The 30nt complementary region of piR-FTH1 spans between exon 1 and exon 2. This suggests that piR-FTH1 can only regulate the *Fth1* mRNA in post-transcriptional level but not at the transcriptional level. When we searched for the piR-FTH1 origin, we found that piR-FTH1 is a non-repeat piRNA molecule and this sequence matches in four different places within the genome. (chr13:23270583–23270613, chr3:72978292–72978322, chr5:17354403–17354433 and chrX:147133831–147133861). The piR-FTH1 sequence aligned in the intronic region in Chr 3 but in other chromosomes, piR-FTH1 sequence aligned with intergenic region. When we analyzed the Chr3 region, we found that piR-FTH1 sequence (reverse complementary sequence) completely aligned with the ferritin heavy chain 1 pseudogene 23 (FTHIP23) (40,41). Given that there are multiple sites of generation, we cannot exactly indicate if it is generated from one or more of the potential sites. However, it's likely that piR-FTH1 is generated from *Fth1* pseudogene, and regulates the *Fth1* protein coding gene expression as explained below. There are two different mechanistic ways the piRNAs can regulate the mRNA expression: (i) if the mRNA sequence contains the transposons insertion, those mRNA can be regulated by piRNA and (ii) if the piRNAs derived from pseudogenes and cis-natural antisense transcripts, they can regulate the expression of their endogenous gene. Since *Fth1* mRNA do not possess any transposon sequence in their CDS region, the possible mechanism should be that pseudogene (FTHIP23) derived piR-FTH1 regulates the expression of *Fth1* mRNA. This prediction would match well with previous studies in *Drosophila* (42,43). piR-FTH1-*Fth1* can be a model for regulation of protein-coding gene via pseudogene-derived piRNA in human somatic cells.

As mentioned piR-FTH1 has a base-pair complementarity (30 nt) in the coding region (CDS) of the *Fth1* mRNA.

This high level of base of complementarity observed suggests that the piR-FTH1 should be able to directly regulate *Fth1* mRNA function. To investigate whether piR-FTH1 mediated regulation of *Fth1* expression is a naturally occurring control mechanism in cells, we checked for the existence of any correlation between the expression levels of piR-FTH1 and *Fth1* mRNA in various human cell lines. Human cell lines (MDA-MB-231, A2008, A549, PC3, MCF-7 and HEK-293) were grown in 6 well plates and total RNA were isolated from cells. *Fth1* mRNA levels were quantified by RT-qPCR using specific set of primers. The *Gapdh* was used as a reference gene. The TaqMan small RNA assay was performed to measure the piR-FTH1 level with custom designed specific hydrolysis probes and using U6 snoRNA as a reference. In the tested human cancer cell lines (MDA-MB-231, A2008, A549, PC3 and MCF-7), piR-FTH1 was expressed at low levels but *Fth1* mRNA was overexpressed at significantly higher levels (Figure 1B and C). However, in case of the non-malignant human cell line HEK-293, both piR-FTH1 and *Fth1* RNAs were expressed at a level, which we considered as the basal level and were used to normalize those two RNA levels in the cancer cells that were used. We used another non-malignant human cell line RF3 (dermal fibroblast cells) to support that HEK-293 is showing 'basal level' of *Fth1* and piR-FTH1 expression (supplementary information Figure S1). In fact, among the tested human tumor cell lines, we found a high inverse correlation ($R^2 = 0.80$) between the piR-FTH1 and *Fth1* level (Figure 1D). These finding suggests that *Fth1* mRNA is most likely post-transcriptionally regulated by piR-FTH1. Then we investigated if the piR-FTH1 is indeed a piRNA. This piRNA (piR-FTH1) was first identified by high throughput RNA sequencing analysis from the short RNAs that were co-immunoprecipitated with PIWI (HIWI) protein from human testis sample (44). In order to confirm that the identified piR-FTH1 sequence is a real piRNA sequence, we performed the periodate oxidation and alkaline β elimination. The piRNAs have 2'-*O*-methylation, which are resistant to periodate-treatment and remain susceptible to TaqMan small RNA assay, whereas miRNAs and siRNAs contain a terminal 2',3'-hydroxyl group that are modified after the treatment. Periodate treatment therefore selectively enriches for piRNAs. To perform the periodate oxidation and alkaline β elimination, HEK-293 cells were grown in six-well plates and small RNAs were isolated from cells by using mirVana™ RNA Isolation Kit. The isolated RNA was divided into two portions. One portion subjected to periodate oxidation and alkaline β elimination while other portion used as a control. As shown in Figure 1E, we detected the piR-FTH1 and piR-58320 in both prior to periodate treatment and after the treatment in small RNAs isolated from HEK-293 cells and the level of both piRNAs was relatively similar. However, miRNA-382 level was significantly reduced after the periodate treatment and alkaline elimination compared to the level before the treatment. It should be noted that the RNA pool subjected to β -elimination will have an enrichment of RNAs which have protected 2'-OH in the 3'-end or other 3'-end modifications. In this study we used the miR-382 as our negative control and piR-58320 used as a positive control. This study confirmed that piR-FTH1 have 2'-*O*-methylation that make the piR-FTH1 re-

