A novel human endogenous retroviral protein inhibits cell-cell fusion

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While common in viral infections and neoplasia, spontaneous cell-cell fusion, or syncytialization, is quite restricted in healthy tissues. Such fusion is essential to human placental development, where interactions between trophoblast-specific human endogenous retroviral (HERV) envelope proteins, called syncytins, and their widely-distributed cell surface receptors are centrally involved. We have identified the first host cell-encoded protein that inhibits cell fusion in mammals. Like the syncytins, this protein, called suppressyn, is HERV-derived, placenta-specific and well-conserved over simian evolution. In vitro, suppressyn binds to the syn1 receptor and inhibits syn1-, but not syn2-mediated trophoblast syncytialization. Suppressyn knock-down promotes cell-cell fusion in trophoblast cells and cell-associated and secreted suppressyn binds to the syn1 receptor, ASCT2. Identification of the first host cell-encoded inhibitor of mammalian cell fusion may encourage improved understanding of cell fusion mechanisms, of placental morphogenesis and of diseases resulting from abnormal cell fusion.

### Results

#### Amino acid sequence, genomic structure and expression of HERV-Fb1

The suppressyn coding sequence is derived from the env-coding region of a HERV-F (type b) family member: HERV-Fb1 (Fb1) (Fig. 1a). Fb1 transcripts can be detected in placental tissues from early and late gestation and its protein product is a 160 aa polypeptide (Fig. 1b). The predicted 18 kDa HERV-Fb1 translation product is part of the surface subunit (SU) and contains a putative signal sequence (39aa) and a premature stop codon that truncates the protein product prior to the SU-transmembrane (TM) cleavage site. The TM subunit of other HERV envelope genes commonly contains an immunosuppressive domain (ISD) that neither the TM nor the ISD present in suppressyn. The translation product contains no predicted N-linked glycosylation sites and a single O-linked site (Fig 1b). All of these characteristics suggest that suppressyn may function differently from other HERV envelope-derived proteins. Typical of endogenous retroviral proteins, the Fb1 sequence is found in several locations in the human genome, although its full length coding sequence is present only in a reverse orientation on chromosome 21q22.3. The Fb1 DNA and suppressyn amino acid sequences are highly conserved through simian evolution (Supplementary Fig. S1a online and unpublished observations) suggesting Fb1 is not a pseudogene. Suppressyn protein was identified by immunoprecipitation-western immunoblotting or direct immunoblotting using a combination of polyclonal and monoclonal anti-suppressyn antibodies in human placental samples.
and in trophoblast cell lines (Fig. 1d, Supplementary Fig. S2 online). Both methods identify cell-associated and secreted forms of the protein. In cell lysates, suppressyn was identified at a molecular mass of 14 kDa, identical to that of recombinant suppressyn and consistent with that predicted after truncation of its putative signal peptide. Suppressyn in cell supernatants had an unexpected size of approximately 15–16 kDa. Suppressyn contains no predicted N-glycosylation sites, and exhibits no change in migration after O-glycosidase treatment (Fig. 1e), suggesting the size difference between cell-associated and secreted suppressyn may result from other types of O-linked glycosylation or alternate protein modifications. A polyclonal antibody generated against a suppressyn-specific C-terminal 17-mer (Fig. 1b and Supplementary Fig. S2 online) detected intracellular expression of suppressyn in extravillous trophoblast (EVT) cells isolated from first and third trimester human placental samples, mirroring the expression of the
EVT-specific non-classical MHC product, HLA-G1,12 (Figs. 1f, g). Additional detection of suppressyn in the human chorionic gonadotropin (hCG) positive, syncytiotrophoblast layer of placental floating villi (Figs. 1f, h) is consistent with RNA expression patterns previously observed through *in situ* hybridization8. Although syn1 is typically detected at the cell surface, the cell-type expression patterns of suppressyn mimic those of syn1 protein in human placenta13,14.

