

The human Piwi protein Hiwi2 associates with tRNA-derived piRNAs in somatic cells

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Received March 26, 2014; Revised June 26, 2014; Accepted June 26, 2014

ABSTRACT

The Piwi-piRNA pathway is active in animal germ cells where its functions are required for germ cell maintenance and gamete differentiation. Piwi proteins and piRNAs have been detected outside germline tissue in multiple phyla, but activity of the pathway in mammalian somatic cells has been little explored. In particular, Piwi expression has been observed in cancer cells, but nothing is known about the piRNA partners or the function of the system in these cells. We have surveyed the expression of the three human Piwi genes, *Hiwi*, *Hili* and *Hiwi2*, in multiple normal tissues and cancer cell lines. We find that *Hiwi2* is ubiquitously expressed; in cancer cells the protein is largely restricted to the cytoplasm and is associated with translating ribosomes. Immunoprecipitation of *Hiwi2* from MDAMB231 cancer cells enriches for piRNAs that are predominantly derived from processed tRNAs and expressed genes, species which can also be found in adult human testis. Our studies indicate that a Piwi-piRNA pathway is present in human somatic cells, with an uncharacterised function linked to translation. Taking this evidence together with evidence from primitive organisms, we propose that this somatic function of the pathway predates the germline functions of the pathway in modern animals.

INTRODUCTION

Piwi-domain containing Argonaute proteins are conserved among eukaryotes and archaea (1–4). The eponymous

member of the Piwi subclade was identified in *Drosophila* as a factor necessary for germ cell maintenance (5), and Piwi orthologues have since been found to be highly expressed in the developing germline tissues of a wide variety of animal species (4). Model organisms with impaired Piwi function generally show no obvious defects outside the germ line: for example, in laboratory mice, deletion of any one of the three murine *Piwi* genes (*Miwi*, *Mili* and *Miwi2*) results in male sterility accompanied by transposon derepression in differentiating gametes (6–10), but mice are viable and appear healthy. Piwi proteins, like their Ago protein cousins, partner with small RNAs (11–16); Piwi-interacting RNAs (piRNAs) are typically 24 to 31 nt long, are produced in a Dicer-independent manner and have reported links to a multitude of functions (reviewed in (4)). Unlike miRNAs, piRNAs are extremely diverse, with at least hundreds of thousands of mature species transcribed from thousands of genomic loci, many of which are large asymmetric and syntenic clusters (7,11,17).

Retrotransposon suppression in the developing germline is the best understood function of the Piwi-piRNA pathway. Piwi proteins and retrotransposon-derived piRNAs act together during distinct periods of germ cell development to suppress retrotransposon activation both transcriptionally and post-transcriptionally (reviewed in (18)). In laboratory mice lacking *Mili* or *Miwi2*, increases in retrotransposon transcription are linked to loss of genomic cytosine methylation on these elements (8,9); this has contributed to the now well-accepted idea that piRNAs act as guides for epigenetic modification and suppression of homologous sequences in the germline (19).

Studies on the Piwi-piRNA pathway outside the germline are limited, but there is evidence for somatic function in a number of organisms. Somatic piRNAs have been cloned from *Drosophila*, rhesus macaque and mouse tissues (20–

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24), including mouse hippocampal neurons where they associate with Miwi (25). Somatic piRNAs have also been implicated in long-term memory formation *via* epigenetic mechanisms in the neurons of the model sea slug *Aplysia* (26). Expression of the effector Piwi proteins has also been observed in somatic tissues across phyla. Orthologues of Piwi are expressed in the stem cells and regenerative tissues of a number of primitive organisms including jellyfish (27), sponges (28), planarians (29,30), polychaete worms (31) and colonial ascidians (32), implying a conserved function for Piwi in stem cell maintenance beyond the germline. Piwi proteins have been found in mammalian somatic cells, in particular, mouse haematopoietic cells (33,34) and human cancer cells (35–38), while piRNAs have separately been reported in both tumours and cancer cell lines (39,40). It has been suggested that epigenetic silencing by the Piwi-piRNA pathway could be responsible for the aberrant hypermethylation of genes commonly seen in malignant cells (41).

Given the evidence for somatic Piwi expression, and particularly the evidence for expression in cancer cells, we set out to establish which of the human Piwi proteins might partner with piRNAs in cancer cells, and to seek clues to the function of the Piwi-piRNA pathway in somatic cells. We find that the human Piwi orthologue *Hiwi2* is ubiquitously expressed in both normal human somatic tissues and cancer cell lines, albeit at a lower level than in the testis. In MDAMB231 breast cancer cells, *Hiwi2* localises to the cytoplasm and associates with the translational apparatus. Contrary to the hypothesis that the Piwi-piRNA pathway is involved in the epigenetic aberrations of cancer cells, we find that *Hiwi2*-bound piRNAs are not derived from retrotransposons or hypermethylated CpG islands; rather they are predominantly derived from tRNAs, and to a lesser extent from expressed and unmethylated genes. The tRNA-derived and gene-derived piRNAs can also be found in the adult human testis, suggesting a function for *Hiwi2* that is common to germline and soma.

MATERIAL AND METHODS

Quantitative RT-PCR

Total RNA from human adult testis (Ambion), somatic tissues (Human Total RNA master panel II, Clontech) and cell lines was reverse transcribed and subject to quantitative PCR in Sybr Master Mix (Roche) with 20 nM primer. Ribosomal protein Rpl13a was used as a reference gene. Primer sequences are as follows: *Hiwi* F: AC GCTGCATATTCAGGATAGA, *Hiwi* R: GACAGTGA CAGATTTGGCTCTC, *Hili* F: CGCATTATGTCTGT GTTCTCAA, *Hiwi* R: AAGCGATTCTCCTGCCTTAG, *Hiwi2* F: AATGCTCGCTTTGAACTAGAGAC, *Hiwi2* R: ATTTTGGGGTAGTCCACATTAATC, *Rpl13a* F: CCTGGAGGAGAAGAGGAAAGAGA, *Rpl13a* R: TG TCATACCAGGAAATGAGC.

