

# An Lnc RNA (GAS5)/SnoRNA-derived piRNA induces activation of TRAIL gene by site-specifically recruiting MLL/COMPASS-like complexes

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## ABSTRACT

**PIWI-interacting RNA (piRNA) silences the transposons in germlines or induces epigenetic modifications in the invertebrates. However, its function in the mammalian somatic cells remains unknown. Here we demonstrate that a piRNA derived from Growth Arrest Specific 5, a tumor-suppressive long non-coding RNA, potentially upregulates the transcription of tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), a proapoptotic protein, by inducing H3K4 methylation/H3K27 demethylation. Interestingly, the PIWI1/4 proteins, which bind with this piRNA, directly interact with WDR5, resulting in a site-specific recruitment of the hCOMPASS-like complexes containing at least MLL3 and UTX (KDM6A). We have indicated a novel pathway for piRNAs to specially activate gene expression. Given that MLL3 or UTX are frequently mutated in various tumors, the piRNA/MLL3/UTX complex mediates the induction of TRAIL, and consequently leads to the inhibition of tumor growth.**

## INTRODUCTION

PIWI-interacting RNAs (piRNAs) are a distinct class of 26–31 nucleotide (nt) single-strand non-coding RNAs. They were initially identified as an inhibitor for the transposable elements (TEs) in the germline of invertebrates and mammals and play an important role in maintaining the

genomic stability (1,2). The precursor of piRNA in the germline is a single-strand RNA produced from particular intergenic repetitive elements known as piRNA clusters or piRNA loci and then processed into mature piRNAs through a ‘Ping-Pong’ amplification cycle which is mainly completed by AUB and Ago3 (3). The biogenesis of piRNAs and inhibition of TEs simultaneously take place during the cycle. The biogenesis of piRNAs is dicer-independent and the piRNAs precursors are not double-strand RNAs or hairpin structures, which is different from that for microRNA (miRNA) and small interfering RNA (siRNA). Conventional piRNAs that are loaded into PIWI proteins can regulate the target RNA to silence its expression by the base-pairing recognition.

PIWI is a subgroup of Argonaute protein family which can directly bind small RNAs and plays an important role in RNA-induced gene-silencing processes. Four PIWI proteins, including PIWI1/2/3/4, have been identified in human cells (4). The PIWI proteins are expressed predominantly in the germline and have been shown to participate in self-renewal, development and transposons silencing (5,6). However, PIWI proteins have also been found in various human somatic cells (7,8). Recent studies have reported that piRNA expression can be found in the ovarian somatic cells and neuron cells in invertebrates, as well as in many other mammalian somatic cells (9–12), which greatly expands the scope of piRNA existence. The function analysis indicates that piRNAs regulate the expression of target genes across many dimensions. Primarily, piRNA is shown to cleave the target RNA and mediate its degradation (3). Nevertheless, it has been shown that piRNAs induce de

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novo DNA methylation both in the testis of mice and in the neurons of *aplysia* (10,13). PiRNAs also promote the heterochromatin formation through regulating the trimethylation of H3K9 and are able to transmit this epigenetic silencing memory from one generation to the next (14,15). In most cases, piRNA plays a role in suppressing gene expression for genome stability. However, it has also been revealed that piRNA can activate the gene expression through the globally epigenetic regulation *in cis* in the subtelomeric heterochromatin of *Drosophila* (16). Although this report has found that piRNA upregulates H3K4me3 and inhibits H3K27me3, it also shows that the H3K9me3 and other histone modifications, such as histone acetylation, are also changed. Further, the molecular mechanism of how the piRNA mediates the recruitment of various complexes for epigenetic activation remained uncertain.

Breast cancer is one of the most common cancers worldwide. Although at present surgery and chemotherapy are able to help majority of the patients reach remission and have significantly increased the survival rate, breast cancer is still a severe threat to women and leads to a low life quality. Therefore, identification of more targets for the prevention and treatment of breast cancer is still a major challenge. For further understanding the pathogenesis of breast cancer, we performed a deep sequence analysis in search of more functional small non-coding RNAs. We found that there was a distribution in the 26–30 nts, which is distinctive from the peak of miRNAs in the 21–24 nts. Our further identification indicates that this group of small RNAs is of the characteristics of piRNAs. Some of these piRNAs were generated from snoRNAs or the long non-coding RNA (lncRNA). Although the expressions of piRNAs in mammalian somatic cells including cancer cells have also been reported (17,18), the function mechanism of piRNAs in these cells has not yet been elucidated. In this report, we have identified a novel pathway for piRNA to affect the epigenetic modifications of histone. As a result, one of snoRNA-derived piRNAs specifically upregulated the expression of tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), an important ligand for Death Receptor 4 (DR4) and Death Receptor 5 (DR5) could induce apoptosis and inhibit the growth of cancer cells.

## MATERIALS AND METHODS

### The collection of breast cancer samples

This research was approved by the Ethics Review Board of Sun Yat-Sen University and the Ethics Review Board of The First Affiliated Hospital of Sun Yat-Sen University. The written informed consent was provided by study participants and/or their legal guardians.

Paired tumor and adjacent normal breast epithelial tissues were collected from a total of 29 female patients with breast carcinoma from The First Affiliated Hospital of Sun Yat-Sen University, and subsequently frozen and stored in liquid nitrogen. None of the patient received preoperative neoadjuvant chemotherapy or radiotherapy, or bared other diagnosed cancer. All cases were diagnosed at stage I–II with histological confirmation. The basic characteristics of patient donors are outlined in Supplementary Table S1. All

patients were clinically free of overt metastasis at the time of tissue collection.

### Small RNA deep sequencing and annotation; microarray analysis

The malignant breast epithelial tissues were collected as described above. The RNAs extracted from three donors were mixed with equal amount (10 µg per donor) by adjusting RNA to the same concentration. Small RNA fraction of 18–40 nts in length from the total RNA was prepared and sequenced by Beijing Genomics Institute (Shenzhen, China).

Gene expression profiles were analysed with the Whole Human Genome Microarray (Human Genome U133 Plus 2.0, Affymetrix). Data normalization and analysis were processed by DAVID Gene ID Conversion Tool (<http://david.niaid.nih.gov>).

### Cell culture, transfection, plasmid and cell-line construction

Human MCF7 cells and HEK293T cells were obtained from ATCC and grown at 37°C in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal calf serum (Invitrogen), 100 units/ml of penicillin and 100 µg/ml of streptomycin (Invitrogen). Lipofectamine 2000 (Invitrogen) and Lipofectamine RNAiMAX (Invitrogen) were used for transfection of plasmids or small RNAs respectively by following the instruction of manufacturer. The peripheral blood mononuclear cells (PBMCs) were isolated from healthy human donors through Ficoll gradient centrifugation, followed by culturing in the RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 100 units/ml of penicillin and 100 µg/ml of streptomycin (Invitrogen).

The templates of clones were from genomic DNA or cDNA of MCF7 cells and were used to amplify the target genes by PCR. The PCR fragments were digested by restriction enzymes and then inserted into pcDNA3.1 vectors. The accuracy of all the clones was confirmed by DNA sequencing. The primers and the sites for restriction enzymes were listed in the Supplementary Table S2.

To construct the cell lines of piRNA overexpression, MCF7 and MDA-MB-231 cells were infected by the recombinant lentiviruses which expressed pi-sno75 because of the insertion of SNORD75 precursor at the downstream of CMV promoter in pHIV-cPPT-GFP, a lentiviral vector.

### RNA extraction and quantitative RT-PCR analysis

The total RNAs from cells or clinical tissues were extracted by Trizol (Invitrogen) according to the instructions of manufacturer. Reverse transcription (RT) reactions were conducted with oligodT or specific primers (Supplementary Table S2) using PrimeScript RT reagent Kit (TAKARA). piRNA quantitative detection was performed according to method of stem-loop PCR, which was primarily used for small RNA detection, such as miRNA. And the qRT-PCR products for piRNA were confirmed by DNA sequencing. Quantitative real-time PCR assay was then performed according to the instruction of manufacturer (SYBR Pre-

mix ExTaq, TaKaRa) on a CFX96 Real-Time System (Bio-Rad). Primers for RT-qPCR were listed in Supplementary Table S2. Human Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as internal controls.