**Fb1** gene knock-down induces cell fusion. To identify a potential function for this novel HERV-derived human placental protein, we used siRNA knock-down and the BeWo cell line. Suppressyn-specific mRNA and protein knock-down (50–60% mRNA knock-down; Figs. 2b, c) in BeWo cells using distinct siRNA (siFb1a and siFb1b) or their respective scrambled siRNA controls induced a significant increase in cell fusion when compared to parental cells or to those treated with control siRNA (Figs. 2a, d). hCG transcripts and secreted protein were increased 2.5-fold (mRNA) and 1.5-fold (protein) 72 hours after suppressyn knock-down (Supplementary Fig. S3a, b online). Human placental lactogen (hPL) mRNA expression and progesterone secretion were likewise increased at 72 hours; estriol was undetectable at all time points (Supplementary Fig. S3c, d online). These data show that, in addition to inhibiting syn1-mediated fusion, suppressyn may also promote differentiation of BeWo cells. Trophoblast fusion and trophoblast differentiation have been shown in cell lines (BeWo) and in *ex vivo* models to be linked, but distinct processes15,16. Inhibition of fusion using Fb1 siRNAs was also visualized immunocytochemically using antibodies against the intercellular tight junction protein, zona occludens-1 (ZO-1, Fig. 2a, lower panels). Unlike most human trophoblast cell lines, BeWo cells syncytialize poorly when grown under standard culture conditions but differentiate into multinucleate, syncytiotrophoblast-like cells and secrete the syncytiotrophoblast cell differentiation marker, hCG, in response to forskolin17. Although some controversy remains, this secretory product may help to distinguish these cells from trophoblast giant cells18–20. BeWo cells spontaneously translate cell-associated and soluble suppressyn (Fig. 1d) and cell-associated syn1 (Supplementary Fig. S2d online). Although forskolin exposure increases suppressyn transcript in BeWo cells, it also decreases ASCT2 levels (data not shown). Still, suppressyn knock-down promotes cell fusion in the absence of forskolin. We therefore hypothesize that endogenous suppressyn exerts tonic control of syn1-mediated BeWo cell fusion.

*Figure 2 | Fb1 knock-down increases cell fusion in syn1 expressing cells.* Fb1-specific mRNA knock-down in BeWo choriocarcinoma cells using distinct siRNA (siFb1a and siFb1b) or their respective scrambled siRNA controls. Fb1 knock down induced a significant increase in cell fusion (a, d). (a) ZO-1-FITC and Hoechst 33342 immunocytochemistry was performed 72 hours after siRNA exposure and images are depicted in the lower row. The upper row shows matched, phase-contrast images. Scale bar represents 100 μm. Quantitative RT-PCR, immunoprecipitation and immunoblotting confirm decreases in (b) Fb1 mRNA (50–60% knock-down) and (c) protein (cell-associated and secreted) in siRNA treated BeWo cells. Expression (b) is normalized to unexposed samples cultured for similar time periods (48 or 72H). (d) Mean fusion indices for siRNA-exposed and control cells (error bars represent standard deviations; n = 4 × 5 fields). Cell fusion was assessed using phase contrast microscopy and quantitated using cell fusion indices. All observations were performed at a final magnification of 200X and total nuclei were counted per field using Leica MetaMorph image analyzing software. The number of fused syncytiotrophoblast cell aggregates and the number of nuclei in each aggregate was counted manually and fusion indices were defined as \[(N - S) / T \] × 100. N is the number of nuclei in syncytia, S is the number of syncytia, and T is the total number of nuclei counted. The fusion index quantitates the percentage of fusion events in a cell population. Black bars - Fb1 siRNA exposed; hatched bars-control siRNA exposed. Data in (b, d) are representative of three independent experiments performed in duplicate. *p < 0.05 and **p < 0.01 compared to matched siRNA control. Statistical comparisons used Kruskal-Wallis and Mann-Whitney U-testing with (d) or without (b) Bonferroni corrections.
Suppressyn inhibits syn1-induced cell fusion and binds to the syn1 receptor, ASCT2. A trophoblast cell line, HTR8/SVneo<sup>21</sup>, that does not spontaneously syncytialize and exhibits low to undetectable endogenous expression of suppressyn and syn1 (Fig. 1d, Supplementary Fig. S2 online) was stably transfected with a vector driving Fb1 expression (HTR8-Fb1). Control cells and two distinct HTR8-Fb1 clones (HTR8-Fb1.1 and HTR8-Fb1.2) were then transiently-transfected with vectors driving the expression of syn1 or syn2 and analyzed for cell size (FSC) and granularity (SSC) using flow cytometry. The presence of large, multinucleated (more granular) syncytialized cells increased in direct proportion to the amount of transfected syn1 and syn2 in HTR8 (parent) and HTR8-V (vector only control) cells and continuous expression of suppressyn in stably-transfected HTR8-Fb1.1 and HTR8-Fb1.2 cells abrogated syn1- but not syn2-induced cell fusion (Figs. 3a–d). Transient expression of syncytins in transfected cells was verified using western immunoblotting, excluding the possibility of inter-vector inhibition of transcription in doubly-transfected cells, supporting the concept that suppressyn modulates trophoblast fusion, and suggesting that suppressyn does not inhibit syn1 production or maturation<sup>23–26</sup> (Supplementary Fig. S4a, b online).