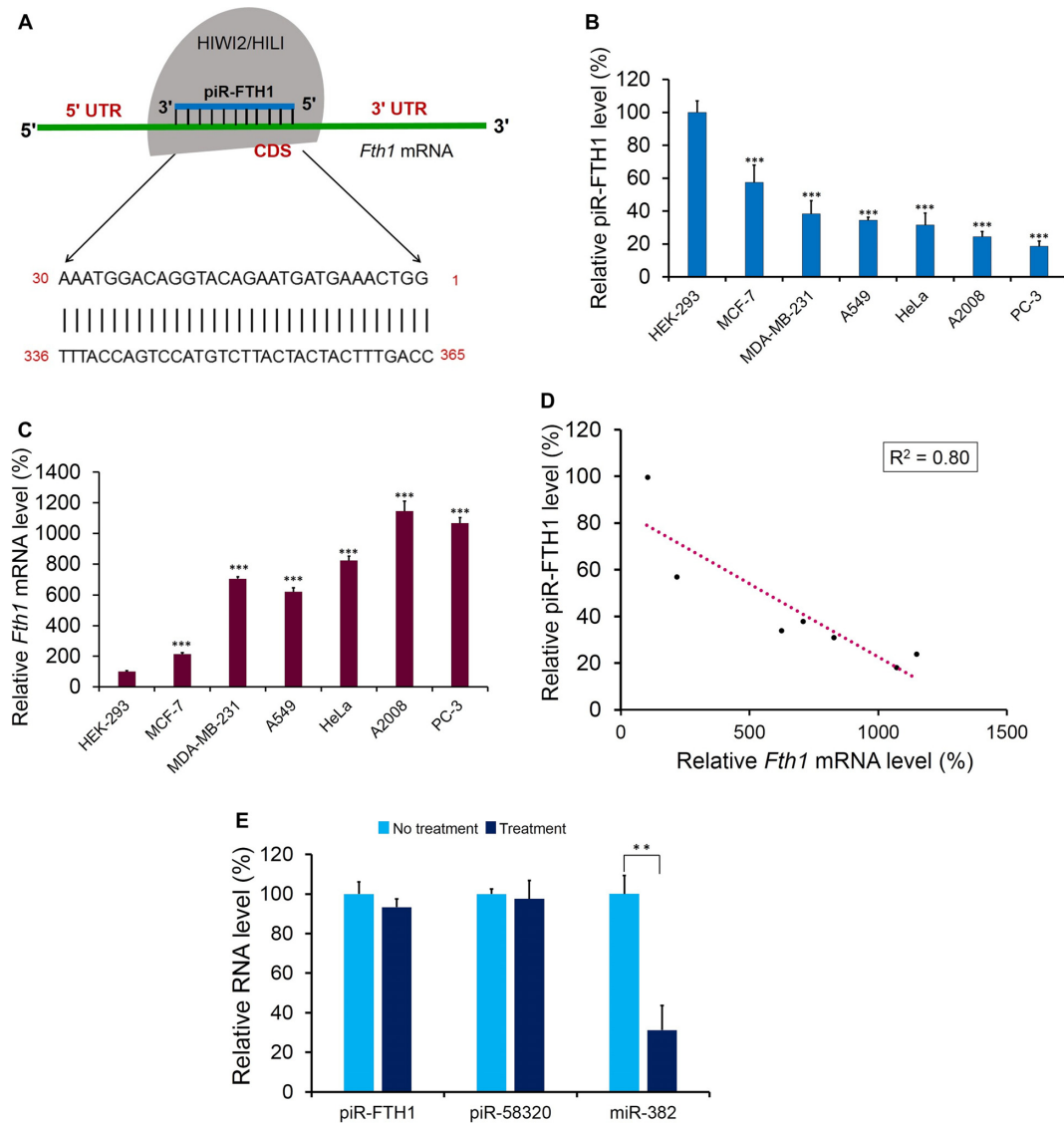


Figure 1. piR-FTH1 level inversely correlated to *Fth1* expression. (A) Schematic representation of piR-FTH1 binding in the coding region of *Fth1* mRNA. (B) Bar graph representing the relative *Fth1* mRNA level to *Gapdh* was generated by RT-qPCR. The MDA-MB-231, MCF-7, HEK-293, A549, A2008, PC3, and HeLa cell lines were grown in six-well plates and the isolated total RNA were subjected to RT-qPCR reaction with a specific set of primers. The level of *Fth1* in HEK-293 cells was considered as a basal level of expression and was used for normalization. The human cancer cells showed elevated levels of *Fth1* expression compared to HEK-293 cells. (C) The relative piR-FTH1 level in human cancer cells and non-cancer cells were measured by TaqMan small RNA assay using specific hydrolysis probes. The U6 snoRNA was used as a reference. The tested cancer cells showed reduced piR-FTH1 expression compared to non-cancer HEK-293 cells. (D) A plot representing the correlation between the expression of *Fth1* mRNA and piR-FTH1 level in the tested human cancer cells. (E) The relative level of piR-FTH1, piR-58320 and miR-382 were detected by TaqMan small RNA assay prior and after periodate oxidation and alkaline β -elimination. Total small RNA was isolated from HEK-293 cells and subjected to alkaline β -elimination followed by periodate oxidation. The piR-58320 was used as a positive control and miR-382 was used as a negative control. The presence of 3' end 2'-*O*-methylation in piR-FTH1 provide the resistance for periodate oxidation and alkaline β -elimination. The results are presented as the mean \pm SEM ($n = 3$) of three independent experiments. The statistical significance was calculated by *t*-test analysis (** $P < 0.01$; *** $P < 0.005$).

sistant to periodate oxidation and alkaline β elimination, clearly establishing that the sequence under investigation (piR-FTH1) is a truly a piRNA molecule and contains the 3'-end 2'-*O*-methylation.

Fth1 is a target of post-transcriptional repression by piR-FTH1

To test post-transcriptional regulation mediated by piR-FTH1, we decided to use the MDA-MB-231 cell line as

a model system because multiple studies have shown that *Fth1* is overexpressed in the triple negative breast cancer (MDA-MB-231) cells and that the overexpression led to a decrease in drug sensitivity (33,45). We treated the MDA-MB-231 cells with 50 nM of piR-FTH1 and scramble piR-FTH1. The cells treated with scramble piR-FTH1 acted as a control for this experiment. The level of synthetic piR-FTH1 after transfection was quantified by TaqMan small RNA assay. We observed that piR-FTH1 was increased 2.2-fold relative to endogenous piR-FTH1 after synthetic

piR-FTH1 transfection (supplementary information Figure S2). After 24 hrs of treatment with piR-FTH1 and scramble piR-FTH1, *Fth1* mRNA levels were quantified by RT-qPCR (Figure 2A). A significant (approximately 90%) amount of knockdown of *Fth1* mRNA level was observed with 50 nM piR-FTH1 treatment *in cellulo*. To identify the optimal time for silencing efficiency, MDA-MB-231 cells were treated with piR-FTH1 and the *Fth1* mRNA levels were quantified after 24, 48 and 72 hrs respectively. The *Fth1* mRNAs were depleted by almost 90% after 24 hrs of treatment and the knockdown effect lasted up to 72 hrs, the farthest time point measured (supplementary information Figure S3). The endogenous level of FTH1 protein was measured by western blotting after 24 hrs treatment with piR-FTH1 and scramble piR-FTH1 (Figure 2B) using GAPDH as a reference. Treatment with 50 nM piR-FTH1 showed 75% reduction in FTH1 level. The western blot was subjected to densitometric analysis by ImageJ software (supplementary information Figure S4). Down-regulation of FTH1 protein level was further evidenced by immunocytochemistry of the MDA-MB-231 cells with anti-FTH1 antibodies (Figure 2C). The piR-FTH1 treated cells showed clear loss in staining intensity when compared with control cells. Further immunocytochemical analyses showed the cytoplasmic localization of FTH1 in MDA-MB-231 cells. The mechanism of piR-FTH1 mediated down-regulation of *Fth1* can be due to mRNA degradation, translation arrest or a combination of both. However, the reduction in both FTH1 protein and *Fth1* mRNA level resulting from the piR-FTH1 treatment suggests that piR-FTH1 knocks down FTH1 level by degrading *Fth1* mRNA.

