Supporting Information

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SI Materials and Methods

Gene Synthesis and Plasmid Construction. ClustalW alignments and majority consensus sequences of the chimpanzee endogenous retrovirus-1 (CERV1) envelope, CERV2 envelope, and RhERV2 envelope genes were derived using MacVector software. All envelope sequences were collected from Ensembl using TBLASTN according to their homology with Mo-MLV protein sequences first, and then CERV consensus sequences were used to blast the rhesus macaque genome to ensure the recovery of all homologs. Overlapping oligonucleotides were designed using Genedesign from The Johns Hopkins University (http://www.genedesign.org). The target oligonucleotide length and overlap melting temperature was set to 60 nucleotides and 56 °C, respectively. EcoRI and NotI sites were added to oligonucleotides designed to construct the 5′ and 3′ ends of the envelope genes for cloning into the pCAGGS vector.

For envelope gene construction, two sequential polymerase chain reactions were carried out. The first contained a mixture of the overlapping oligonucleotides prepared by combining 1 μL of each 100-μM stock solution and then diluting 10-fold. A 50-μL PCR contained 3 μL of the diluted oligonucleotide mixture and gene synthesis was carried out for 20 cycles using Phinzymes Phusion (1 min 98 °C, 1 min 50 °C, and 2 min 72 °C). The second PCR contained 2 μL of crude product from the first in a 50-μL reaction with 200-nM external primers. Amplification proceeded for 25 cycles (1 min 98 °C, 1 min 55 °C, and 2 min 72 °C) and the resulting product was gel-purified using a Qiagen gel extraction kit and cloned into pCAGGS with EcoRI and NotI. Subcloning between individual clones was necessary to eliminate errors in three of the envelopes using the following restriction enzymes: SpeI for CERV1 envelope, DraI for RhERV2 envelope A, and BsmI and NsiI for RhERV2 envelope B.

Retroviral huCTR1 expression vectors were constructed by cloning CTR1 into either pLNCX2 (Clontech) or pLNCX2 constructs containing an HA tag using XhoI and NotI (forward primer: 5′-TATATACTCGAGATGGATCATTCCCACCATATGG-3′ reverse primer: 5′-TATATAGCGGCCGCTCAATGGCAATGCTCTGTGATATCC-3′). In the case of CTR1 mutant constructs, PCR products were first cloned using XhoI and NotI into pCR3.1 (Invitrogen) containing an HA tag sequence 5′ to the cloning site and then subcloned to pLNCX2 using SnaBI and NotI. Hamster CTR1 was amplified from Chinese hamster ovary (CHO) cDNA using primers designed against untranslated regions that are conserved between mouse and rat CTR1 (forward primer: 5′-TATATACTCGAGGGGATCCAGTTCTGAGAGGAAGAC-3′ reverse primer 5′-TATATAGCGGCCGCAGACAGGAGAGAGRAGGGATGATTGG-3′). Following cloning into pCR3.1 using XhoI/NotI and sequencing, primers were designed to amplify only the ORF of hamster CTR1 (forward primer: 5′-TATATACTCGAGATGGGGATGAACCATATGGGC-3′ reverse primer 5′-TATATAGCGGCCGCCTTAATGGCAATGCTCTGTGATGTCC-3′) and this product was cloned into pLNCX2 using XhoI and NotI. The haCTR1 coding sequence has been deposited in GenBank (accession number HQ290320).

An MLV vector expressing a neomycin resistance gene under a CMV promoter (CN) was used to generate CERV2-enveloped particles for the first round of library screening. CN was constructed by the removal of CMV-GFP from CNCG using BstBI/NotI, followed by Klenow polymerase treatment and blunt-ended ligation.

Complementary DNA Library Construction. The LMN8iresZeo cDNA retroviral plasmid library was generated as follows. Total RNA was extracted from 5×10^6 HeLa cells using TRIzol (Invitrogen), purified with two sequential chloroform extractions, precipitated in isopropanol, and washed in 70% ethanol. Messenger RNA was purified from total RNA using Qiagen Oligotex polyA+ resin. Complementary DNA containing SfiI sites was synthesized and amplified using Clontech SMART primers and Invitrogen size exclusion columns were used to dispose of small fragments. Following precipitation and SfiI digestion, cDNA was ligated overnight to LMN8iresZeo. The ligation reaction was transformed into DH10B *Escherichia coli* and grown under selection in soft agar for 72 h.

