Supporting Information

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SI Text

Human Tissue Samples. Ten percent formalin-fixed, paraffin-embedded human tissue samples were obtained from diagnostic biopsies or resection specimens from patients at the hospitals Luis Tisné, San José, and Barros Luco Trudeau (Santiago, Chile). The samples were used in agreement with the ethical guidelines approved by the ethical committee of the hospitals and our institutions.

5′ RACE. The sequence at the 5′ end of the ASncmtRNAs was determined as described before (1). Briefly, total RNA from HFK cells was digested with 25 μg/mL of RNase A for 10 min at room temperature (RT) and the double-stranded stem was recovered after phenol extraction. The cDNA was synthesized with primer 11 or 12 (Fig. 2*B*) and tailed at the 3′ end with dCTP and terminal deoxynucleotidyl transferase (TdT) (Promega). PCR amplification was carried out with primer 11 or 12 and the anchor primer (Invitrogen). The amplicons were purified and both strands were sequenced.

ISH of Human Biopsies. For tissue samples, ≈5-μm-thick serial paraffin sections were collected on silanized slides (DAKO) and deparaffinized by immersion in 2 consecutive xylene washes, 5 min each. One section was stained with hematoxylin and eosin. The other sections were rehydrated in two 3-min washes of 100 and 95% ethanol each and once in DEPC-treated distilled water for 5 min (1, 2). Sections were then incubated in 2.5 μg/mL of Proteinase K (Invitrogen) at room temperature for 20 min in a humid chamber and afterward washed twice for 3 min in DEPC-treated water, immersed in 96% ethanol for 10 s, and air dried (2, 3). Hybridization was carried out with digoxigenin-labeled probes 1 and 13 for detection of the SncmtRNA and probes 2 and 3 for the ASncmtRNAs (Table S1). Hybridization mixtures contained 35 pmoles/mL of each probe in hybridization solution as described before (1, 2). Hybridization was carried out at 37 °C overnight in a humid chamber. Samples were then washed twice in 2× SSC at room temperature for 10 min and once in 0.5× SSC at 37 °C for 30 min. After rinsing briefly in PBS, sections were incubated for 2 h at RT with anti-digoxigenin antibody conjugated to alkaline phosphatase (Roche Applied Science), diluted 1:500 in blocking solution. Samples were then washed twice in PBS for 3 min and once for 1 min in alkaline phosphatase buffer (100 mM Tris·HCl, 100 mM NaCl and 50 mM $MgCl_2$ pH 9,5). The color reaction was performed as described before (2). Samples were mounted in Entellan (Merck) or Faramount (DAKO).

S1 Protection Assay. S1 protection was carried out by using single-stranded digoxygenin-labeled probes complementary to ASncmtRNAs-1 and -2, generated by asymmetric PCR using as template ds PCR products obtained using primers 4 and 17 for ASncmtRNA-1 (160-bp amplicon) and 4 and 2 for ASncmtRNA-2 (245-bp amplicon). Asymmetric dig-labeling PCRs contained 50 ng template, 10 mM primer 4 only, 200 mM dATP, dCTP, and dGTP, and 100 mM dig-11-dUTP (Roche Applied Science). PCR was carried out at 95 °C for 5 min, followed by 50 cycles of 30 s at 94 °C, 30 s at 60 °C, and 30 s at 72 °C, and a final 10 min elongation step at 72 °C. For S1 protection, a 50-ng dig-labeled probe was ethanol precipitated either alone or in the presence of 100 mg of total HUVEC RNA overnight at −20 °C. After centrifugation and washing, pellets were resuspended in 50 mL of S1 hybridization buffer (40 mM Pipes pH 6.4, 1 mM EDTA, 0.4 M NaCl, and 80% formamide). Hybridization mixes were incubated at 100 °C for 5 min and 17 h at 45 °C (for ASncmtRNA-1 probe) or 60 °C (for ASncmtRNA-2 probe). Fifty milliliters of 10× S1 reaction buffer (0.5 M sodium acetate pH 4.5, 2.8 M NaCl, and 45 mM ZnSO4) and 400 mL of nuclease-free water were then added. One-tenth of each reaction mix was precipitated and the remaining 9/10 were incubated at 37 °C for 30 min in the presence of 100 units of S1 nuclease. Reaction mixtures were precipitated, sedimented, and products were run on native 2.5% agarose gels and transferred overnight in 20× SSC onto Hybond-XL membranes (Amersham Pharmacia Biotech). Membranes were UV cross-linked and incubated in blocking buffer (PBS containing 0.05% tween 20 and 5% skim milk) for 30 min at RT and afterward in a 1:2,000 dilution of alkaline phosphatase-coupled anti-digoxigenin antibody (Roche Applied Science) for 3 h at RT. After three 5-min washes in PBS/0.05% tween 20, membranes were developed for 15 min in BCIP/NBT substrate (KPL).

