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A SnoRNA-derived piRNA interacts with human interleukin-4 pre-mRNA and induces its decay in nuclear exosomes

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ABSTRACT

PIWI interacting RNAs (piRNAs) are highly expressed in germline cells and are involved in maintaining genome integrity by silencing transposons. These are also involved in DNA/histone methylation and gene expression regulation in somatic cells of invertebrates. The functions of piRNAs in somatic cells of vertebrates, however, remain elusive. We found that snoRNA-derived and C (C′)/D′ (D)-box conserved piRNAs are abundant in human CD4 primary T-lymphocytes. piRNA (piR30840) significantly downregulated interleukin-4 (IL-4) via sequence complementarity binding to pre-mRNA intron, which subsequently inhibited the development of Th2 T-lymphocytes. Piwil4 and Ago4 are associated with this piRNA, and this complex further interacts with Trf4-Air2-Mtr4 Polyadenylation (TRAMP) complex, which leads to the decay of targeted pre-mRNA through nuclear exosomes. Taken together, we demonstrate a novel piRNA mechanism in regulating gene expression in highly differentiated somatic cells and a possible novel target for allergy therapeutics.

INTRODUCTION

piRNAs are 26–31-nt small non-coding RNAs that are associated with Piwi proteins and used to be considered as germline-specific (1–5). Recently, it has been reported that piRNAs or piRNA-like molecules are widely expressed in somatic cells (6–11). These have also been identified in human cancer cells and cancer cell lines (12–16). piRNAs are further divided into two classes based on their associated biogenesis pathway: the processing pathway in somatic cells and the ping-pong cycle in germ cells (9–10,17–19). Basically, piRNAs are distinct from other small RNAs based on their length, 2′-O-methyl modified 3′ termini and interaction with PIWI proteins (1–4,20–21).

The Piwi protein, which is a member of a subfamily of the Piwi/Argonaute family, was first described in *Drosophila* (22,23) and plays a key role in germline development and gametogenesis (24,25). The human genome encodes four PIWI-like proteins, namely, Hiwi (Piwil1), Hili (Piwil2), Hiwi2 (Piwil4) and Hiwi3 (Piwil3) (26,27). Several lines of evidence have shown that the Piwi protein is involved in the function and mechanism of Piwi-interacting RNAs (piRNAs) (1,28–29). Similar to other small RNA pathways, the key players in the piRNA pathway are PIWI proteins directly interacting with small RNAs that guide proteins in sequence-specific binding to its targets (30). Studies on piRNA function have mostly focused on transposon silencing in the germline (10,25,30–33). Recently, the role of piRNAs in somatic cells has been extensively explored. The somatic function of PIWI/piRNA was initially determined using ovarian somatic cells of *Drosophila* (10,22). In addition, piRNA influences long-term memory plasticity in *Aplysia* (8). A few studies have suggested that piRNA–PIWI plays a role in the pathogenesis of human cancer, al-

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though the underlying mechanism remains to be clarified (12–15,34). These results demonstrated that piRNAs exhibit broader functions outside the germline.

Small non-coding RNAs are critical regulators of gene expression (35). The miRNAs and siRNAs regulate gene expression by sequence-specific cleavage, deadenylation or translational repression of target mRNAs (36,37). Compared to siRNAs and miRNAs, piRNAs are relatively the least investigated class of small RNAs. In addition to inducing the degradation of transposon RNA, accumulating evidence has indicated that piRNAs are involved in epigenetic regulation (27). The piwi–piRNA complex interacts with epigenetic factors such as HP1a and histone methyltransferase Su (var) 3–9, and are therefore involved in histone methylation and gene expression in both germline and somatic cells, resulting in the suppression of gene expression (38–42). In the germline, piRNA is also involved in DNA methylation on non-transposon loci such as *Rasgrf1* (43). In the neurons of *Aplysia*, the PIWI–piRNA pathway directs the CpG methylation of the CREB2 promoter, which results in the transcriptional silencing of CREB2 (8). In addition, piwi–piRNA also promotes euchromatic histone modification and acts as an epigenetic activator (44). A recent report indicated that piRNA is involved in cleaving *Masc* mRNA in the early silkworm embryos through the ping-pong mechanism, which results in sex determination (45). In addition, piRNA complementarily targets the *nos* 3′-untranslated region and plays important roles in the cytoplasmic decay of maternal mRNAs, depending on CCR4-mediated deadenylation in the early embryo (46). Although more piRNA-related pathways in regulating gene expression have been identified, its underlying mechanisms in somatic cells are not fully understood. Here, we demonstrate a novel piRNA mechanism in somatic cells and its role in lymphocyte differentiation.

MATERIALS AND METHODS

Cell culture and transfection

HEK293T cells were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA) and cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (Invitrogen), 50 U/ml penicillin and 50 U/ml streptomycin. The chemically synthesized piRNAs or siRNAs (RiboBio, Guangzhou, China) were transfected into the 293T cells using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol.

Primary CD4 T-lymphocytes were enriched from peripheral blood mononuclear cells (PBMCs) using a CD4 T MicroBead kit (Miltenyi Biotec.) and then sorted by using SORP FacsAria II. The naïve ($CD4^+/CD45RA^+/CCR7^+$) T-lymphocytes were purified from enriched CD4 T lymphocytes by flow cytometric cell sorting (CD4-APC, 555349; CD45RA-FITC, 555488; CCR7-PE, 552176, BD). The cells were subsequently cultured in RPMI 1640 media supplemented with 10% fetal calf serum, 50 U/ml penicillin and 50 U/ml streptomycin. The naïve cells were activated with plate-bound anti-CD3 (2μg/mL) (MAB100, R&D) and anti-CD28(2μg/mL) (MAB342, R&D) antibodies with IL-2 (10 ng /ml) (202-1L, R&D), IL-4 (20 ng/ml) (200-04,PeproTech) and neutralizing antibodies such as anti-IFN-γ (5 μg /ml) (MAB285, R&D) and anti-IL-12 (5μg /ml) (AB-219-NA, R&D Systems). Human primary CD4 T lymphocytes were transfected by using Lipofectamine RNAimax (Life Technologies) according to the manufacturer's protocol. The cells were collected for qRT-PCR or western blotting analysis at 48 hrs after transfection.

Plasmid construction

The piR30840 precursor construct containing the snord63 hosted intron and flanking exons was amplified from human genomic DNA and inserted into the pEGFP-c1 vector as described previously (47,48). The 3′UTR and 5′UTR fragments of the *il-4* gene were amplified by using specific primers and cloned into the multiple cloning site of the pMir-reporter vector (Promega). The promoter fragment (ranging from positions -665 to 64) of the *il-4* gene was inserted into the multiple cloning site of the pGL3 Luciferase reporter vector (Promega).

The IL4 (full length) construct was generated by polymerase chain reaction (PCR) amplification, followed by insertion into the pcDNA3.1 vector. The expression vector containing the IL-4 coding regions (CDs) was constructed by amplifying IL-4 mRNA using RT-PCR, and the fragment was inserted into the multiple cloning site of the pcDNA3.1 vector. The splicing adaptor sequence was amplified and then the fragments were introduced into the constructed IL-4 CDs by fragment overlap using PCR amplification. Meanwhile, the cleaving sites were also introduced into the intronic region. Subsequently, In1, In2, In3 or In2s constructs were generated by direct insertion of the intronic fragments into the cleaving sites, respectively. All the mutations in the pIL4m, In2m, In2w3b or In2sm constructs were generated via PCR-based mutagenesis. The 5′ end HA or Flag-tagged Piwil4, Ago4, Mtr4 or Prp4 constructs were generated via insertion of sequences corresponding to the CD of these genes into the pcDNA3.1 vector, respectively.

Deep sequencing

Total RNA was extracted from the primary human CD4 T lymphocytes using the TRIzol reagent (Invitrogen), according to manufacturer's protocol. The quality of the RNA samples were evaluated by using a Nanodrop 2000c Spectrophotometer (Thermo) and then size-fractionated on a 15% polyacrylamide gelelectrophoresis (PAGE) gel to collect the 18–40 nt fraction. The 5′ and 3′ RNA adaptors were ligated to the RNA pool, followed by RT-PCR to generate the sequencing libraries. PCR products were purified and then sequenced using a HiSeq 2000 sequencing system (BGI Tech).

RNA pull-down and mass spectrometry analysis

The CD4 T lymphocytes were transfected with 5′-biotinylated synthesized piRNA (single strand), a random sequence (single strand) or miRNA (double strand), respectively. Two days later, CD4 T lymphocytes were collected in an extraction buffer and fragmented using an ultrasonicator. After separating the lysate by centrifugation at 14 000

rpm for 15 min (5417R, Eppendorf), the cell extract was incubated with streptavidin-coated microbeads overnight at 4°C, following the manufacturer's protocol (μMACS, Miltenyi Biotec.). The proteins interacting with the piRNAs were pulled down using magnetic separator, followed by three washes. The proteins were resolved on PAGE, followed by silver staining. Gels fragments that contained bands of the protein of interest were excised and analyzed by mass spectrometry.

RIP-qPCR

HEK293T cells were transfected with constructs HA-Piwil4 and pIL4 or pIL4m, respectively. Two days later, the 293T cells were collected and lysed in RIP buffer, following the manufacturer's protocol (Magna RIP kit, Millipore). The cell lysates were then separated by centrifugation at 14 000 rpm for 15 min at 4°C. The anti-HA antibody (M180-3, MBL) was mixed with the magnetic beads protein A/G and incubated with constant rotation for 30 min at room temperature. Subsequently, the anti-HA antibody and protein A/G-coated magnetic beads were then mixed with the lysates and incubated at 4°C for 4 h. The beads were then precipitated using a magnetic separator, followed by six washes of cool RIP buffer. The immunoprecipitated RNA samples were then extracted and reversely transcribed using an RT reagent kit (Invitrogen) with specific primers, followed by qPCR. A CFX 96 Real-Time System (Bio-Rad) was used for quantitative real-time PCR (qRT-PCR) amplification. Results were normalized to input RNA levels and plotted as fold enrichment relative to that of the IgG control.