Nuclear/cytoplasmic fractionation and western blotting

MDAMB231 cells ($\sim 10 \times 10^6$) were pelleted by centrifugation at 4°C. Cytosolic proteins were recovered by lysing the cell pellet in 400 μ l Buffer A (10 mM Hepes, 10 mM KCl,

1.5 mM MgCl₂, 0.1 mM EDTA, 1 mM dTT, with protease inhibitor cocktail) for 15 min on ice before addition of 40 μ l of 1% NP-40. The cell lysate was then centrifuged at 13 000 $\times g$ and the supernatant collected as the cytoplasmic fraction. The pellet was washed twice with 500 μ l Buffer A with centrifugation at 13 000 $\times g$, then lysed in 150 μ l Buffer C (50 mM Tris-HCl (pH 7.5), 0.5% Triton X-100, 137.5 mM NaCl, 10% Glycerol, 5 mM EDTA, 0.5% SDS, with protease inhibitor cocktail). The nuclear lysate was sonicated and cleared by centrifugation at 17 000 $\times g$ for 15 min. Separation of the cytoplasmic and nuclear fractions was verified by western blot for β -tubulin (anti- β -tubulin, Sigma T5201) and histone H2b (anti-H2B, Abcam ab1790). Western blot for *Hiwi2* used the anti-Piwi4 antibody (Abcam ab21869). Western blot secondary antibodies were fluorescently labelled and membranes were visualised on a Licor Odyssey.

Immunocytochemistry

MDAMB231 cells on gelatin-coated coverslips were fixed in 4% PFA and blocked before incubation with anti-Piwi4 (Abcam ab21869) followed by Cy3-AffiniPure Donkey anti-Rabbit IgG, and visualisation on a Carl Zeiss LSM 700 Upright Confocal microscope. DNA was stained with Hoechst-33342.

Polysome profiling

Preparation of sucrose density gradient fractionated polysomes was performed as previously described (65). Briefly, cells treated with either 200 μ g/ml puromycin or no treatment were lysed and the cleared lysate applied to 17–50% sucrose gradients before ultracentrifugation at 210 000 $\times g$. Fractions were collected along the gradient and protein precipitated from the fractions with methanol before chloroform extraction. Identification of polysome-containing fractions was achieved by western blot for ribosomal protein Rps6 (anti-Rps6, Cell Signal 5G10).

Generation of Flag-*Hiwi2* expressing cell line

MDAMB231 cells were transfected with a linearised Flag-*Hiwi2*-piRESneo construct using Lipofectamine 2000. Cells were re-plated after 24 h in a medium containing 1000 μ g/ml G418. After approximately 3 weeks, colonies containing >100 cells were isolated and expanded. Verification of Flag-*Hiwi2* expression in clonal lines was achieved by western blot for the Flag epitope (anti-Flag M2, Sigma F1804). No clone demonstrated high expression of the tagged protein—all clones showed a modest Flag signal on western blot; the clone with the highest Flag *Hiwi2* expression was chosen for immunoprecipitation experiments.

Flag-*Hiwi2* immunoprecipitation

Approximately 1×10^8 wild-type and Flag-*Hiwi2* expressing MDAMB231 cells were harvested and lysed by resuspension in 200 μ l MagnaRIP (Millipore) lysis buffer. Flag-*Hiwi2* was immunoprecipitated with anti-FLAG M2

antibody using the MagnaRIP protein and RNA immunoprecipitation kit (Millipore). Half of the immunoprecipitation was reserved for protein isolation and co-immunoprecipitated RNAs were isolated from the remainder according to the manufacturer's instructions. Co-immunoprecipitated proteins were eluted from the reserved fraction by incubation in 1 mg/ml 3x Flag peptide solution (Sigma) in MagnaRIP wash buffer (Millipore).

Mass spectrometry

Proteins co-immunoprecipitated with Flag-Hiwi2 were separated on a 4–12% Bis-Tris PAGE gel (Invitrogen) and visualised using SYPRO Ruby stain (BioRad). The entire lane was excised from the gel and analysed by one-dimensional MS on either a Thermo LTQ FT or Applied Biosystems QSTAR Pulsar using trypsin digest and ESI TRAP identification of peptides. Peptides were identified by Mascot (Matrix Science) using the IPI-Human-v3.58 database, with fragment tolerance of 0.4 Daltons (monoisotopic) and parent tolerance of 4.0 parts per million (monoisotopic). Protein coverage, peptide number and significance were assessed using Scaffold 3 (Proteome Software). Any peptides with significant coverage in the wild-type MDAMB231 immunoprecipitation were removed from the list of Hiwi2 co-immunoprecipitated proteins. Results presented are an average of protein coverage from biological replicate experiments.

Small RNA sequencing

Libraries for deep sequencing were prepared from 3 μ l of a total 5 μ l of co-immunoprecipitated RNA, and from ~500 ng total RNA from MDAMB231 cells and adult testis, using the Small RNA Expression Kit with barcoded primers (Life Technologies). A single-emulsion PCR was used to couple the barcoded libraries to P1-coated beads, and sequencing was performed using 35 bp chemistry on a SOLiD machine (version 3.0).

SOLiD sequencing data were mapped using the Lifescope small RNA pipeline (Life Technologies) with zero mismatches, and filtered for rRNA. Each tag was mapped against an expanded small RNA dataset including miRNA (miRbase 19), snoRNA and tRNA (hsa19 tRNAdb); unmapped tags were then mapped against the entire human genome (hg19). Multimappers were not binned to the default Lifescope filter; Lifescope output comprised a single best-mapped position for each tag. The Miwi2 IP E16.5 testis dataset 8 was downloaded from the Gene Expression Omnibus (accession GSM319957). Adapters were trimmed using cutadapt prior to mapping to the mouse genome (mm10) using Bowtie with an 18 base seed, up to three mismatches and a maximum sum of mismatched quality values of 70.

Mapped tags were annotated hierarchically using the Ensembl perl API and custom perl scripts, with preference given to sense-oriented transcripts and to small/noncoding RNA over protein coding genes, in the following order: Human: the miRNA/snoRNA/tRNA dataset above, UCSC hg19 nested repeat dataset, UCSC hg19 piRNA dataset, Ensembl Homo sapiens core database (build

67_37); Mouse: miRNA/tRNA, UCSC mm10 nested Repeat dataset, Ensembl Mus musculus core database (build 72_38).

Gene expression and cytosine methylation analysis

Gene expression analysis of MDAMB231-Flag-Hiwi2 cells was performed on 1 μ g total RNA using an Affymetrix® GeneChip® Human Gene 1.0 ST Array. Gene signals were calculated as the mean intensities from all probes across the gene. Cytosine methylation analysis was performed on 1 μ g genomic DNA using the Illumina® Infinium Human-Methylation27 BeadChip. Data were processed in BeadStudio software from Illumina; CpG sites are assigned a β value that is calculated from the intensity of methylated and unmethylated signals. Thresholds of $\beta \geq 0.5$ and $\beta \leq 0.2$ were used to designate loci as methylated and unmethylated, respectively.

Gene ontology analysis

Functional analysis of genes with ≥ 50 mapped piRNAs in both the Hiwi2 IP and adult testis was carried out in Ingenuity Pathways Analysis (Ingenuity Systems), to obtain broad ontologies, and also independently in GOrilla (<http://cbl-gorilla.cs.technion.ac.il/>), to obtain both broad and specific ontologies. The Ensembl homo sapiens gene database (GRCh37.p11), filtered to include only protein-coding genes, was used as a reference set for both analyses.

