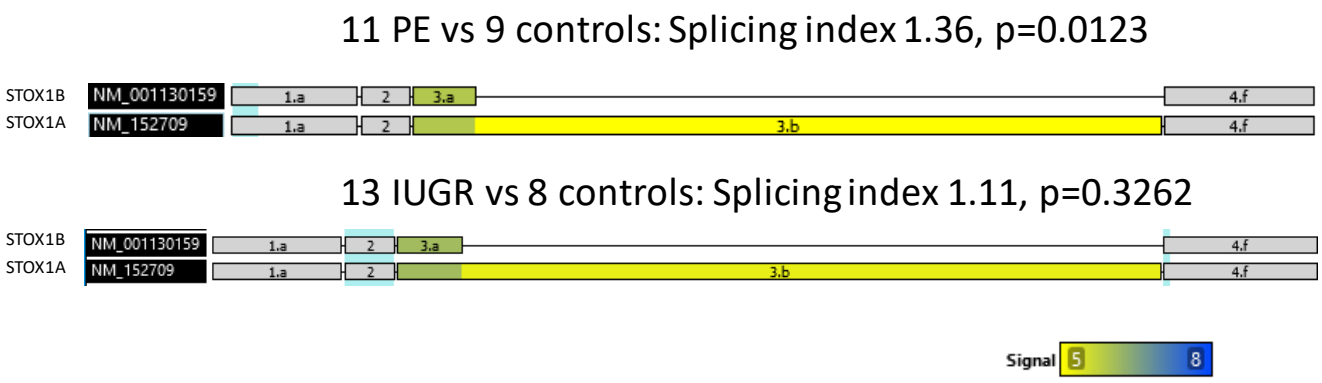
iScience, Volume *23*

Supplemental Information

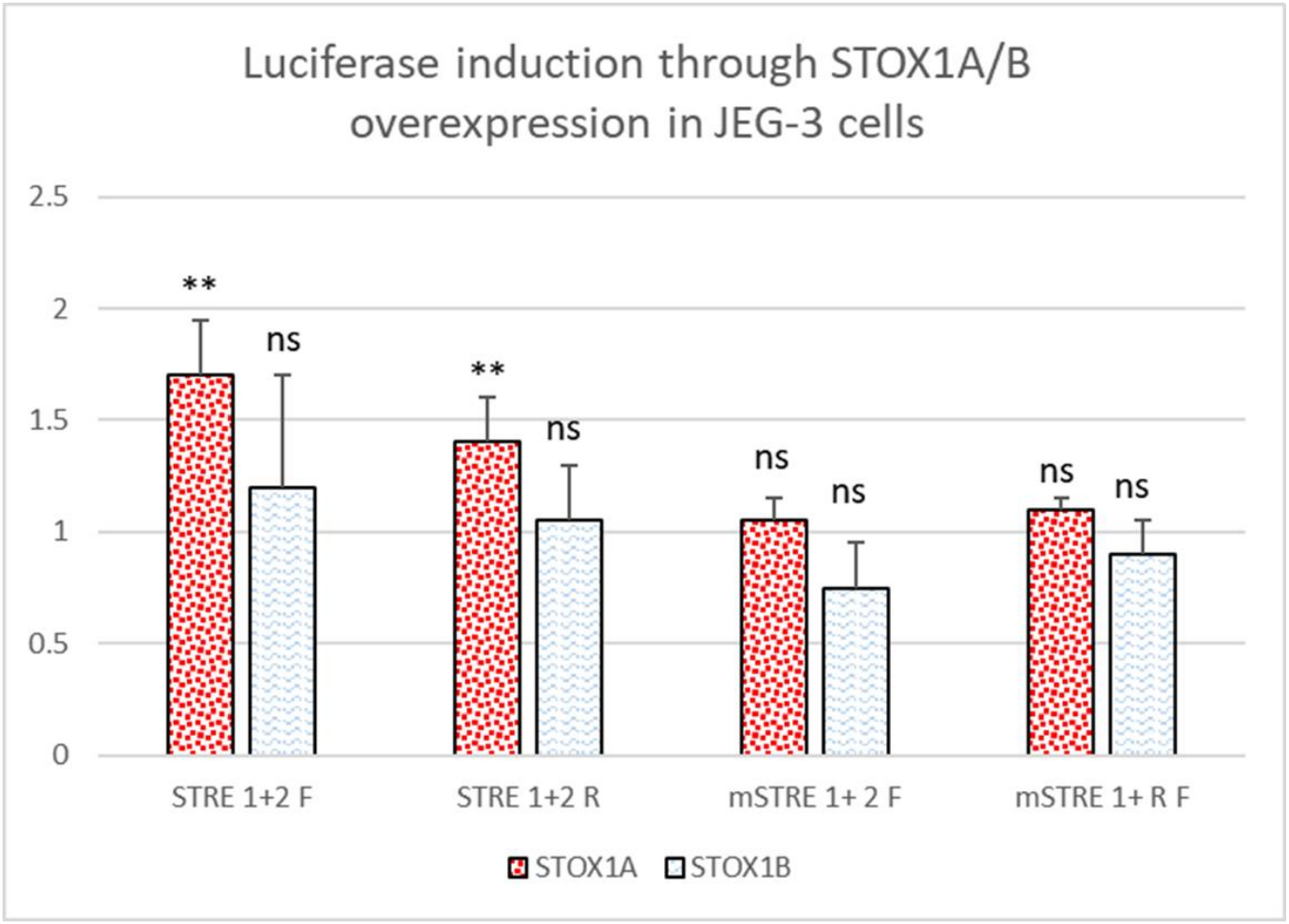
Molecular Mechanisms of Trophoblast Dysfunction Mediated by Imbalance between STOX1 Isoforms