Reverse transcription at Low deoxyribonucleoside triphosphate (dNTPs) (RTL-P)

The 3′end methylation of piR30840 was detected by RTL-P, followed by PCR according to Dong *et al*., with some modifications (49). Briefly, small RNAs were isolated using the *mir*Vana microRNA isolation kit (Ambion) and ligated to a 3′RNA adapter using a T4 RNA ligase (NEB). The ligation product was then reverse-transcribed into complementary DNA (cDNA) using a low (0.4 mM) or high (40 mM) dNTP concentration with or without anchored RT primers that were designed to anchor the modified nucleotide. The cDNA was subsequently PCR amplified using specific primers. The PCR products were then electrophoresed in a 2% agarose gel.

RNA extraction and quantitative RT-PCR

piRNA quantification was performed according to the method described previously with some modifications (50). Small RNAs were isolated using the *mir*Vana microRNA isolation kit (Ambion). Small RNA samples were polyadenylated at 37°C for 20 min in a 20-μl reaction volume containing 0.5 μg of RNA and 1.0 U of poly (A) polymerase (NEB). The poly (A)-tailed small RNAs were reversely transcribed by using anchored oligodT primers. Real-time qPCR analysis of piRNA expression was performed using piRNA-specific primers. Cycle threshold (CT) values of the piRNAs were normalized against an actin internal control and plotted as relative transcript abundance.

Total RNAs were isolated from 293T or CD4 T lymphocytes. For IL-4 mRNA detection, total RNA (2.0 μg) was treated with DNase (M6101, Promega) and reversely transcribed with Superscript III (18080-051, Life Technologies) using oligodT primers. RNA transcript levels were measured by qRT–PCR using a SYBR Green PCR Master Mix using gene-specific primers listed in Supplementary Table S1. All experiments were performed in three biological replicates.

Cytokine antibody array

Human primary CD4 T-lymphocytes were nucleofected with the precursor of piR30840 or control. After 48 h, the cells were collected and lysed in lysis buffer according to the manufacturer's protocol (RayBiotech, Atlanta, GA, USA). The cell lysates were then separated by centrifugation at 14 000 rpm for 15min at 4°C and subjected to the Human Cytokine Antibody Array 5 antibody chip (RayBiotech).

Northern blot and western blot analyses

For the detection of piRNA, 20 μg of total RNA was extracted from CD4 T-lymphocytes with Trizol and loaded onto a 10% denaturing PAGE gel. The procedure of northern blot described by Varallyay *et al*. was followed, with minor modifications (51). The membranes were hybridized with a ^{32}P-labeled oligonucleotide probe complementary to the piRNA (probe sequences are listed in Supplementary Table S1).

For IL-4 mRNA detection, 20 μg of total RNA was separated on 1.2% formaldehyde-agarose gels, transferred to Amersham Hybond-N$^+$ membranes (GE Healthcare) and hybridized with ^{32}P-labeled oligonucleotides probes or *in vitro*-transcribed probes. The membranes were exposed on Phosphorimager screens and analyzed by using the software, Quantity One (Bio Rad).

The procedure used was as previously described (52). Anti-IL-4 (AP5241b, ABGENT) and anti-PIWIL4 (Ab87939, Abcam) were used as primary antibodies. Actin was used as a loading control. Scanned images were quantified using Quantity One (Bio-Rad).

Co-immunoprecipitation

The 293T cells were cultured in a 60-mm-diameter plate and transfected with various plasmids. Forty-eight hours later, the cells were collected and disrupted using a lysis buffer containing a protease inhibitor mixture (Sigma) for 30 min at 4°C. The cell lysates were separated by centrifugation at 14 000 rpm for 15 min at 4°C. Anti-HA agarose beads (A2059, Sigma) were mixed with the cell lysates and incubated at 4°C for 4 h or overnight. The anti-HA antibody (M180-3, MBL) or normal mouse IgG was mixed with the magnetic beads protein A/G and incubated with constant rotation for 30 min at room temperature. The beads were then washed three times with cold lysis buffer and eluted with a gel loading buffer. The immunoprecipitated samples were analyzed by SDS-PAGE, followed by western blotting.

Anti-AGO4 (ab85077, Abcam) was used to detect endogenous AGO4.

Adoptive transfer

The Animal Ethical and Welfare Committee of Sun Yat-sen University reviewed the protocols used in the present study. The NOD/Shi-scid, IL-2Rγ KO (NOG) male mice were purchased from the Central Institute for Experimental Animals (Japan) and its genotype was confirmed by PCR. All mice were maintained under specific pathogen-free conditions in accordance with ethical guidelines for animal care of Sun Yat-sen University. After growing for eight weeks, the mice were whole-body irradiated with 2.0 Gy. The human $CD34^+$ cells, which were isolated from PBMCs of healthy individuals by using a $CD34^+$ MicroBead kit (Miltenyi Biotec.), were then transferred into the NOG mice via intravenous injection. Two months later, the synthetic ago-pir30840 (modified with 2′-O-Me and 5′ cholesterol) or control was injected into the NOG mice intravenously.

Intracellular staining analyzed with FACS

The cells were collected and fixed with formaldehyde according to the manufacturer's protocol (Cytofix/Cytopeam Plus, BD, USA). The cells were then permeabilized and stained with anti-IL-4-APC (17–7049, eBioscience) and anti-IFNγ−FITC (11-7319-82, eBioscience), followed by analysis with the BD LSRFortessa^{TM} cell analyzer (BD Biosciences).

Chromatin immunoprecipitation (ChIP) assay

The cells were collected and fixed with formaldehyde to crosslink. The procedure was used according to the manufacturer's protocol (Magna ChIP G, 17–611, Millipore). Anti-H3K9me3 antibody (ab8898, Abcam) or normal IgG was used to immunoprecipitate chromatin fragments. Real-time PCR was performed using the SYBR Green PCR reagent kit (TaKaRa).

Patient samples and measurement of cytokine levels

Blood samples were collected from clinically defined asthma and healthy volunteers at the First Affiliated Hospital or Sun Yat-sen Memorial Hospital, Sun Yat-sen University (Guangzhou, China). Samples were obtained from subjects after receiving informed consent. The Ethical Committee of the First Affiliated Hospital or Sun Yat-sen Memorial Hospital of Sun Yat-sen University approved this protocol. Serum levels of IgE or IL-4 were measured using commercially available ELISA kits (NeoBioscience, Shenzhen, China), following the manufacturer's instructions. Primary CD4 T-lymphocytes were enriched from peripheral blood mononuclear cells (PBMCs) by using the CD4 T MicroBead kit (Miltenyi Biotec.).

Statistical analysis

Values are reported as means± the standard error. Values of $P < 0.05$ were considered significant. Statistical significance between two samples was determined by using the two-tailed student's *t*-test or Wilcoxon test.

RESULTS

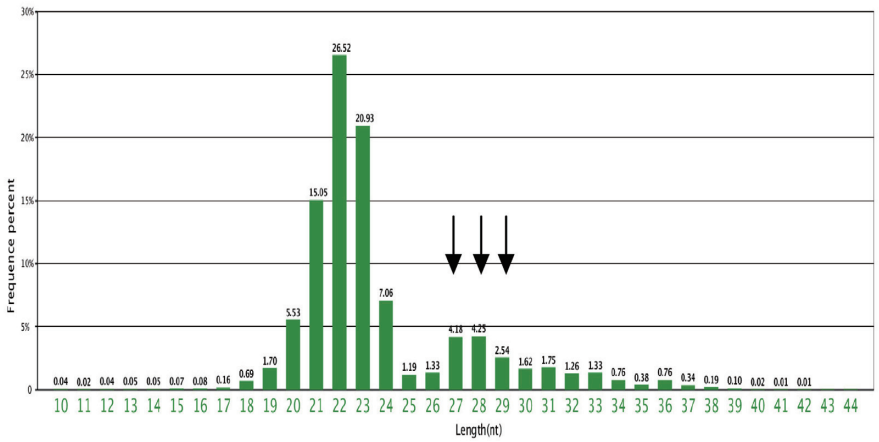
Detection of piRNAs in human CD4 T lymphocytes

To systematically investigate small RNA expression in human primary CD4 T-lymphocytes, deep sequencing of small RNAs was performed. In addition to an apparent peak at 21–24 nt for miRNAs, we unexpectedly found a peak at 26–30 nt (Figure 1A). The abundantly expressed small RNAs within this peak were described as piRNAs in GenBank (Figure 1B). Sequence alignment analysis showed that the most highly expressed piRNAs are uniquely derived from snoRNA and contain the conserved sequences C/D′ or C′/D boxes of snoRNAs (Figure 1C and D), in accordance with the findings of previous reports (16,53). These boxed C/D motifs might be important for protein binding and required for the function of this class piRNAs (54,55). The expression of piR30840 in CD4 T-lymphocytes was confirmed by northern blot analysis (Figure 1E). To investigate piRNA associated factors, pull-down of RNA–protein complexes by using biotinylated RNA was performed to purify proteins. Several proteins bound to piR30840 more strongly than the control RNA. RNA-bound proteins were identified by mass spectrometry. We found that Piwil4 and Ago4 are among the piRNA-binding proteins (Figure 1F). The expression of Piwil4 and Ago4 in CD4 T lymphocytes was then identified by qRT-PCR. Piwil4 expression in CD4 T lymphocytes was further confirmed by western blot analysis (Supplementary Figure S1). In addition, we summarized the NCBI GEO Profiles database, which showed that Piwil4 is a moderately expressed gene in human CD4 T lymphocytes (Supplementary Figure S2). RIP-qPCR assay indicated that Piwil4 directly interacted with piR30840, whereas no interaction between Piwil4 and miR-181a as control was observed (Figure 1G). Similarly, enrichment of piR36170 as control increased (Supplementary Figure S1D). Furthermore, the RTL-P assay showed that piR30840 in human CD4 T-lymphocytes has the 2′-O-methyl group at its 3′-end, which is one of the characteristics of piRNAs and might be involved in preventing these from exonucleolytic degradation or required for piRNA function (Figure 1H) (49). Together, these findings indicate that the small RNAs derived from snoRNAs possess the features of piRNAs (8,20–21). In addition, piR30840 was most abundant in CD4 T-lymphocytes, instead of CD8 T-lymphocytes, B lymphocytes or monocytes (Figure 1I). However, the expression level of the piR30840 homolog was significantly lower in CD4 T-lymphocytes of mice (data not shown). Importantly, compared to the reads of some miRNAs that play an important role in CD4 T-lymphocytes, piR30840 was also highly expressed in CD4 T-lymphocytes (Figure 1J) (56–59). Together, these findings suggested that piR30840 could perform some specific functions in human CD4 T-lymphocytes.

