Supplementary Material

Supplementary material is presented in PDF format and includes: detailed material and methods, two figures and figure legends, two tables, and references that are pointed in Table S1 (this table is a copy of Table 1, but including an extra column with references where *CDH1* mutations were identified).

Supplementary Material and Methods

Plasmids construction – CDH1 mini-gene

The CDH1 WT mini-gene (WTint) was obtained by introducing an intron 15 minimal sequence between exons 15 (L34788.1) and 16 (L34789.1) of *CDH1* (NM_004360.3; ENST00000261769). This intronic sequence was obtained using the http://www.cbs.dtu.dk/services/NetGene2/ online tool loaded with different portions of intron 15 sequences. The canonical donor and acceptor splice-sites of *CDH1* intron 15 were predicted to allow correct splicing even when deleting the intronic sequence between c.2439+111bp and c.2440-180bp. The intronic regions, upstream of c.2439+111bp and downstream of c.2440-180bp were obtained by PCR from NCI-N87 cell line genomic DNA and later reconnected. Nonsense 1003 C>T mutation was then introduced by site-directed mutagenesis (SDM) using primers indicated in Table S1.

Plasmids construction – modified pIRES vector with sup-tRNA^{Arg}

To ensure each would have both genes, allowing the monitorization of sup-tRNA activity through E-cadherin expression, a new multiple cloning site (MCS) was first created in a region 919bp upstream the CMV promoter, after removing the original pIRES-EGFP2

MCS with blunt restriction enzymes AfeI and SmaI and the resulting ends were re-ligated. A SmaI restriction site was created in pIRES-EGFP2 vector by SDM (Table S1). The new MCS was designed (Figure 1a and Figure S1), amplified by PCR, digested with SmaI and HpaI (New England BioLabs, Ipswich, MA) and introduced in the SmaI restriction site.

Then, an arginine tRNA sequence [chr15.trna4 (87679308-87679380), Genomic tRNA Database - hg18 - NCBI Build 36.1 Mar 2006], flanked by 111bp upstream and 157bp downstream (primers sequence in Table S1), was amplified and inserted in the new MCS from pIRES-EGFP2 vector through AfeI and SacI (New England Biolabs, Ipswich, MA) restriction sites. The nonsense mutation was created in the anticodon of sup-tRNA^{Arg} by SDM (Table S1).

Vectors containing both sup-tRNA and CDH1 sequences were cloned through NdeI and NotI (New England Biolabs, Ipswich, MA) restriction sites.

All restriction reactions were purified from 1% agarose gel with illustra GFX PCR DNA and Gel Band Purification kit (GE Healthcare, Little Chalfont, UK). Purified fragments were cloned into vectors by T4 ligase (Invitrogen, Carlsbad, CA) and expanded through One Shot® Stbl3^{TM} Chemically Competent *E. coli* (Invitrogen, Carlsbad, CA) according with the manufacturer's instructions.

Protein extraction and Western blotting

Cells were lysed in cold catenin lysis buffer (1%Triton X-100, 1% Nonidet P-40 in PBS) supplemented with a protease inhibitor cocktail (Roche, Mannheim, Germany) and a phosphatase inhibitor cocktail (Sigma, St. Louis, MO). Protein concentration was

46 determined by Bradford-modified assay (DC Protein Assay, Bio-Rad Laboratories).

47 For analysis of total cellular proteins, 35μg of protein extracts were separated by 7.5%
48 sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and
49 electroblotted onto a Hybond nitrocellulose ECL membrane (GE Healthcare, Little
50 Chalfont, UK). Membranes were blocked with 5% non-fat milk and PBS 0.5% Tween-20
51 in for 30 minutes and incubated with antibodies against E-cadherin SHE78-7 (Enzo Life
52 Sciences Inc.) (1:1000), α-tubulin (1:10000, Sigma) and GFP (1:500, SantaCruz
53 Biotechnology Inc.) for 1h at RT. Membranes were washed with PBS 0.5% Tween-20, and
54 incubated with the secondary antibody appropriate anti-rabbit IgG or anti-mouse IgG
55 conjugated to horseradish peroxidase (GE Healthcare, Little Chalfont, UK). Signal was
56 detected by ECL chemiluminescence (GE Healthcare, Little Chalfont, UK). Band intensity
57 quantification was determined using densitometry software (Quantity One, version 4.6.2,
58 Bio-Rad Laboratories).