To assure we were not documenting endoreduplication, a common characteristic of end-differentiated EVT, particularly trophoblast giant cells<sup>23–26</sup>, we transfected mixed cultures of equal numbers of CSFE (green)- and Cell Vue Claret (red)-stained HTR8, HTR8-V, HTR8-Fb1.1 and HTR8-Fb1.2 with a vector driving syn1 expression (Supplementary Fig. S4c online). Non-fused cells in all cultures continued to display distinct red or green fluorescence at 72 hours post-transfection; fused cells in the HTR8 and HTR8-V (Fb1-negative) controls displayed a mixture of green and red fluorescence, indicating fusion rather than endoreduplication.

Although suppressyn does not contain the SDGGGX<sub>2</sub>DX<sub>2</sub>R conserved motif (Supplementary Fig. S1b online) thought to be essential for syn1/hASCT-2 interactions<sup>27</sup> nor the heptad repeat sequences that characterize previously described synthetic inhibitors of syn1-mediated fusion<sup>28</sup>, syn1 inhibition is still most likely to occur at the level of the receptor. Immunoprecipitation of suppressyn co-precipitates ASCT2 and immunoprecipitation of ASCT-2 co-precipitates suppressyn in doubly transfected cells (Fig. 3e), indicating direct binding of suppressyn to ASCT-2. The high molecular weight form of ASCT bound by Fb1 is likely a glycosylated multimer<sup>29</sup>. Purification of surface biotinylated proteins from HTR8, HTR8-V and HTR8-Fb1 revealed ASCT-2 on the cell surface of all tested cells, as expected<sup>29</sup>, and surface-expressed suppressyn only on HTR-Fb1 cells (Fig. 3f). Since suppressyn lacks a transmembrane domain, the detection of this ASCT-2 binding HERV product at the cell surface suggests that it is ligand-bound and promotes a potential mechanism for syn1 antagonism.

Figure 3 | Suppressyn inhibits syn1- but not syn2-induced trophoblast cell fusion and suppressyn binds to the syn1 receptor ASCT2. HTR8 trophoblast cells were stably transfected with vectors driving the expression of Fb1 (HTR8-Fb1.1 and 1.2) or vector alone (HTR-V). Cells were then transiently transfected with increasing amounts of a vector driving expression of syn1 (a) or syn2 (c), counterstained with hematoxylin and analyzed using phase contrast microscopy. To calculate fusion indices, cells were resuspended and analyzed by flow cytometry (b and d). Relative cell fusion was defined as the number of fused syn1- or syn2-transfected cells (gated by flow cytometry) divided by the number of fused cells in parent (HTR8) and in stable vector- or Fb1-transfected (HTR8-V, HTR8-Fb1.1, HTR8-Fb1.2), but syncytin non-transfected, cells cultured under identical conditions (indicated as negative) controls displayed a mixture of green and red fluorescence, at 72 hours post-transfection. (f) HTR8, HTR8-V and HTR8-Fb1 cells were surface biotinylated and proteins purified using the Pierce cell surface protein isolation kit. Equal amounts of cytoplasmic and biotinylated surface protein were subsequently analyzed with standard SDS-PAGE and immune detection. Data in (e) and (f) are representative of three independent experiments.
Figure 4 | Secreted suppressyn binds ASCT2 and inhibits syn1-induced trophoblast cell fusion. (a) Syn1 transfected HTR8 cells were cultured in the presence of increasing amounts of recombinant secreted suppressyn for 24 hours prior to fusion assessment. Relative cell fusion was assessed by flow cytometry as in Fig 3b but was defined as the number of fused cells (gated by flow cytometry) in suppressyn peptide-exposed cells normalized to the number of fused cells in syn1-transfected HTR8 cells exposed to a control peptide. **p < 0.01 compared to 0 μg/ml sample. Statistical comparisons were made using Kruskal-Wallis and Mann-Whitney U-testing with Bonferroni corrections. Data are representative of three independent experiments performed in duplicate. (b, c) Transwell (0.4 μm; Becton Dickenson 353493) cell culture models (upper chamber; HTR8-V or HTR8-Fb1, lower chamber; HTR8 parent cell) were used to assess the effects of secreted suppressyn on non-transfected ASCT2-expressing target cells. Structural changes (*high and low molecular weight suppressyn forms) were observed in HTR8 cells after exogenous exposure to suppressyn (b). Coprecipitation experiments demonstrate direct binding of ASCT2 and suppressyn (c). Data in (b) and (c) are representative of three independent experiments. *Co-precipitated ASCT2.
Secreted suppressyn binds ASCT2 and inhibits cell fusion. We hypothesized that secreted suppressyn may exert effects on nearby trophoblast cells as well as on the secreting cell itself. To test the former hypothesis, syn1-transfected HTR8 parent cells were exposed for 24 hours in culture to suppressyn protein purified from JEG3-Fb1-flag supernatants and cell fusion was assessed as in Fig. 3b. Exposure to exogenous suppressyn reduced syn1-induced fusion in a dose-dependent fashion, reaching a level of nearly 50% suppression at a suppressyn concentration of 1 μg/ml (Fig. 4a).