To further confirm the post-transcriptional gene silencing of *Fth1* mRNA by piR-FTH1, we inhibited the piR-FTH1 with an antisense molecule and measured the *Fth1* level in the cells. Our rationale was that if the antisense molecule blocked the activity of piR-FTH1, that would result in an increase in *Fth1* expression. We chose the HEK-293 cells for this experiment because HEK-293 cells express the piR-FTH1 at high level and *Fth1* at low level. We designed an antisense molecule for piR-FTH1 (anti-piR-FTH1) and a scramble version of anti-piR-FTH1 as a control for the experiment. The HEK-293 cells were grown in 6 well plates and treated with Anti-piR-FTH1 and scramble anti-piR-FTH1. After 24 hrs, total RNA was isolated from the cells and subjected to RT-qPCR and TaqMan small RNA assay to measure *Fth1* mRNA level and piR-FTH1 level respectively. As shown in Figure 2D and E, anti-piR-FTH1 treatment decreased the level of piR-FTH1 level and increased the *Fth1* level in HEK-293 cells compared to the control, which was treated with the scramble version of anti-piR-FTH1. This study clearly demonstrated the piR-FTH1 mediated post-transcriptional repression of *Fth1*.

piR-FTH1 functions in MDA-MB-231 cells is mediated by HILI and HIW12

The critical question we sought to address was if the piRNA-mediated knockdown that we observed followed a pathway that was different from the one used by miRNA and siRNA. The post-transcriptional regulation of mRNA by piRNAs involves endonucleolytic cleavage

of mRNA or induction of mRNA degradation after complementary base-pair recognition through the piRNA-induced silencing complexes (piRISC) (10). RISC is a multi-ribonucleoprotein complex and Argonaute protein is one of its key components (46,47). There are eight members of the Argonaute family proteins found in human and they are classified into two major subfamilies: one is the AGO subfamily and the other one is the PIWI subfamily (48–50). The PIWI subfamily consists of four types of proteins (PIWIL1/HIWI, PIWIL2/HILI, PIWIL3 and PIWIL4/HIW12) (49). It is known that miRNAs and siRNAs bind to the AGO subfamily of proteins while piRNAs bind to the PIWI family of proteins (2,51). To establish that the chemically synthesized piR-FTH1 that were transfected in the cells use the HIWI family proteins for their functional role, we first planned on knocking down the HIWI family proteins and then treat with the piR-FTH1 in HIWI depleted cell lines. We reasoned that if the piR-FTH1 activity is dependent upon a specific member of the HIWI protein family, then transfection of piR-FTH1 will diminish the down-regulation of *Fth1* mRNA due to lack of specific HIWI family protein member in the HIWI depleted cells. In this study we chose the non-methylated version of piR-FTH1 (piR-FTH1(NM)) as our control because the 3' end 2'-O-methylation is the only chemical characteristic feature that is present in the piRNAs and absent in other small non-coding RNAs. The MDA-MB-231 cells were treated with piR-FTH1(NM) and scramble piR-FTH1(NM). Then total RNA was isolated from cells after 24 hrs and subjected to RT-qPCR to measure the *Fth1* mRNA level. As shown in Figure 3A, the piR-FTH1(NM) treatment knocked down the *Fth1* mRNA approximately 62% and 90% with 50 nM and 300 nM respectively in the MDA-MB-231 cells. However, the complete knockdown of *Fth1* was observed with 50 nM piR-FTH1. We reasoned that the piR-FTH1(NM) very likely acted as an antisense molecule at high concentration (300 nM). This result clearly indicated the crucial role of methylation in piRNAs

In order to investigate the specific HIWI pathway, we systematically knocked down the HIWI, HILI, HIW12 and AGO2 proteins by transfecting the MDA-MB-231 cells with HIWI, HILI, HIW12, AGO2 and control siRNAs respectively. The cells that were treated with control siRNAs acted as a control for this experiment. The knockdowns were confirmed by RT-qPCR (Figure 3B) and western blotting (Figure 3C). Then the HIWI knocked down and AGO2 knocked down MDA-MB-231 cells were transfected with piR-FTH1, scramble piR-FTH1, piR-FTH1(NM) and scramble piR-FTH1(NM). Then total RNA was isolated from cells after 24 hrs and the relative level of *Fth1* mRNA was measured by RT-qPCR. Individual knockdown of the HIW12 and HILI depleted cells showed 45% and 37% of *Fth1* mRNA expression respectively after piR-FTH1 treatment (Figure 3D). However, knockdown of HIWI protein and AGO2 did not show a significant rescue effect on *Fth1* mRNA expression. However, the AGO2 depleted cells showed the 82% rescue of *Fth1* mRNA after piR-FTH1(NM) treatment, presumably due to the loss of AGO2 protein. Interestingly, the piR-FTH1(NM) treatment did not show significant rescue in *Fth1* mRNA expression in HIWI depleted cells. This study demonstrated that piR-