1. Villegas J, et al. (2007) Expression of a novel non-coding mitochondrial RNA in human proliferating cells. *Nucleic Acids Res* 35:7336–7347.
2. Henke RT, Kim SE, Maitra A, Paik S, Wellstein A (2006) Expression analysis of mRNA in formalin-fixed, paraffin-embedded archival tissues by mRNA in situ hybridization. *Methods* 38:253–262.

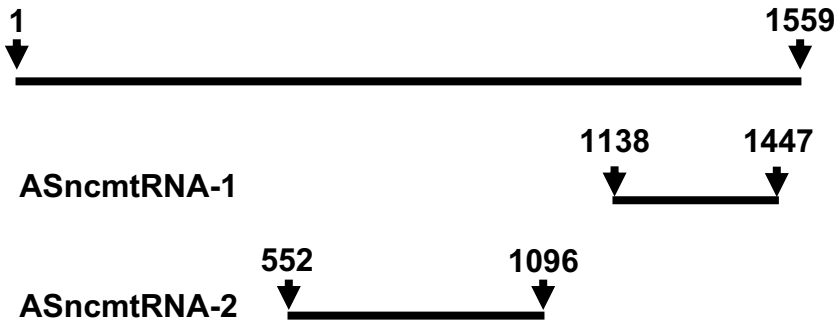

Fig. S1. Position of the IRs of the ASncmtRNA-1 and ASncmtRNA-2 with respect to the 16S mtrRNA. The IR of the ASncmtRNA-1 corresponds to positions 1,138 to 1,447 of the 16S mtrRNA. The IR of the ASncmtRNA-2 corresponds to positions 552 to 1,096 of the same transcript.

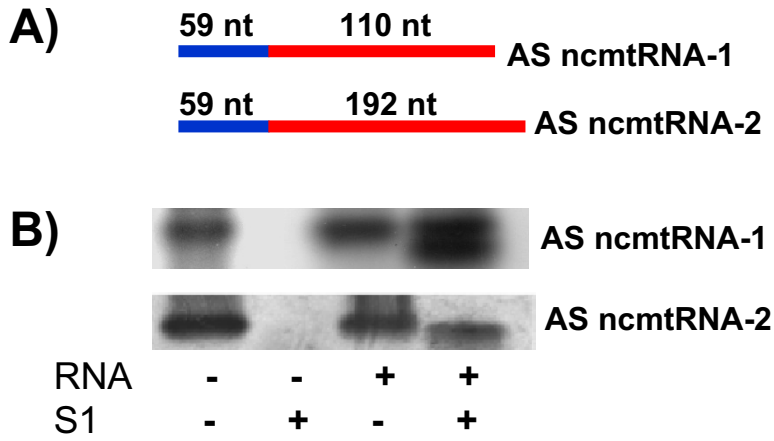

Fig. S2. S1 Protection Assay. (*A*) Schematic of Dig-labeled ssDNA probes against ASncmtRNA-1 and -2, generated by assymetrical PCR. The probes span the linker region between the antisense 16S mtrRNA (red bars) and the IR (blue bars) of the ASncmtRNAs-1 and -2. (*B*) S1 protection assay for ASncmtRNA-1 (*Upper*) and ASncmtRNA-2 (*Lower*). The lower band present in the ASncmtRNA-1 assay probably corresponds to the 16S mtrRNA.