Transwell culture cells models (upper chamber: HTR8-V or HTR8-Fb1; lower chamber: Fb1 negative HTR8 parent cells) were used to assess the effects of secreted suppressyn on non-transfected ASCT2-expressing target cells. HTR8 target cells were lysed for protein isolation after 3 days of physically-separated co-culture with Fb1-expressing or control cells. In cultures exposed to exogenous suppressyn, co-precipitation experiments demonstrated direct binding of ASCT2 and suppressyn and bound ASCT2 exhibited the same structural changes (degradation and multimer formation) observed in cells endogenously expressing ASCT2 (Figs. 4b, c). That the secreted and cell-associated suppressyn forms in primary placental tissues and in trophoblast cell lines exert similar effects on ASCT2 and inhibit cell fusion suggests that this novel suppressor of cell fusion may utilize intracellular, autocrine and paracrine pathways.

Discussion

We are aware of no previous reports of a protein encoded by a mammalian genome that specifically inhibits cell fusion. The implications of this finding to human health and disease may be far-reaching. In the placenta, cytotrophoblast progenitor cells syncytialize into a continuous, multinucleated layer of terminally-differentiated fused cells that line the intervillous space; this layer is called the syncytiotrophoblast. The syncytiotrophoblast sheds cell-derived microparticles into the maternal circulation, likely via activation or apoptosis, but certainly through processes that appear to be tightly regulated. While the exact control mechanisms for syncytiotrophoblast turnover remain undefined, it is clear that syn1 is involved in formation of syncytiotrophoblast and that several diseases of pregnancy, including intrauterine growth retardation (IUGR) and pre-eclampsia, are characterized by abnormal placental development and/or villous turnover. The possibility of central involvement of a placental inhibitor of syn1-induced syncytialization (i.e., suppressyn) in these disorders is enticing and indicates an important pathway for future investigations. While we have here described the cell fusion effects of suppressyn in syncytiotrophoblast models, syn1 and suppressyn are also detected in invasive EVT. Like abnormal syncytialization, shallow placental invasion is also characteristic of preeclampsia and IUGR, but also of spontaneous pregnancy loss. While the role of syn1 in placental invasion remains incompletely defined, the co-expression of syn1 and suppressyn at this maternal-fetal also promotes intriguing hypotheses. For example, the presence of suppressyn in EVT could be involved in the inhibition of EVT end-differentiation into non-invasive, HLA-G positive, non-secretory trophoblast giant cells.

In a remarkable example of convergent evolution, several mammalian species, including rodents, lagomorphs, Carnivora, and humans have co-opted endogenous retroviral envelope proteins for use in normal placental development. Also remarkable is the finding that, despite widely varying sequences and independent acquisition, human syn1 and rabbit syn-Ory1 can use the same neutral amino acid transporter, ASCT-2, as a receptor mediating cell fusion; the receptors for the murine synA and Carnivoral syncytin (syncytin-Car1) have not been identified. Our demonstration of direct binding of suppressyn to ASCT-2 supports an important role for suppressyn in human fusion events and predicts that a search for similar retrovirally-derived fusion-control proteins in other species is likely to be fruitful.