piR30840 downregulates the expression of IL-4

To investigate the function of piR30840, we overexpressed the precursor of piR30840 in primary CD4 T-lymphocytes and detected its effect on the cytokine expression via a human cytokine antibody array. The results showed that piR30840 significantly decreased the expression of some cy-

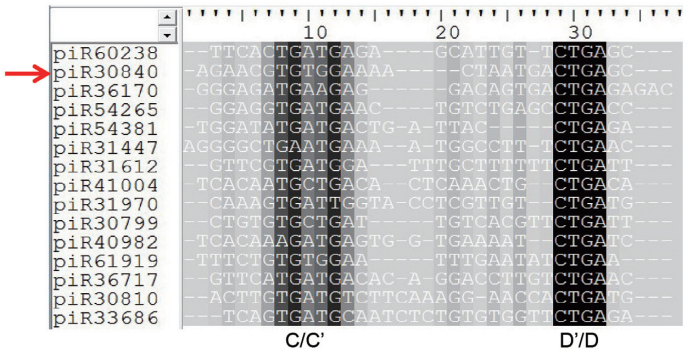
A



B

GenBank	Sequence	piRNA	Reads	snoRNA
DQ594126.1	TTCACTGATGAGAGCATTGTTCTGAGC	piR60238	36642	SNORD85
DQ570728.1	AGAACGTGTGGAAAACTAATGACTGAGC	piR30840	33204	SNORD63
DQ598104.1	GGGAGATGAAGAGGACAGTGACTGAGAGAC	piR36170	6422	SNORD62B
DQ587153.1	GGAGGTGATGAACTGTCTGAGCCTGACC	piR54265	5954	SNORD57
DQ587269.1	TGGATATGATGACTGATTACCTGAGA	piR54381	4018	SNORD20
DQ571335.1	AGGGGCTGAATGAAAATGGCCTTTCTGAAC	piR31447	3045	SNORD6
DQ571500.1	GTTCGTGATGGATTTGCTTTTTTCTGATT	piR31612	2691	SNORD51
DQ572892.1	TCACAATGCTGACACTCAAACTGCTGACA	piR41004	1106	SNORD71
DQ593636.1	TCTACTGAACTGCCATGAGGAAACTGC	piR33748	467	SNORD100
DQ571858.1	CAAAGTGATTGGTACCTCGTTGTCTGATG	piR31970	386	SNORD4A
DQ570687.1	CTGTGTGCTGATTGTCACGTTCTGATT	piR30799	286	SNORD43
DQ571387.1	AGGTGAGCGCTTTGCGCAGTGATGAC	piR31499	283	SNORD84
DQ572870.1	TCACAAAGATGAGTGGTGAAAATCTGATC	piR40982	261	SNORD17
DQ595807.1	TTTCTGTGTGGAATTTGAATATCTGAA	piR61919	260	SNORD1B
DQ598648.1	GTTCAGTGATGAGGCCTGGAATGTGCGCTG	piR36714	173	SNORD83B
DQ598651.1	GTTCATGATGACACAGGACCTTGTCTGAAC	piR36717	134	SNORD107
DQ570698.1	ACTTGTGATGTCTTCAAAGGAACCACTGATG	piR30810	118	SNORD42B
DQ593744.1	CTGCAGTGATGACTTTCTTAGGACACCTTT	piR33856	44	SNORD58A
DQ595186.1	TTGCTGTGATGACTATCTTAGGACACCTTT	piR61298	32	SNORD58C
DQ593574.1	TCAGTGATGCAATCTCTGTGTGGTTCTGAGA	piR33686	30	SNORD91A

C



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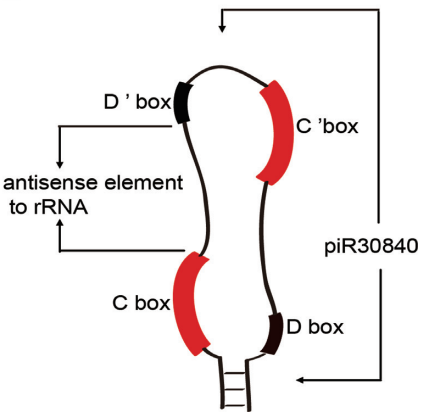


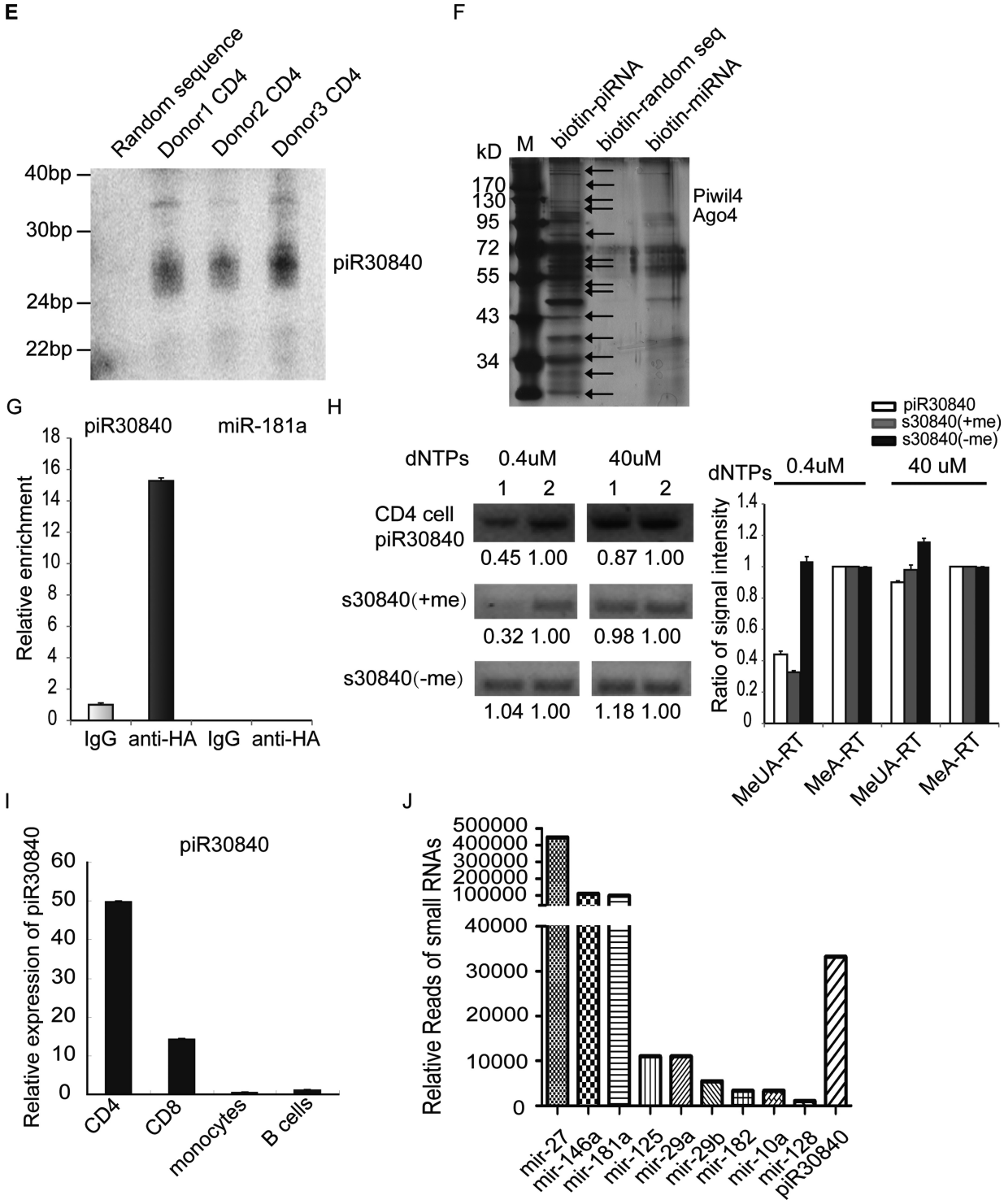


Figure 1. Identification of piRNAs in human primary CD4 T-lymphocytes. (**A**) Deep sequencing shows the distribution of small RNAs in the activated CD4 T—lymphocytes. The arrows indicate piRNA peaks. The length cutoff is 18–40 bp. (**B**) Sequences of the abundant piRNAs. Selected piRNAs were annotated in GenBank. (**C**) Sequence alignment analysis of specific snoRNA-derived piRNAs. Conserved boxes are highlighted. (**D**) Schematic demonstration that the piRNA (piR30840) is derived from snoRNA. Cleavage sites are indicated by arrows. (**E**) One highly abundant piRNA (piR30840) was analyzed by northern blotting using a piR30840 antisense probe. (**F**) RNA pull-down assay using a piRNA, a scrambled sequence or a miRNA. The differential bands (indicated by arrows) were excised for mass spectrometry analysis. The locations of piRNA-associated Piwil4 and Ago4 are indicated by arrows. (**G**) Co-immunoprecipitation of Piwil4 with piRNA. The Piwil4-expressing plasmid was transfected into the PBMCs. HA-tagged Piwil4 was immunoprecipitated, and the associated piRNA was analyzed by qRT-PCR, with miR-181a as a control. The data represents three independent experiments. (**H**) Detection of 2′-O-methylation at the 3′-ends of piRNA by RTL-P. RT-PCR products were generated by using an unanchored (MeUA-RT) (1) or anchored (MeA-RT) RT primer (2) in the RT reaction at different concentrations of dNTPs. The data showed the average value of three independent experiments (right panel). (**I**) Expression of piR30840 in human CD4 T-lymphocytes, CD8 T-lymphocytes, B cells or monocytes. (**J**) Deep-sequencing reads of piR30840 compared to various miRNAs in the CD4 T lymphocytes.