TaqMan assay of tRF-piRNAs

tRF-piRNAs were validated using custom TaqMan small RNA assays (Life Technologies) against the following RNA sequences (from human tRNAdb loci): GCAUUGGUGG UUCAGUGGUAGAAUUCU (chr16.trna25-GlyGCC); UCCCUGGUGGUCUAGUGGGUUAGGAUUCGG (chr1.trna59-GluCTC); GCCCGGAUAGCUCAGU CGGUAGAGCAUCAGACUU (chr11.trna5-LysTTT); GUUCCGUAGUGUAGUGGGUCAUCACGUU (chr5.trna15-ValAAC); GUUCCGUAGUGUAGCGG UUAUCACAUU (chr19.trna13-ValCAC). hsa-miR-145 was used as a reference. tRNAs were reverse transcribed using the TaqMan® MicroRNA Reverse Transcription kit, and qPCR performed using TaqMan® Universal PCR Master Mix II.

RESULTS

Hiwi2 is widely expressed outside the germline

Activity of the Piwi-piRNA system in somatic cells would require expression of both a Piwi protein and piRNAs. We surveyed the expression of mRNAs encoding the three human Piwi members, *Hiwi*, *Hili* and *Hiwi2*, in total RNA from a panel of normal human tissues and human tumour cell lines representing a diverse range of tumour types including breast, colon, squamous cell carcinoma and leukaemia (Figure 1A). *Hiwi* expression was below the limit of detection in most normal tissues and cell lines; *Hili* was expressed at very low levels in all normal tissues tested, but

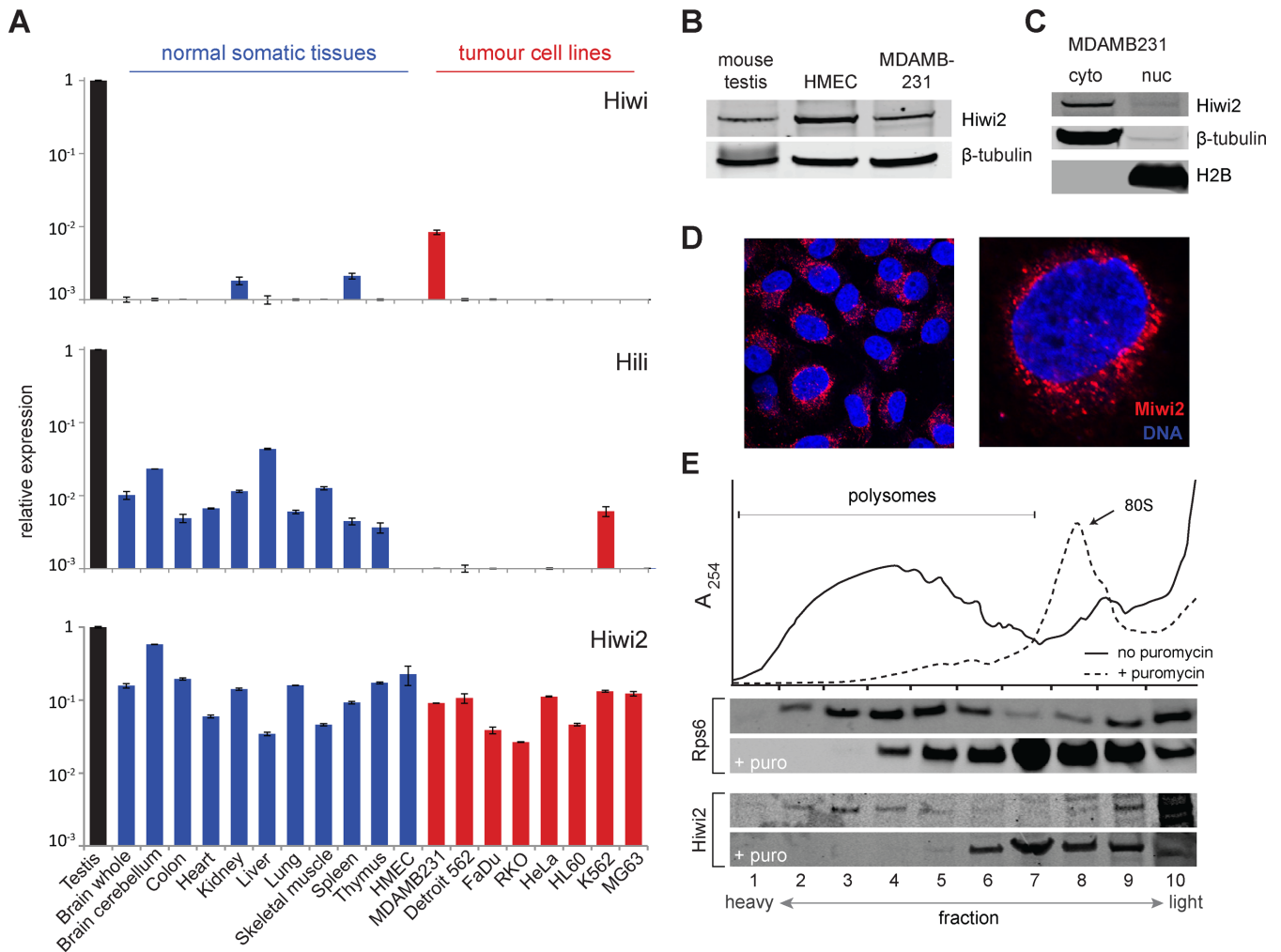


Figure 1. Hiwi2 is expressed in normal human somatic cells and cancer cells. (A) qRT-PCR data showing *Hiwi*, *Hili* and *Hiwi2* expression in adult testis (black), normal somatic tissues (blue) and tumour cell lines (red). Expression levels are shown relative to adult testis. (B) Western blot showing Hiwi2 expression in human mammary epithelial cells and the breast cancer cell line MDAMB231, and Miwi2 expression in P21 murine testis. (C) Western blot showing Hiwi2 expression in cytoplasmic and nuclear fractions prepared from MDAMB231 cells. (D) Immuno-fluorescence staining of native MDAMB231 cells captured at low (left) and high (right) magnification showing Hiwi2 concentrated in perinuclear granules in the cytoplasm. (E) Polysome profiling using sucrose density gradient fractionation showing puromycin-sensitive Hiwi2 association with polysomes. Western blot for ribosomal protein Rps6 (top) demonstrates the presence of polysomes in heavier fractions; these dissociate and move to lighter fractions with puromycin treatment (+puro). Western blot for Hiwi2 (bottom) shows an association of Hiwi2 with the polysome-containing fractions that is disrupted with puromycin treatment.

in only one of the cell lines. In contrast, *Hiwi2* was ubiquitously expressed across normal somatic tissues, at around 10% of the level seen in the human (18 year old) testis, and at similar levels in every tumour cell line tested. *Hiwi2* protein expression was confirmed in the breast cancer cell line MDAMB231 and normal human mammary epithelial cells by western blot, using an antibody against the murine orthologue Miwi2 that also recognizes Hiwi2 (see Supplementary Figure S1). *Hiwi2* protein from both normal and malignant breast cells gives a similar signal to that of Miwi2 in the testis of an adolescent (P21) mouse (Figure 1B); this represents about a third of the level of expression seen in neonatal testis where expression is thought to be at its peak (see Supplementary Figure S2).