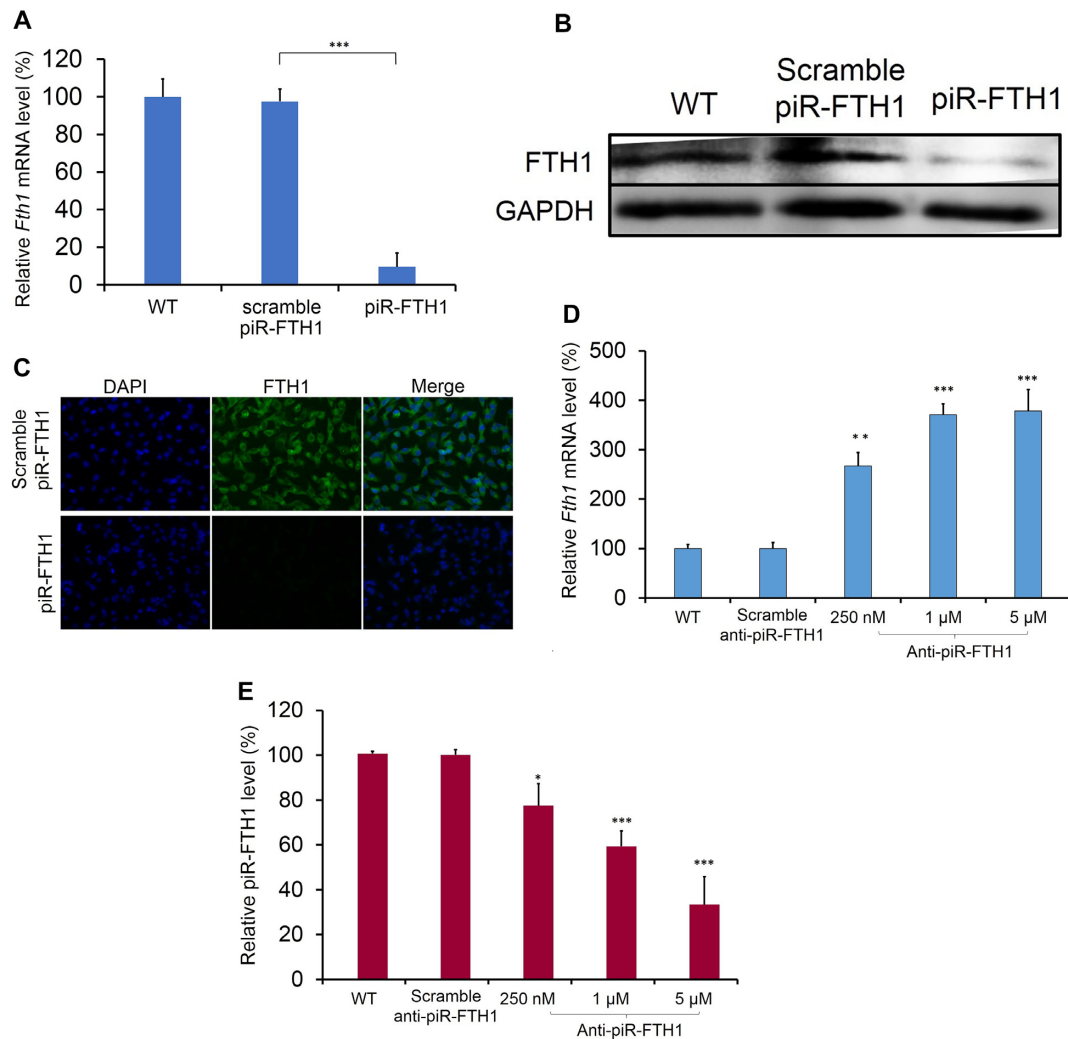


Figure 2. piR-FTH1 post-transcriptionally represses the *Fth1* mRNA. (A) The histogram representing the relative *Fth1* mRNA level to *Gapdh* mRNA 24 hrs after the piR-FTH1 and scramble piR-FTH1 transfection into MDA-MB-231 cells. The data were generated by RT-qPCR. WT represents wildtype without any treatment and control represents the cells treated with scramble piR-FTH1. The 50 nM piR-FTH1 treatment knocked down approximately 90% of *Fth1* mRNA level. (B) Western blot of the FTH1 protein expression level when MDA-MB-231 cells treated with piR-FTH1 and scramble piR-FTH1. The endogenous FTH1 and GAPDH level detected by anti-FTH1 antibody and anti-GAPDH antibody respectively. GAPDH used as a reference. (C) Immunocytochemistry of FTH1 in MDA-MB-231 cells with treatment showing cytoplasmic staining in the center panel, DAPI (4',6-diamidino-2-phenylindole) in the left panel and merged images in the right panel. The FTH1 knockdown with piR-FTH1 treatment confirmed by loss of signal in FTH1 panel. (D) The relative level of piR-FTH1 to U6 snoRNA were quantified by TaqMan small RNA assay after Anti-piR-FTH1 treatment in HEK-293 cells. Control cells were treated with scramble Anti-piR-FTH1. (E) The histogram representing the relative level of *Fth1* mRNA level to *Gapdh* after anti-piR-FTH1 and scramble anti-piR-FTH1 treatment in HEK-293 cells. The data were generated by RT-qPCR. The anti-piR-FTH1 treatment increased the *Fth1* mRNA level by inhibiting the piR-FTH1 function. The results are presented as the mean \pm SEM ($n = 3$) of three independent experiments. The statistical significance was calculated by *t*-test analysis. (* $P < 0.005$; ** $P < 0.01$, *** $P < 0.05$).

FTH1 specifically utilized the HIWI proteins for their function. In the HIWI knockdown studies, we did not check the functional important of PIWIL3 in silencing function. The PIWIL3 is only found in human and it is expressed only in oocytes (52). In addition, it is reported the PIWIL3 expression found in gastric cancer, but there is no supporting evidence to indicate that PIWIL3 is involved in the cancer progression (53). Since the knowledge regarding the PIWIL3 expression pattern and their functional importance are lacking, we did not test the PIWIL3 in our experiments.

To investigate the combined effect of HILI and HIWI2 on piR-FTH1 function, we performed a simultaneous double knockdown of HILI and HIWI2 to check if there was

a complete recovery of *Fth1* mRNA expression with piR-FTH1 treatment. Unfortunately, when we performed the double knockdown of HILI and HIWI2, it was lethal to the cells. We observed more than 75% cell death in the HILI and HIWI2 knockdown cells, although, we did not observe any detectable cell death in control siRNA treated cells under similar conditions. Mainly HIWI family proteins were overexpressed in breast cancer tissues and breast cancer cell lines and the depletion of HILI and HIWI2 can activate apoptosis and lead to cell death (45). Because high level of the double knockdown was lethal, we reasoned that a partial knockdown may not lead to cell death but still allow us to detect *Fth1* mRNA rescue. Thus, we performed a partial