While a recent exciting description of syn1 expression and function in osteoclast fusion adds to the very limited evidence for tissue expression of syn1 among healthy organs other than the placenta, in diseased tissues, syn1 has been implicated in neoplastic cell fusion and ASCT2 appears to play roles in cancer aggression and in viral fusion. ASCT2 has been identified as the receptor for a diverse family of retroviruses, including baboon endogenous retrovirus, avian reticuloendothelial virus, feline endogenous viruses (RD114), and type D simian retroviruses. Syn1 can pseudotype HIV-1 viral cores, likely through interactions with RD114 (ASCT2). We hypothesize that suppressyn-mediated inhibition of signaling through ASCT2 could help to explain the fairly robust ability of the human placenta to delimit vertical viral transmission, at least for a subset of exogenous pathogens.

Finally, it is intriguing to speculate on the possible function of Fb1 in its ancestral exogenous retrovirus. Several retroviruses have well-described mechanisms by which they downregulate the host cell surface receptors mediating their entry after infection has occurred. Like those that share ASCT2, many other retroviruses have endogenous counterparts that inhibit superinfection by other interference group members, including HERV-W, that shared preference for ASCT2-mediated entry into host cells. A similar role has been described for a murine gene called Fv4 that is derived from a murine leukemia virus (MuLV) Env-like sequence and can function in cellular resistance to MuLV infection. Like our description of the effects of suppressyn on the syn1 receptor, ASCT2, interference by Fv4 has been hypothesized occur at the level of the MuLV receptor.

Methods

Cell lines and human tissues. The human choriocarcinoma cell lines JEG3, JAR and BeWo were obtained from ATCC. The endometrial Ishikawa cell line was generously supplied by Dr. Susan Nagel at The University of Missouri. The human trophoblast cell line, HTR8/SVneo, was kindly provided by Dr. Charles H. Graham, Department of Anatomy & Cell Biology, Queen’s University, Toronto, Canada. With the exception of BeWo cells, which were maintained in Kaitgn’s Modification medium (F-12K, Invitrogen) supplemented with 15% fetal calf serum (FCS), all cells were grown in D-MEM containing 10% fetal bovine serum (Invitrogen). Early and term placental tissues for immunohistochemical analyses were obtained from women at 7–12 weeks of gestation undergoing dilation and evacuation or those undergoing an uncomplicated delivery at 37–41 weeks of gestation. Normal paraffin-embedded human pancreas (T2234188) and tests (T2234260) tissue sections were purchased from Biochain. All human tissue specimens were obtained under University of Missouri or University of the Ryukyus IRB-approved protocols.

Immunohistochemistry and antibodies. The anti-suppressyn polyclonal antibody used in immunohistochemistry, immunoprecipitation and western immunoblotting was generated in rabbits against a synthetic C-terminal KLH-conjugated 17 aa polypeptide (EDIKRQAIKAKASKP; Fig. 1b) and purified over a peptide affinity column loaded with the immunizing peptide (Genemed Synthesis, Inc.). The monoclonal antibody was raised against a C-terminal 10-mer (PRHHHEEQKL; Fig. 1b; AbMart). The commercially available antibodies for immunohistochemistry included: 1) monoclonal anti-FLAG M2 (F3165; Sigma-Aldrich), 2) monoclonal anti-Myc-tag (05-7324; Millipore) 3) polyclonal anti-human chorionic gonadotropin (AB936; Chemicon), 4) monoclonal anti-human HLA-G (ab152453; Abcam), 5) polyclonal anti-human syn1 (sc-50369; Santa Cruz Biotechnology-1), 6) monoclonal anti-beta actin (MAB1501; Millipore), 7) monoclonal anti-GEF (04363-24; Nakalai tesque), 8) monoclonal anti-ZO-1-FITC (33-9111; Zymed), 9) polyclonal anti-GFP (2500), negative control rabbit IgG (identical concentration, 731642; DAKO), anti-human HLA-G (1:5000, and negative control
mouse IgG (identical concentration, 0.15–0.005; Jackson ImmunoResearch Laboratories) antibodies occurred overnight at 4 °C. Sections were washed and processed using a Histofine SAB peroxidase kit (424021, 424031; Nichirei) or diaminobenzidine (DAB). Samples were visualized using standard microscopy.