#### Northern blot and western blot

Northern blot with locked nucleic acid probes labeled by [ $\gamma$ - $^{32}$ P] ATP was conducted to determine piRNAs and their precursors as described previously (19). The blotting membranes were then exposed to a phosphorimager for overnight and scanned with a Typhoon Trio plus scanner (GE Healthcare). The probe sequences were listed in Supplementary Table S2.

Western blot was conducted to analyse the expression of proteins as described previously (20). Rabbit anti-TRAIL antibody (CST) and rabbit anti- $\beta$ -actin (CST) were used as primary antibodies. The horse radish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody was used as the secondary antibody, followed by ECL detecting and exposing to X-ray film for visualization.

#### RT at low dNTP concentrations followed by PCR

The RT at low dNTP concentrations followed by PCR (RTL-P) method is used for the detection of 2'-O-methylated sites in the 3'-end of RNA (21). For the detection of the modification of pi-snoRNAs, the total RNA was isolated using the Trizol reagent (Ambion) and 500 ng RNA was ligated to a 3' RNA adapter using T4 RNA ligase (Takara). The ligation product was then reverse-transcribed using a low (0.4  $\mu$ M) or high (40  $\mu$ M) dNTP concentrations with or without anchored RT primers that were designed to anchor the modified nucleotide. The complementary DNA (cDNA) was subsequently amplified by PCR with specific primers under the same reaction conditions. The PCR products were then equally loaded and separated on 1.5% agarose gels, stained with GelRed dye (Biotium) and visualized by UV. It is notable that the RT-PCR product from the 3' modification of piRNA was low when the RT reaction was done with an unanchored RT primer at a low concentration of dNTPs (0.4  $\mu$ M). Otherwise, there was no differences in the product despite of the dNTP concentration or primers (anchored or unanchored) used in the RT reaction (20).

#### RNA immunoprecipitation

To detect whether the small RNAs were associated with the PIWI proteins, PBMCs were homogenized using immunoprecipitation (IP) lysis buffer plus RNase inhibitor RNaseOUT (40 units/ml, Invitrogen). The mixtures were then incubated with anti-PIWIL1 (SAB4200365, Sigma), anti-PIWIL4 (Abcam), or pre-immune IgG antibody and ProteinA/G beads overnight at 4°C. After centrifugation and then washing three times, the RNA-protein complex-associated beads were dissolved in 1 ml of Trizol (Invitrogen), and RNA was extracted as described above, followed by qRT-PCR analysis.

#### Chromatin immunoprecipitation assay

To detect the interaction between the DNA and proteins, Chromatin immunoprecipitation (ChIP) experiment was performed with the EZ-Magna ChIP<sup>TM</sup> A/G Chromatin Immunoprecipitation Kit (17-10086, Millipore) according to the instructions of manufacturer. The following antibodies were used: anti-PIWIL1, anti-PIWIL4, anti-H3K4me3(07-030, Millipore), anti-H3K9me3(07-442, Millipore), and anti-H3K27me3 (07-449, Millipore).

#### Co-immunoprecipitation

HEK293T cells were transfected with pcDNA3.1-based constructs containing interested genes using Lipofectamine 2000 (Invitrogen) and were lysed 48 h later with IP lysis buffer. The lysates were incubated with 40  $\mu$ l of anti-HA beads (Sigma) from 4 h to overnight at 4°C. Then the bead-protein mixtures were centrifuged and washed three times with lysis buffer. The immunoprecipitated samples were analysed by immunoblotting as described previously with the following primary antibodies: anti-FLAG (rabbit polyclonal, MBL) or anti-HA (mouse monoclonal, MBL) (20).

#### Apoptosis analyses

An Annexin V-FITC Apoptosis Detection Kit (KeyGen Biotech, Nanjing, China) was used for detecting apoptosis according to the instructions of manufacturer. The MCF7 cells were labeled by Annexin-V and propidium iodide (PI) and then were detected by an LSR Fortessa flow cytometer (Becton Dickinson). Of note, since the fluorescence of doxorubicin would interfere with the PE fluorescence channel, only Annexin V was chosen as the apoptosis indicator when the samples were treated with doxorubicin.

#### Immunohistochemistry

Paraffin-embedded sections of xenografts taken out from NOD/SCID mouse were deparaffinized in xylene and ethanol (100, 90 and 75%, respectively), then rehydrated before analysis. Slides were boiled in 3000 ml EDTA (pH = 8.0) at high pressure for 5 min for epitope retrieval and endogenous peroxidase was quenched with 0.5% hydrogen peroxide. Slides were washed in PBS, blocked with 5% normal goat serum and incubated overnight at 4°C with a rabbit polyclonal anti-human TRAIL-specific IgG (Abcam, Ab9959 and Ab2435, UK) at 1:150. DAKO ChemMate EnVision K500711 Kit containing a biotinylated secondary antibody and avidin-conjugated horseradish peroxidase was used to identify antibody binding. Diaminobenzidine was used as substrate for the horseradish peroxidase. Slides were counterstained with hematoxylin.

#### In vivo experiments

All animal procedures were conducted in accordance with the protocols generated by the Institutional Animal Care and Use Committee at the Sun Yat-Sen University. For subcutaneous xenografts, 4- to 6-week-old female NOD/SCID mice were operated to implant the 17 $\beta$ -estradiol pills before injecting into the mammary fat pad with  $1 \times 10^7$  cells in

a 150  $\mu$ l suspension. Each mouse was injected with MCF7 or MDA-MB-231 cells expressing pi-sno75 or empty vector control into the left and right mammary fat pad respectively to avoid confounding factors from chemotherapy drug administration. Doxorubicin (2 mg/kg) was injected into the tail vein weekly for 4 weeks when the subcutaneous tumor has reached 5 mm in diameter or 3 weeks after the MCF7 or MDA-MB-231 cells injection.

### Small interfering RNAs

The chemically synthesized siRNAs were purchased from RiboBio (Guangzhou, China) and GenePharm (Shanghai, China). A combination of three siRNAs was routinely used for one target gene. The efficiency of siRNAs-mediated knockdown was shown in the Supplementary Figures S6 and S7 and the siRNAs sequences were listed in Supplementary Table S2.

### Statistical analysis

Quantitative real-time PCR assay was performed according to the instruction of manufacturer (SYBR Premix Ex-Taq, TaKaRa) on a CFX96 Real-Time System (Bio-Rad). The data of qPCR in this report are processed by the software of Bio-Rad CFX Manager (Version1.5). Results of experiments are expressed as mean  $\pm$  SD. Student's paired/unpaired *t*-test was used to determine significance respectively.  $P < 0.05$  indicated significant difference. A canonical correlation analysis was performed to evaluate the association between pi-sno75 and TRAIL expression variables by using the Comprehensive R Archive Network (<http://CRAN.R-project.org/>).

## RESULTS

### GAS5-derived small RNA (pi-sno75) specifically upregulates TRAIL expression in breast cancer cells

To investigate the role(s) that small RNAs could play in the biogenesis and progression of breast cancer, a small RNA deep sequencing was conducted in the malignant breast epithelial tissues. Analysis of the deep-sequencing data showed that the major composition of the sequence reads was miRNAs. In addition, we found a group of non-annotated sequences at the size of 26–31 nts, which corresponds to the length of piRNAs (Supplementary Figure S1A). By comparing with the published small RNA deep-sequencing data of breast cancer cells and cancer-adjacent cells, we found that our data were consistent with theirs (22,23). One of them was illustrated in the Supplementary Figure S1B. Among the highly expressed small RNAs within 26–31 nts from different data bases, we noticed that some were derived from a long non-coding RNA named Growth Arrest Specific 5 (GAS5) (Figure 1A). GAS5 has long been considered as a tumor suppressor and is comprised of 12 exons and 11 introns. Ten box C/D snoRNAs are produced from its introns and five of them harbor piRNA-like molecules from our deep sequencing data (Figure 1A and B) (24,25). These RNAs retained the intact conserved motifs of C/D' or C'/D boxes (Figure 1B) (26). The cleaving sites for generating these

small RNAs were primarily located in the middle of the snoRNA sequences, in accordance with previous reports (27,28). As these snoRNA-derived small RNAs have not been documented in Genbank, they are therefore named pi-snoRNAs in this report.