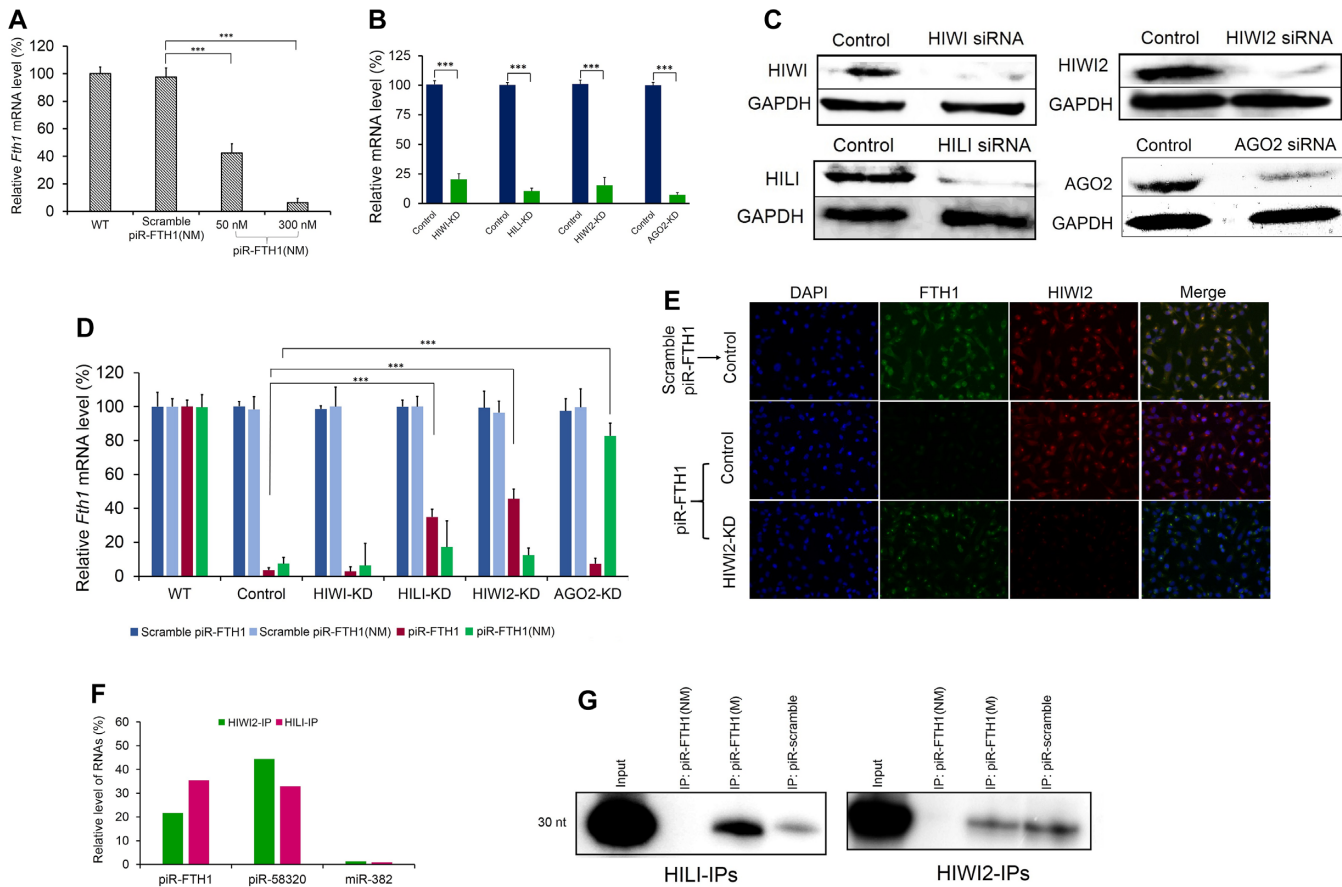


Figure 3. piR-FTH1 associates with HIWI and HILI and utilizes the HIWI pathway for its silencing function. (A) The relative level of *Fth1* mRNA level to *Gapdh* 24 hrs after piR-FTH1(NM) treatment in MDA-MB-231 cells was quantified by RT-qPCR. The cells treated with scramble piR-FTH1(NM) act as a control for this experiment. (B) Histogram representing the relative *Hiwi*, *Hili*, *Hiwi2* and *Ago2* mRNA levels to *Gapdh* mRNA 24 hrs after the HIWI, HILI and HIWI2 and AGO2 siRNA treatment were generated by RT-qPCR. The control cells were treated with control siRNAs. (C) Western blot analysis confirmed HIWI, HILI, HIWI2 and AGO2 knockdown after transfection with HIWI, HILI, HIWI2 and AGO2 siRNAs in MDA-MB-231 cell lines. (D) Histogram representing the relative *Fth1* mRNA level to *Gapdh* mRNA 24 hrs after the piR-FTH1(50 nM) and piR-FTH1(NM) (300 nM) transfection in the HIWI, HILI, HIWI2 and AGO2 depleted MDA-MB-231 cell lines were generated by RT-qPCR. The cells treated with scramble piR-FTH1(50 nM) and scramble piR-FTH1(NM) (300 nM) considered as a control for piR-FTH1 and piR-FTH1(NM) respectively. The HILI and HIWI2 knockdown rescued the *Fth1* mRNA expression after piR-FTH1 confirmed the HIWI family protein dependent functional role of piR-FTH1. (E) Immunocytochemistry of FTH1 in control and HIWI2 depleted MDA-MB-231 cell, DAPI (4',6-diamidino-2-phenylindole) in the first panel from left, FTH1 stained with green in the second panel from left, HIWI2 stained with red in the third panel from left and merged in the right panel. The presence of clear FTH1 staining signal in HIWI2 depleted cells after piR-FTH1 treatment confirmed the importance of HIWI2 in piR-FTH1 function. (F) The relative piR-FTH1 and piR-58320 in HILI and HIWI2 immunoprecipitated RNA from HEK-293 cells was quantified by TaqMan small RNA assay. The levels of piR-FTH1, piR-58320 and miR-382 were normalized to exogenous piR-48164. 10 pmol of a known synthetic piRNA (piR-48164) was added to the IP as an exogenous RNA control. The levels of piR-FTH1, piR-58320 and miR-382 in total RNA pool were used as controls to calculate the relative amount of those RNA level in the IPs. This was done by considering each of the values of piR-FTH1, piR-58320 and miR-382 in total RNA pool as 1 and normalizing the measured amount of those RNAs in the IP samples to calculate the relative percentage of each of the RNAs. The piR-58230 was used as a positive control and miR-382 was used as a negative control for this experiment. The endogenous piR-FTH1 loaded into HILI and HIWI2 proteins. (G) The synthetically made transfected piR-FTH1 and scramble piR-FTH1 were detected in HILI (left panel) and HIWI2 (right panel) IPs from MDA-MB-231 cell lysate. piR-FTH1(NM) served as a negative control. The input denotes the amount of radiolabeled RNAs (piR-FTH1, scramble piR-FTH1 and piR-FTH1(NM)) were transfected into the cells. The results are presented as the mean \pm SEM ($n = 3$) of three independent experiments. The statistical significance was calculated by *t*-test analysis. (***) $P < 0.005$.

knockdown of both HILI and HIWI (less than 50%) by reducing the amount of siRNA used during the knockdown assay and observed 18% rescue of *Fth1* mRNA level after piR-FTH1 treatment and did not detect any significant cell death in the partially HILI and HIWI2 depleted cells (supplementary information Figure S5).

The immunocytochemistry staining performed with anti-HIWI2 and anti-FTH1 antibodies further supported that the HIWI2 is important for piR-FTH1 function. As shown

in Figure 3E, HIWI2-KD (HIWI2-knockdown) cell lines showed clear loss of staining intensity for HIWI2 protein compared to the control. This observation further confirmed the knockdown of HIWI2. A clear signal for FTH1 protein in HIWI2-KD cell lines compared to control even after piR-FTH1 treatment demonstrated the significant role of HIWI2 in piR-FTH1 function. Moreover, we observed the cytoplasmic localization of HIWI2 proteins by immunocytochemistry. Post-transcriptional gene silencing happens

in the cytoplasmic region of the cells, and thus for the knockdown of the *Fth1* mRNA, HILI and HIWI2 are expected to be in the cytoplasmic space. Generally, HIWI and HILI reside in the cytoplasmic region while HIWI2 is present in the nucleus (31,54,54,55). But a recent study reported that HIWI2 is highly expressed in breast cancer tissues and is in the cytoplasmic region of MDA-MD-231 cells (35) which supports our observation as well as the role of HIWI2 in piR-FTH1 functions.

To confirm that piR-FTH1 physically interacts with HILI and HIWI2 protein, we performed the immunoprecipitation using HILI or HIWI2 specific antibodies. To establish that endogenous piR-FTH1 associated with HIWI proteins, we chose the HEK-293 cells due to the high level of piR-FTH1 expression. HEK-293 cells were grown in 6 well plates and whole cell lysate was isolated from cells. Then HIWI2 and HILI proteins were immunoprecipitated with HIWI2 and HILI antibodies. Following that co-immunoprecipitated RNAs were released from proteins and subjected to TaqMan small RNA assay. As shown in Figure 3F, we observed the loading of endogenous piR-FTH1 into HILI and HIWI2. We observed the loading of piR-58320 (a positive control) into HILI and HIWI2 proteins as well. However, we did not detect the miR-382 (a negative control) in HILI-IPs and HIWI2-IPs. In order to establish that transfected synthetic piR-FTH1 also interacts with HIWI family proteins, we radiolabeled the synthetic piR-FTH1 to distinguish the endogenous piR-FTH1 from synthetic piR-FTH1. In this experiment we used the scramble piR-FTH1 as a positive control due to the presence of methylation and piR-FTH1(NM) as a negative control. Then labelled piR-FTH1, scramble piR-FTH1 and piR-FTH1(NM) were transfected into the MDA-MB-231 cells and endogenous HIWI2 and HILI was immunoprecipitated with anti-HILI and anti-HIWI2 antibodies. As shown in Figure 3G, the co-immunoprecipitated labelled piR-FTH1 was detected in anti-HILI and anti-HIWI2 IPs. We used the mouse IgG and Rabbit IgG as our negative control for immunoprecipitation experiment (supplementary information Figure S6). We observed a small presence of the transfected and the scramble piR-FTH1 in the anti-HILI and anti-HIWI2 pool which might be due to the presence of 3' end 2'-O-methylation in scramble piR-FTH1, allowing it to be recognized by HIWI family proteins as a piRNA molecule. Importantly, transfected piR-FTH1(NM) did not show any signal in anti-HILI and anti-HIWI2 IPs.