Given that Miwi2 plays a pivotal role in the epigenetic silencing of transposons in developing germ cells, we wondered whether it might play a similar role in cancer cells,

where characteristic genomic hypomethylation is associated with widespread retrotransposon transcription (42). Direct involvement in targeted epigenetic silencing would require that Hiwi2 be located in the nucleus. However, western blot of nuclear and cytoplasmic fractions of MDAMB231 cancer cells reveals that the bulk of somatic Hiwi2 resides in the cytoplasm (Figure 1C), and immunofluorescence shows Hiwi2 concentrated in perinuclear granules (Figure 1D). In murine fetal germ cells, Miwi2 is also reportedly present in cytoplasmic granules, but this is overshadowed by its abundance in the nucleus (8); we observe essentially no nuclear staining for Hiwi2 in MDAMB231 cells (Figure 1D).

In the mouse germline, it has been shown that Mili and Miwi are exclusively cytoplasmic, and that Miwi associates with the translational machinery (8,43). We asked if cytoplasmic Hiwi2 in somatic cells is similarly associated with translation, by performing sucrose gradient fraction-

ation of MDAMB231 lysates followed by western blotting with the Hiwi2 antibody. We found that a proportion of Hiwi2 protein associates with denser polysomal fractions in a puromycin-sensitive manner (Figure 1E), indicating that Hiwi2 interacts with actively translating ribosomal complexes in the cancer cells.

Hiwi2 binds small RNAs in MDAMB231 cells

The presence of Hiwi2 protein in somatic cells implies the presence of partner piRNAs, but available antibodies do not immunoprecipitate Hiwi2 from human cells. Therefore we established stable MDAMB231 clones that express a Flag-tagged version of Hiwi2; further analysis was performed on a line that showed only a modest increase in total Hiwi2 protein, to avoid any off-target effects associated with over-expression (see Supplementary Figure S3).

Immunoprecipitation with anti-Flag antibody isolated Hiwi2 from MDAMB231 lysates, along with a number of other proteins (Figure 2A). MS analysis of the Hiwi2 IP shows that somatic Hiwi2 interacts with a diverse range of proteins, only some of which have previously been reported as Piwi partners (Table 1). Heat shock proteins were the most prominent partners, consistent with an association with translating ribosomes (44); we also identified several other translation-associated proteins. Tudor domain containing proteins, which commonly associate with Piwi proteins in the germline (45), were notably absent.

We extracted RNA from duplicate MDAMB231 Flag-Hiwi2 immunoprecipitations (Hiwi2 IP) and used it to construct small RNA libraries for SOLiD deep sequencing. We also prepared small RNA libraries from MDAMB231 total RNA, and commercially available human adult testis RNA, for comparison. After filtering we obtained 3 894 997 reads from the Hiwi2 IP that mapped to the human genome reference without error. The length distribution and broad annotation of the Hiwi2-bound small RNAs is shown in Figure 2B, and those of unselected MDAMB231 small RNAs in Figure 2C. Both the repertoire and the size distributions of the Hiwi2 IP RNAs are very different from the overall small RNA population in the cell line, consistent with enrichment for particular RNA species: the Hiwi2 IP distribution is bimodal and shifted toward larger, pi-sized RNA species heavily dominated by tRNAs, whereas the total MDAMB231 sample has a modal length of 23, consistent with microRNA being the dominant species. Control immunoprecipitations using wild-type MDAMB231 lacking Flag-Hiwi2 failed to enrich for any small RNAs.

We also find that the population of Hiwi2-associated piRNAs in MDAMB231 cells is distinctly different from the Miwi2-associated piRNAs in the prepachytene mouse testis reported by Hannon and colleagues (8). Since tRNAs were not specifically reported in the prepachytene dataset, we used our pipeline to re-map these data; the resultant distribution of annotations is nearly identical to that in the original report (Figure 2D). Most prepachytene Miwi2-associated piRNAs are retrotransposon derived, but we find no enrichment for retrotransposon sequences in the MDAMB231 Hiwi2 IP; conversely, most MDAMB231 Hiwi2 IP piRNAs are derived from tRNAs, but we find very few tRNAs in the prepachytene Miwi2 IP dataset. Further-

more, the MDAMB231 Hiwi2 IP did not recover piRNAs from the large intergenic clusters characteristic of pachytene testis piRNAs (46); this is unlikely to be an artefact of our library preparation or mapping strategy, as we readily detect these intergenic clusters in the adult testis library (Figure 2E). Despite the absence of the intergenic clusters, we do detect the 3'UTR-associated piRNA clusters previously observed in *Drosophila* ovaries, *Xenopus* eggs and mouse testis (47); we also find these 3'UTR clusters in the human testis sample (Figure 2F). This raises the possibility of a retrotransposon-independent function for Piwi that is common to germ and somatic cells.

Hiwi2-associated genic piRNAs derive from unmethylated genomic regions

A small but significant proportion of Hiwi2-associated piRNAs are derived from the sense strands of protein-coding genes (6% of all reads, a 3-fold enrichment over unselected small RNAs). Using an arbitrary threshold of ≥ 20 piRNAs per gene, we find that there are 2681 and 1968 genes producing piRNAs in the Hiwi2 IP and testis, respectively, with a representative number of each (672 in both) coming from 3'UTRs. The overlap between MDAMB231 and testis is highly significant for both gene and 3'UTR sets ($\chi^2 = P < 0.0001$; Figure 3A).

Miwi2 is required for faithful maintenance of methylation and transcriptional repression of retrotransposons in developing male gametes (8,9). Retrotransposon sequences were not enriched in our Hiwi2 IP, but given the known function of piRNAs in suppressing homologous sequences, we considered that Hiwi2-bound piRNAs might perform a similar function in cancer cells; consequently we sought to determine whether the gene-derived Hiwi2 piRNAs were associated with hypermethylated and silent loci in cancer cells. We used Affymetrix GeneChip Human Gene 1.0 ST expression arrays and Illumina Infinium 27K arrays to determine expression state and methylation state in MDAMB231-Flag-Hiwi2 cells. In total, 11 981 genes for which we obtained expression data could be unambiguously categorised by the Infinium arrays as either methylated (4338) or unmethylated (7643); 34 995 piRNAs mapped to methylated genes whereas 184 907 piRNAs mapped to unmethylated genes (Figure 3B). The differences in distribution when considering those genes producing zero, few (1–19) or many (>20) piRNAs indicate that unmethylated genes are significantly more likely to produce many piRNAs, and methylated genes are more likely to produce none at all (Figure 3C). The increased production of piRNAs from unmethylated loci is not merely a function of transcription, as we find no correlation between transcript levels and piRNA abundance (Figure 3D).