59

60 *Immunocytochemistry*

61 Cells were seeded on glass coverslips and grown to at least 80% confluence. Then, cells
62 were fixed in formaldehyde (4% in PBS) for 20 minutes, washed and incubated with NH_4Cl
63 (50 mM) and were then permeated with PBS-0,2 % Triton X-100. Coverslips were then
64 blocked with 5% BSA (Sigma, St. Louis, MO) in PBS for 30 min at room temperature
65 (RT), and incubated with primary antibodies anti-E-cadherin SHE78-7 (mouse, 1:100, Enzo
66 Life Sciences Inc.), diluted in 5% BSA-PBS for 1h at RT. After washing with PBS,
67 coverslips were incubated with anti-mouse AlexaFluor 594 secondary antibody (1:500,

Invitrogen, Carlsbad, CA) for 1h at RT in the dark. Coverslips were washed in PBS and mounted with Vectashield/DAPI (Vector Laboratories Inc). Images were captured using a Zeiss Imager Z1 fluorescence microscope, equipped with ApoTome, and supplied with Axiovision Rel 4.8 software (Carl Zeiss Inc).

Cell sorting and flow cytometry

To collect GFP-expressing population, cells were then washed and re-suspended in PBS-0,02%EDTA-0,5%BSA (freshly made). $5x10^5$ cells were taken and washed twice in the same re-suspension buffer and submitted to cytometer reading. To collect E-cadherin-expressing population, detached cells were washed with PBS-$CaCl_2$-3%BSA and incubated for 30 min in the primary antibody anti-E-cadherin HECD1 (mouse, 1:100, Zymed Laboratories Inc.) at 4ºC. Cells were then washed twice and incubated for 15 min with anti-mouse Alexa Fluor 647 secondary antibody (Invitrogen, Carlsbad, CA), at 4ºC in the dark. Finally, cells were washed with PBS-$CaCl_2$-3%BSA-0,02%EDTA and submitted to cytometer reading.

For analysis of E-cadherin and GFP expressing cells by flow cytometry, detached cells were washed with PBS-CaCl2-3%BSA and incubated for 1h in the primary antibody anti-E-cadherin HECD1 (mouse, 1:100, Zymed Laboratories Inc.) at 4ºC. Cells were then washed twice and incubated for 30 min with anti-mouse Alexa Fluor 647 secondary antibody (1:400, Invitrogen, Carlsbad, CA), at 4ºC in the dark. Cells were washed with PBS-CaCl2-3%BSA-0,02%EDTA, then with PBS, fixed with 2% formaldehyde and submitted to reading in a FACS Calibur flow cytometer (BD Biosciences). Flow cytometry results were analysed by FlowJo software version 8.7.3.

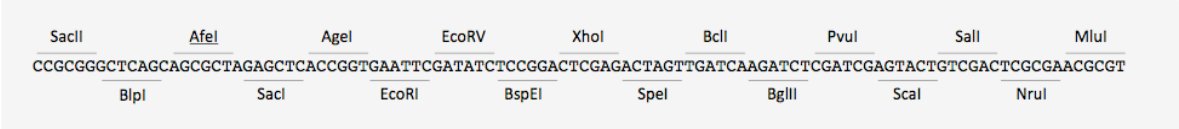

Figure S1. New multiple-cloning site (MCS). The new MCS was designed and inserted 919bp upstream of the CMV promoter. A PCR amplified gDNA fragment containing the Arg tRNA gene sequence plus 111bp (upstream) and 157bp (downstream) flanking regions was then inserted into the new MCS. This was intended to avoid interference between the tRNA (Pol III) and CMV promoters (Pol II), and to allow for the combined expression of tRNA and *CDH1* from the same plasmid.