Table 1. The effect of piR30840 on cytokine expression by using a human cytokine antibody array

RayBio® Cytokine Antibody Arrays – Human Cytokine Antibody Array 5

	pEGFP	piR30840		pEGFP	piR30840
Pos	42.74	42.74	TPO	3.44	2.40
Neg	0.00	0.00	VEGF	34.85	27.29
ENA-78	16.00	12.52	PDGF-BB	45.19	50.01
G-CSF	2.10	1.05	Leptin	27.60	19.78
GM-CSF	2.37	0.71	BDNF	33.27	39.13
GRO	33.60	34.44	BLC	9.64	5.58
GRO-alpha	3.84	0.26	CK beta 8-1	8.52	4.61
I-309	12.39	5.78	Eotaxin	28.64	20.64
IL-1alpha	7.66	3.64	Eotaxin-2	21.49	16.29
IL-1beta	6.01	4.67	Eotaxin-3	6.44	4.76
IL-2	4.32	2.66	FGF-4	15.03	12.69
IL-3	14.92	15.84	FGF-6	16.60	8.45
IL-4	4.08	1.92	FGF-7	20.52	19.15
IL-5	2.71	0.54	FGF-9	20.58	19.88
IL-6	3.69	1.11	Flt-3 Ligand	5.64	2.73
IL-7	1.94	0.85	Fractalkine	8.86	2.98
IL-8	42.80	52.21	GCP-2	8.70	0.04
IL-10	15.14	5.66	GDNF	18.66	5.89
IL12-p40	3.67	2.78	HGF	4.52	0.23
IL-13	2.22	1.70	IGFBP-1	11.95	6.49
IL-15	18.39	10.04	IGFBP-2	28.78	23.38
IFN-gamma	5.72	4.66	IGFBP-3	22.25	7.61
MCP-1	23.46	16.98	IGFBP-4	18.25	7.34
MCP-2	5.86	5.24	IL-16	20.57	18.89
MCP-3	9.83	6.29	IP-10	37.31	30.55
M-CSF	9.75	7.70	LIF	33.33	20.69
MDC	12.13	5.95	LIGHT	6.35	0.50
MIG	5.43	2.13	MCP-4	5.02	0.00
MIP-1 beta	17.81	18.72	MIF	28.27	2.44
MIP-1-delta	1.92	1.55	MIP-3-alpha	1.99	0.00
RANTES	50.75	57.01	NAP-2	43.11	33.23
SCF	14.77	9.18	NT-3	39.97	23.70
SDF-1	3.70	1.89	NT-4	12.04	0.04
TARC	31.71	21.40	Osteopontin	22.49	2.02
TGF-beta 1	20.57	10.91	Osteoprotegerin	13.53	3.15
TNF-alpha	19.98	14.42	PARC	7.77	1.27
TNF-beta	28.34	20.28	PIGF	10.84	1.48
EGF	41.50	48.39	TGF-b 2	43.76	37.13
IGF-1	5.45	3.34	TGF-b 3	8.47	0.00
Angiogenin	3.99	2.05	TIMP-1	27.58	1.84
Oncostatin M	12.92	8.94	TIMP-2	35.72	36.80

Human primary CD4 T-lymphocytes were nucleofected with the precursor of piR30840 or control. The RayBio cytokine antibody array was used to examine cytokine expression in primary CD4 T lymphocytes.

tokines, including those involved in the development and maintenance of Th2 T-lymphocytes such as interleukin-4 (IL-4) and IL-5 (Table 1). By analyzing the array data and computational prediction, IL-4 was selected for further analysis. qRT-PCR, northern blotting and western blotting further confirmed that the chemically-synthesized piR30840 (s30840) downregulated IL-4 expression in CD4 T-lymphocytes, as well as in PBMCs (Figure 2A, B, C and Supplementary Figure S3A). Overexpression of piR36170 did not show a similar result in the control (Supplementary Figure S3B) and s30840 showed no effect on the expression of CCR7 and GAPDH (Supplementary Figure S3C and D). When we used the piRNA antisense inhibitor to inhibit endogenous piR30840 in the CD4 T lymphocytes, the expression of IL-4 was significantly upregulated in a dose-dependent fashion (Figure 2D and Supplementary Figure S3E). Taken together, these results demonstrated that piR30840 downregulates the expression of IL-4 in CD4 T lymphocytes.

The IL-4 intron is the target of piR30840

As small RNA regulates gene expression mostly through sequence complementary to the non-CDs of the targeted gene (46,60), we generated several constructs in which the expression of *firefly luciferase* was affected by the promoter, 3′untranslated regions (3′UTR) or 5′untranslated regions (5′UTR) of the *il-4* gene, respectively (Supplementary Figure S4A). The results showed that the observed decrease in IL-4 expression by piR30840 was not due to its interaction with the sequences in these regions (Supplementary Figure S4B–D). Alternatively, as piRNAs are involved in the modification of chromatin state (39,40), we used ChIP-qPCR to detect histone methylation in the regions of the *il-4* promoter or intron, and the result is negative (Supplementary Figure S4E). We then generated a construct that con-

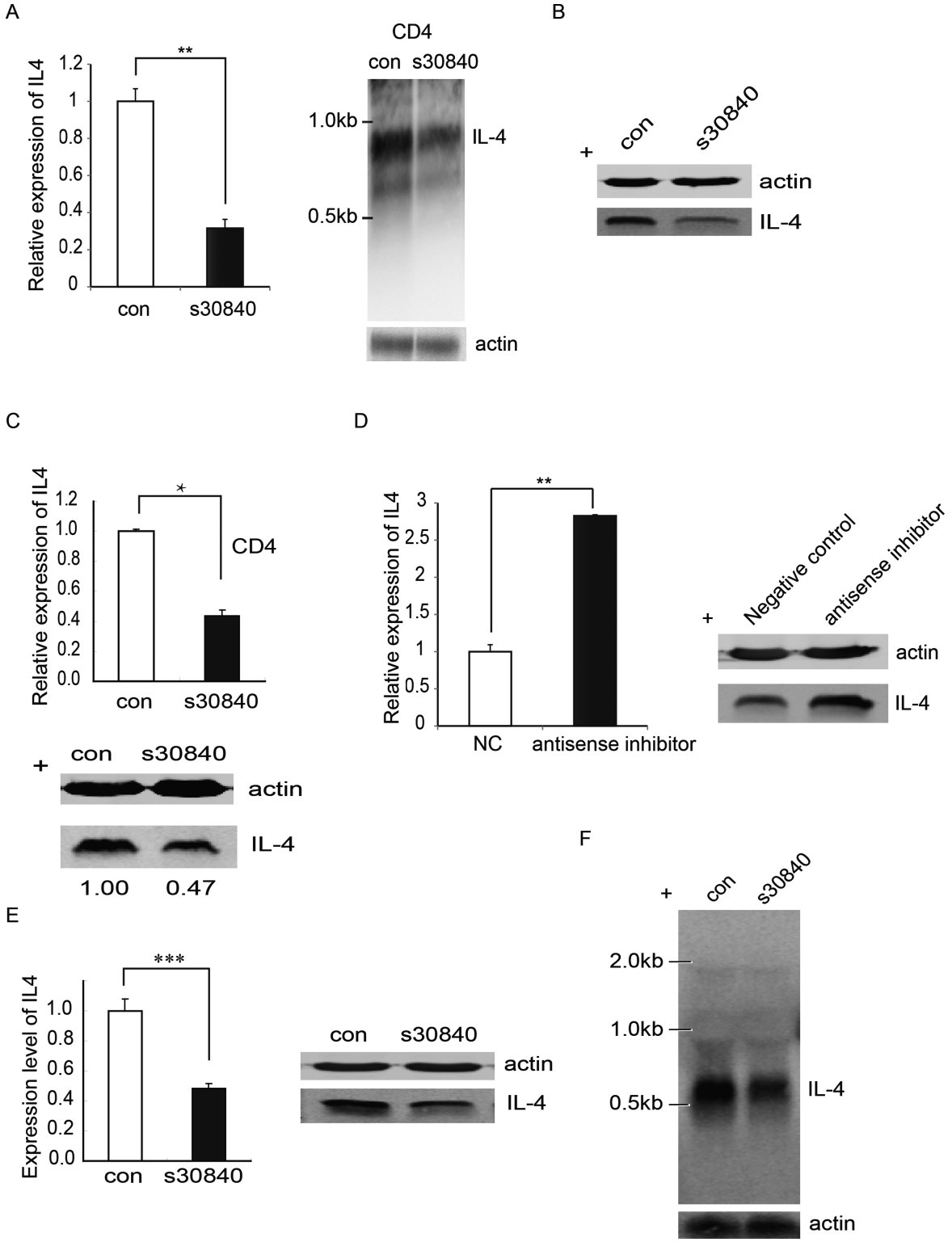


Figure 2. piR30840 significantly decreases IL-4 expression. (**A** and **B**) s30840 decreases IL-4 expression in purified CD4 T lymphocytes from PBMCs. IL-4 expression was detected by qRT-PCR (left panel), northern blotting (right panel) (A) and western blotting (B). The data represents three independent experiments. Con represents the synthesized random sequence for control. s30840 represents the synthesized piR30840. $P < 0.05$. (**C**) s30840 decreases IL-4 expression in activated CD4 T lymphocytes. IL-4 expression was detected by qRT-PCR (upper panel) and western blotting (lower panel) analyses. The data represents three independent trials. $P < 0.05$. (**D**) Antisense inhibitor piR30840 antisense upregulates IL-4 in purified CD4 T lymphocytes from PBMCs. IL-4 expression was detected by qRT-PCR (left panel) and western blot (right panel) analyses. The data represents three independent trials. $P < 0.05$. (**E** and **F**) The construct containing all the IL-4 exons and introns (construct pIL4) was co-transfected with s30840 into 293T cells. IL-4 expression was detected by qRT-PCR (left panel) and western blotting (right panel) (E), as well as by northern blotting (F) analyses. The data represents three independent experiments. $P < 0.01$.