Using Ingenuity Pathway Analysis, we find that the genes producing ≥ 50 piRNAs in both MDAMB231 and testis are enriched for a range of ontologies, including development, growth and proliferation (Figure 3E shows the top 20 most significant ontologies; a full list is presented in Supplementary Table S1). A more detailed analysis with GOrilla (48) shows that many of the significantly enriched ontologies involve protein translation and related functions (Supplementary Table S2).

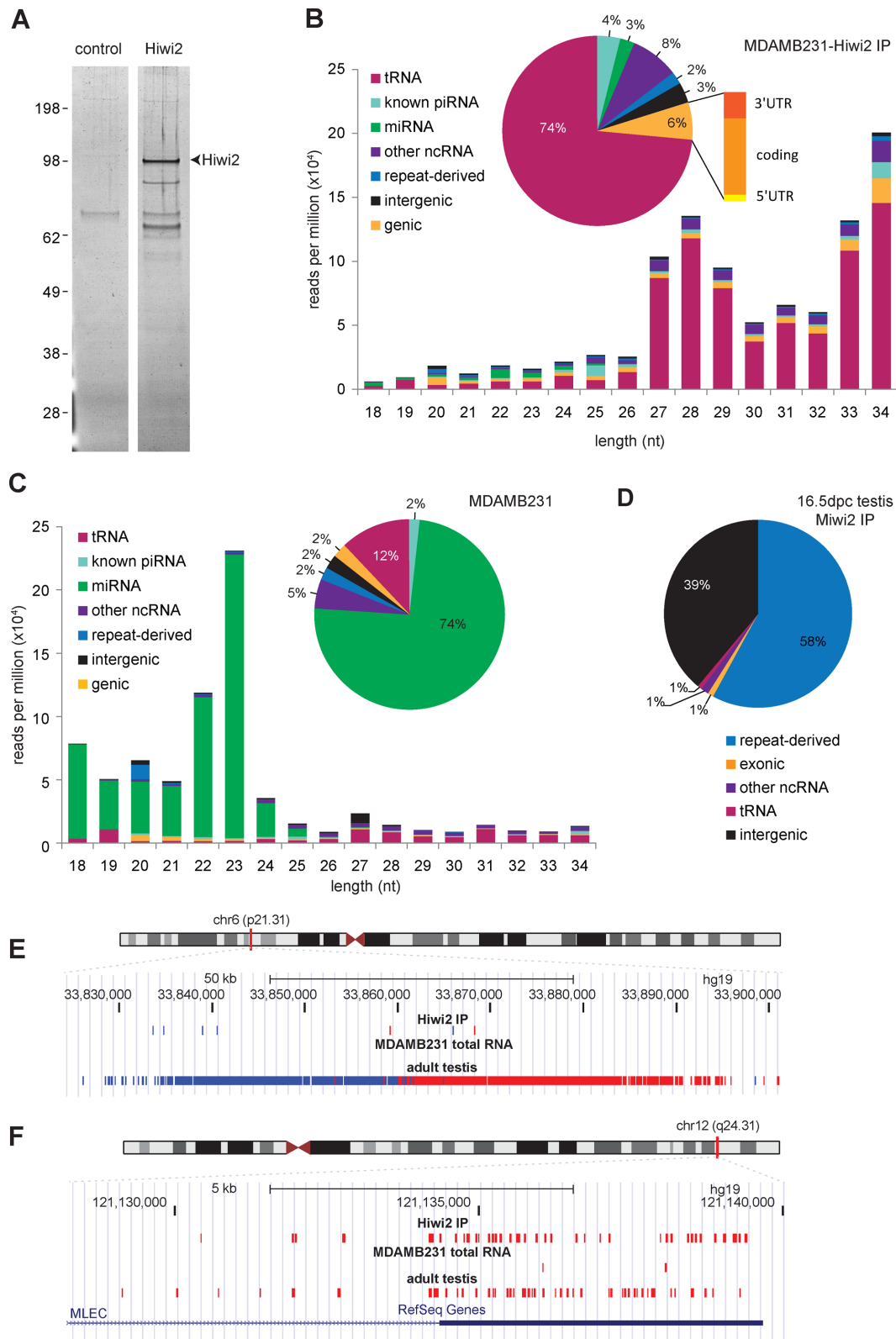


Figure 2. Hiwi2 immunoprecipitation from MDAMB231 cells captures a unique set of piRNAs. **(A)** SYPRO Ruby-stained PAGE gel showing proteins immunoprecipitated with Flag M2 antibody from native MDAMB231 cells (left) and MDAMB231 cells stably expressing Flag-Hiwi2 (right). Proteins that co-immunoprecipitate with Hiwi2 are listed in Table 1. **(B)** Length distribution (main graph) and annotations (pie chart) of small RNAs co-immunoprecipitated with Flag-Hiwi2. The genic class is expanded to show the relative number of piRNAs mapping to 5'UTRs, exons and 3'UTRs. **(C)** Length distribution and annotations of small RNAs cloned from MDAMB231 total RNA. **(D)** Annotations of Miwi2-bound piRNAs from E16.5 mouse testis (8), re-mapped to specifically include tRNAs. **(E, F)** UCSC Genome Browser snapshots show custom track examples of the absence of Hiwi2-bound somatic piRNAs from intergenic piRNA clusters **(E)** but their presence at 3'UTR clusters **(F)**; small RNAs from the adult testis dataset map to both intergenic and 3'UTR clusters. Reads mapping to the positive strand are in red, negative strand in blue.