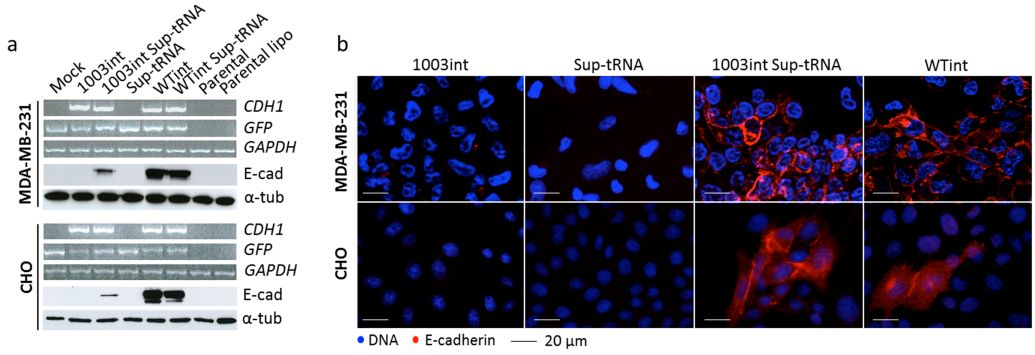

Figure S2. Suppressor-tRNA restores E-cadherin expression in MDA-MB-231 and CHO cell lines. (**a**) MDA-MB-231 and CHO cells transiently transfected with pIRES-EGFP2 modified vectors: mock-MCS, 1003int, 1003int_sup-tRNA^{Arg}, Sup-tRNA^{Arg}, WTint, WTint_sup-tRNA^{Arg}, 1003cDNA, 1003cDNA_sup-tRNA^{Arg}, WTcDNA, WTcDNA_sup-tRNA^{Arg} and parental cells, treated and not treated with Lipofectamine2000. RNA expression was analysed by RT-PCR for *CDH1* transcripts, using GFP expression transfection control, and GAPDH as endogenous RNA control (upper panel). Protein expression was detected by WB, using α-tubulin as endogenous

control (lower panel); (**b**) Immunocytochemistry in cells stably transfected with 1003int_sup-tRNA^{Arg}, showing E-cadherin expression localized at the membrane, while control cells 1003int do not have any expression (amplification 40x).

Table S1. Primers used for PCR amplifications.

PCR	Primer	Sequence
CDH1 mini-gene	CDH1_Nhe_F	5'-GCTAGCATGGGCCCTTGGAGCCCAG-3'
	Pr2R	5'-CTCGAGCTAGTCGTCCTCGCCGCCTCCGTACAT-3'
Arginine-tRNA	tRNAarg_F	5' – CAGTGATATCCCAACCTCCCCTTCTCAAG – 3'
	tRNAarg_R	5' – AGCAAGTCCCTGCGAGATTTCCGGAACTG – 3'
SDM CDH1_1003	1003sdm_F/R	5' – GTCACCACTGGGCTGGACtGAGAGAGTTTCC – 3'
SmaI_SMD	SmaI_F/R	5' – CCCCAAGTTCccGGGTGAAGGCCCAGGGCTCGCAGC – 3'
SDM Sup-tRNA^{Arg}	SuptRNA_1003_F/R	5' – GATAAGGCGTCTGACTTCaGATCAGAAGATTGCAG – 3'

Table S2. Nonsense mutations described in HDGC, LBC families and EODGC cases that could be targeted by suppressor-tRNAs (complete data with references).

	Gene Domain	Protein Domain	*CDH1* Mutation	Normal codon	Original amino acid	Generated PTC	References
HDGC	Exon 2	Signal	c.59G>A	TGG	Trp	TAG	[1]
	Exon 2	Signal	c.70G>T	GAG	Glu	TAG	[2]
	Exon 3	Precursor	c.187C>T	CGA	Arg	TGA	[3] [4] [5]
	Exon 3	Precursor	c.190C>T	CAA	Gln	TAA	[2]
	Exon 3	Precursor	c.283C>T	CAG	Gln	TAG	[6] [7]
	Exon 4	Precursor	c.489C>A	TGC	Cys	TGA	[5]
	Exon 5	Extracellular	c.586G>T	GGA	Gly	TGA	[2]
	Exon 7	Extracellular	c.1003C>T	CGA	Arg	TGA	[8] [3]
	Exon 8	Extracellular	c.1023T>G	TAT	Tyr	TAG	[9]
	Exon 10	Extracellular	c.1507C>T	CAG	Gln	TAG	[6] [10]
	Exon 12	Extracellular	c.1792C>T	CGA	Arg	TGA	[4] [11] [3]
	Exon 12	Extracellular	c.1913G>A	TGG	Trp	TAG	[6]
	Exon 13	Extracellular	c.2095C>T	CAG	Gln	TAG	[9] [3]
	Exon 14	Cytoplasmic	c.2275G>T	GGA	Gly	TGA	[12]
	Exon 14	Cytoplasmic	c.2287G>T	GAG	Glu	TAG	[9]
EODGC	Exon 12	Extracellular	c.1792C>T	CGA	Arg	TGA	[13]
LBC	Exon 3	Precursor	c.283C>T	CAG	Gln	TAG	[14]

Footnote: TAG = amber; TGA = opal; TAA = ochre; arginine = Arg; glutamine = Gln; glycine = Gly; glutamic acid = Glu; tryptophan = Trp; tyrosine = Tyr; and cysteine = Cys.

Supplementary References (Table S2)

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