Cell Lines and Retroviral Transduction/Infection. CHO cells and all derivatives were cultured in Ham's F12 media supplemented with 10% FBS and 1 mM glutamine. *Pan troglodytes verus* skin fibroblasts AG06939 (Coriell) were grown in MEM-α with 20% FBS and 2 mM glutamine. All other cell lines were grown in DMEM supplemented with 10% FBS. All media was supplemented with 10 μg/mL gentamycin. The CHO-CTR1 and CHO-745-CTR1 cell lines were generated by transduction with LNCX2-based retroviral vectors expressing untagged or tagged CTR1 proteins and selection with 1 mg/mL G418.

All retrovirus pseudotypes and the packaged retroviral cDNA library were generated by polyethyleneimine cotransfection of 293T cells overnight in 100-mm dishes with plasmids expressing Moloney murine leukemia virus GagPol (6 μg), an envelope glycoprotein [1 μg for vesicular stomatitis virus (VSV)-G, 6 μg for all other envelopes], and a minimal MLV provirus containing a reporter gene or a selectable marker (6 μg). Supernatants were collected 48 h posttransfection and purified through 0.22-μm filters. All infections were allowed to proceed overnight and 5-μg/mL polybrene was used in the infection of all cells, with the exception of CHO-745. CERV2-enveloped virions carrying CN or LHCX (Clontech) vectors used for library screening were titrated by colony forming units using HeLa selected with 1 mg/mL G418 or 100 μg/mL hygromycin, respectively. For all other infections, MLV vectors carrying GFP (CNCG) or DsRed Express (CNCR) were used and infectivity measured by flow cytometry.

Receptor Screen. Twenty pools of 2×10^5 CHO-745 cells were transduced with the HeLa cDNA library constructed in the LM8iresZeo MLV vector at 0.5 zeocin resistant colony-forming units per cell. Following selection with 200 μg/mL zeocin, two groups of 10^5 cells from each pool were challenged with CERV2-enveloped virions carrying a MLV vector that contained a CMV-Neo cassette at an MOI of 3.5. Following selection with 1 mg/mL G418, colonies were pooled in 20 separate groups corresponding to the original 20 pools of pgsA-745 that were transduced with separated aliquots of the HeLa expression library. Thereafter, 2.5×10^4 cells from each of these pools of colonies were independently challenged with CERV2-enveloped virions carrying the LHCX MLV vector at a multiplicity of infection of 1.0. After selection with 100 μg/mL hygromycin, genomic DNA was extracted from surviving G418 and hygromycin-resistant cells using the Qiagen DNeasy kit and used as PCR template with primers designed to anneal to DNA flanking the LMN8iresZeo cloning site (forward primer: 5′-ATACACGCCGCCCACGTGAAGG-3′ reverse primer 5′-GCTTCCTTCACGACATTCAACAGACC-3′). The resulting PCR products were cloned and sequenced using the Invitrogen TOPO zero-blunt cloning kit.

Virus-Like Particle Binding Assay. GFP-labeled virus particles were generated by cotransfection of 293T cells with 5 μg of an MLV Gag-GFP expression plasmid and 5 μg pCAGGS-CERV2 envelope or 5 μg pCAGGS. The virion-containing supernatant was harvested 2 d posttransfection and passed through a 0.22-μm filter. CHO cells (10^5) or a derivative expressing humanCTR1 were seeded 2 d before binding onto poly-D-lysine coated coverslips. To assess the effect of copper on virion binding, cells were incubated either with or without 20 μM $CuCl_2$ for 2 h at 37 °C. Cells were then incubated in media containing 20 mM hepes pH 7.2 at 4 °C for 20 min, followed by a 1-h incubation at 4 °C with 1 mL viral supernatent supplemented with 20 mM hepes pH 7.2, 5 mg/mL polybrene, and either with or without 20 μM $CuCl_2$. The cells were washed three times with filtered PBS at 4 °C before fixation for 20 min at room temperature with 4% paraformaldehyde. The cells were then washed with PBS, DAPI stained, and visualized on an Olympus IX70-based DeltaVision suite (Applied Precision). A *z*-series of images that captures the entire thickness of the cell monolayer was acquired and projected onto a single image and the number of fluorescent particles was counted for 15 randomly selected cells under each condition.