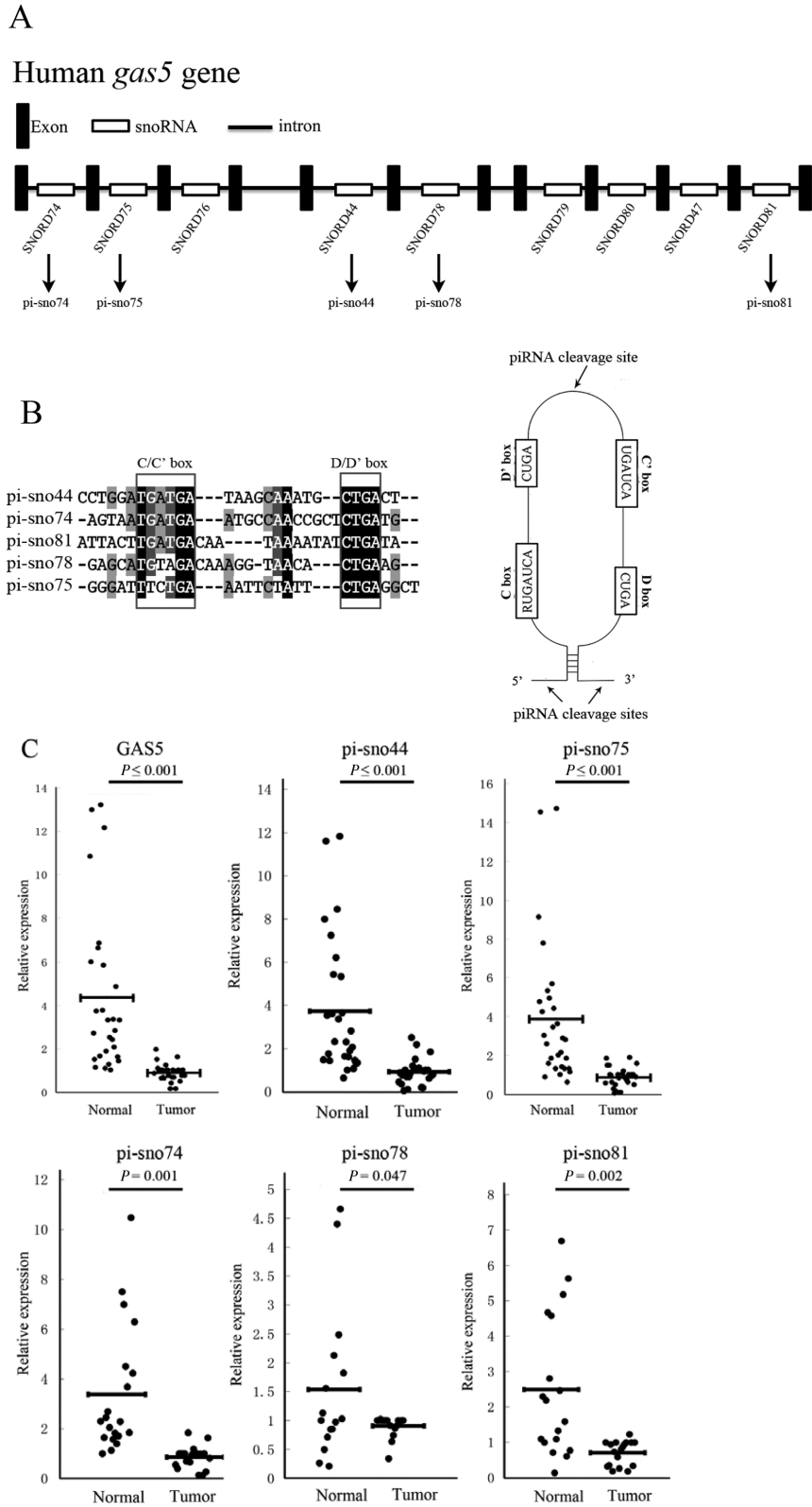
The quantitative analysis of GAS5-derived small RNAs from clinical breast samples showed that the expressions of pi-snoRNAs, as well as the corresponding snoRNAs, were significantly lower in cancer tissues than in adjacent normal tissues (Figure 1C and Supplementary Figure S2A), which was in consistence with that of GAS5 (24). To investigate the function of these small RNAs in breast cancer cells, we transfected a mixture of the five chemically synthesized single-stranded piRNAs, including pi-sno44/74/75/78/81, into MCF7 cells and detected the gene expression by microarray. Approximate 290 genes whose expression changed significantly were filtered with a fold-change  $> 2$  (Figure 2A and Supplementary Table S3). We noticed that a group of piRNA-affected genes was related to the cancer-associated gene ontology (GO) terms, such as cell death or proliferation biological processes. Among these genes, TRAIL (also known as TNFSF10 and Apo2L) was significantly upregulated by the piRNAs' mixture. TRAIL is a tumor-specific suppressor that induces apoptosis in a broad spectrum of cancer cells but not in normal cells (29).

To determine which piRNA(s) upregulated the TRAIL expression, we transfected MCF7 cells with five GAS5-derived piRNAs respectively. The qRT-PCR results showed that only pi-sno75 increased the mRNA level of TRAIL significantly (Figure 2B). This enhancement was dose dependent (Figure 2C). The TRAIL protein level was also potently increased by pi-sno75 (Figure 2D). However, pi-sno75 did not increase many other cancer-associated genes whose expressions were also changed in the microarray result (Figure 2E). These findings indicated that pi-sno75 specifically increased the TRAIL expression.

### Characteristics of GAS5-derived piRNAs in breast cancer cells

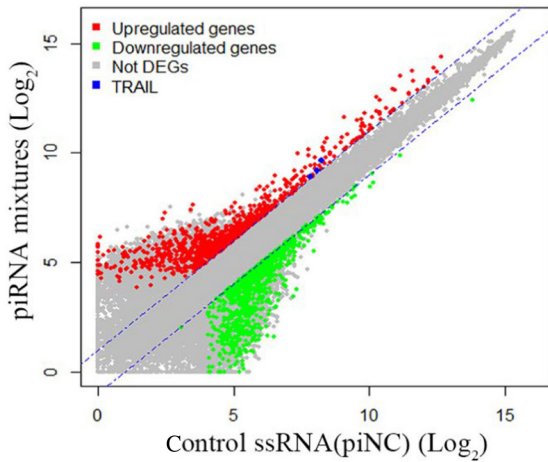
Further, the total RNAs were isolated from the clinical breast cancer tissue including the adjacent normal tissue, followed by northern blot analysis, followed by northern blot analysis. The expression of GAS5-derived small RNAs can be confirmed (Figure 3A and Supplementary Figure S2B). Conversely, the expressions of PIWIL1 and PIWIL4, the piRNA binding proteins in Homo sapiens, can be detected in the MCF7 cells with western blotting through specific antibodies (Supplementary Figure S3). RNA immunoprecipitation assay showed that GAS5-derived small RNAs can specifically bind with PIWI proteins, but snoRNA or miRNA cannot (Figure 3C and D). Importantly, the result of RTL-P experiment showed that pi-sno75 had a modification of 2'-O methylation at the 3' terminus (Figure 3B) (21), which was a feature of piRNA (30,31). Together, we have defined these GAS5-derived small RNAs as piRNAs.

As the sequences of piRNAs could be precisely aligned within the sequences of snoRNAs and the cleavage sites for the production of piRNAs were not random, Fibrillar (FBL) or NOP56, which were important for the bio-

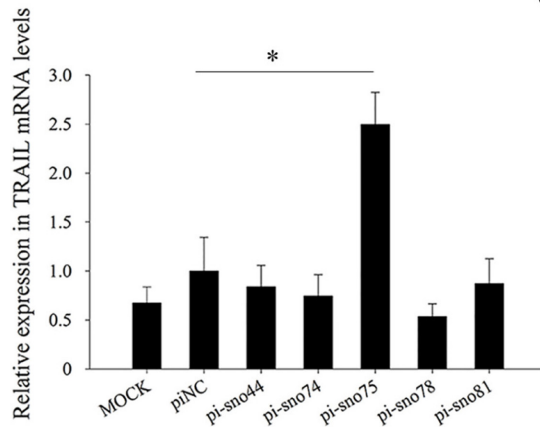


**Figure 1.** GAS5-derived small RNAs are low in breast cancer cells. (A) Graphical representation of GAS5. There are 11 introns, 10 snoRNAs and 5 pi-snoRNAs. (B) Sequences of the small RNAs derived from GAS5's snoRNAs were analysed and the conserved motifs were annotated. (C) Comparison of the expression of GAS5 and small RNAs in breast cancer tissue and normal tissue adjacent to carcinoma. The RNAs were quantitated by real-time RT-PCR and paired *t*-test was applied ( $n = 29$ ).