tained all exons and all introns of the *il-4* gene (Supplementary Figure S5A). After transfection into the 293T cells, we found that s30840 significantly decreased IL-4 expression (Figure 2E and F). To identify the target sites of piR30840, we generated a construct containing IL-4 exons only (Supplementary Figure S5A) and found that piR30840 failed to decrease IL-4 expression (Supplementary Figure S5B). Because introns of genes are correlated with pre-mRNA degradation (61,62), and several putative binding sites of piR30840 were predicted in the introns of IL-4 based on the middle region between the conserved C (C')/D' (D) boxes (Figure 3A), we generated several IL-4-expressing constructs that contained the first, second, third intron or fragment of the *il-4* gene, respectively (Supplementary Figures S5A and S6A). piR30840 did not decrease IL-4 expression using the constructs containing In1 or In3, but significantly reduced IL-4 expression using the construct harboring In2, which included the putative binding site of piR30840 (Figure 3B–E). When a mutation was introduced to the piR30840-binding site in In2, no impairment of IL-4 expression due to piR30840 was observed (Figure 3F). Introduction of mutations into piR30840 (30840mut) that was correspondingly complementary to the mutation in the In2m resulted in a decrease in the expression of IL-4 in the In2m (Figure 3G), suggesting that direct binding was required in the regulation. Furthermore, when a mutation of the piR30840-binding site was generated in the pIL-4 (Supplementary Figure S5A), piR30840 also lost its effect on IL-4 expression (Supplementary Figure S5C and D). Similarly, the expression of IL-4 using the construct harboring the IL-4 mutant was downregulated by 30840mut (Supplementary Figure S5E). Moreover, we generated a minigene of *il-4* (In2s and In2sm), which only contained a short fragment of the second intron, encompassing the binding site or harboring a mutation of the binding site (Supplementary Figure S6A). As shown in Supplementary Figure S6B and C, piR30840 downregulated the expression of IL-4 using the construct harboring the In2s, but not the In2sm that harbored the mutant. However, the expression of IL-4 using the construct In2sm was significantly reduced by 30840mut (Supplementary Figure S6D). piR30840 was derived from snoRNA63 and overexpression of snoRNA63 should impart a similar effect on piR30840. To validate the function of snoRNA63, the construct of the piR30840 precursor which includes the fragment of snoRNA63 was transfected with plasmid pIL4, In2 or In2s respectively and the expression of IL-4 was similarly decreased by the precursor of piR30840 (Supplementary Figure S7). Taken together, these results demonstrated that piR30840 decreases IL-4 expression by sequence-specific interaction with its binding site in the second intron of the *il-4* gene. In terms of evolution, the binding site of piR30840 did not appear until the emergence of the hominidae (Supplementary Figure S8 and Supplemental statement).

piR30840 mediates the decay of IL-4 pre-mRNA

Because mature mRNA in the cytoplasm usually does not contain introns, we hypothesized that piR30840 could affect the pre-mRNA of the *il-4* gene in the nucleus (Figure 4A). The present study showed that s30840 reduced IL-4 pre-mRNA in CD4 T-lymphocytes (Figure 4B and Supplementary Figure S9A). To further validate this effect, a reporter construct containing the full length *il-4* gene (Supplementary Figure S5A) was co-transfected with s30840 into 293T cells and piR30840 still decreased IL-4 pre-mRNA (Figure 4C and Supplementary Figure S9B). These findings suggest that piR30840 mediates pre-mRNA decay. Previous reports have showed that Piwil4 and Ago4 are localized in the nucleus (63–65). Our data indicated that Piwil4 predominantly localized to the nucleus (Supplementary Figure S9C). In addition, as the RNA pull-down assay indicated that piRNA interacts with Piwil4 and Ago4 (Figure 1F), we sought to determine whether Piwil4 interacts with Ago4. Co-immunoprecipitation confirmed that Piwil4 binds to the endogenous Ago4 (Figure 4D). To explore the possibility that Piwil4 and Ago4 are involved in piRNA-mediated pre-mRNA degradation, we co-transfected s30840 and siRNAs specific to Piwil4 or Ago4 into primary CD4 T-lymphocytes. The knockdown effect of siRNA was detected by qRT-PCR (Supplementary Figure S10). The piRNA-mediated decreased expression of IL-4 was rescued by the depletion of Piwil4 or Ago4 (Figure 4E). To determine whether Piwil4/Ago4 binds to the piRNA-complementary site in the intron of IL-4, we co-transfected constructs expressing Piwil4 and pIL-4 or pIL4m into 293T cells. RIP-qPCR analysis indicated that Piwil4 significantly enriched the IL-4 pre-mRNA expressed from pIL4, but not that from pIL4m (Figure 4F). The addition of s30840 further enhanced the enrichment of Piwil4-associated IL-4 pre-mRNA (Figure 4G). These results demonstrated that the piR30840/Piwil4 complex interacts in a sequence-specific fashion with IL-4 pre-mRNA and participates in the decay of IL-4 pre-mRNA.

Alternatively, using the λN-5BoxB tethering assay previously described (66), we generated an *il-4*-expressing construct by using a 5-BoxB sequence in the short fragment of the second intron, as well as several fusion protein-expressing constructs to connect the λN peptide, which is a high-affinity binding factor for the 5-BoxB sequence with Piwil4, Ago1, Ago2, Ago3 or Ago4, respectively (Figure 4H). After co-transfection, the λN peptide could guide the fusion proteins to specifically bind to the *il-4* intron harboring the 5-BoxB sequence. Our data showed that λN-Piwil4 or λN-Ago4 co-transfected with In2s-5BoxB downregulated IL-4 (Figure 4I). In contrast, λN-Ago1, λN-Ago2 and λN-Ago3 co-transfected with In2s-5BoxB had no effect on IL-4 expression (Supplementary Figure S11A–C). These data further indicated that, guided by piR30840, these piRNA-associated proteins mediated IL-4 pre-mRNA decay.

piR30840 induces the degradation of IL-4 through nuclear exosomes

As previous reports indicate that Trf4/Air2/Mtr4 polyadenylation (TRAMP) complexes are involved in pre-mRNA degradation (67,68), we sought to determine whether TRAMP complexes played a role in the function of piRNA by using gene-specific siRNA to knockdown components of TRAMP complexes such as Trf4, Air2 and Mtr4. The expression of IL-4 was restored by the

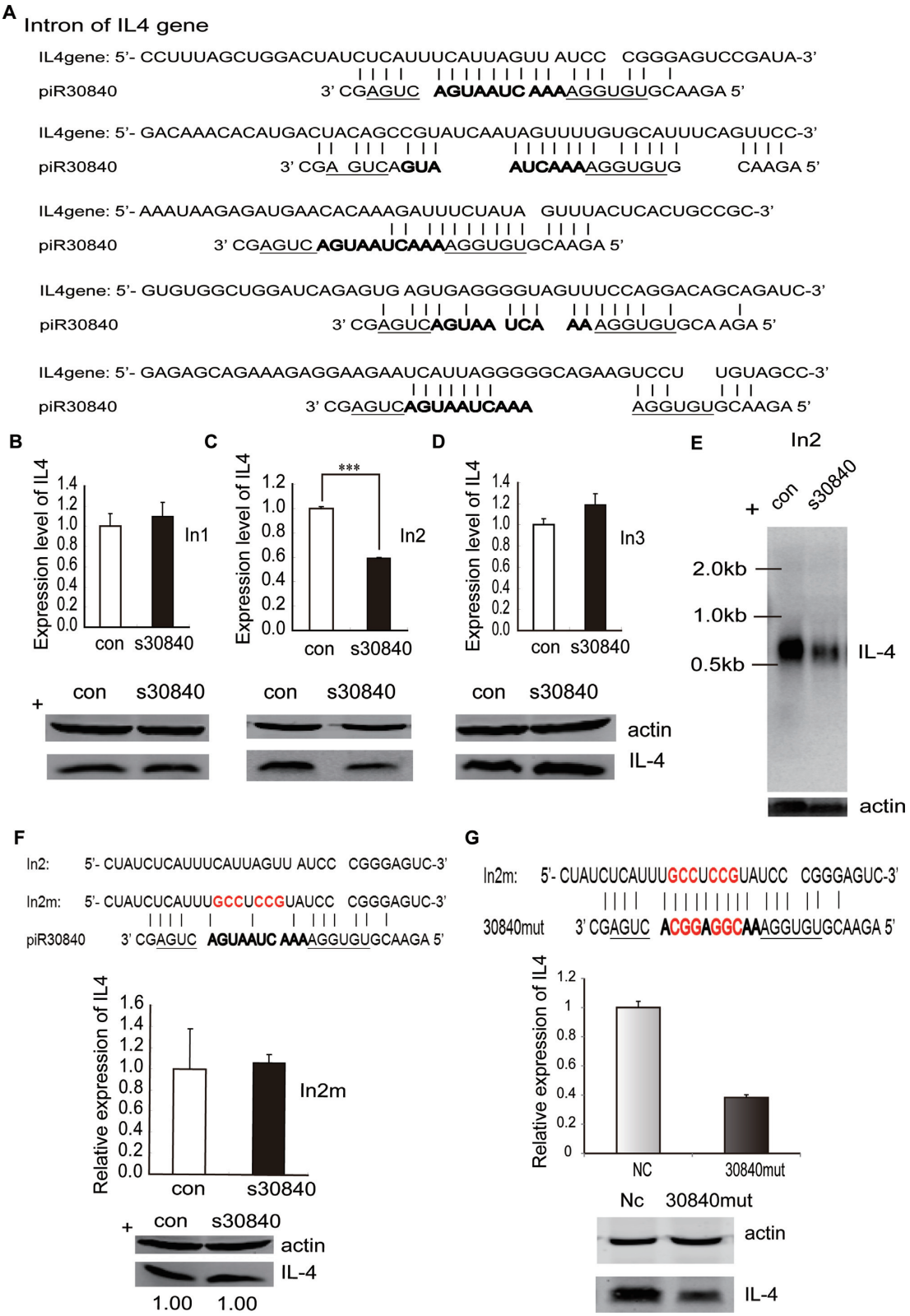
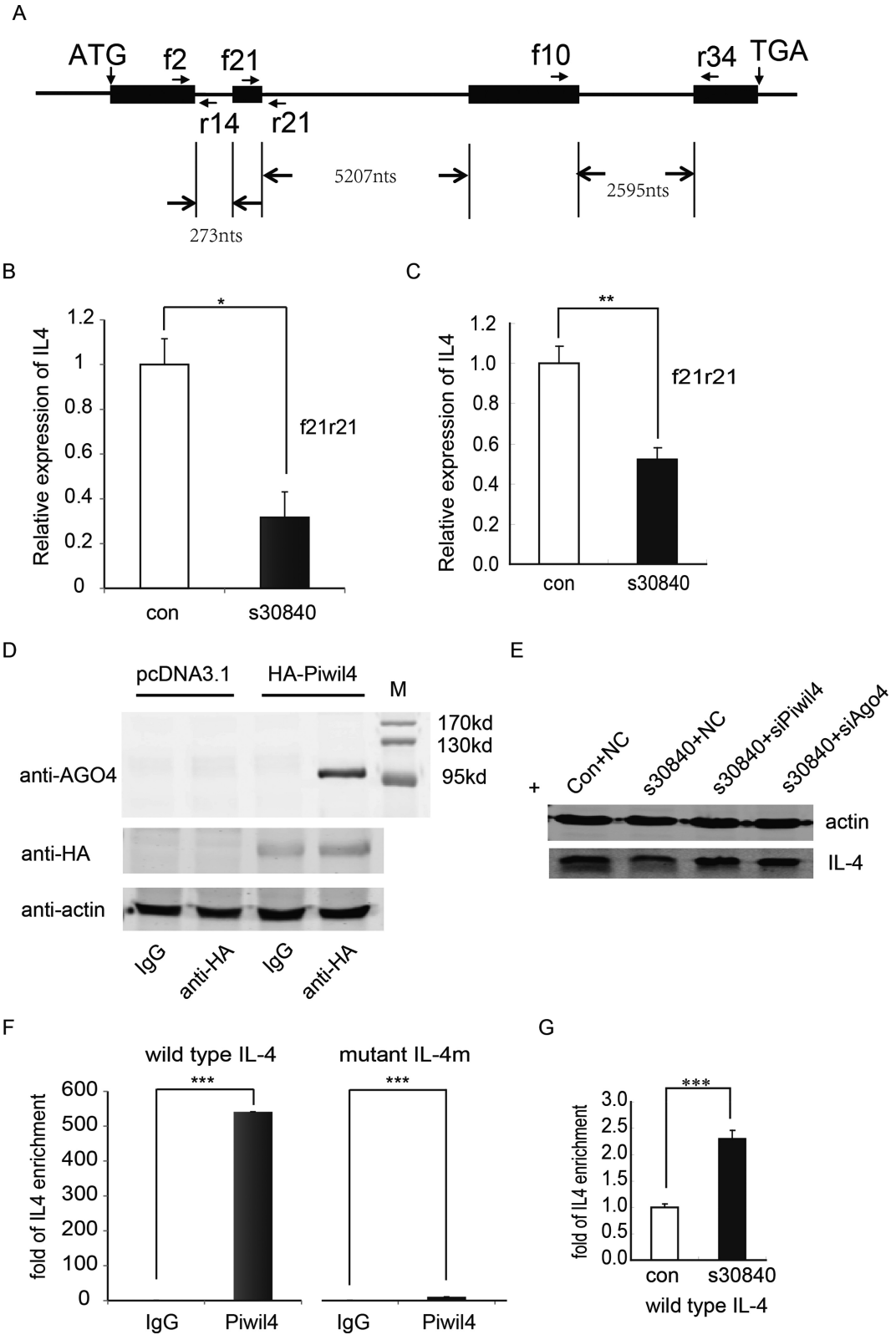