RNA Interference. A Dharmacon siGENOME smart pool was used to knock down CTR-1 expression by targeting the following sequences within the CTR1 ORF: 5′-AGGCAGUGGUAGUGGAUAU-3′, 5′-CUGCGUAAGUCACAAGUCA-3′, 5′-GGAACCAUCCUUAUGGAGA-3′, and 5′-CAUAUGGGGAUGAGCUAUA-3′. Lipofectamine 2000 was used to cotransfect 10^5 cells with 10 pmol of pooled siRNA and 300 ng of DsRED expression plasmid (to monitor transfection efficiency) according to the manufacturer's instructions (Invitrogen). The transfection was repeated the following day and 8 h later, cells were trypsinized and seeded at 10^4 cells per well for infection the following day. To validate siRNA activity, transfections were repeated as described above except 500 ng of pCR3.1-HA-CTR1 was included in the first of the two siRNA transfections. Cells were collected for Western blot in SDS/PAGE loading buffer 2 d after the first transfection.

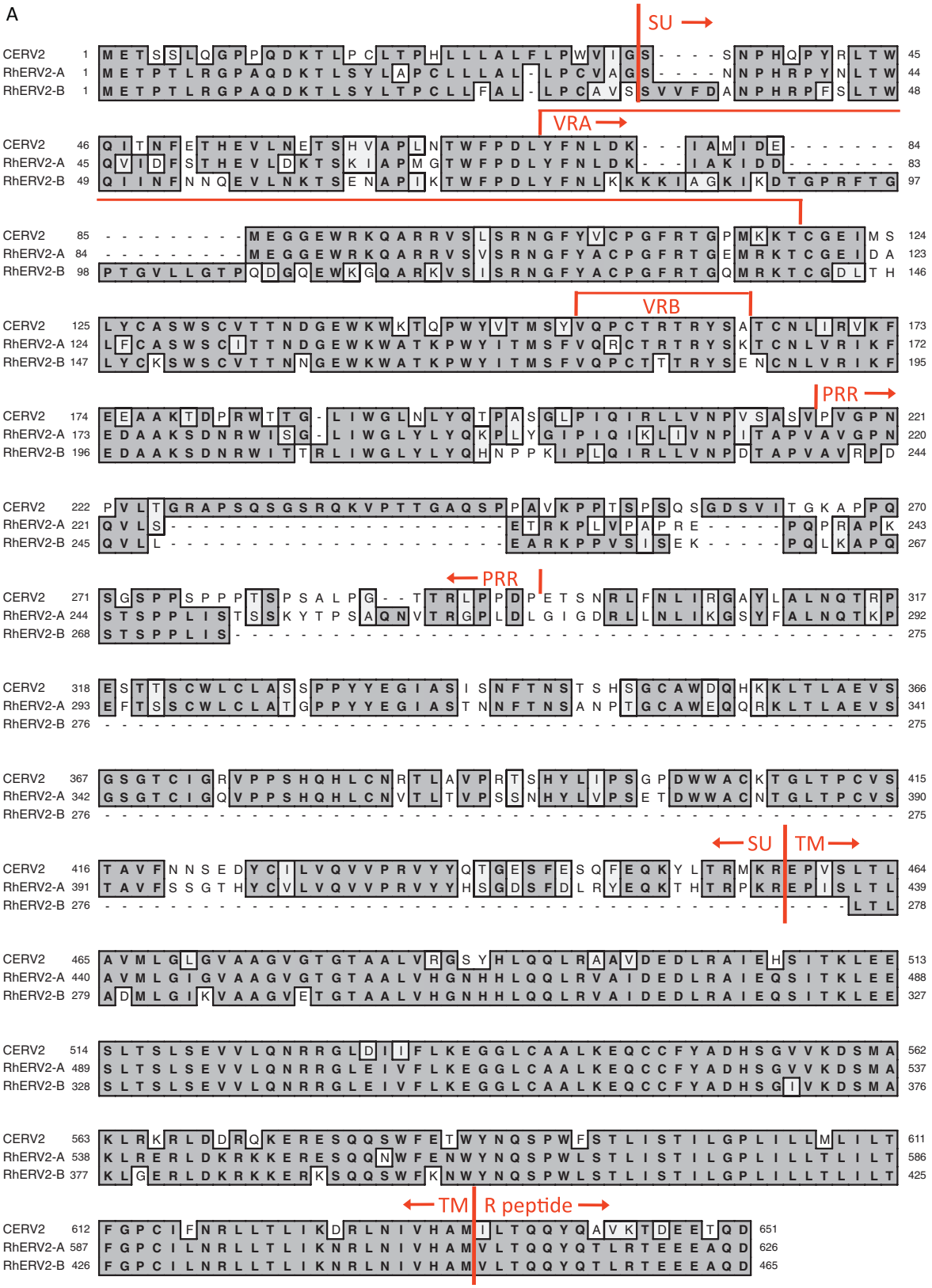

Fig. S1. (Continued)