Specificity of the primary polyclonal anti-suppressyn antibody used in immunohistochemistry was confirmed using a blocking peptide competition assay (BPC). The suppressyn polyclonal antibody was incubated with 0- or 500-fold mol excess peptide (EDIKRQQAKAKASKP, the sequence used for antibody generation) overnight at 4 °C; both antibodies were then used for standard immunohistochemistry as described above (Supplementary Fig. S2 online).

Immunoprecipitation and western immunoblot analyses. Whole cell extracts from cell lines or placental tissues were prepared in cell lysis buffer (50 mM Tris HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, supplemented with a protease inhibitor cocktail (11–873–580–001; Roche). Twenty to five hundred micrograms of extract were used for immunoprecipitation with 2 microgram of polyclonal anti-suppressyn antibody. Purified antigens or antigens purified by immunoprecipitations were captured with 50 μl of protein G-agarose (A-2220; Sigma) according to the manufacturer’s recommendations. Captured proteins were separated by standard SDS–PAGE and analyzed by immunoblotting with specific primary antibodies (above) and peroxidase conjugated goat anti-rabbit IgG (432460; Pierce) or anti-mouse IgG (432430; Pierce) secondary antibodies. Antibodies were detected using chemiluminescence (ECL Prime, RPN2232; GE Healthcare).

Vectors and constructs. Fbl, syn1, syn2 and ASCT2 were reverse transcribed and amplified from long term placental mRNA or RNA from trophoblast cell lines. Further amplification used restriction enzyme site-linked primers and fragments were cloned into the pGEM-T Easy vector (Promega) for sequencing. Clones with 100% sequence identity to c21orf105, syn1 (NM_014950.3), syn2 (BC068585) or ASCT2 (BC000062) were inserted into a pFlag-EGF vector that replaced the CMV promoter of the pFLAG-CMV expression vector (ED908, Sigma Aldrich) with an elongation factor 1 (EF1) promoter. A separate ASCT2 vector replaced its flag tag with a myc tag; Flag-tag fused or -unfused suppressyn fragments were transferred to a mammalian expression vector with a CAG promoter to obtain stable suppressyn-expressing cell lines. Cloning primer sequences and 5’ sequence positions are listed in Supplementary Table S1 online.

siRNA gene knock-down. BeWo cells at 60% confluency were transfected using 1.5 μl of lipofectamine 2000 (Invitrogen) and 20 pmol siRNA in 500 μl of Opti-MEM medium. After 6 hours, medium was changed and incubation continued for 72 hours. siRNA sequences are listed in Supplementary Table S1 online.

Immunocytochemistry. siRNA treated cells were grown on 24 well plates for 72 h. Cells were then fixed in PBS-4% paraformaldehyde. After blocking in PBS-1% BSA, cells were immobilized with a mouse anti-ZO-1-FITC conjugated antibody at 4 °C overnight. Cells were washed and counterstained for 5 min at room temperature with Hoechst33342 (0.5 μg/ml). Exposed cells were examined with an EVOS fl (AMF-50) for 60 min. Cells were washed and incubated for 10 min using TrypLE Express (Invitrogen). Cells were transferred to a 100 mm dish and incubated for 30 min at 37 °C. Cells were trypsinized for 10 min using TrypLE Express (Invitrogen) and counted by flow cytometry.

Fluorescent activated flow cytometry. Syn1 transfected cells were stained with the polyclonal anti-synctin-1 antibody (1: 50) for 60 min. Cells were exposed to the secondary DyLight™ 488 Donkey anti-rabbit IgG antibody (1: 200, #406404; Biolegend) at room temperature for 60 min and then analyzed by flow cytometry (BD FACS Calibur).

Surface Biotinylation. Cell surface biotinylation was performed using a cell surface protein isolation kit (Pierce; 89881).

Statistical analyses. Data are expressed and plotted as means +/− standard deviations. Means were compared using Mann-Whitney U-tests or Kruskal-Wallis tests followed by Mann-Whitney U-tests with Bonferroni corrections as appropriate (described in figure legends). Statistical significance was defined as P < 0.05 or P < 0.01, as indicated.

HERV-Fbx1 gene and protein accession number: AB610407. Additional experimental and statistical details can be found online in the supplemental materials.

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