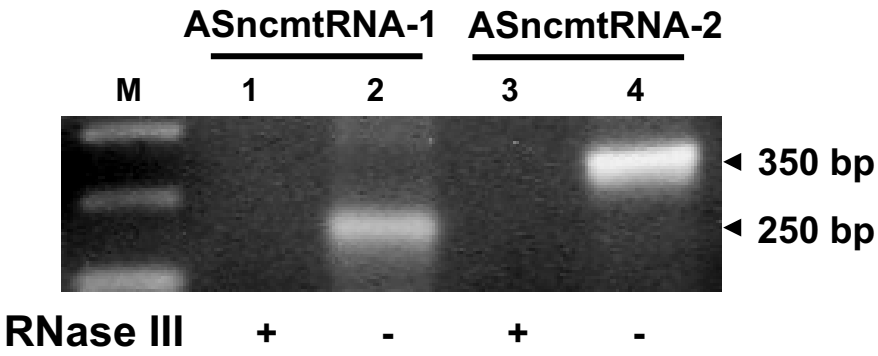

Fig. S3. RT-PCR amplification of linker regions of the ncmtRNAs is abolished after RNase III digestion. Total HUVEC RNA (1 μg) was incubated for 30 min with or without 1 unit of *E. coli* RNase III (Ambion) in a final volume of 10 μL, according to manufacturer's instructions. Products were amplified by RT-PCR as described in *Materials and Methods*. The 250-bp amplicon corresponding to the ASncmtRNA-1 (lane 2) was obtained with primers 4 and 3; the 350-bp amplicon corresponding to ASncmtRNA-2 (lane 4) was obtained with primers 4 and 6. (see Fig. 2*A*). Lane *M*, 100-bp ladder.