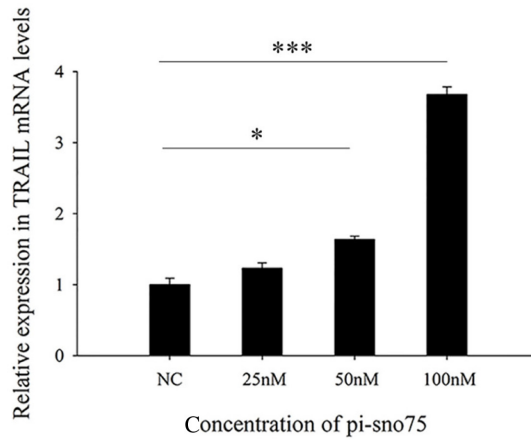
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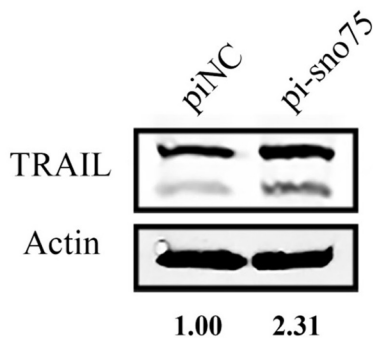
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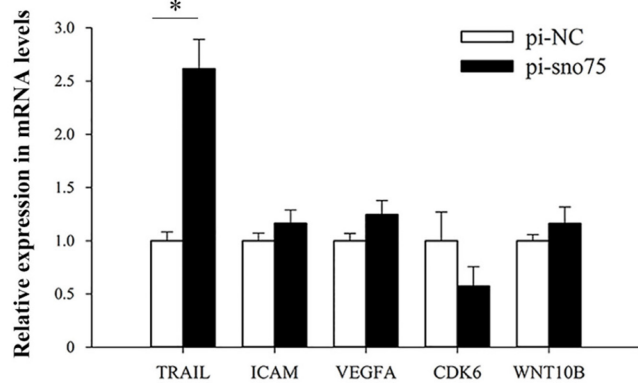
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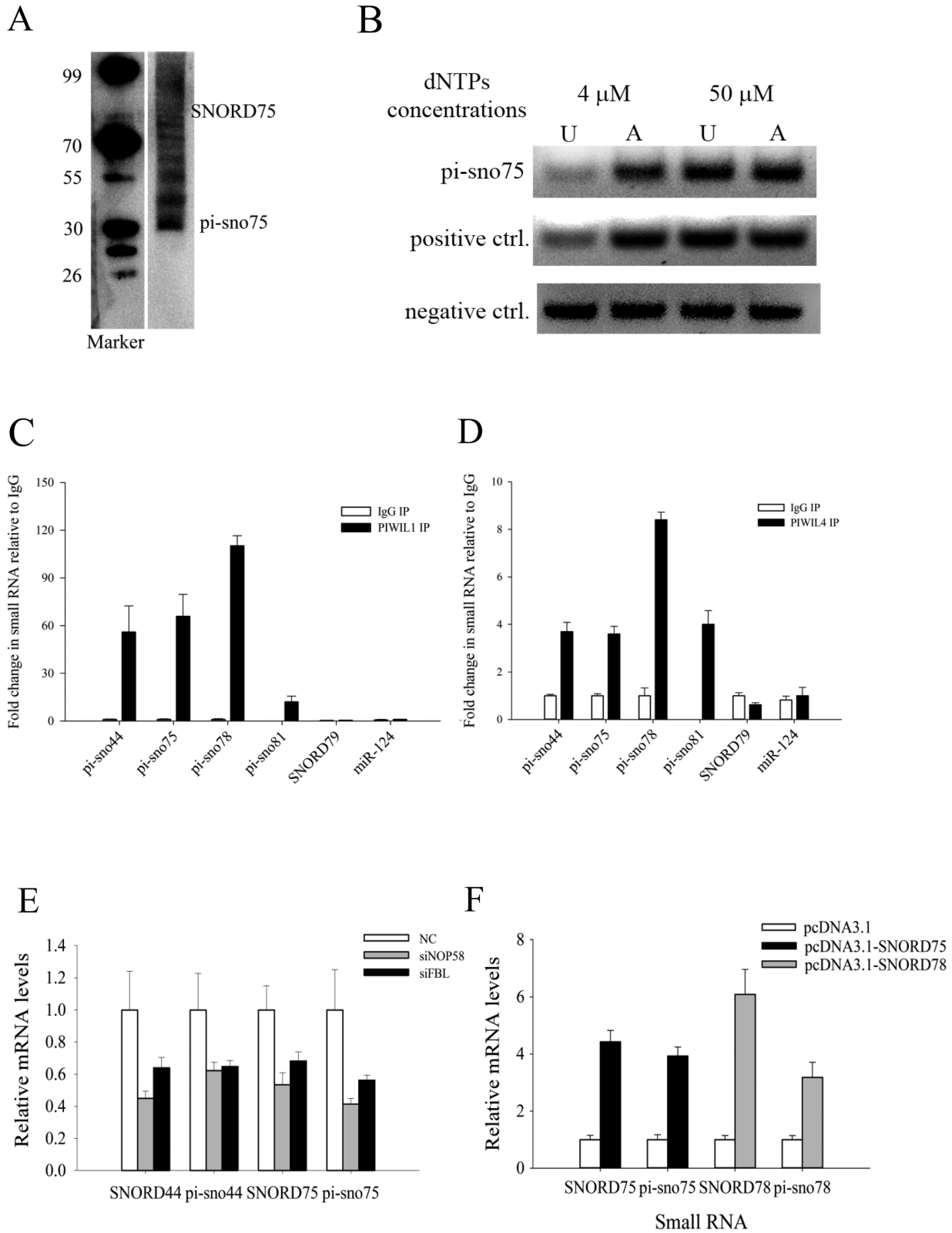
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**Figure 2.** Gas5-derived small RNA (pi-sno75) specifically upregulates TRAIL expression in breast cancer cells. (A) The mRNA profiling microarray analysis. The gene expression scatterplot of the piRNAs mixture group versus that of the control group was shown. Significantly downregulated genes were shown in green, while upregulated genes in red. (B) MCF7 cells were transfected with piRNAs derived from GAS5 respectively for 48h, TRAIL mRNA levels were analysed by quantitative RT-PCR (qRT-PCR) and normalized to GAPDH levels. (C) MCF7 cells were transfected with pi-sno75 at different concentrations. The qRT-PCR was performed to analyse the effect of pi-sno75 on TRAIL expression. (D) MCF7 cells were transfected with pi-sno75 and piNC for 48h, western blot was performed to analyse TRAIL protein expression. (E) In MCF7 cells transfected with pi-sno75, the mRNA levels of TRAIL and other genes were determined by qRT-PCR. Bar graphs in (B), (C) and (E) represent mean  $\pm$  SD ( $n = 3$ ).



**Figure 3.** Identification of GAS5-derived pi-sno75 in breast cancer cells. (A) Northern blot for SNORD75 and pi-sno75 expressions in the clinical breast cancer and adjacent normal tissue sample. (B) The detection of 2'-O methylation at the 3' end of piRNA by the RTL-P method. The RT reaction was performed with an unanchored or anchored RT primer at different concentrations of dNTPs. The positive control sample was the synthetic RNA molecule containing a 2'-Ome at 3' terminal and the negative control was the synthetic RNA molecule without any modifications. (C, D) RNA-IP with antibody against PIWIL1 (C) or PIWIL4 (D). The associated RNAs were quantified by qRT-PCR. RNA from IgG IP was set as the control. (E) After NOP58 or FBL knockdown by siRNA in MCF7 cells, both pi-snoRNAs and the corresponding snoRNAs were detected by qRT-PCR. (F) MCF7 cells were transfected with pcDNA3.1, pcDNA3.1-SNORD75 or pcDNA3.1-SNORD78, respectively. The expressions of pi-snoRNAs and snoRNAs were detected by qRT-PCR. Bar graphs in (C)–(F) represent mean  $\pm$  SD ( $n = 3$ ).

genesis of snoRNAs and the small nucleolar ribonucleoproteins (snoRNPs) complex assembly (32), were knockdown by siRNAs. The decrease of FBL and NOP56 reduced the expressions of snoRNAs and piRNAs (Figure 3E). In addition, overexpression of snoRNAs significantly increased the expressions of the piRNAs accordingly (Figure 3F). These data indicated that snoRNAs were the precursors for the generation of piRNAs.