Aurélien Ducat, Betty Couderc, Anthony Bouter, Louise Biquard, Rajaa Aouache, Bruno Passet, Ludivine Doridot, Marie-Benoîte Cohen, Pascale Ribaux, Clara Apicella, Irène Gaillard, Sophia Palfray, Yulian Chen, Alexandra Vargas, Amélie Julé, Léo Frelin, Julie Cocquet, Camino Ruano San Martin, Sébastien Jacques, Florence Busato, Jorg Tost, Céline Méhats, Paul Laissue, Jean-Luc Vilotte, Francisco Miralles, and Daniel Vaiman

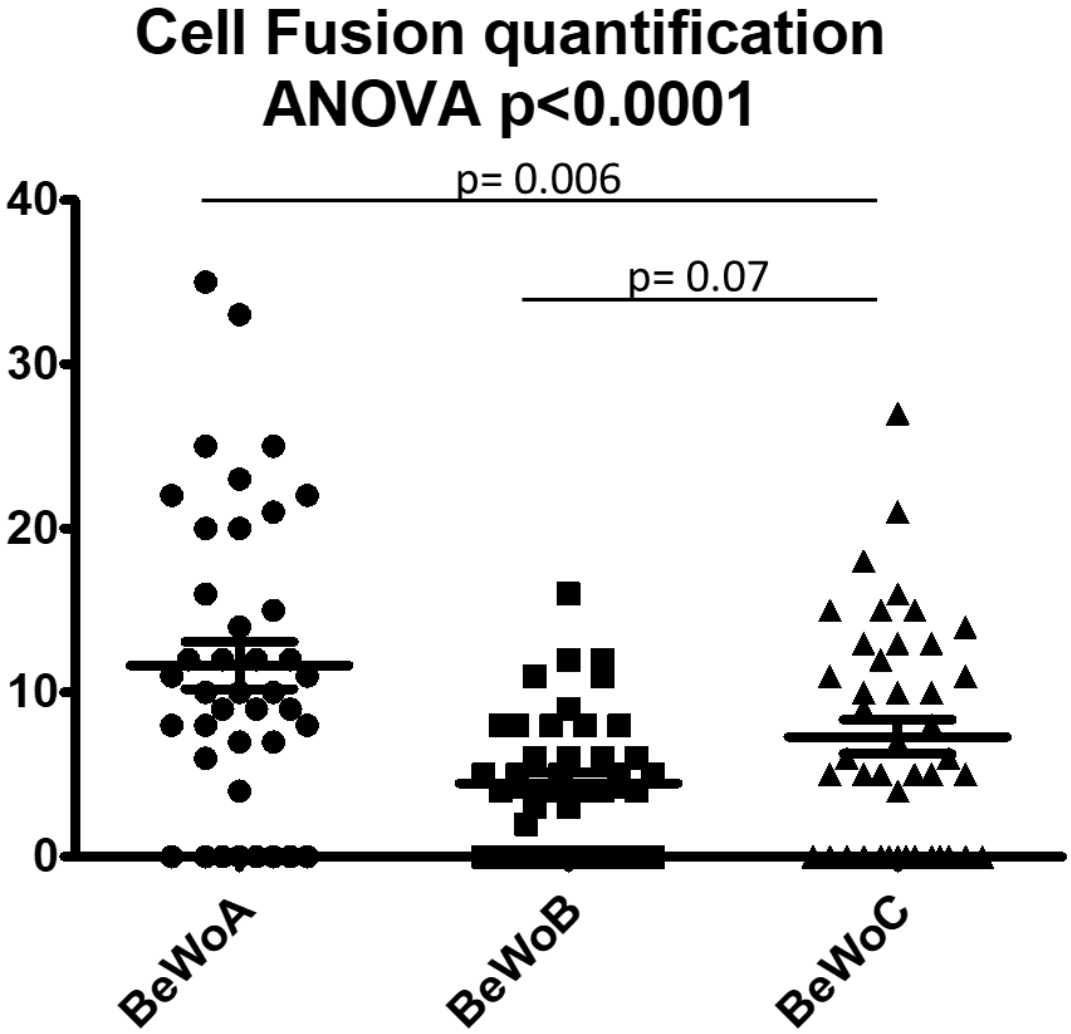


Supplementary Figure S1, Structure of STOX1 major isoforms STOX1A and STOX1Bn and splicing index in preeclampsia (PE) versus controls and in Intra-Uterine Growth Restriction (IUGR) versus controls, Related to Figure 2b and paragraph 6 of the Discussion in the main text. The p values were provided following TAC® Affymetrix software analysis from ClariomD microarrays (manuscript in preparation)

Construction STRE21F	GCTAT**GGTGYGGAMA**GCGC**CATYTCACGG**AGAT**GGTGYGGAMA**GCGC**CATYTCACGG**AGAGC
Construction STRE21R	GCTCTCCGTGARATGGCGCTKTCCRCACCATCTCCGTGARATGGCGCTKTCCRCACCATAGC
Construction mSTRE21F	GCTAT**GATGYTGAMA**GCGC**CACYTCATGG**AGAT**GATGYTGAMA**GCGC**CACYTCATGG**AGAGC
Construction mSTRE21R	GCTCTCCATGARGTGGCGCTKTCARCATCATCTCCATGARGTGGCGCTKTCARCATCATAGC



Supplementary Figure S2, Luciferase constructions with normal and mutant STRE elements (Related to Figure 1): upper part, presentation of the constructions encompassing WT and mutant variants of STRE1 and STRE2. Lower part, luciferase assays analyzing the constructions by transfections in JEG3 cells (related to Figure 1). Tested by Student T-tests compared to control conditions ** $p<0.01$.



Supplementary Figure S3, Visual evaluation of cell fusion in BeWo cells overexpressing either STOX1A or STOX1B (Related to Figure 9). P values were calculated by Student Neuman Keuls pos-hoc test after one-way ANOVA (pool of three independent experiments).

Supplementary Table S1: qPCR oligonucleotides used in the study. Related to Figure 1.