The results indicated that endogenous piR-FTH1 is bound by HILI and HIWI2 proteins and regulates the expression of *Fth1*. Therefore, we reasoned that if we knockdown the HIWI family proteins that should result in increased expression of *Fth1*. To test this, we performed HIWI family protein (HIWI, HILI and HIWI2) knockdown experiments by transfecting the siRNAs for HIWI, HILI and HIWI2 respectively. The control cells were treated with control siRNAs. Twenty-four hours after transfection, total RNAs were isolated from cells and *Fth1* mRNA level were quantified by RT-qPCR in HIWI, HILI and HIWI2 depleted HEK-293 cells. We observed that HIWI2 knockdown increased the *Fth1* mRNA level 1.3-fold compared to control (supplementary information Figure S7). However, we did not observe any significant change in *Fth1* mRNA level

Table 1. The IC₅₀ values of doxorubicin for piR-FTH1 treated MDA-MB-231 cells and scramble piR-FTH1 treated MDA-MB-231 cells

	IC ₅₀ (nM)
WT	618.05 ± 5.23
Scramble piR-FTH1 treated cells	480.28 ± 8.47
piR-FTH1 treated cells	25.36 ± 3.18

after the HILI knockdown. It might be due to the fact that *Fth1* expression is controlled in the cells by other factors, such as, miR-200, which has been reported to control the *Fth1* expression in order to maintain the proper level of *Fth1* in the cells (56). Overall, our studies indicate that HIWI2 and HILI are the two major HIWI family proteins which are important for piR-FTH1 functions, suggesting that piR-FTH1 likely forms piRISC via binding with HILI or HIWI2.

Up regulation of piR-FTH1 in MDA-MB-231 cells increases sensitivity to chemotherapy

Ferritin is an iron storage protein and a critical component for iron homeostasis. It is composed of two subunits, the ferritin light chain (FTL, L-subunit, 19 kDa) and the ferritin heavy chain (FTH1, H-subunit 21 kDa) (57,58). FTH1 has ferroxidase activity, and it is essential for iron uptake (59). Ferritin is differentially over-expressed in multiple malignancies, including, breast cancer, hepatocellular carcinoma, lymphoma, and pancreatic cancers (60–62). FTH1 plays a protective role against the chemotherapeutic reagents, and can protect both normal and cancer cells (62,63). It has been suggested that the targeted repression of ferritin may be a useful strategy to overcome drug resistance in cancer chemotherapy. Therefore, ferritin down-regulation can potentially enhance the impact of chemotherapeutic agents for cancer treatment. In the present study we discovered that FTH1 expression can be repressed by piR-FTH1 treatment in the MDA-MB-231 TNBC cells. To assess whether piR-FTH1 induced down-regulation of FTH1 is associated with increased sensitivity to chemotherapy, a cell viability assay (MTS assay) was performed on the MDA-MB-231 TNBC cells. First, MDA-MD-231 cells were treated with piR-FTH1 twice and the designated MDA-MB-231 cells were treated with the control scramble piR-FTH1 twice. Subsequently, both group of cells were treated with increasing concentrations of the chemotherapeutic agent doxorubicin. The piR-FTH1 treated cells showed the IC₅₀ value of 25.36 ± 3.18 nM, while the scramble piR-FTH1 transfected cells showed an IC₅₀ value of 480.28 ± 8.47 nM (Table 1). The piRNA-mediated down-regulation of FTH1 increased the drug (doxorubicin) sensitivity by a remarkable 20-fold compared to the control sequence treated cells (Figure 4). Given Doxorubicin's known dose dependent cardiotoxicity a 20-fold improvement in IC₅₀ is highly significant for its more consistent use as a chemotherapeutic.

The presence of 3'-end methylation in piRNAs increases its stability in serum.

The instability of siRNAs and miRNAs under *in vivo* physiological conditions has been considered as one of the main

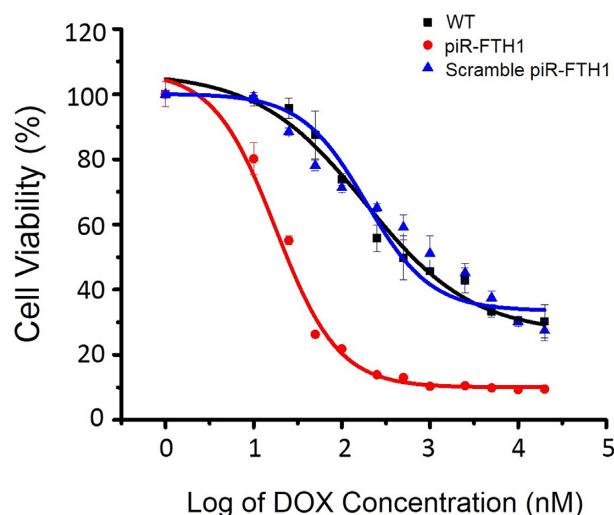


Figure 4. Cell viability assay showed that treatment of piR-FTH1 significantly reduces the IC₅₀ of doxorubicin for MDA-MB-231 cells. The cells treated with scramble piR-FTH1 act as a control for this experiment. The results are presented as the mean \pm SEM ($n = 9$) of three independent experiments with triplicate in each.

problems for its therapeutic applications. It has been reported that chemical modification of specific nucleotide motifs improves the resistance against nucleases (64,65). Since the Ribose 2'-OH group is not essential for recognition or mRNA cleavage process by activated RNAi machinery, it is the most common site for modification (66). The most common 2'-OH chemical modification is the substitution of 2'-OH with other chemical group such as 2'-*O*-methyl, 2'-F, and 2'-H (42,64,65). It has been reported that 2'-*O*-methyl modification increases the resistance against enzymatic digestion and thermal stability (67). Given that the naturally occurring piRNAs are known to contain the 3'-end 2'-*O*-methyl modification, it provides them with an inherent advantage regarding their biological stability. To evaluate the biological stability of piRNAs, we performed a degradation assay in the presence of fetal bovine serum (FBS). We tested a piR-FTH1 sequence containing a 3'-end 2'-*O*-methyl modification, while the piR-FTH1(NM) sequence did not contain any modification. The piR-FTH1 and piR-FTH1(NM) were incubated in FBS solution at 37°C and aliquots were collected at different time intervals. As shown in Figure 5A, piR-FTH1(NM) (right panel) was completely degraded after 30 min incubation with serum. In contrast, 2'-*O*-methyl modification was able to protect piR-FTH1 for at least 8 hrs of incubation with serum (left panel), showing that the 2'-*O*-methyl group in piRNAs can protect against serum RNases. We calculated the amount of parent band present in the different time intervals by quantifying its band intensity by the ImageJ software. Then we calculated the half-life of these RNA in the serum by fitting the amount of RNA vs. time in the decay curve. We observed that piR-FTH1 have higher half-life of 316 min compared to piR-FTH1(NM) (half-life of \sim 12 min) (Figure 5B). The improvement of >25-fold in the inherent stability of piR-FTH1 in serum can be beneficial for using as a therapeutic molecule in controlling gene expression.