### Pi-sno75 significantly induces epigenetic alterations on TRAIL promoter

Recent studies revealed that small RNAs can activate genes expression and modify the epigenetic status of the target genes (16,33,34). Meanwhile some reports also indicated that PIWI could regulate gene expression by altering the chromatin state (9,35,36). We initially tried to search for the possible binding sites of pi-sno75 on the region of TRAIL gene. A highly complementary binding site was predicted at position -169 bp of the transcription start site (TSS) on the TRAIL promoter through an online program RNAhybrid [Minimum Free Energy (MFE) -27.4 kcal/mol] (Supplementary Figure S4) (37). In addition, five other potential targets of pi-sno75 were also predicted with lower putative binding affinities (Figure 4A). Chromatin immunoprecipitation (ChIP) was conducted to detect the epigenetic states of TRAIL gene. The results showed that the H3K4me3 level was significantly increased and H3K27me3 level was significantly decreased in the area of -169 bp site of TRAIL promoter in the MCF7 cells with pi-sno75 overexpression (Figure 4B and D). However, no significant change was detected in H3K9me3 level and H3K9Ac level in the condition of pi-sno75 overexpression (Figure 4C and Supplementary Figure S5A). This pattern of histone modifications, H3K4 methylation and H3K27 demethylation, favored the activation of gene expression (38). Meanwhile, both PIWIL1 and PIWIL4 were associated with the area at -169 bp site of TRAIL promoter under the overexpression of pi-sno75 (Figure 4E and F). In addition, PIWIL1 or PIWIL4 knockdown by siRNA reduced TRAIL expression upregulated by pi-sno75 (Figure 4G). Taken together, these data indicated that the piRNA/PIWIL1 or piRNA/PIWIL4 complex specifically interacted with the area at -169 bp site on TRAIL promoter and was required for the upregulation of TRAIL expression by pi-sno75.

To further investigate the molecular mechanism of pi-sno75 upregulating TRAIL, we performed an RNAi screening of the genes with catalytic activities of H3K4 methylation or H3K27 demethylation to identify the essential factor(s) involved in this process (38). The screening results suggested that the knockdown of three H3K4 methyltransferases, including MLL3, SET7/9 and SMYD3, and one H3K27 demethyltransferase, UTX (KDM6A), significantly counteracted pi-sno75 upregulating TRAIL (Figure 4H and I, Supplementary Figures S6 and S7). As WDR5, RbBP5, Ash2L and CGBP were important cofactors in the catalytic process of MLL-family-mediated H3K4 methylation, the results from the siRNAs specific for them showed that only WDR5 knockdown significantly inhibited the function of pi-sno75 (Figure 4J and Supplementary Figures S7 and S8) (39,40).

### Pi-sno75/PIWIL complex specifically interacts with WDR5 and recruits MLL3/h-COMPASS-like complex to the region of TRAIL promoter

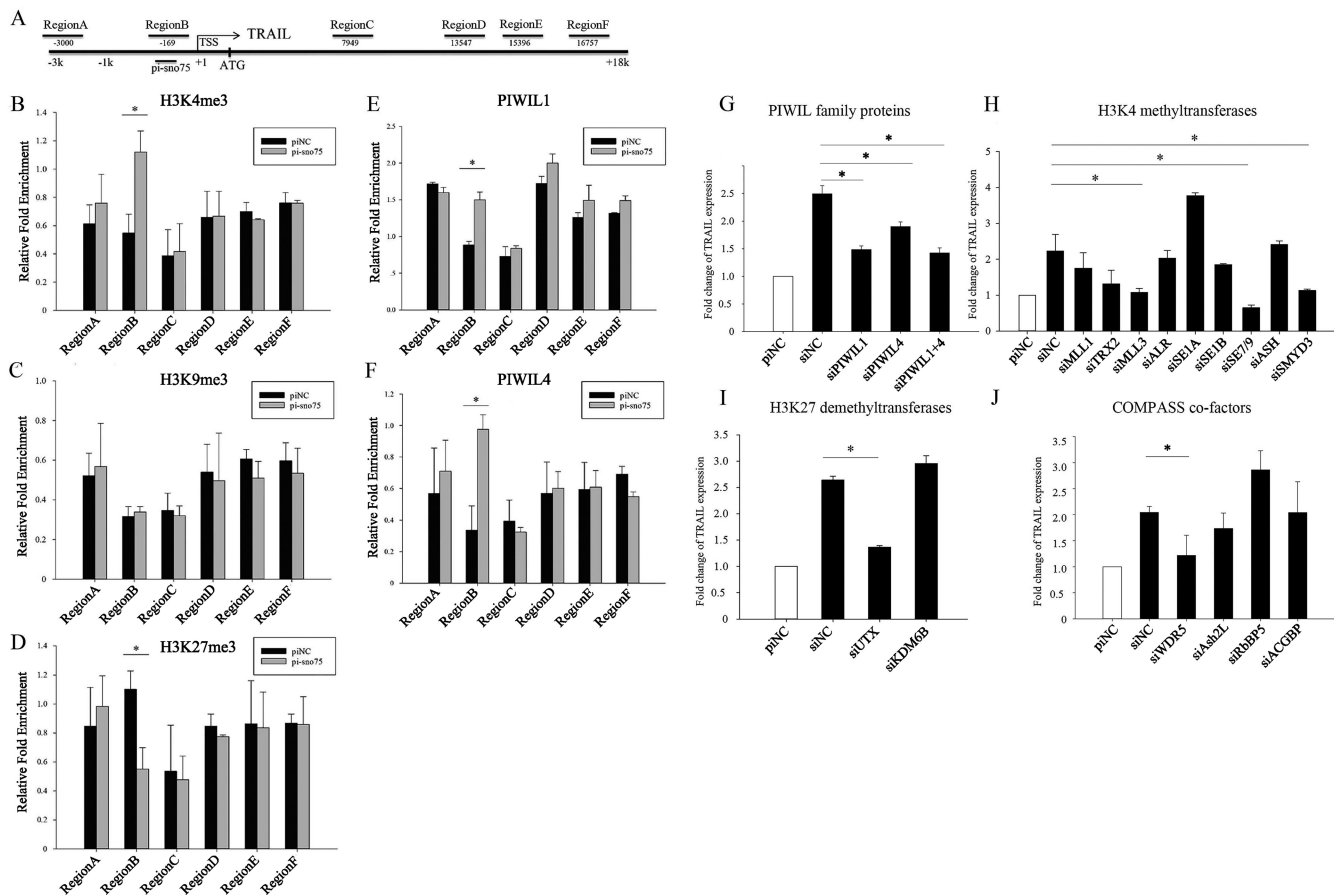
The significant effect of MLL3, UTX or WDR5 knockdown on pi-sno75 regulating TRAIL indicated that MLL3/hCOMPASS-like complex played an important role in this process (Figure 4H–J) (39,41–43). These results prompted us to examine the physical associations between PIWIL1/PIWIL4 and the histone modification-related proteins. Co-immunoprecipitation (Co-IP) experiments showed that PIWIL1 or PIWIL4 directly and stably interact with WDR5 (Figure 5A and B). However, there were no apparent associations between PIWIL1/4 and RbBP5 or Ash2 (Supplementary Figure S9 A–D). Meanwhile we found that PIWIL1 and PIWIL4 interacted with each other (Figure 5C), and therefore we just examined the interaction between PIWIL1 and the components of h-COMPASS-like complex in the following experiments. We also found that the interaction between PIWIL1 and WDR5 was not dependent on RNA (Figure 5D). The interactions between PIWIL1 and WDR5 in the presence and absence of MLL3 or UTX were analysed further (Figure 5E). The results suggested that the binding between WDR5 and PIWIL1 was direct and stable. However, the connections between PIWIL1 and MLL3 (C-terminal 3993aa~4911aa) or UTX were dependent on the existence of WDR5 (Figure 5F and G). The endogenous Co-IP between WDR5 and PIWIL1 further confirmed their binding (Figure 5H). WDR5 can be enriched at the promoter region of TRAIL gene by pi-sno75 overexpression (Figure 5I). In addition, PIWIL1 knockdown would reduce the enrichment of WDR5 (Figure 5K). However, PIWIL1's enrichment was not affected in the absence of WDR5 (Figure 5J), which indicated binding of WDR5 was dependent on PIWIL1.