Fig. S1. Consensus CERV/RhERV envelope proteins. (*A*) Alignment of the consensus CERV2, RhERV2-A, and RhERV2-B Env proteins. Features typical of γ-retroviral envelope proteins are indicated. The SU/TM and TM/R peptide boundaries and the variable regions (VR) A and B are predicted based on homology with MLV sequences; the signal peptide cleavage site (or the start of SU) was predicted using Signal P software. Note the deletion in RhERV2-B sequences that removes the domain between the proline rich region (PRR) and the N terminus of the TM protein. (*B*) The consensus CERV1 Env protein. Features typical of γ-retroviral envelope proteins are indicated, where possible, based on conservation with the MLV Env sequence and signal peptide prediction.

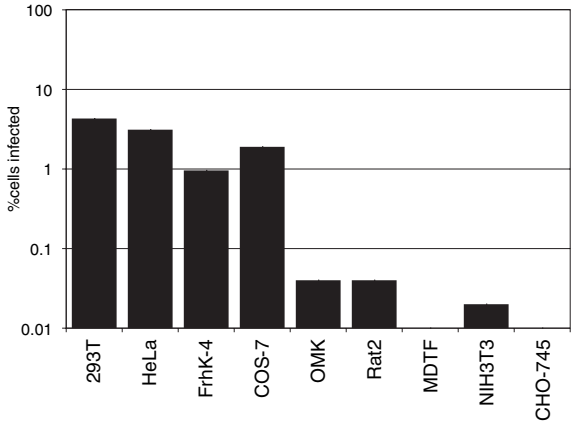

Fig. S2. Infection of mammalian cells by CERV1 pseudotyped MLV particles. MLV particles (50 μL) carrying a GFP reporter and pseudotyped with the CERV1 envelope protein were used to infect 10^4 cells. Cell lines from a variety of species were used: human (293T, HeLa), rhesus macaque (FrhK-4), African green monkey (COS-7), owl monkey (OMK), rat (Rat2), mouse (MDTF), NIH 3T3, and hamster (CHO-745). Cells were analyzed by FACS 2 d after infection.