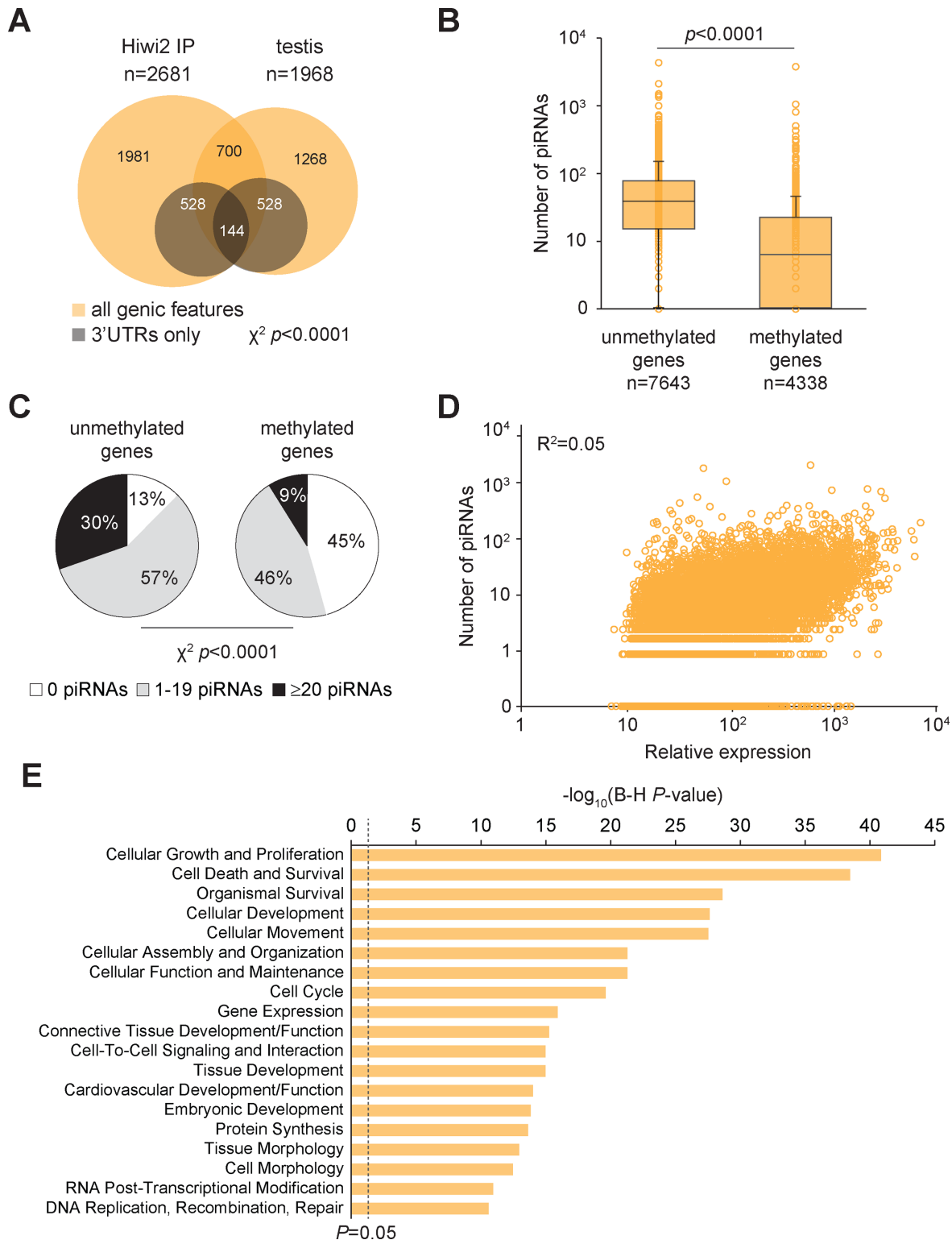


Figure 3. Hiwi2-associated genic piRNAs are derived from unmethylated genes. **(A)** Venn diagram showing overlap of genes with ≥ 20 mapped piRNAs from the Hiwi2 immunoprecipitation and in adult testis. Genes with ≥ 20 piRNAs mapping to the 3'UTR are shown in grey; those with ≥ 20 piRNAs mapping across all coding regions are shown in orange. The overlap for both 3'UTRs and all coding regions is highly significant (χ^2 test, $P < 0.0001$). Genes targeted by Hiwi2-bound piRNAs are significantly more likely to be unmethylated: **(B)** box-and-whisker plots show the number of piRNAs mapping to unmethylated versus methylated genes (Student's t -test, $P < 0.0001$) and **(C)** pie charts show the number of unmethylated versus methylated genes with no, few (1–19) or many (≥ 20) mapped piRNAs (χ^2 test, $P < 0.0001$). **(D)** Scatter plot showing that the number of Hiwi2-bound piRNAs is not related to the expression level of the parent gene in MDAMB213-Flag-Hiwi2 cells ($R^2 = 0.05$). **(E)** The 20 most significant ontological functions produced by IPA for genes with ≥ 50 mapped piRNAs in both the Hiwi2 IP and adult testis. The scale shows the $-\log_{10}$ of the Benjamini–Hochberg corrected P -value; the dashed line denotes corrected $P = 0.05$.

Table 1. Proteins identified by MS in Hiwi2-Flag immunoprecipitations.

Identified proteins	Gene	Class	M.W. (kDa)	Average % coverage
Piwi-like protein 4 (Hiwi2)	PIWIL4	Piwi protein	97	32
Elongation factor 1-alpha 1	EEF1A1	Translational machinery	50	1
Ribosomal protein S27a	RPS27a	"	18	5
Myosin-6	MYO6	Structural proteins	224	1
Tubulin, alpha ubiquitous chain	TUBA1B	"	46	18
Tubulin beta	TUBB	"	48	20
Tubulin beta-2C chain	TUBB2C	"	50	18
Tubulin beta-6 chain	TUBB6	"	47	7
RuvB-like 1	RUVBL1	ATPase, DNA helicases	50	6
RuvB-like 2	RUVBL2	"	51	9
BAG family molecular chaperone regulator 2	BAG2	Heat shock proteins and chaperones	24	14
Chaperonin-containing TCP1, subunit 8	CCT8	"	59	2
Heat shock 90 kDa protein 1, alpha isoform 1	HSP90AA1	"	98	3
Heat shock 70 kDa protein 1	HSPA1A/B	"	70	10
HSPA5 protein	HSPA5	"	72	7
Isoform 1 of heat shock cognate 71 kDa protein	HSPA8	"	71	24
60 kDa heat shock protein, mitochondrial	HSPD1	"	61	2
DnaJ homolog subfamily A member 1	DNAJA1	"	45	2
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	Catabolic enzymes	36	3
Isoform M1 of pyruvate kinase isozymes M1/M2	PKM2	"	58	2
26S protease regulatory subunit	PSMC1	"	49	5

Somatic Hiwi2 preferentially binds processed tRNA fragments

The most striking finding from our annotation of Hiwi2-bound small RNAs is the predominance of tRNA-derived RNAs (Figure 2B). The tRNA species we find do not appear to be degradation products; rather they are processed tRNA fragments (tRFs) with specific signatures. Almost three-quarters (73.5%) of Hiwi2-bound piRNAs in the MDAMB231 cells were tRFs (Figure 2B); we have called them tRF-piRNAs. Almost 95% of the tRF-piRNAs are derived from only nine specific tRNA isotypes representing just five amino acids: glycine, glutamic acid, lysine, aspartic acid and valine (Figure 4A); most other tRNA isotypes are also represented in the total number of tRF-piRNAs, but collectively these contributed less than 5% of all tRF-piRNA reads. We chose five of the most abundant tRF-piRNAs for independent validation using custom TaqMan assays capable of amplifying only the processed tRF and not the corresponding full-length tRNA. We were able to detect all five tRFs in MDAMB231 total RNA and testis (Figure 4B) indicating that these fragments are not degradation products from full-length tRNA generated during the IP or library preparation procedures.