Meanwhile, PIWIL1 or PIWIL4 could be specifically associated with SMYD3, but not with SET7/9, indicating that SMYD3 was involved in the pi-sno75 regulation processes as well (Supplementary Figure S9E and F). Moreover, although WDR5 was able to interact with Gcn5 which is a histone acetyltransferase (44), Gcn5 knockdown had no effect on the pi-sno75 upregulating TRAIL in breast cancer cells, which further indicated that histone acetylation did not participate in this process (Supplementary Figure S5B). Taken together, pi-sno75/PIWIL can upregulate the expression of TRAIL by specifically interacting with WDR5 and recruiting the whole hCOMPASS-like complex and with SMYD3 to the promoter region of TRAIL gene.

### pi-sno75 has the anti-tumor activity both *in vitro* and *in vivo*

TRAIL has been well characterized as a specific tumor cell killer and serves as an effective anti-tumor agent (29). The upregulation of TRAIL expression by pi-sno75 suggests that the pi-sno75 could be of anti-tumor activity. Compared with the control, there was no apparent apoptosis in MCF7 cells transfected with pi-sno75 by Annexin-V/PI staining detected with FACS (Figure 6A). The result was in consistency with the observation that most breast cancer cells were relatively resistant to TRAIL-induced apoptosis (45). To





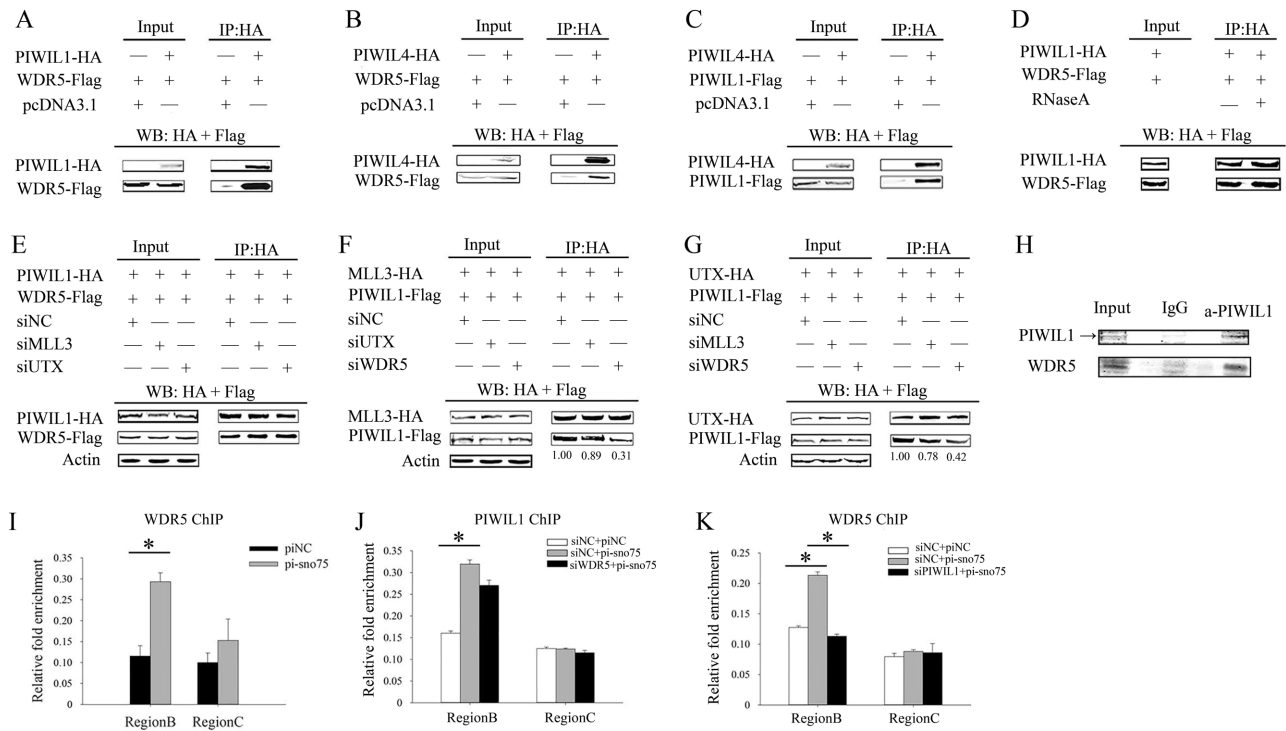
**Figure 4.** pi-sno75 significantly affects the histone modifications of TRAIL promoter. (A) Schematic representation of the TRAIL gene and the predicted piRNA-targeting locations relative to the TSS. (B–D) Association of H3K4me3, H3K9me3 or H3K27me3 at the TRAIL gene with the overexpression of pi-sno75 was assayed by ChIP-qPCR. The relative enrichment was calculated by normalizing the quantity of TRAIL DNA against the quantity of input. (E and F) Association of PIWIL1 or PIWIL4 at the TRAIL promoter with the overexpression of pi-sno75 was assayed by ChIP-qPCR. (G–J) RNAi screening of various histone modifiers and PIWIL family proteins on the impact of pi-sno75 regulating TRAIL. GAPDH was set as the internal reference. Data in bar graphs in (B–J) represent mean  $\pm$  SD ( $n = 3$ ). \*, statistically significant,  $\leq 0.05$ .

avoid the tolerance for apoptosis, we treated the pi-sno75-transfected cells with doxorubicin (45,46). In comparison with a control small RNA, a significant increase in apoptosis took place in MCF7 cells treated with pi-sno75 under the same stimulation of doxorubicin (Figure 6B). The activity of pi-sno75 inducing cell death was counteracted by DR4 and DR5 knockdown (Figure 6C, middle panel). A similar phenomenon was also observed by the WDR5 knockdown (Figure 6C, low panel), further supporting the involvement of WDR5 and h-COMPASS complex.

In order to study the *in vivo* anti-tumor activity of pi-sno75, the stable MCF7 and MDA-MB-231 cell lines that overexpressed the high level of pi-sno75 were constructed. Immunohistochemistry data indicated that the TRAIL levels were higher in the MCF7 and MDA-MB-231 xenografts expressing pi-sno75 than that in cell lines expressing the empty vector, (Figure 6D and E). When the stable breast cancer cell lines were transplanted into the NOD/SCID female mice, there was initially no difference in the sizes of cell xenografts between pi-sno75 and vehicle expression. After the palpable tumors reached to 5 mm in diameter, the mice were treated with doxorubicin (2 mg/kg) weekly, which

reduced the growth rate of all the xenografts. Four weeks after the first time of drug administration, the sizes of tumors with pi-sno75 expressing vector were much smaller than that of the control. Both MCF-7 (Figure 6F) and MDA-MB-231 (Figure 6G) cell xenografts showed a similar phenomenon. Combined together, the animal experiment results revealed that pi-sno75 significantly suppresses the xenografts in combination with chemotherapy agent *in vivo*.