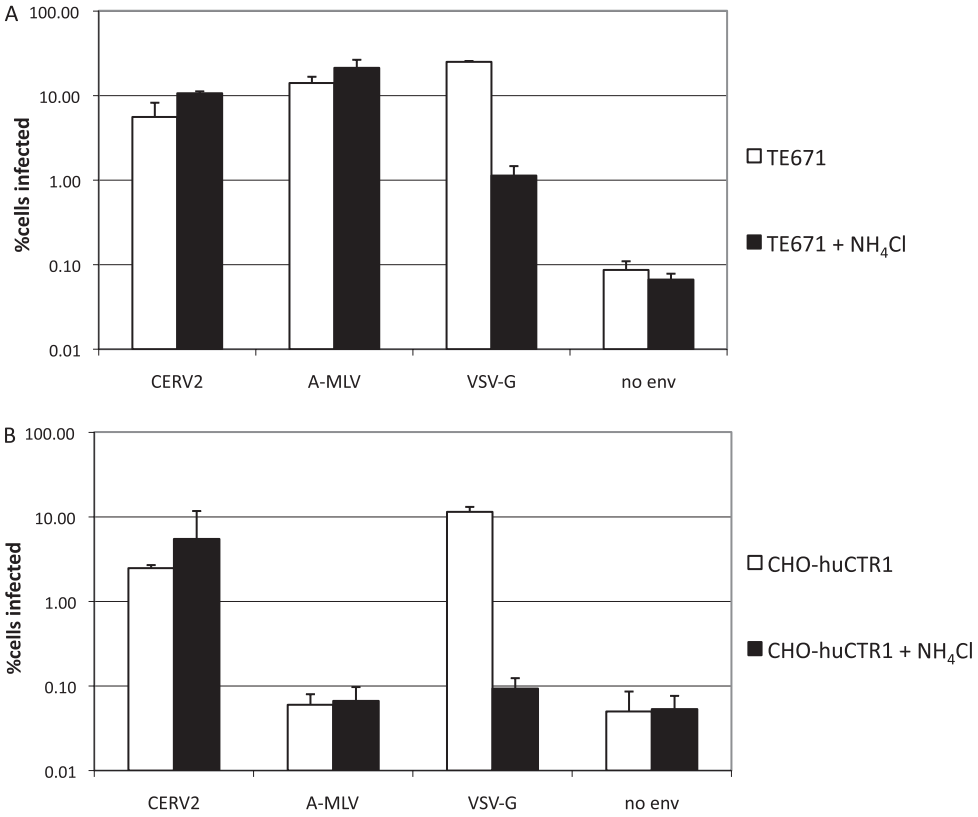

Fig. S3. CERV2 entry is endosomal pH-independent. TE671 (*A*) or CHO-huCTR1 (*B*) cells were infected by CERV2, A-MLV, or vesicular stomatitis virus (VSV)-G enveloped MLV-GFP in the presence or absence of 30 mM NH_4Cl following a 2-h preincubation under the same condition. After 3 h of infection, the virus was removed and cells were incubated for an additional 2 h in fresh media with 30 mM NH_4Cl before the media were replaced with fresh media lacking NH_4Cl.