The abundance of particular tRF-piRNA species in the MDAMB231 Hiwi2 IP is not correlated to the number of genomic loci by which they are encoded (Figure 4A), nor do they appear correlated to the relative expression of the mature tRNA isotypes in MDAMB231 cells (49), so their prominence is not a function of gene dosage or expression level of the tRNA from which they are derived. However we did find a strong correlation between the tRF-piRNAs in the total MDAMB231 small RNA library and those in the IP library ($R^2 = 0.83$; Figure 4C), which indicates that the native relative abundance of tRF-piRNAs is captured by the immunoprecipitation procedure. We also found that many of the MDAMB231 tRF-piRNAs are present in the total adult testis library (Figure 4D) and their abundance in both datasets is highly correlated ($R^2 = 0.80$).

When considering the most abundant tRF-piRNAs individually, it is clear that each species has a specific size distribution, consistent with selective processing (Figure 4E). The exception is the aspartyl-tRNA isotype (Asp-GTC), which gives a broad size distribution, and also is the only dominant tRF-piRNA to be processed from the 3' end of the tRNA: all other tRF-piRNAs are derived from the 5' end of the tRNA (Figure 4F). We also find that Asp-GTC tRF-piRNAs are virtually absent from the adult testis sample, while all the other dominant species are represented (Figure 4F). All of the most abundant tRF-piRNAs contain a conserved GG dinucleotide at position 17/18 or 18/19; although this is a common feature of tRNAs in general, it is noteworthy as this dinucleotide is required for the translational repressive effect of exogenous tRFs *in vitro* (50).

DISCUSSION

Since the discovery of piRNAs, the function of the Piwi-piRNA pathway in the developing animal germline has been studied extensively. But despite numerous reports of Piwi expression in somatic cells (reviewed in (51)), and cancer cells (reviewed in (52)), the function of the system in the soma has been obscure. Here we have found that one of the human Piwis, *Hiwi2*, is widely expressed at low levels across somatic tissues, and is also expressed at similar levels in human cancer cell lines. By studying one cancer cell line in detail, we find evidence for a conserved function of the Piwi-piRNA pathway in the regulation of protein translation. Taken together, the protein partners of Hiwi2, its cytoplasmic location and the derivation of piRNAs from expressed genes and specific tRNAs in MDAMB231 cells and testis, all point to a role in translation regulation. The findings also suggest that if Hiwi2 functions in translational control in somatic cells, it may also do so in the germline.

We began this study with the idea that Hiwi2, given its known role in *de novo* methylation and repression of retrotransposons, could be responsible for aberrant methylation in cancer cells, which typically exhibit regional cytosine hy-