To further determine the correlation between the expression of pi-sno75 and the expression of TRAIL in breast normal and cancer tissues, the breast cancer samples from 29 cases were collected. The RNA was extracted from the cancer tissue or normal tissue respectively and subjected to the qRT-PCR analysis for TRAIL mRNA. The results revealed that the cancer cells displayed much lower expression of TRAIL than that in the normal tissue cells (Figure 7A), which is in consistency with the tendency of pi-sno75 (Figure 1C). A canonical correlation analysis (CCA) showed a positive correlation between the pi-sno75 and TRAIL variables (Figure 7B). Within the 29 cases of clinical samples, the CCAs produced a statistical significance correla-



**Figure 5.** Interactions between PIWI proteins and histone modification-related proteins. (A) Co-immunoprecipitation (Co-IP) assay of PIWIL1 and WDR5 by anti-HA agarose beads followed by immunoblots with indicated antibodies (middle). (B) Co-IP assay of PIWIL4 and WDR5. (C) Co-IP assay of PIWIL1 and PIWIL4. (D) Co-IP assay of PIWIL1 and WDR5 after RNase treatment by anti-HA agarose beads followed by immunoblots. (E) Co-IP assay of PIWIL1 and WDR5 in the presence or absence of MLL3 or UTX by anti-HA antibodies followed by immunoblots. (F) Co-IP assay of PIWIL1 and MLL3 in the presence or absence of WDR5 or UTX. (G) Co-IP assay of PIWIL1 and UTX in the presence or absence of WDR5 or MLL3. (H) Endogenous co-IP assay of PIWIL1 and WDR5 in MCF7 cells by using anti-PIWIL1 antibody. (I) association of WDR5 with the TRAIL promoter under the overexpression of pi-sno75 was detected by ChIP-qPCR. The relative enrichment was calculated by normalizing the quantity of TRAIL DNA against the quantity of input. (J) The association of PIWIL1 with the TRAIL promoter in the absence of WDR5 was detected by ChIP-qPCR. (K) The association of WDR5 with the TRAIL promoter in the absence of PIWIL1 was detected by ChIP-qPCR. Bar graphs represent mean  $\pm$  SD ( $n = 3$ ). \*, statistically significant,  $\leq 0.05$ .

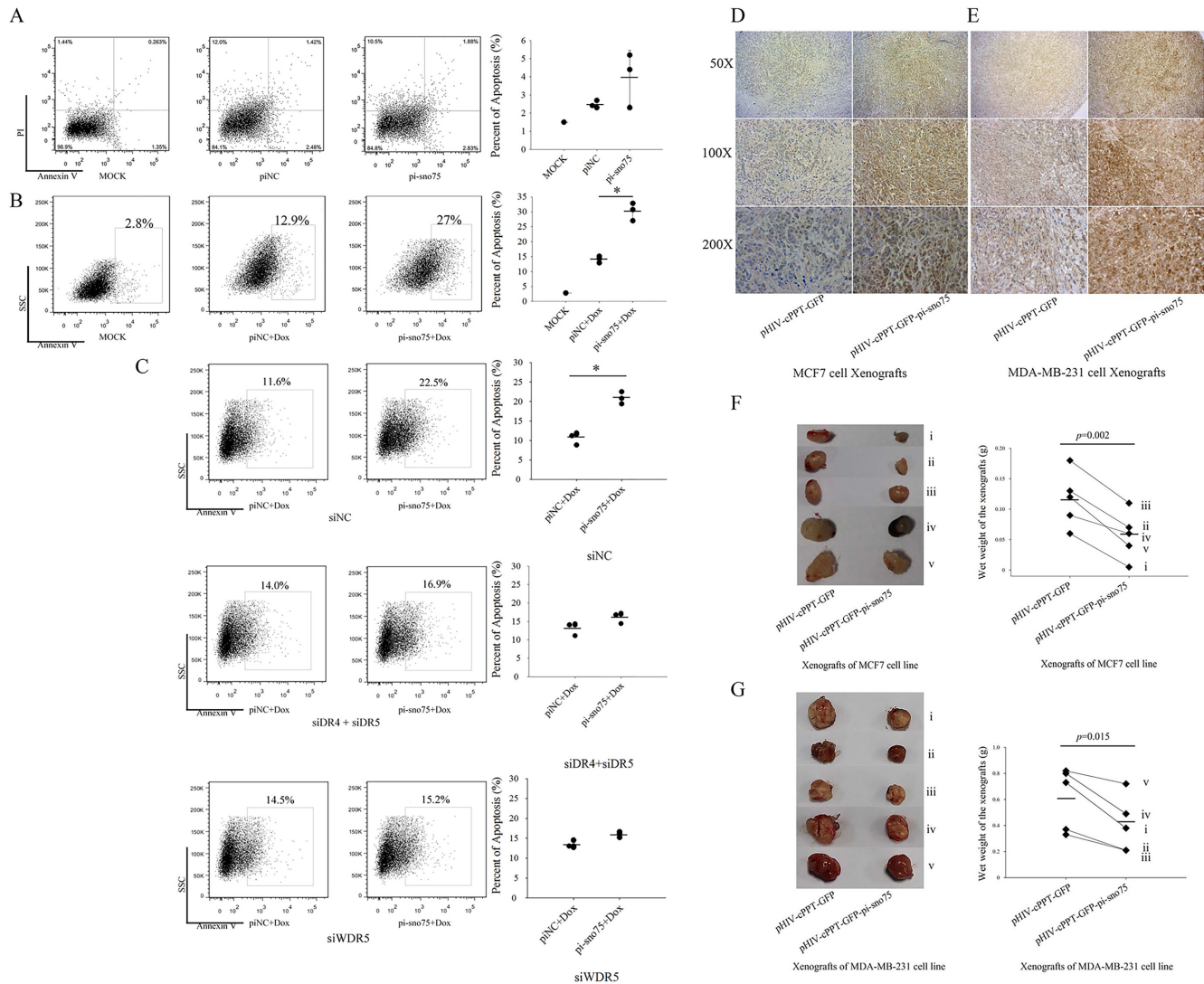
tion (Corr = 0.77,  $P < 0.0005$ ) and explained the most of the variables of TRAIL in breast cancer cells [Wilks's  $\lambda = 0.3949890$  (a value of 0 means perfect discrimination),  $F = 7.093640$ ,  $df1 = 4$ ,  $df2 = 48$ ,  $P < 0.0005$ ]. Collectively, in breast cancer cells, the decrease of TRAIL mRNA is significantly correlated with the decrease of pi-sno75 expression.

## DISCUSSION

In this report, we found a cluster of five of  $\sim 28$  nt small RNAs was generated from the GAS5-derived snoRNAs. These small RNAs were of the characteristics of piRNAs. All of these snoRNA-derived piRNAs contain the conserved C/D box motifs. Interestingly, the cleavage site for these piRNA is not random but conserved. However, not all the snoRNAs in GAS5 could produce piRNAs, and the formation of mature snoRNA is an important step for the generation of these GAS5-derived piRNAs. Nevertheless, the factors for cleaving mature snoRNA and producing piRNA remain to be determined. We have demonstrated that the expression of pi-sno75 in the breast cancer cells is much lower than that in the adjacent normal tissues. Overexpression of pi-sno75 in cancer cells leads to the upregulation of TRAIL, which can trigger the extrinsic pathway of apoptosis by binding its receptors DR4 and DR5. Further inves-

tigation of mechanism has indicated that pi-sno75 guides the recruitment of MLL3/COMPASS-like complexes to the promoter region of TRAIL, inducing H3K4 methylation and H3K27 demethylation and subsequently transcription activation.

In mammalian cells, H3K4 methylation is catalyzed by a group of SET-domain-containing methyltransferases such as SET1A/B, MLL1-4, SMYD3 etc., while H3K27 methylation is primarily catalyzed by polycomb repressive complex 2 including EZH2 (38). Interestingly, COMPASS (Complex proteins associated with SET1) has been identified to function as an H3K4 methyltransferase and activate the gene expression and play an important role in the embryonic development. Moreover, a demethyltransferase UTX, which is specific for H3K27 demethylation, has been found to be directly associated with the MLL2(ALR)/3-COMPASS-like complex, indicating that H3K4 methylation and H3K27 demethylation can be simultaneously regulated (39). In addition to MLL2/3 and UTX, other co-factors such as WDR5, Ash2L, RbBP5 and Dyp30 are also associated with COMPASS-like complex (39,41). Our data have found that MLL3/hCOMPASS-like complex is recruited to the promoter region of TRAIL, an important proapoptotic gene, and plays an important role for piRNA/PIWIL to activate gene expression. In addition to