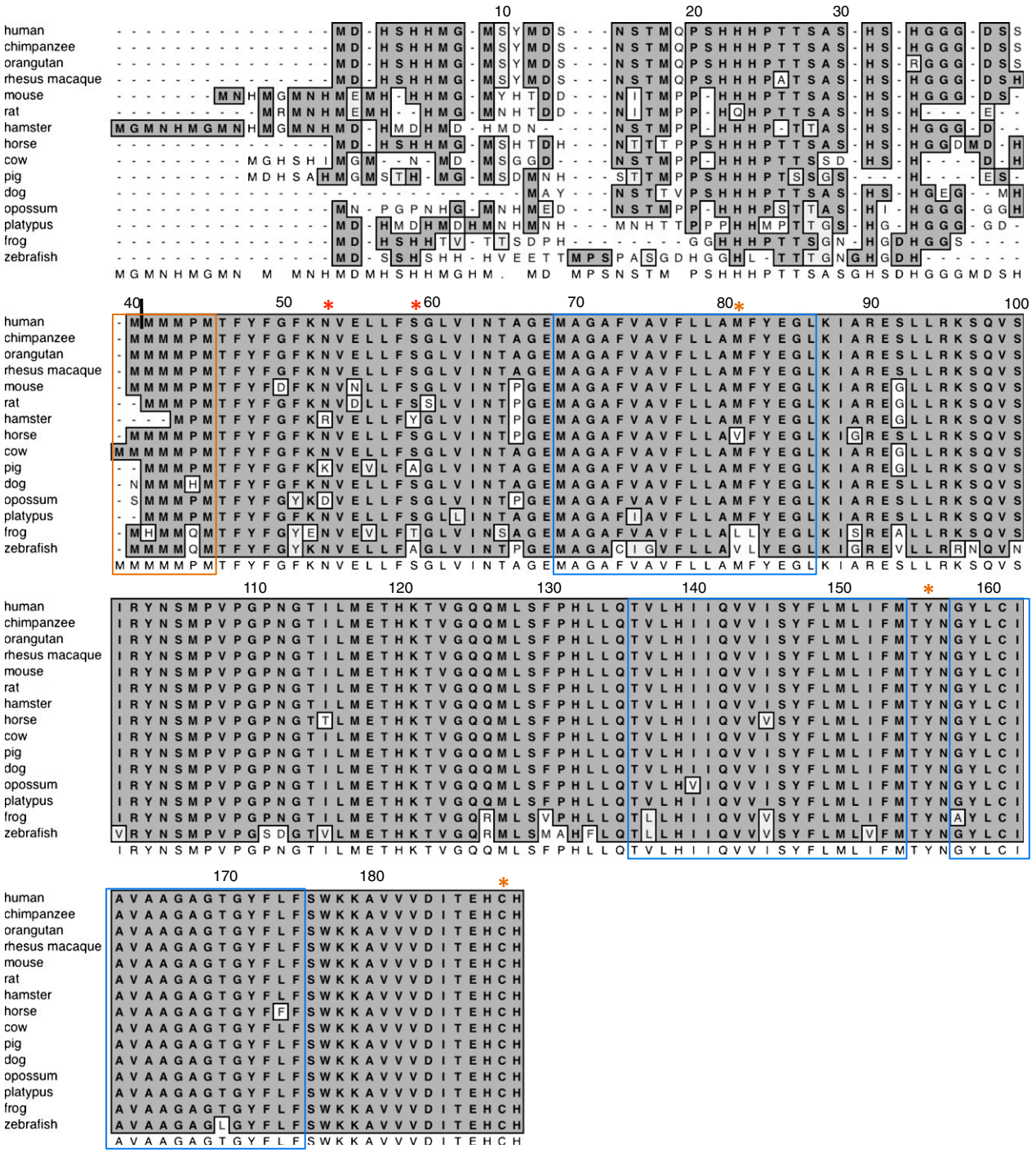

Fig. S4. Alignment of CTR1 proteins. Numbering is according to the human CTR1 amino acid sequence. The MMMMXM copper-coordination motif is marked by a copper colored box, transmembrane domains are marked by blue boxes, and residues mutated in the studies described herein are indicated by asterisks. The hamster CTR1 sequence was determined by cloning the gene from CHO cell cDNA. All other sequences were previously annotated in the National Center for Biotechnology Information gene database or were collected by a TBLASTN search of the nonredundant nucleotide database. Sequences from chimpanzee, rhesus macaque, horse, dog, platypus, and opossum are annotated in the sequence database as predicted transcripts. In the case of dog CTR1, a start codon at a position homologous to that found in other species is lacking; therefore, an amino acid sequence translated from a downstream hypothetical start codon is displayed.

Table S1. FACS analysis of CHO cells expressing N-terminally HA-tagged CTR1

	MFI	Percent positive cells
wt huCTR1	161.04	62.47
40del	154.35	84.43
M81I	323.65	94.37
Y156A	106.8	41.18
C189S	339.29	27.9
3Mdel	108.55	37.51
S59Y	342.47	79
N53R/S59S	92.22	39.98
wt hamster CTR1	160.16	73.89
ham 3Madd	91.81	45.82
ham R(53)N	135.38	58.32
ham Y(59)S	152.83	63.32
ham R(53)N/Y(59)S	125.04	52.42
ham 3Madd/R(53)N/Y(59)S	203.25	78
LNCX2	9.41	1.17
wt huCTR1 (unstained)	3.26	0.45

Cells were incubated with a mouse αHA monoclonal antibody followed by an AlexaFluor488 conjugated secondary antibody and subjected to FACS analysis. Mean fluorescence intensity (MFI) of the entire population, and the percentage cells with fluorescence greater than a cutoff gate that was selected based on the background fluorescence of stained empty vector (LHCX) transduced cells and unstained controls is indicated.