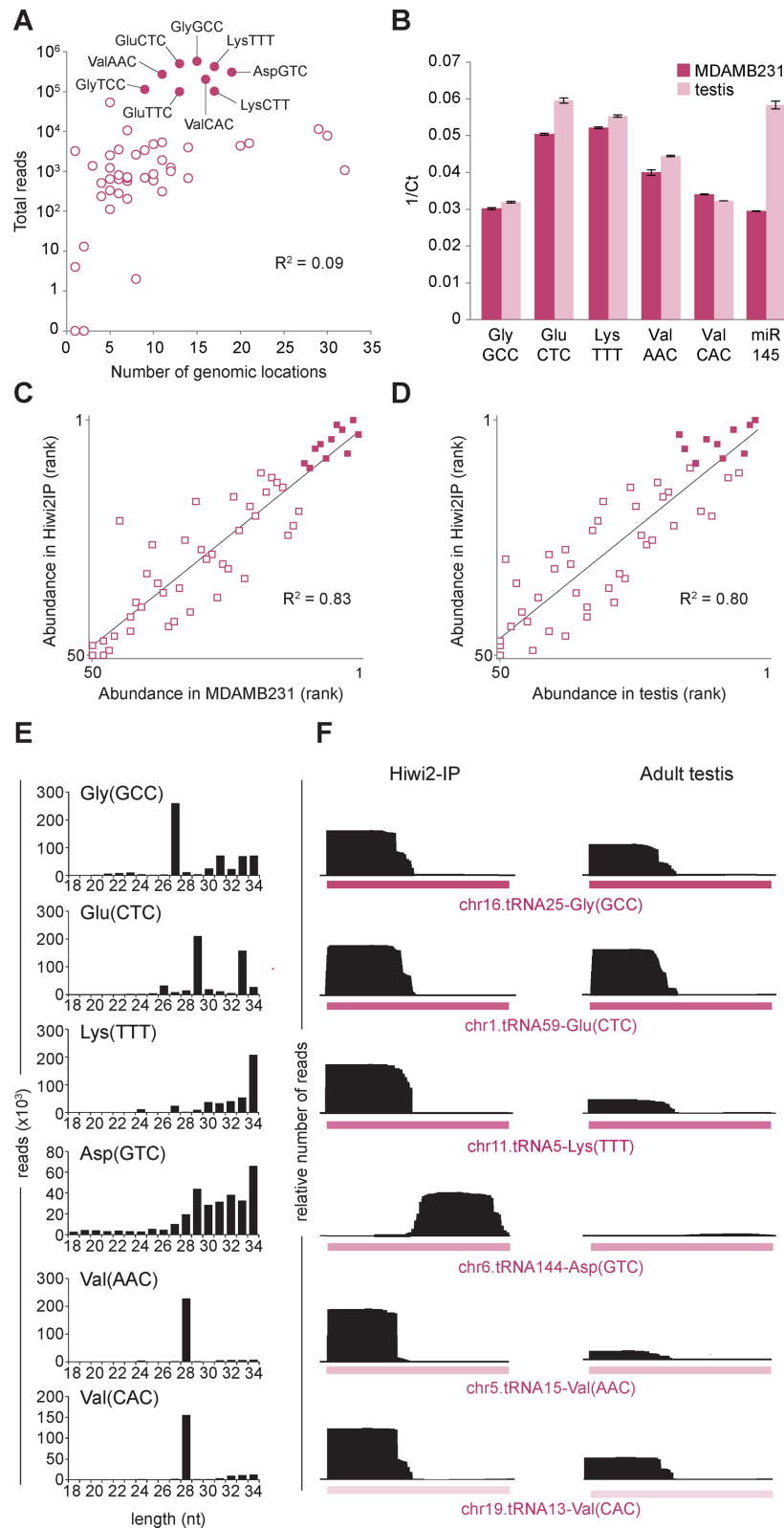


Figure 4. tRNA-derived piRNAs dominate the Hiwi2-IP and are also present in the adult testis. **(A)** Scatter plot showing the abundance of each tRF-piRNA in the Hiwi2 IP, plotted against the number of its genomic copies. The nine tRF-piRNAs with $\geq 10^5$ reads are highlighted. **(B)** TaqMan validation of tRF-piRNA expression in MDAMB231 cells and human adult testis. Taqman quantification of mir-145 is shown for comparison. **(C)** Scatter plot showing the ranked abundance of tRF-piRNAs in the Hiwi2 IP versus that in MDAMB231 cells. **(D)** Scatter plot showing the ranked abundance of tRF-piRNAs in the Hiwi2 IP versus that in the human adult testis. **(E)** Size distributions of the six most abundant Hiwi2-bound tRF-piRNAs and **(F)** the six tRF-piRNAs from Hiwi2 IP and testis mapped to a representative genomic locus.

permethylation and silencing concomitant with a global loss of 5-methylcytosine that is largely attributable to loss of epigenetic repression at retrotransposons and other repeats (53). We considered that somatic Hiwi2 might capture genic sequences by accident, and silence them when acting to repress the widespread retrotransposon activation. We thus sought evidence for Hiwi2-bound piRNAs that may drive gene silencing in cancer cells. However, we found no enrichment of retrotransposon-derived piRNA sequences in the Hiwi2 IP, and no association between methylation of a locus and abundance of the piRNAs derived from it. On the contrary, the majority of Hiwi2-bound piRNAs are derived from unmethylated loci, and their abundance has no apparent relationship to the abundance of the parent transcript. Derivation of piRNAs from unmethylated and expressed genes is inconsistent with a role for Hiwi2 in gene silencing, but may be consistent with a role in post-transcriptional gene regulation. Many of the genes producing large numbers of piRNAs were common between the Hiwi2 IP and adult testis, which raises the possibility that the piRNAs may have a function that is common to germline and soma. Genes producing piRNAs cluster in a range of highly significant ontologies, in which cell growth and morphology functions are prominent. Functions related to RNA and protein biosynthesis, metabolism and transport are also among the most significant.

Gene-derived piRNAs, whatever their function, were vastly outnumbered in the Hiwi2 IP dataset by tRNA-derived RNA fragments (tRF). The tRFs are a class of small RNA that have been observed in a variety of settings and, like piRNAs, appear to be a phylogenetically ancient species of small regulatory RNA. They have been found in humans and other mammals (54), plants (55), yeast (56), protozoa (57) and even archaea (58,59). tRFs are classified according to their processing signature, which essentially relates to the part of the tRNA from which they are cleaved (54). All but one of the tRFs in our dataset are 5'tRFs, and can be distinguished from oxidative stress-induced, angiogenin-dependent tRNA 'halves' on the basis of their length (60). The relationship, if any, between tRF-piRNAs and gene-derived piRNAs is yet to be explained.

The generation of mammalian 5'tRFs has been reported to be Dicer-dependent in some studies and Dicer-independent in others (61–63), and this is likely a reflection of the heterogeneity in this growing class of small RNAs; they vary in size and sequence composition and can also be influenced by culture or growth conditions. The Hiwi2 tRF-piRNAs identified in this study are a case in point: they differ in their processing features, although none of the most abundant show the size or 5'U preference characteristic of Dicer processing. The principal unifying feature of the tRF-piRNAs we observe is their association with Hiwi2.

Our findings point toward a role for Hiwi2 in translational control. First, Hiwi2 partners predominantly with tRF-piRNAs (Figures 2B and 4); although their mechanisms of action are poorly understood, available evidence strongly implicates tRFs as translational repressors, with both sequence-dependent and -independent modes of action (50,58,63). Second, Hiwi2 resides in the cytoplasm where it partners with actively translating ribosomes (Figures 1C, D and 2E) and other proteins involved in transla-

tion, such as eEF1-alpha and the heat shock proteins (Table 1). Association of Hiwi2 with piRNAs derived from sense strands of active genes, independent of the genes' transcript levels (Figure 3D), is also consistent with a role for Hiwi2 in translational regulation. The murine Piwi proteins Miwi and Mili have both previously been linked to translational regulation in developing gametes (43,64); thus the function of the Piwi-piRNA pathway in translational control is likely to be conserved across species, and perhaps also through germline and soma.

While this study is the first report of tRF association with a Piwi protein in an animal, there is precedent in the protozoa: shorter 18–22 nt 3'tRFs are found complexed with the growth-essential Piwi protein Twi12 in *Tetrahymena thermophila*, although their biological function remains uncharacterised (57). In mammalian cells, 5'tRFs associate only weakly with the classic Ago proteins (62), and in most species protein partners of 5'tRFs have not been identified. A significant exception is the archaeon *Haloferax volcanii*, where 5'tRFs are found complexed with actively translating ribosomes (58); these 5'tRFs are the size of piRNAs (26nt) and they act to repress translation in a stress-dependent manner. These data point toward a primitive translational control function of tRF-piRNAs that acts in the regulation of gene output in response to changing environmental conditions. But are the archaeal tRFs also complexed with archaeal Piwi proteins? This has not been studied, but our findings here suggest that these may well be.

Here we presented evidence that a Piwi-piRNA pathway may have a role in translational regulation in human somatic cells *via* Hiwi2 and its associated piRNAs. Correlation with human testis piRNAs suggests that this function may also be present in human germ cells. Taken together with evidence regarding Piwi and small RNAs in primitive organisms, our data suggest an ancient and conserved function for the pathway that may predate the now prominent transposon-repressive function in the metazoan germline.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

The authors thank: Gunter Meister for providing the Hiwi2-Flag construct, Richard Saffery and Nick Wong for assistance with Infinium arrays, the Ramaciotti Centre for Gene Function Analysis and the Bioanalytical Mass Spectrometry Facility at UNSW for assistance with expression arrays and mass spectrometry, Rupert Shuttleworth for assistance with scripting and annotation, and Grace Wei for assistance with polysome profiling. J.E.C. is an ARC DE-CRA Fellow. T.P. is an NHMRC Senior Research Fellow. C.M.S. and G.H. are ARC Future Fellows.

FUNDING

National Health and Medical Research Council (NHMRC) of Australia [APP1025210 to C.M.S.]; in part by the Australian Research Council (ARC) [DP130103027 to G.H.]. Funding for open access charge: National Health and Medical Research Council.

Conflict of interest statement. None declared.

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