Figure 3. piR30840 downregulates IL-4 pre-mRNA via binding to its intron region. (**A**) The putative binding sites of piR30840 in the introns of IL-4 pre-mRNA. (**B**–**D**) IL-4 constructs containing the first (In1) (B), second (In2) (C) or third intron (In3) (D) were co-transfected with s30840 into 293T cells respectively. IL-4 expression was then analyzed by qRT-PCR (upper panel) and western blotting (lower panel). Statistical significance between two samples was determined by using *t*-test. $P < 0.01$. (**E**) The construct In2 was co-transfected with s30840 or control. The RNA expression of IL-4 was analyzed by northern blotting. (**F**) The construct In2m was co-transfected with s30840. IL-4 expression was examined by qRT-PCR and western blotting analyses. (**G**) The construct In2m was co-transfected with the piR30840 mutation (30840mut). IL-4 expression was determined by qRT-PCR and western blotting analyses.


A
ATG
f2
f21
f10
r34
TGA
r14
r21
5207nts
2595nts
273nts
B
Relative expression of IL4
f21r21
con
s30840
C
Relative expression of IL4
f21r21
con
s30840
D
pcDNA3.1
HA-Piwil4
M
170kd
130kd
95kd
anti-AGO4
anti-HA
anti-actin
IgG
anti-HA
IgG
anti-HA
E
Con+NC
s30840+NC
s30840+siPiwil4
s30840+siAgo4
actin
IL-4
F
wild type IL-4
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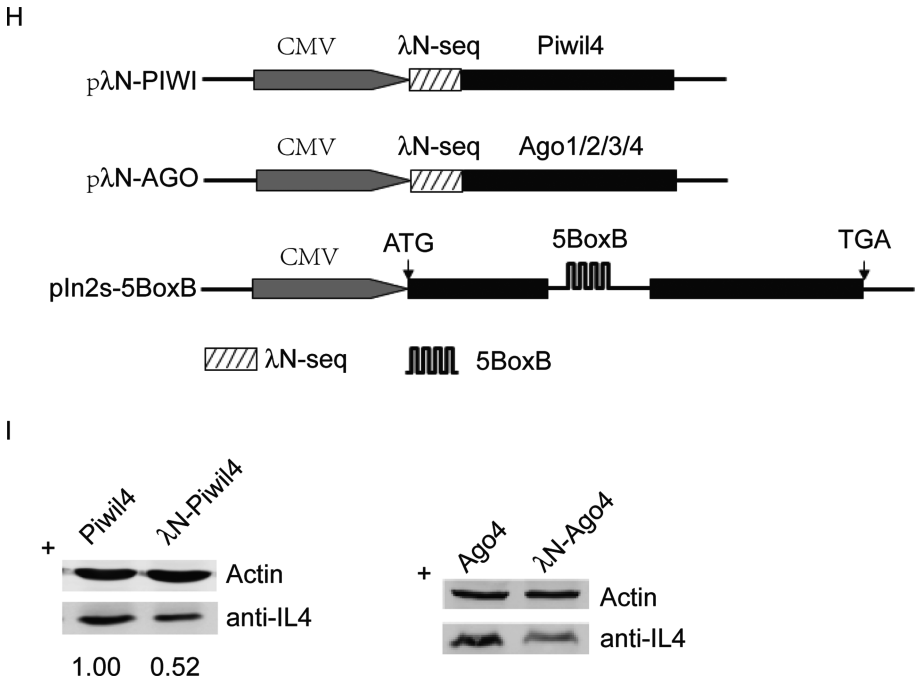

Figure 4. piRNA-guided Piwil4 and Ago4 participate in IL-4 pre-mRNA decay. (**A**) Schematic showing the primers used in detecting *il-4* pre-mRNA. The primers f2/r14 and f21r21 were used to detect IL-4 pre-mRNA. (**B** and **C**) The effect of piR30840 on the IL-4 pre-mRNA. Primers were specific to the intron and exon of IL-4 (Figure 4A). The IL-4 pre-mRNA was examined in the purified CD4 T lymphocytes from PBMCs (B). The construct pIL4 was co-transfected with s30840 or control into the 293T cells. IL4 pre-mRNA was detected via qRT-PCR (C) analysis. Statistical significance between two samples was determined by using the student's *t*-test. $P < 0.05$. The data represents three independent experiments. (**D**) The interaction between Piwil4 and Ago4 in 293T cells was detected by co-immunoprecipitation. Actin was used as loading input control. (**E**) The s30840 was co-transfected with *piwil4- or ago4*-specific siRNAs into CD4 T lymphocytes, respectively. IL-4 expression was detected by western blotting. The data represents three independent trials. (**F**) The Piwil4-expressing construct was co-transfected with the pIL4 or pIL4m construct into the 293T cells. The Piwil4-associated IL-4 pre-mRNA was detected by RIP-qPCR. Statistical significance between two samples was determined by using the student' *t*-test. $P < 0.01$. The data represents three independent experiments. (**G**) The Piwil4-expressing construct was co-transfected into 293T cells with the pIL-4 construct, with or without s30840. Piwil4-associated IL-4 pre-mRNA was detected by RIP-qPCR. $P < 0.01$. The data represents three independent trials. (**H**) Schematic graph showing the tethering assay system. pIn2s-5BoxB contains five 19 nt BoxB hairpins in the intron of IL-4, which can potently interact with various λN-PIWI or λN-AGO fusion proteins. (**I**) The construct containing In2s-5BoxB was co-transfected with the construct piwil4/ λN-piwil4 or ago4/λN-ago4. The effect on IL-4 expression was analyzed with western blotting.

depletion of Trf4 or Air2, but slightly by the depletion of Mtr4, possibly because of the abundant expression of Mtr4 (Figure 5A). Co-immunoprecipitation results showed that Piwil4 interacts with Mtr4, which indicated that Piwil4/Ago4 interacts with the TRAMP complex (Figure 5B). A previous study indicated that Mtr4 interacts with the tri-snRNP protein, Prp4 (68). Similarly, Piwil4 was immunoprecipitated with Prp4 (Figure 5C), suggesting that the complex of Piwil4 and piRNA may be involved in regulating pre-mRNA processing.