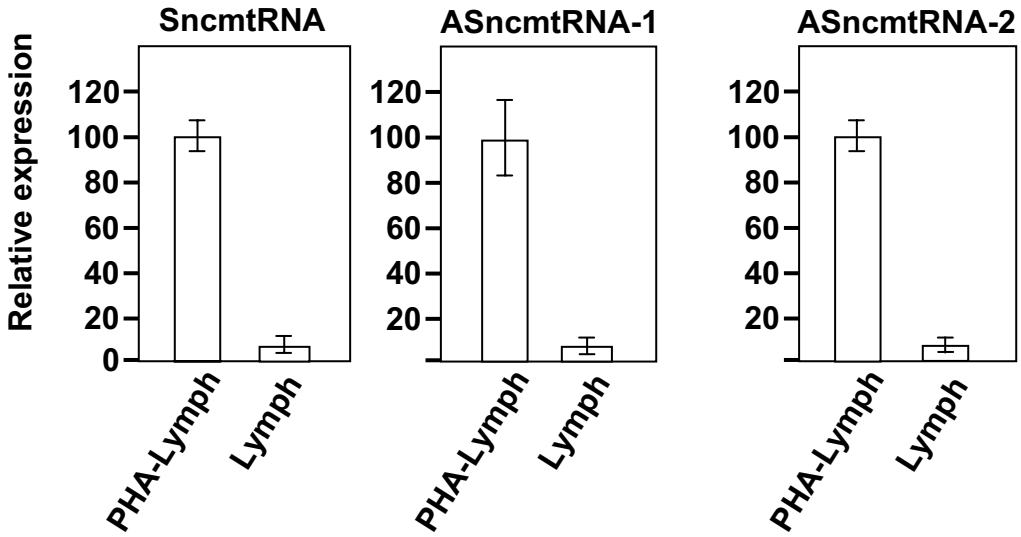

Fig. S4. Relative expression of the SncmtRNA, ASncmtRNA-1, and ASncmtRNA-2 was determined in resting lymphocytes (lymph) and PHA-stimulated lymphocytes (PHA-lymph) after 48 h of culture. The results were normalized to GAPDH mRNA expression. The Ct values of GADDH in resting lymphocytes were 24, 25, and 25. In PHA-stimulated lymphocytes the Ct values of GAPDH were 24, 24, and 25 in 3 different experiments.

