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# Detection of PIWI and piRNAs in the mitochondria of mammalian cancer cells

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ABSTRACT

Piwi-interacting RNAs (piRNAs) are 26–31 nt small noncoding RNAs that are processed from their longer precursor transcripts by Piwi proteins. Localization of Piwi and piRNA has been reported mostly in nucleus and cytoplasm of higher eukaryotes germ-line cells, where it is believed that known piRNA sequences are located in repeat regions of nuclear genome in germ-line cells. However, localization of PIWI and piRNA in mammalian somatic cell mitochondria yet remains largely unknown. We identified 29 piRNA sequence alignments from various regions of the human mitochondrial genome. Twelve out 29 piRNA sequences matched stem-loop fragment sequences of seven distinct tRNAs. We observed their actual expression in mitochondria subcellular fractions by inspecting mitochondrial-specific small RNA-Seq datasets. Of interest, the majority of the 29 piRNAs overlapped with multiple longer transcripts (expressed sequence tags) that are unique to the human mitochondrial genome. The presence of mature piRNAs in mitochondria was detected by qRT-PCR of mitochondrial subcellular RNAs. Further validation showed detection of Piwi by colocalization using anti-Piwil1 and mitochondria organelle-specific protein antibodies.



## 1. Introduction

Piwi family proteins and their cognate small non-coding RNAs (Piwi-interacting RNAs, piRNAs), are essential for the differentiation of male primordial germ cells [1,2]. It is believed that they protect the genome by facilitating transcriptional and post-transcriptional silencing of transposable elements via heterochromatization and endonucleolytic cleavage, respectively [3–5].

The piRNAs are small noncoding RNAs of 26–31 nucleotides in length, and are generally processed from their longer precursors that are transcribed from repetitive elements, introns and 3′UTR regions [1]. During processing, Piwi family proteins are involved in cleavage of the longer piRNA precursors [1,2].

Consistent with their association with piRNAs, Piwi family proteins, generally localized in the cytosol and nucleus, are associated with DNA methylation, transposon repression, and translational repression, in germ line cells and somatic follicular cells of the *Drosophila* ovary [5,6]. Piwi family proteins and piRNAs have also been identified in higher eukaryotes and the mammalian Piwi ortholog has been implicated in infertility and cancer [6–9]. However, despite increased recognition of Piwi and piRNAs in the pathogenic associations, piRNA sequence location and Piwi localization to mitochondria remains largely unknown [10–12].

In this study, we utilized both computational and experimental analyses to study the possible presence of Piwi and piRNA transcripts in mitochondria from human normal and cancer cell lines. Using small RNA-Seq databases, we identified mitochondrial piRNA transcripts which comprise a previously unknown subset of mitochondrial small non-coding RNAs. Considering piRNAs have their longer precursors [1,2], we also discovered multiple long

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mitochondria-unique expressed sequence tags (ESTs) that overlapped the piRNA transcripts. Further support for mitochondrial presence of piRNA and Piwi, was provided by qRT-PCR and Western blot in mitochondrial subcellular fractions. To our knowledge, this is the first report on localization of piRNAs and Piwi proteins in mammalian cancer cell mitochondria.

## 2. Material and methods

### 2.1. Mapping piRNAs and mitochondrial small RNA-Seq to mtDNA

In order to locate piRNA sequences in mtDNA, we mapped known piRNA sequences [13] to the human mitochondrial genome [GenBank: NC_001807], which was downloaded from UCSC Genome Browser (GB) web site (genome.ucsc.edu). Carmell and colleagues experimentally identified the piRNA sequences [13]. Using those sequences [13] [GenBank: DQ539889 to DQ569912] from NCBI GenBank, we found 29 piRNAs (Fig. 1) that formed perfect matches with the mtDNA genomic sequence by using BLAST (Table S1). Moreover, 12 out of the 29 identified piRNAs also perfectly matched sequence fragments from seven tRNAs (tRNA-Phe, -Val, -Leu, -His, -Ser, -Glu, and -Thr).

We used small RNA-Seq datasets (from mitochondrial subcelluar fractions) to measure read depths of the 29 piRNA regions in mitochondria. Based on the multiple datasets, we inspected the read depths of the piRNAs. The small RNA-Seq dataset of HeLa and HEK293 cells was downloaded from NCBI Gene Expression Omnibus (GEO, ncbi.nlm.nih.gov/geo) under the accession number GSE32185 [10] (see read length distribution in Fig. S1). The small RNA-Seq dataset of 143B cell line was obtained from NCBI GEO under the accession number GSM763531 [12]. We then filtered the small RNA-Seq reads by using FASTQ Groomer (galaxyproject.org) [14], with default options and a minimum Phred quality score of 20. The small RNA-Seq reads of the three cell lines were mapped to the human mitochondrial genome [GenBank: NC_001807] by using the default options of the Burrows–Wheeler Alignment tool (BWA) [15] and SAMtools [16]. Read depths at mitochondrial DNA (mtDNA) genomic positions were obtained using the default options of BEDTools [17], and are visualized in Fig. 2A. To determine the expression of each 5′ and 3′ tRNA fragment (Fig. 2B and Table S2), we calculated the average read depth for each fragment. The expression of the 5′ fragment was measured by the average read depth of the 26 bases in the middle of the tRNA 5′ half region. The expression of the 3′ fragment was measured by the average read depth of the 26 bases in the middle of the tRNA 3′ half region.