Exosomes have been detected in the nucleus and are associated with TRAMP-mediated pre-mRNA decay (67,68). To determine whether exosomes played a role in the Piwil4/piRNA-induced pre-mRNA decay, we used siRNA to knockdown the components of exosomes. Figure 5D showed that the piRNA-mediated decreased expression of IL-4 was restored by the depletion of Rrp6, Rrp44 or both, suggesting that piRNA-guided Piwil4 interacts with TRAMP complexes and subsequently promotes *il-4* pre-mRNA degradation in the nuclear exosomes (Supplementary Figure S12).

piR30840 affects the development of Th2 lymphocytes

To further examine the biological effect of piR30840, we transfected the activated naïve cells with s30840 or piRNA antisense inhibitor under the culture conditions for Th2 development and observed a downregulation of IL-4 (Figure 6A) or conversely enhanced (Figure 6B). The effect of piR30840 on IL-4 expression *in vivo* was subsequently examined. We found that piR30840 downregulated the expression of IL-4 mRNA in the human T-lymphocytes isolated from irradiated NOG (NOD/Shi-scid, IL-2Rγ KO) mice, which were first humanized by adoptive transfer of human $CD34^+$ cells for 8 weeks, followed by injection of agopir-30840 intravenously (Figure 6C). These findings suggested that piR30840 inhibition in IL-4 may be a potential therapeutic strategy for human allergic diseases. Furthermore, the purified naive CD4 T-lymphocytes were activated and transfected with s30840, then cultured *in vitro* with Th2 skewed condition for 7 days. piR30840 significantly inhibited the differentiation of Th2 cells (Figure 6D). Moreover, purified human CD4 naive T-lymphocytes were activated and transfected with piRNA antisense inhibitor, then cultured in the presence of various cytokines and neutralizing antibodies for 2 days. Subsequently, we adoptively

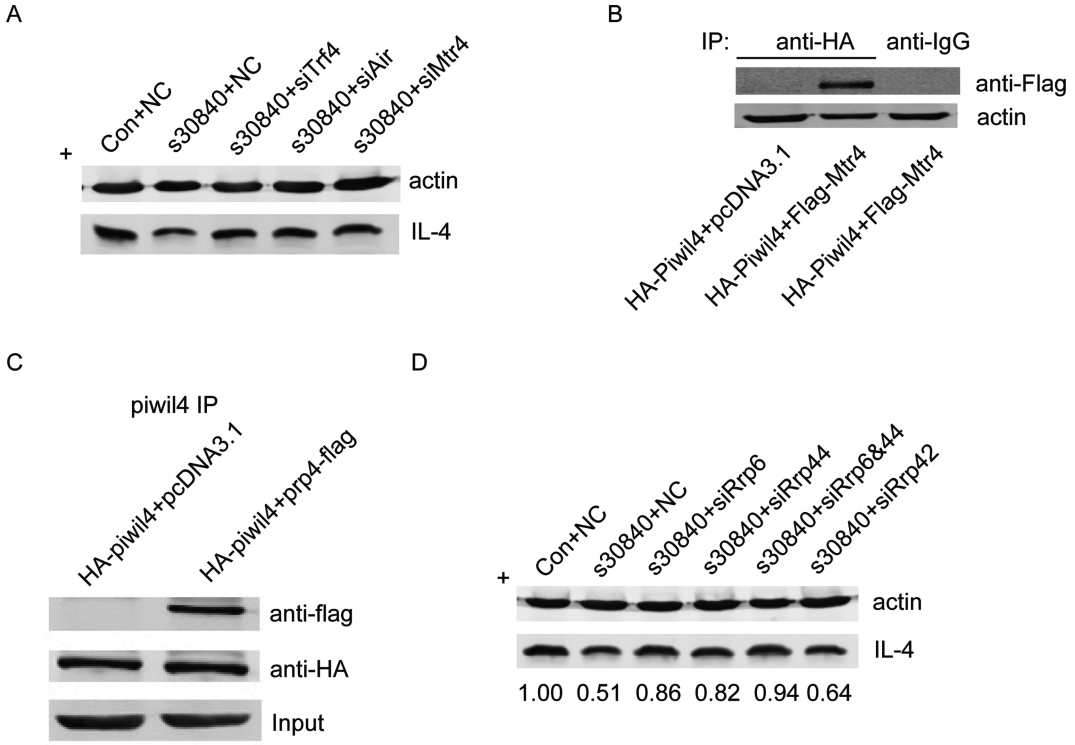

Figure 5. TRAMP complex and exosome are involved in the decay of IL-4 pre-mRNA in the nucleus. (**A**) s30840 was co-transfected with siRNAs specific for trf4, air or mtr4 into CD4 T lymphocytes, respectively. The IL-4 expression was analyzed by western blotting. The data represents three independent experiments. (**B**) The interaction between Piwil4 and Mtr4 in 293T cells was detected by co-immunoprecipitation. Actin was used as loading control. (**C**) Piwil4 interacting with Prp4 was detected by co-immunoprecipitation in 293T cells. (**D**) s30840 was co-transfected with siRNA specific for rrp6, rrp44 and rrp42 into primary CD4 T-lymphocytes. The expression of IL-4 was detected by western blotting. The data represents three independent trials.

transferred these into the humanized mice. The inhibition of piR30840 potently promoted the generation of human Th2 cells in the humanized mice (Figure 6E). Together, piR30840 regulates the development of Th2 lymphocytes *in vitro* and *in vivo* by regulating IL-4 expression.

piR30840 is inversely correlated with the expression of IL-4 in asthma patients

To investigate the clinical relevance of inhibition of piRNA on IL-4, the samples of asthma patients or healthy individuals were collected and serum levels of IgE and IL-4 and piR30840 in CD4 T lymphocytes were detected. IgE and IL-4 were highly expressed in patients and normal levels of IL-2 and IL-7 were detected in the controls (Figure 7A, B and Supplementary Figure S13A and B). The expression of piR30840 was significantly lower in patients (Figure 7C). For the control, no difference in the expression of piR36170 between healthy individuals and patients was observed (Supplementary Figure S13C). The altered expression of piR30840 was inversely correlated to the expression level of IL-4 in asthma patients ($r = 0.63$) (Figure 7D).

DISCUSSION

We have identified a large amount of snoRNA-derived piRNAs in human primary CD4 T-lymphocytes, which is a group of highly differentiated somatic cells, and have proven that these possess important biological functions, thereby, suggesting a multifaceted role for piRNA that is beyond that observed in germlines and early embryos. The existence and differential expression of piR30840 were confirmed by RT-qPCR and northern blot analyses, which showed the characteristics of classical piRNAs. The results of RTL-P proved that piR30840 presented a modified 2′-O-methylation at its 3′ end (21,49). RNA immunoprecipitation (RIP) also confirmed the interaction between PIWIL proteins and small RNAs. To examine the expression of PIWILs in human CD4 T-lymphocytes, we detected the mRNA and the proteins levels of PIWILs in CD4 T-lymphocytes. Importantly, its function was inhibited by the knockdown of Piwi protein by using siRNAs, further supporting that piR30840 possessed features of piRNAs (Figure 4E).

Our findings indicate that piR30840 in human CD4 T-lymphocytes decreased the expression of an important cytokine through sequence-specific binding to its pre-mRNA intron. Various miRNAs are involved in the regulation of lymphocyte development and differentiation (56–57,59). Some of their expression levels are much lower than that of piR30840. These miRNAs regulate gene function through the canonical pathway of miRNAs. Our findings revealed a new molecular mechanism for piRNA and possibly an important and universal pathway for regulating gene expression, which is different from that of piRNA expressed in the germline or other somatic cells (8,40–41,45–46,69–70). Although it has been reported that *piwi* genes are dis-