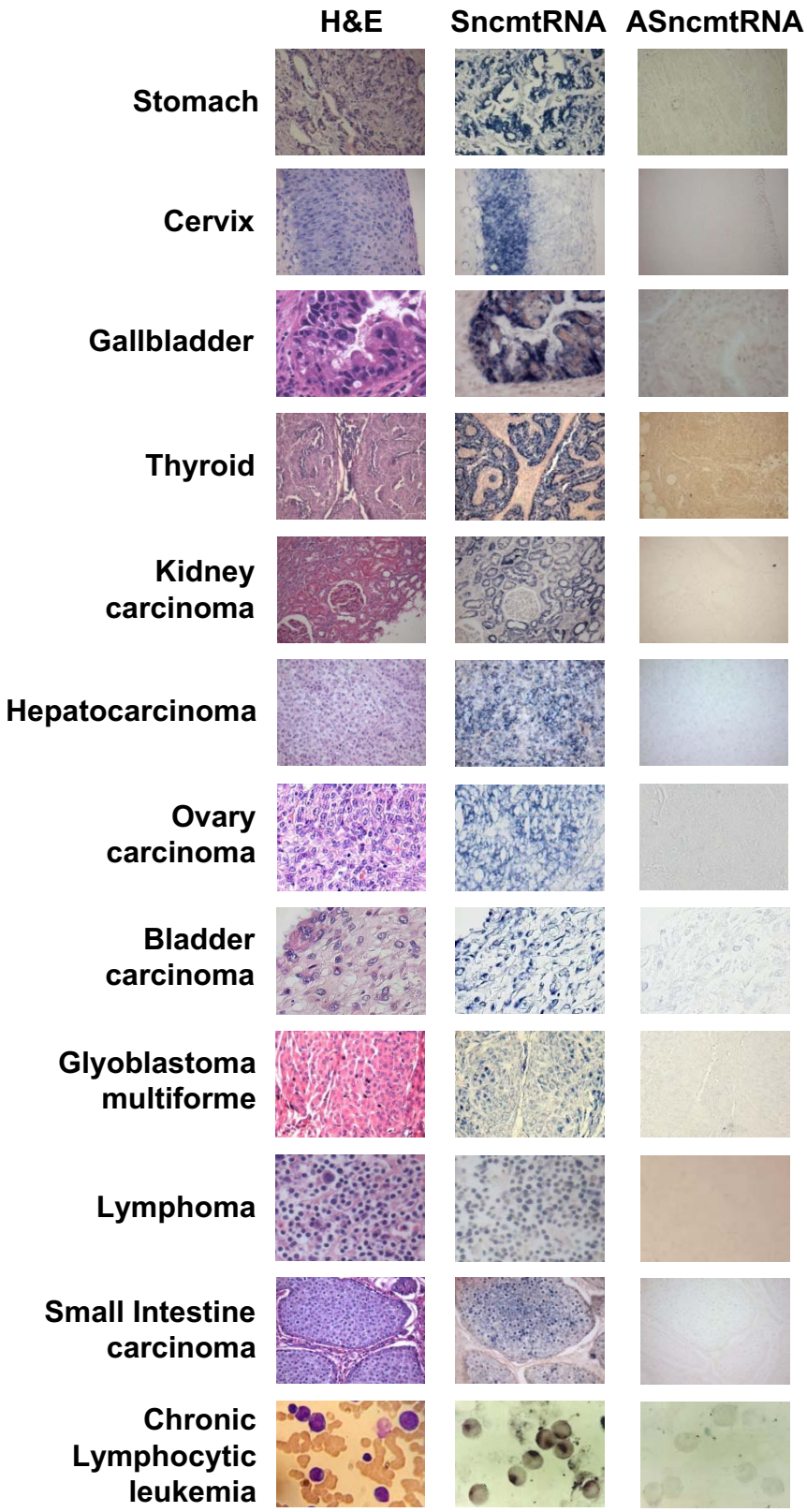

Fig. S5. Expression of SncmtRNA and ASncmtRNAs in tumor biopsies. Serial paraffin-embedded tissue sections of the indicated tumor biopsies were subjected to ISH to determine the expression of SncmtRNA and the ASncmtRNAs using probes 1 and 2. In each case, a parallel section was stained with H&E (magnification 20×). The blood smear of a chronic lymphocytic leukemia was stained with MGG (100×).

Table S1. Sequence and position of primers

Primer	Sequence	Position*
P1 (R)	5′-AGTGATTATGCTACCTTTGCACGGT	912–934
P1 MM(R)†	5′-AGAGACCATGCGCCTTTACACCGGT	912–934
P2 (F)	5′-ACCGTGCAAAGGTAGCATAATCACT	912–934
P3 (F)	5′-TAGGGATAACAGCGCAATCCTATT	1,260–1,283
P4 (R)	5′-CCGTAAATGATATCATCTCAACTT	1,500–1,522
P5 (F)	5′-AACCTCCGAGCAGTACATG	1,167–1,185
P6 (F)	5′-GAACTCGGCAAACCTTACC	801–819
P7 (F)	5′-AGCCCAATATCTACAATCAACC	706–727
P8 (F)	5′-TTGGACCAATCTATCACCCTA	596–616
P9 (F)	5′-TAGGCCTAAAAGCAGCCACCAA	501–522
P10 (R)	5′-AAACCTAGCCCCAAACC	5–22
P11 (R)	5′-AATAGGATTGCGCTGTTATCCCTA	1,260–1,283
P12 (R)	5′-GGTAAGATTTGCCGAGTTC	801–819
P13 (R)	5′-ACAGGGAGGAATTTGAAGTAGATAG	1,429–1,453
P14 (R)	5′-GGTTTGTTAGGTACTGTTTGCATTAA	1,076–1,101
P15 (R)	5′-AAGGTGGAGTGGGTTTGGGGC	11–31
P16 (F)	5′-GGGGTCTTAGCTTTGGCTCTCC	213–234
P17 (R)	5′-TGCAGCCGCTATTAAAG	11–31
COX I (F)	5′-GAACAGGTTGAACAGTCTACCCT	368–390
COX I (R)	5′-TTCCGAAGCCTGGTAGGATAAGA	735–757
18S (R)	5′-AGTGGACTCATTCCAATTACA	551–571
18S (F)	5′-GATGCGTGCATTTATCAGATC	208–228
GAPDH (R)	5′-ATGATGTTCTGGAGAGCCC	704–722
GAPDH (F)	5′-ACTCTGGTAAAGTGGATATTGT	173–194
β-actin (R)	5′-GGCGACGTAGCACAGCTTCTCC	712–733
β-actin (F)	5′-AAGAGAGGCATCCTCACCCTG	254–274

*Positions of primers used for the ncmtRNAs are numbered according to their position on the sense 16S mtrRNA.
†Under ISH hybridization conditions (50% formamide) the Tm of primer 1 with 7 mismatches is 14 °C.

Other Supporting Information Files

Dataset S1
Dataset S2