DISCUSSION

The piRNA directs post-transcriptional gene silencing in human somatic cells

In this work, we report that a chemically synthesized naturally occurring piRNA sequence can efficiently down-regulate a target gene in human somatic cells. piRNAs are involved in the post-transcriptional regulation of retrotransposons, mRNAs and viral RNAs (10). The previous studies regarding the piRNA directed post-transcriptional regulation were mostly observed in germline cells, adult testis and ovaries (26–28). For example, *Su(Ste)* locus related piRNA degrades the *Stellate* mRNA in fly testis and in silkworm sex determination role of *fem* piRNA by down-regulating *Masc* mRNA are only reported in germline cells (26–28). An important unaddressed question regarding the piRNA-mediated regulation is if the piRNAs have a role in post-transcriptional gene silencing in human somatic cells. In the present study, we identified a naturally occurring piRNA sequence (piR-FTH1), which has an exact complementarity to a site within the CDS of *Fth1* mRNA that most likely is produced from pseudogene FTH1 (FTH1P23). The piR-FTH1 and *Fth1* can be a model system for regulation of protein-coding gene via pseudogene-derived piRNA in human somatic cells. When we investigated the piR-FTH1 and the target gene *Fth1* as a model system, our data clearly showed that piR-FTH1 repressed the expression of *Fth1* mRNA post-transcriptionally in the MDA-MD-231 TNBC cells. Our RT-qPCR data and western blotting indicated that piR-FTH1 treatment in MDA-MB-231 cells reduce the amount of *Fth1* mRNA as well as FTH1 protein level. This observation implied that piR-FTH1 regulated the *Fth1* mRNA level most likely by degrading the mRNA and not through translation repression. The piRNA-mediated mRNA degradation can occur via two different mechanisms, either through the slicing of mRNA by PIWI or via a deadenylation mechanism. Most of the previous studies reported that piRNAs directed mRNA elimination is promoted by a deadenylase-dependent deadenylation mechanism (26,68). However, a recent reporter based assay demonstrated mRNA degradation by slicing activity of MIWI in mouse testis (39). Therefore, piR-FTH1 degrades the *Fth1* mRNA either through the slicer activity of HIWI or by a deadenylation mechanism, but further experimentation will be needed to clearly delineate the exact and detailed mechanism of such a degradation process.

Use of HIWI mediated pathway makes the function of human piRNA mechanistically distinct

The small non-coding RNAs (siRNAs, miRNAs and piRNAs) associate with different types of AGO family partners for their gene silencing function (1,48). A major question we intended to address in our study was if human piRNAs specifically use the HIWI pathway for their function even though there is an active AGO pathway in the cells which is utilized by miRNAs and siRNAs. Our HIWI protein depletion studies clearly demonstrated that piR-FTH1 specifically utilized the HIWI pathway and not an AGO pathway for their functional role. In addition, the immunoprecipitation studies demonstrated that both endogenous piR-

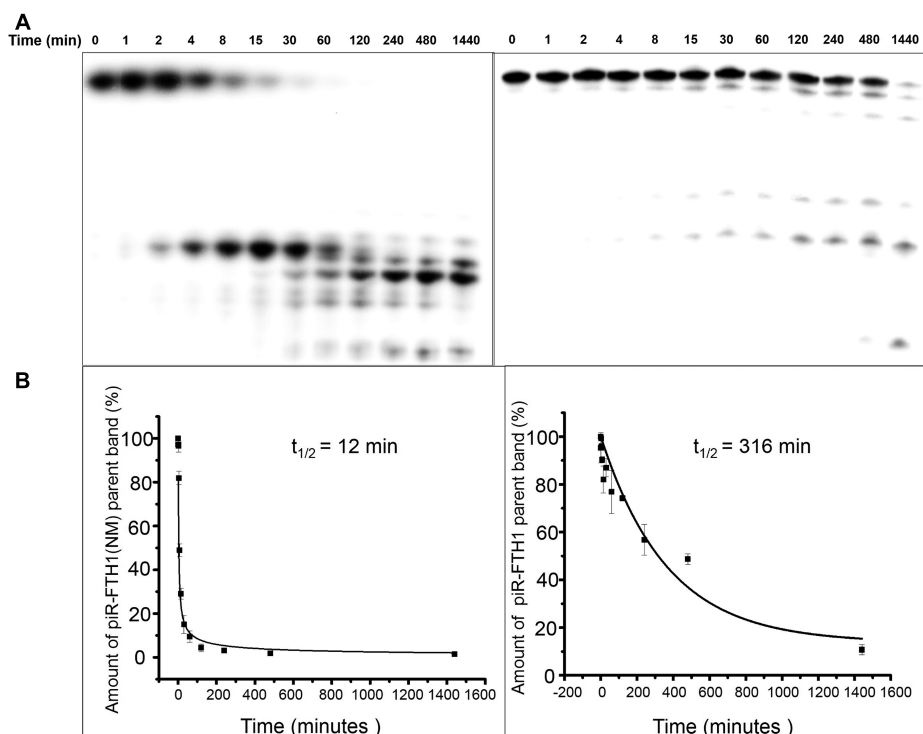


Figure 5. piR-FTH1 show higher stability in serum. (A) The gel picture representing the stability of piR-FTH1(right panel) and piR-FTH1(NM) (left panel) in the serum with respect to time. The piR-FTH1 lasted for longer time (8 hrs) compare to piR-FTH1(NM). (B) The plots representing the decay of piR-FTH1 (right panel) and piR-FTH1 (NM) (left panel) in the serum. ImageJ software was used to measure the intensity of the parent band signal and was normalized to the amount of parent band at time point $t = 0$. The half-life time ($t_{1/2}$) was estimated by fitting decay curve in Origin. piR-FTH1 have higher half-life time compared to piR-FTH1(NM) due to the presence of 3' end 2'-*O*-methylation.