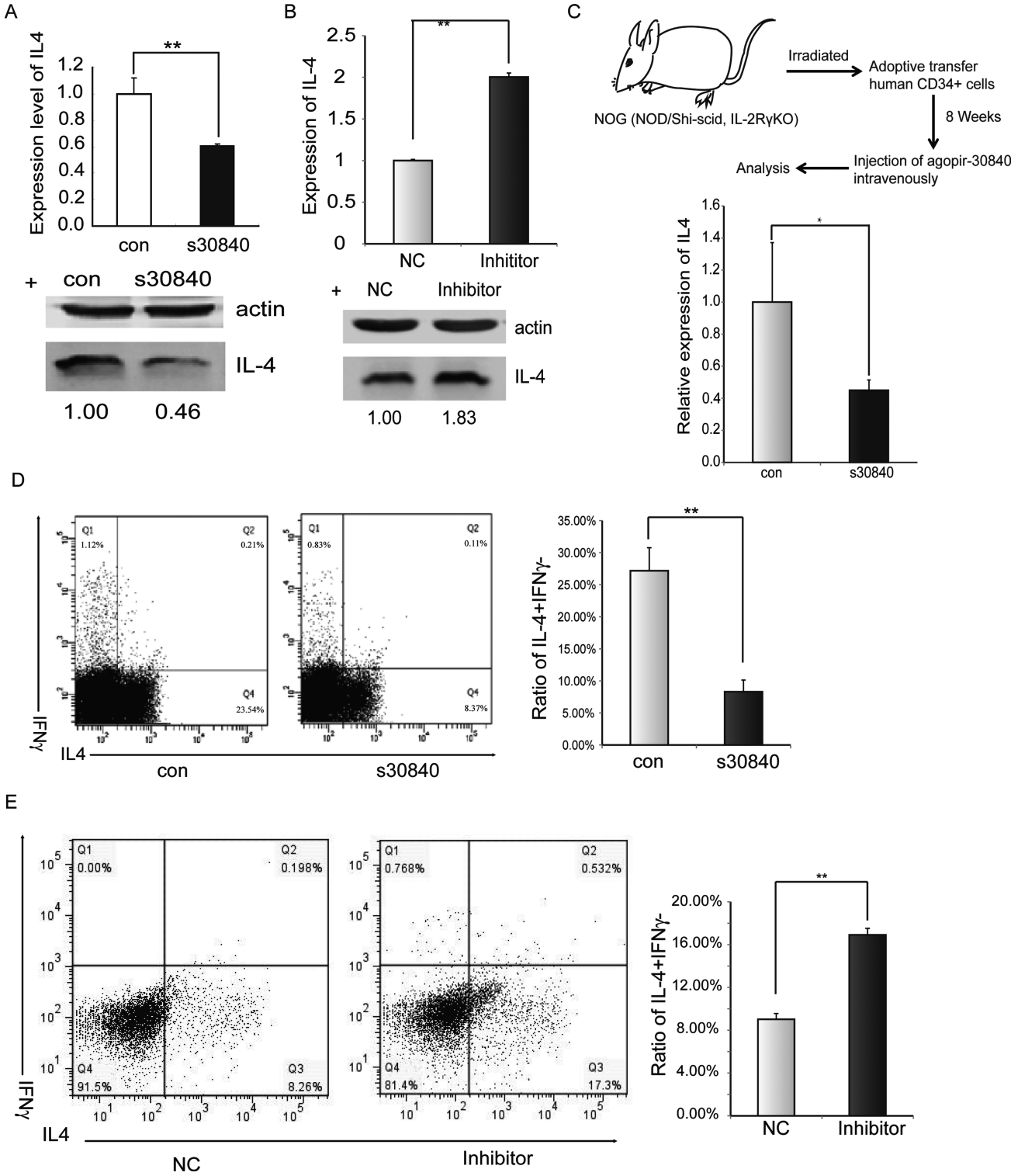

Figure 6. piR30840 regulates the development of Th2 lymphocytes. (**A** and **B**) Human CD4 naïve cells were cultured in a conditioned medium for Th2 development and then transfected with an s30840 (A) or piR30840 inhibitor (B). IL-4 expression was detected by qRT-PCR and western blotting analyses. $P < 0.01$. The data represents three independent experiments. (**C**) Detection of IL-4 expression in human PBMCs isolated from humanized NOG mice. IL-4 expression was detected by qRT-PCR analysis. The data was expressed as the mean of three independent experiments. Wilcoxon test was used to calculate the *P*-value. $P < 0.05$. (**D**) CD4 naïve cells were cultured in the conditioned medium for Th2 development and transfected with s30840 or control. After 7 days, the Th2 T-lymphocytes (intracellular stained as IL-4$^+$ IFN-γ$^-$) were detected with FACS analysis. The data was shown as a mean of six independent experiments. $P < 0.01$. (**E**) The CD4 naïve cells were cultured in a conditioned medium for Th2 development with or without the transfection of inhibitor. We adoptively transferred the naïve cells into the irradiated NOG mice which were first humanized. After 7 days, Th2 T-lymphocytes (intracellularly stained as IL-4$^+$ IFNγ$^-$) were detected by using FACS analysis. The data were expressed as the mean of six independent experiments. Statistical significance between two samples was determined by using the student's *t*-test. $P < 0.01$.

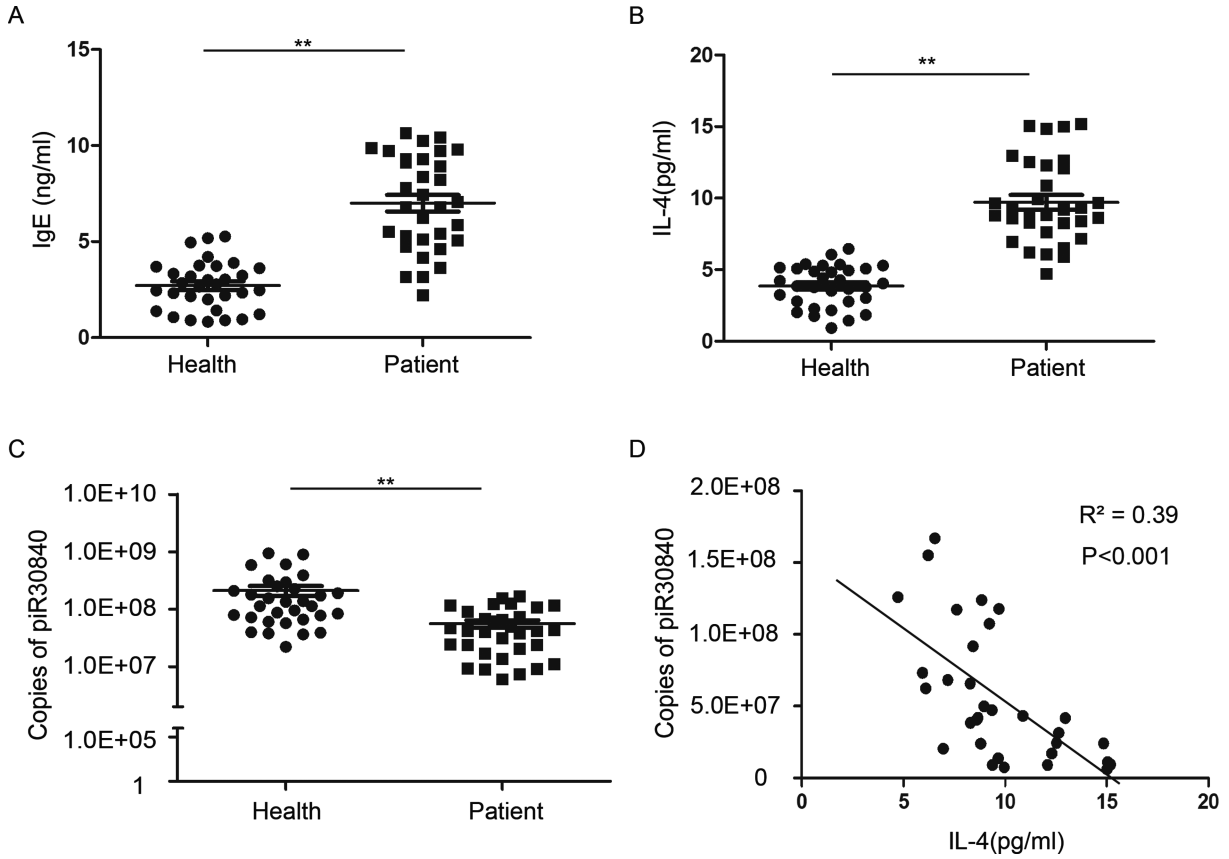

Figure 7. piR30840 is correlated with IL-4 expression in asthma patients. (**A** and **B**) Serum IgE and IL-4 levels of healthy individuals or patients diagnosed with asthma were detected by using ELISA. Statistical significance between two samples was determined by using the student's *t*-test. $P < 0.01$. $n = 32$. (**C**) The copies of piR30840 (10^6 CD4 T lymphocytes) were detected by qRT-PCR. The amount of loading was normalized with β-actin mRNA. $P < 0.01$. $n = 32$. (**D**) The correlation of piR30840 in the CD4 T lymphocytes and IL-4 in patient samples. $R = 0.63$, $P < 0.001$

pensable in hematopoiesis in mice, it does not exclude the possibility that PIWI/piRNA plays important roles in regulating gene expression and differentiation of a subset of T cells (71). In addition, previous expression profiling data showed that *piwi* is less expressed in the mouse CD4 T lymphocytes. However, the present study showed that *Piwil4* is expressed in human CD4 T lymphocytes at a much higher level (Supplementary Figure S2). Conversely, it has been reported that the C/D box snoRNA HBII-52 (SNORD 115) and its fragments (37 to 87 nts) directly bind to the splicing site of mRNA and affect splicing patterns (72,73). Nevertheless, its regulatory mechanism is fundamentally different from that of our findings.

piRNAs originate from diverse transcripts. Small RNAs derived from snoRNA were identified as piRNAs in the present study. The expression of piRNAs was detected in mouse neurons and the reported sequences in existing gene annotations indicate that these piRNAs are fragments of snoRNAs or other abundant RNAs (7). Similar small RNAs derived from other RNA species (tRNA, rRNA, snRNA and snoRNA) have also been identified and have been shown to regulate gene expression or function as signaling molecules (60,74–76). These interesting pools of small RNAs cannot be considered as the products of random RNA cleavage, but seems to be an underestimated source of regulatory molecules. The identified regulatory mechanisms so far, however, are completely different from that reported here. It is interesting that a group of snoRNA-derived piRNAs are highly expressed in human CD4 T-lymphocytes. These piRNAs contain the conserved C/D′ or C′/D box motif. The C/D box motif in the snoRNA has been demonstrated to be important for the processing, stability, nuclear retention, protein binding, nucleolar localization or function of snoRNA (54,77). Therefore, the conserved C/D box motif in the piRNA is possibly required for the interaction with co-factors, distribution or function performance.

The findings generated by studies on nuclear RNA degradation have long been controversial and thus were considered as a series of random events. Nevertheless, increasing evidence has indicated that it is a well-ordered, strictly controlled and reproducible process that regulates cellular transcription and splicing and plays a critical role in controlling RNA quality (61,67–68,78–79). In eukaryotes, the exosome is accompanied by activating complexes known as TRAMP, which is the main component of the RNA quality control system (67,68). TRAMP complexes participate in pre-mRNA degradation (67). In additional, other exonucleases also participate in the degradation of defective RNA molecules in the nucleus (79). Our studies have demonstrated that PIWI/piRNA complexes interacted with TRAMP complexes and exosomes, resulting in

the decay of piRNA-targeted pre-mRNA (Supplementary Figure S12). Furthermore, the association of PIWI with tri-snRNP protein Prp4 suggests that PIWI/piRNA also affects pre-mRNA splicing that generates defective RNAs. Together, these data further provide evidence that after sequence-specific guiding by piRNA, PIWI/piRNA complex mediated RNA decay is an effective process of regulating gene expression in the nucleus.

Th2 cytokines such as IL-4 are critical for various immunological functions, including the maturation of B cells. It is required to complete class-switch to IgE antibody production, which is closely related to atopic disorders such as asthma. Th2 subsets differentiate from CD4 naïve cells and the cytokine IL-4 plays a crucial role in imparting its effect on naïve cells via the IL-4 receptor. In our experiments, we showed that piRNA interacts with Piwil4 and Ago4, and then targets the intron of IL-4, which in turn results in the decay of IL-4 pre-mRNA. This reaction subsequently induces the downregulation of IL-4 in CD4 lymphocytes and inhibits the differentiation of Th2 CD4-T-lymphocytes. This abnormal expression has been reported in pathologic conditions such as asthma. Therefore, our finding has identified a new mechanism for epigenetically regulating the expression of cytokines and the differentiation of T-lymphocytes during physiological or pathophysiological situations and possibly provides a novel therapeutic target for human allergic diseases. Our report also proposes that similar to various miRNAs, piRNAs that are highly expressed in various differentiated somatic cells and tissues could also play important roles during the pathogenesis of human diseases.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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F.D.Z. designed and performed the most experiments, N.Z., H (ong).Z., X.Z., G.G., T.P., H.L., Y.Z., Z.X., B.L., W.G., C.L., L.R. and J.L.(i) participated in some of the experiments such as preparing the cells, constructing the clones, siRNA-knock down gene expressions and detecting promoter activity. Y.G and W.T collected the clinical samples. J.L.(iu) performed the bioinformatics analysis. H.Z. directed and supervised the experiments and interpretations of data. F.D.Z. and H.Z. wrote the manuscript.

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