### 2.2. Identification of uniquely mtDNA-mapped expressed sequence tags (ESTs) containing the 29 piRNAs

Since expressed sequence tags (ESTs) contain non-coding RNA fragments and their precursor fragments [18], we reasoned that the longer ESTs around the piRNAs exist as their precursors (or precursor-like transcripts). We further focused on uniqueness of the ESTs in mtDNA (not in nuclear DNA), since the unique alignment indicates the ESTs are soley transcribed from mtDNA. To assess whether unique mitochondrial ESTs contained the 29 piRNA sequences, we used the GenBank EST (ncbi.nlm.nih.gov/dbEST) alignment information table all.est deposited in the UCSC GB [19], extracting unspliced ESTs uniquely mapped to mtDNA (not nuclear DNA). The alignment was made by BLAT [19]. That comparison of the unique ESTs and the piRNAs (using the UCSC GB table browser tool) resulted in our identification of 4494 mitochondria-specific, unspliced ESTs overlapping (or partially overlapping) the 29 piRNA sequences. In addition, tissue- and cell type-specificities of the piRNA-containing ESTs were obtained from table gbCdnaInfo that includes library description information (e.g., cell- and tissue-type), as extracted from the UCSC Genome Browser database [19].

### 2.3. Western blot analysis

Neuroblastoma Neuro2a cells were fractionated by differential centrifugation, as previously described by Yu et al. [20]. Briefly, after washing with PBS, cells were resuspended in isotonic homogenization buffer (10 mM Tris–HCl (pH 7.4), 250 mM sucrose, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol and protease inhibitor cocktail). After 100 strokes in a Dounce homogenizer, unbroken cells were removed by centrifugation at $30\times g$ for 5 min. The nuclei and mitochondrial fractions were prepared from the resuspended pellets by centrifugation at $80\times g$ for 10 min and $6000\times g$ for 20 min, respectively. The final supernatant was used as the cytosolic fraction.

For Western blot analysis, protein samples were separated by SDS–PAGE, transferred to PVDF membranes, and incubated with primary antibodies against Lamin-B (Abcam), Piwi (Abcam; ab12337 (Anti-PIWIL1 antibody)), β-actin (Abcam), and Tom40 (Santa Cruz Biotech, a mitochondrial marker) antibodies. Following incubation with HRP-conjugated secondary antibodies, immunoluminescence was determined using ECL Prime kits (GE Healthcare). Thus, we used Lamin-B, Tom40, and α-tubulin antibodies as nuclear, mitochondrial, and cytosolic markers, respectively, to examine possible colocalization with Piwi. β-actin was used as a loading control.

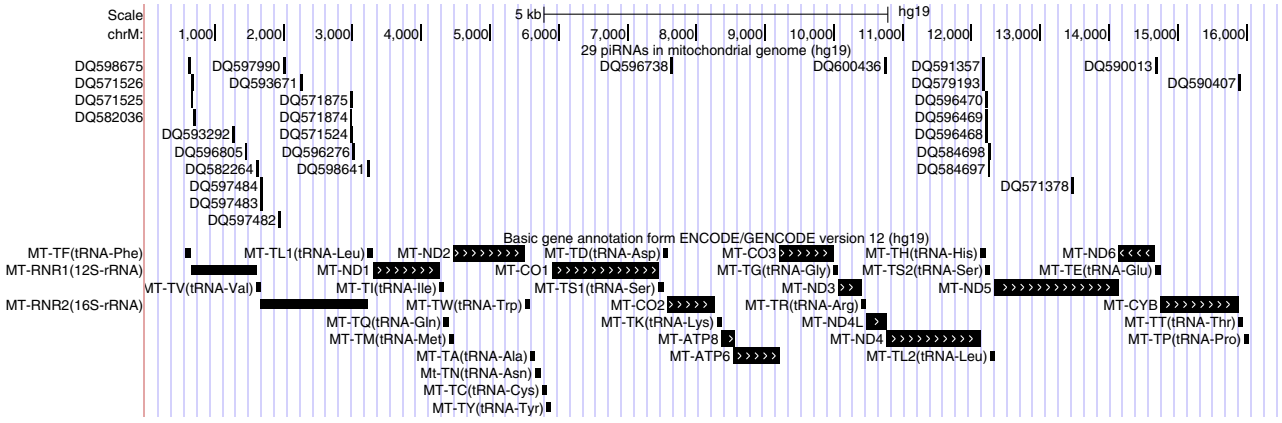


**Fig. 1.** Mitochondrial genomic location of the 29 piRNAs. The panel has two tracks: the 29-piRNAs (upper track) and mtDNA annotations (lower track). The piRNAs are located throughout mitochondrial genome, including rRNA regions, tRNA regions, and protein-coding regions.