FTH1 and synthetic piR-FTH1 load onto the HIWI2 and HILI proteins. It has been shown that human PIWI proteins (HIWI, HILI, PIWIL3 and HIWI2) have similar sequence amongst their PAZ domain but they differ in their binding region with the AGO-PAZ domain (69). Moreover AGO-PAZ bind with ssRNAs as well as ds-RNA containing 2 nt overhangs and ending in 2'-OH at their 3'-end, whereas HIWI-PAZ target ss-RNAs which have 2'-*O*-methylation at their 3'-end due to hydrophobic nature of its binding pocket (70–73). The presence of 3'-end methylation in the synthetically made piR-FTH1 and its single stranded nature allow the piR-FTH1 to act as a target for HIWI-PAZ domains but not for AGO-PAZ domain. However, piR-FTH1(NM) utilize the AGO2 not HIWI family protein for their silencing function due to lack of 3'-end methylation in piR-FTH1(NM). In addition, our systematic HILI, HIWI, and HIWI2 depletion studies revealed that piR-FTH1 specifically selected HILI and HIWI2 as distinct partners for its functional role. This is an interesting finding because presumably piRNAs specifically use their distinct HIWI partners for their function role, unlike miRNAs and siRNAs, as mammalian AGO family proteins do not differentiate between siRNA and miRNA duplexes and both of these duplexes can be loaded onto all four AGO proteins (74). The privileged selection of HILI and HIWI2 by piR-FTH1 most likely is due to the characteristic features of the piRNA sequence. PIWI (homologue to HIWI) bound piRNA sequences showed pronounced tendency to start with 1 uracil (5' U bias) while AGO3 (homologue to HIWI2) associ-

ated piRNA sequences have an adenine at 10th position and no 5' bias (21,72,75). The HIWI binding prefers U at the 5' end, however, the presence of a 5'-guanine nucleotide in piR-FTH1 most likely prevented its binding to HIWI, while presence of the 10th adenine, a ping-pong signature allowed binding with the HILI and HIWI2. Moreover, based on the sequence nature of piR-FTH1, it appears that piR-FTH1 mostly generate from secondary piRNA biogenesis or phased piRNA biogenesis. Because piRNAs produced from primary piRNA biogenesis have 5'-end uracil but the piRNAs generated from secondary piRNA biogenesis have partly bias towards the adenine at 10th positions (3). The piR-FTH1 does not contain uracil at the 5'-end but it has adenine at the 10th position suggesting that it is not produced from primary biogenesis but rather produced by secondary piRNA biogenesis or phased piRNA biogenesis. Based on our experimental evidences we can conclude that synthetic piRNA can utilize HIWI proteins because of the presence of 3'-end methylation that allowed it to be distinguish by the HIWI from AGO and that the 5'-end nucleotide and 10th position adenine nucleotide selected its distinct partners from the HIWI family, namely HIWI2 and HILI. The unique nature of piRNA-HIWI loading may have an advantage in RNAi technology. For example, the HIWI family proteins are differentially expressed in several human cancers and associated with cancer progression (34). We can design synthetic piRNA molecule to target the oncogenic gene depending on the overexpression of one or

more of the HIWI family protein members in that particular cancer.

Repression of the *Fth1* mRNA expression established the therapeutic potential of piR-FTH1

FTH1 is overexpressed in a variety of cancers and is also known to act as an antioxidant (59–62). Because chemotherapeutic drugs such as doxorubicin induce apoptosis by forming reactive oxygen species (ROS) via redox activation, FTH1's elevated level of activity can mute ROS-mediated function of doxorubicin (63,76). Since, FTH1 has ferroxidase activity it converts Fe^{2+} to Fe^{3+} and thus acts against the function of doxorubicin and other ROS producing drug (77). This is suggested to lead to desensitization of the cancer cells against many chemotherapeutic agents. To increase the efficacy of chemotherapeutic molecules, siRNAs and miRNAs have been used in several cancers to knockdown the *Fth1* gene (62,78). In our study, we determined in a set of human cancer cell lines a concomitant down-regulation of piR-FTH1 and overexpression of *Fth1* mRNA. Thus, it is tempting to propose that *Fth1* overexpression is linked to the very small amount of piR-FTH1 expression in the tested human cancer cells. When we used the piR-FTH1 to knockdown the *Fth1* mRNA, our data clearly showed that piR-FTH1-directed down-regulation of *Fth1* mRNA in the TNBC MDA-MB-231 cells increased the doxorubicin sensitivity by 20-fold compared to the control. The major concern using high amounts of chemotherapeutic molecule is associated adverse side effects. Therefore, the increased efficacy of chemotherapeutic molecules at significantly lower concentration may have a beneficial effect towards the cardiotoxicity. Therefore, inhibition of the *Fth1* mRNA by piR-FTH1 should be a viable strategy for improving the efficacy of other anticancer drugs against not only breast cancer but possibly in other cancers as well. One of the potential limitations in using the siRNA and miRNA in therapy is their rapid degradation by nucleases, which resulted in the development of a variety of chemical modifications of the siRNAs and miRNAs to increase their biological stability (79). Interestingly, our stability assay indicated that the presence of 3'-end methylation in piR-FTH1, which is an inherent feature of the piRNAs significantly improved its stability in serum. Moreover, it is reported that 2'-*O*-methyl can be helpful in minimizing the immune response by acting as a competitive inhibitor of TLR7 without losing its gene silencing activity, and thus the naturally occurring 2'-*O*-methyl in piRNA should not elicit any immune response (80). Like miRNAs, we showed that the synthetic version of piRNAs can achieve the biological function mimicking that of the endogenous piRNA. Thus, all the positive characteristic features in piRNA make them a suitable candidate for therapeutic use and provide a viable alternative to siRNA and miRNA down-regulation of gene expression.

Finally, with the high conservation of potential piRNA target sites among placental mammals compared to the surrounding sequences, it can be theorized that piRNA-mediated post-transcriptional regulation might be an evolutionarily conserved process (39,81). Therefore, it is important to study the physiological role of piRNAs' mechanistic relevance when piRNA expression is dysregulated in tumors

and other diseases. Additionally, it is clear that our findings will be useful in developing therapeutic applications of piRNAs.

CONCLUSION

In the present study we report the post-transcriptional regulation of a target gene by piRNAs in somatic cells. We showed that piR-FTH1 down-regulate the *Fth1* mRNA in MDA-MB-231 cells post-transcriptionally. Although, there are many RNA-binding proteins in the cells to bind with small non-coding RNAs and to form RNA induced silencing complex, HIWI knockdown studies distinguished that piR-FTH1 use specific member of HIWI family proteins (HILI and HIWI2) for their function. Also, both exogenous and endogenous piR-FTH1 loads onto HIWI2 and HILI. Therefore, piR-FTH1 is a natural antisense for *Fth1* mRNA that could be used for therapy of FTH1-overexpressing tumors to repress the FTH1 level and increase the chemotherapeutic efficacy. Since the current mRNA knockdown strategies mainly focus on siRNAs and miRNAs, this strategy establishes a novel method for successful down-regulation of mRNAs by a different class of small RNA using a distinctly different mechanism which can be selectively beneficial in certain cancers and perhaps other diseases.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

S. Balaratnam acknowledges the supports from GSS, Kent State University. This work was partially supported by Kent State University.

FUNDING

Funding for open access charge: Kent State University.
Conflict of interest statement. None declared.

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