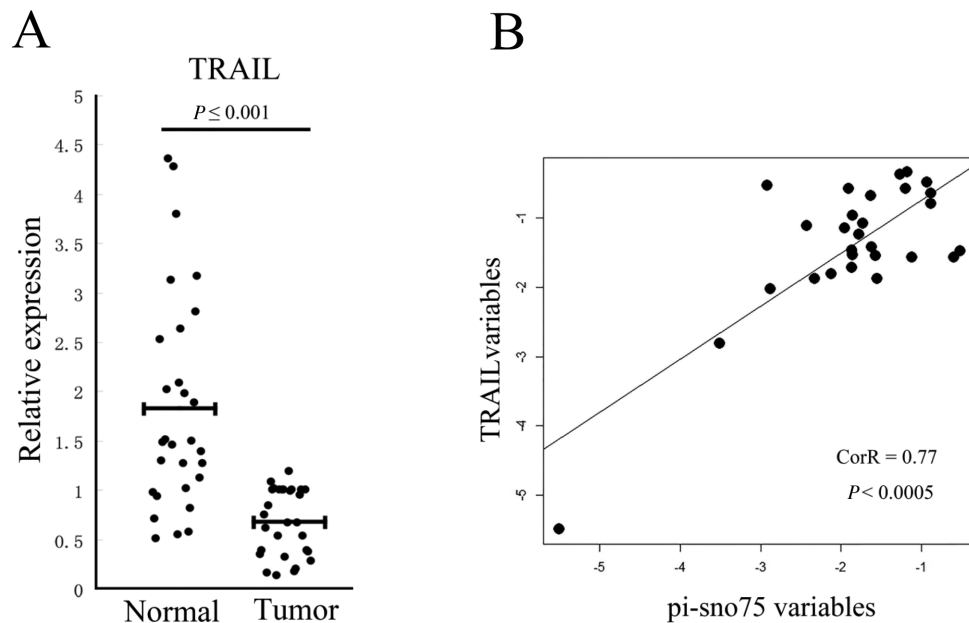


**Figure 6.** Synergistic effect of pi-sno75 and doxorubicin on apoptosis and on the growth of breast carcinoma cell lines *in vitro* and *in vivo*. (A) MCF7 cells were transfected with pi-sno75 or piNC; 48 h later, apoptosis was detected by FACS with Annexin-V/PI staining. (B) MCF7 cells were transfected with pi-sno75 or piNC. After 36 h, doxorubicin (Dox) was added to the cells for 12 h, and apoptosis was analysed by FACS. (C) After DR4 plus DR5 or WDR5 knockdown, impacts of pi-sno75 on apoptosis in MCF7 cells incubated with Dox were analyzed by FACS with Annexin-V staining. (D and E) After tumor formation without drug treatments, Immunohistochemistry (IHC) analysis of TRAIL expression in the MCF7 (D) or MDA-MB-231 (E) xenografts with pi-sno75 overexpression or with control vector. anti-TRAIL antibody was used as the primary antibody. (F and G) Tumor weights of MCF7 xenografts (F) or MDA-MB-231 (G) xenografts with pi-sno75 overexpression or control vector. The paired *t*-test was used. Data represent mean  $\pm$  SD (error bars). \*, statistically significant,  $\leq 0.05$ .

MLL3 and UTX, the knockdown of other histone methyltransferases such as SET7/9 and SMYD3 potentially affects the upregulation of TRAIL by pi-sno75. Moreover, SMYD3 directly binds to PIWI1/4 protein. Their crosstalk with the MLL3/h-COMPASS-like complexes containing WDR5/MLL3/UTX remains to be further determined.

Lee and his colleagues reported that in the presence of retinoic acid (RA) stimulus, the chromatin status of some promoters of HOX family proteins could be remodeled in a manner of H3K4 methylation and H3K27 demethylation (41). They noticed that Ash2L was involved in the process of RA stimulus. However, our data demonstrated that PIWI1/4 directly interacts with WDR5, a core component of COMPASS-like complex, not with Ash2L. WDR5

is a transcription factor and can directly bind H3K4me2 (47). It assists SET1 family proteins in catalyzing H3K4me2 trimethylation (48). Our results suggest that, through recruiting WDR5 and its functional factors including MLL3 and UTX, piRNA/PIWI complex guides the specific locus recognition of COMPASS-like and induces H3K4 methylation and H3K27 demethylation simultaneously to manipulate the bivalent state, and subsequently activate the transcription of TRAIL. Therefore, it is a novel pathway for piRNA/PIWI complex to mediate histone modifications through interacting with the functional cofactors, which serve as a platform to conduct the epigenetic modifiers and then regulate the transcription by changing the chromatin state. However, as the piRNA/PIWI complex could



**Figure 7.** Correlation of TRAIL and pi-sno75 in the breast cancer cells. (A) The mRNA level of TRAIL in breast cancer cells and normal adjacent to carcinoma from 29 patients was analysed with RT-PCR, the expressions were normalized with GAPDH.  $P$  value was calculated using the paired  $t$ -test. (B) Correlation between the expression of pi-sno75 and the TRAIL mRNA level in the cancer cells isolated from the clinical patients.

affect opposing chromatin-modifying regulation on different chromatin locations (16,49), our current model cannot exclude the possibility that piRNA/PIWI may interact with diverse factors according to the certain chromatin contexts or cell status.

GAS5 has been known as a tumor suppressor which was overexpressed in the growth-arrested cells (50). Its expression in breast cancer cells is low. GAS5 can induce apoptosis under certain stimuli, such as starvation, chemotherapeutic drug or ultraviolet treatment, and affect the rate of growth through the regulation of cell cycle (24). However, the molecular mechanism for GAS5 to induce apoptosis and suppress tumor growth has yet to be clarified. Our results suggested that GAS5-derived piRNA, through specifically upregulating the expression of TRAIL, potentially serves as an anti-tumor effector for GAS5. A number of studies have demonstrated that TRAIL activated cell death pathways in variety of cancer cells but not normal cells (29). TRAIL is primarily produced by immune cells to kill tumors. However, the overexpression of TRAIL in cancer cells can also inhibit the tumor growth (51,52). The anti-tumor activity of TRAIL is enhanced by chemotherapy or radiotherapy (29,53). A similar phenomenon has also been observed in our studies (Figure 5A-D). *In vitro* and *in vivo* experiments indicated that the low expression of pi-sno75 could be a fundamental factor responsible for the low expression of TRAIL in breast cancer cells. Our works also propose that much broader roles in cancer cells for other piRNAs, especially these generated from GAS5, merit being further investigated. Importantly, because TRAIL has the relatively specific toxicity in malignant cells, the pi-sno75 could be further developed as a promising anti-tumor agent itself. Moreover, this special regulatory pathway, from the regulation of GAS5 expression to pi-sno75-mediated

TRAIL upregulation, exerts many possible novel targets for anti-tumor.

Recently, many reports have indicated that the regulation of histone methylation/demethylation is frequently disordered in the cancer cells (38,54). It is notable that UTX mutations have been identified in many cancers (55–57). MLL3 deficiency has been reported in leukemia (58), and MLL3 deficiency mice would develop the ureter epithelial tumors (59). The mutations of MLL3 and MLL2 have been widely reported in various tumors (57,60,61). It seems that Trr/MLL2/MLL3 functions as a tumor suppressor to restrict tumor growth and its deletion or mutation contribute to carcinogenesis. From our work, we can propose that the deletion or mutation of functional factors in MLL3/h-COMPASS-like complexes would inhibit the piRNA-mediated upregulation of the proapoptotic TRAIL promoter activity in the human cancer cells.

In summary, our data for the first time have demonstrated a novel pathway for piRNAs to induce the chromatin modifications. The piRNA/PIWI complex, by initially binding with WDR5, subsequently recruits the COMPASS-like complex containing MLL3/UTX to the promoter region of a special gene. Through simultaneous induction of H3K4 methylation and H3K27 demethylation, piRNA/PIWI complex therefore site-specifically guide the transcriptional activation of a special gene (Supplementary Figure S10). This epigenetic regulation is not similar to other histone modifications which usually affect the chromatin state globally. Our works therefore define a novel signal transduction pathway, from the special signal-induced expression of a long non-coding RNA, via piRNA generation and consequently histone modifications, to the specific transcription activation. Similar to protein transcriptional factors, piRNA/PIWI complex could mediate tran-

scriptional regulation through a sequence-specific interaction, and therefore play an important and definitive role in a cascade of regulatory events for gene expression.

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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*Conflict of interest statement.* None declared.

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