Oligo name	Sequence	Bases
ANXA1.f	GCG-GTG-AGC-CCC-TAT-CCT-A	19
ANXA1.r	TGA-TGG-TTG-CTT-CAT-CCA-CAC	21
ANXA2.f	TCT-ACT-GTT-CAC-GAA-ATC-CTG-TG	23
ANXA2.r	AGT-ATA-GGC-TTT-GAC-AGA-CCC-AT	23
BRWD1.f	CCA-GCG-CAT-CGG-TCC-TAT-G	19
BRWD1.r	CTT-CCT-GCA-CCA-AGT-AAA-GAA-GT	23
CAPN6.f	CAG-CAG-ACT-TTT-CTG-TGA-TCC-A	22
CAPN6.r	GGG-GAC-GTT-TCC-ACA-CCA-C	19
GPR146.f	GCA-AGG-CCA-GCA-TGA-CCA-T	19
GPR146.r	GGA-CAC-ATT-GAA-GGG-GAT-CTG	21
HMGN1.f	GCG-AAG-CCG-AAA-AAG-GCA-G	19
HMGN1.r	TCC-GCA-GGT-AAG-TCT-TCT-TTA-GT	23
ITIH5.f	CCT-ACT-GTA-GTA-CAA-CAA-GCC-AG	23
ITIH5.r	TCC-CCA-ATG-CTC-TGT-TCT-CTA-TT	23
PSMG1.f	TCC-TTT-CCT-GAG-AGC-CCT-AAA-A	22
PSMG1.r	TGT-TCT-AGC-AAT-GGA-CAA-CAC-G	22
SEMA6A.f	AAT-CAG-TAT-TTC-GCA-TGG-CAA-CT	23
SEMA6A.r	GCA-ATG-TAG-AGG-GTT-CCG-TTC-A	22
TGM2.f	CGT-GAC-CAA-CTA-CAA-CTC-GG	20
TGM2.r	CAT-CCA-CGA-CTC-CAC-CCA-G	19
WRB.f	TCC-ACA-GTC-AAC-ATG-ATG-GAC-G	22
WRB.r	CTG-TCC-GAG-CTT-TCA-CAT-GGG	21
ERVFRD-1_(Syncytin1.f)	ATG-GAG-CCC-AAG-ATG-CAG	18
ERVFRD-1_(Syncytin1.r)	AGA-TCG-TGG-GCT-AGC-AG	17
ERVW-1_(Syncytin2.f)	CCT-TCA-CTA-GCA-GCC-TAC-CG	20
ERVW-1_(Syncytin2.r)	GCT-GTC-CCT-GGT-GTT-TCA-GT	20
CGA.f	TGC-CCA-GAA-TGC-ACG-CTA-C	19
CGA.r	TTG-GAC-CTT-AGT-GGA-GTG-GGA	21
NOS3.f	TGA-TGG-CGA-AGC-GAG-TGA-AG	20
NOS3.r	ACT-CAT-CCA-TAC-ACA-GGA-CCC	21
CAV1.f	GCG-ACC-CTA-AAC-ACC-TCA-AC	20
CAV1.r	ATG-CCG-TCA-AAA-CTG-TGT-GTC	21

Transparent Methods

1. Human and animal material

Human placental samples previously obtained after Caesarean section outside of labour. Placentas were collected less than half an hour after delivery, two cotyledons were dissected and washed in sterile PBS after removal of the fetal membranes (GascoinLachambre et al., 2010). Several samples of villous trees were placed in Trizol™ prior to RNA extraction. The placentas were collected from four hospital maternity units (Cochin, St Antoine, Institut de Puériculture, Paris, and La Conception, Marseille, France). All protocols have been approved by the local Ethics Committee (No. CPP Am5724-1-COL2991; CODECOH No. DC-2012-1645). All patients have given their written consent for the use of their placenta. The mouse work (female mice, of course) was performed under the local regulations and ethic committees: Animal Care Committee of the Paris Descartes University (agreement no. 02731.02). Placentas were collected at 16.5 days post coïtum and placed in TriZol prior to RNA extraction (Collinot et al., 2018; Doridot et al., 2013).

2. Cell culture

BeWo cells were cultivated in F12 medium (Life Technologies) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin in 6-cm diameter plates, up to 60% confluence and were transfected by using Lipofectamin 2000 Reagent (Invitrogen) with 4 µg pCMX-STOX1-A, 4 µg pCMX-STOX1-B or 4 µg empty pCMX together with 0.4 µg PGK neo, following the provider's recommendations. Cells were passaged at 1:10 dilution into selective medium at 72 hr post-transfection. Selection was continuously applied with Geneticin (G-418) (Invitrogen) at 500 µg/ml concentration for approximately 3 weeks. Resistant clones were grown individually in continual selection and used for further analysis or frozen in DMSO. mRNA was prepared and the expression of STOX1A or B was assessed by qRT-PCR. Three cell lines were retained and called BeWoA, BeWoB and BeWoC (control cell line). A similar experiment was performed for generating an STOX1B-overexpressing cell from JEG-3 cells (grown in DMEM – 10% FBS – 1% penicillin/streptomycin), to isolate a clone overexpressing STOX1B ~3-fold (as evaluated by qRT-PCR), and called B10 (JEG-3B in this paper). All these cell lines, including AA6 (JEG-3A) and BD3 (JEG-3C) JEG-3 cells as well as other control and STOX1A-overexpressing cells were maintained in selective pressure in geneticin G-418 at 500 µg/ml (Rigourd et al., 2008). To evaluate cell fusion, BeWo A, B or C cells were seeded in µ-slide 8-well IbiTreat chamber slides (30'000 cells/well; Ibidi GmbH, Martinsried, Germany) 24 hr before treatment with 20 uM forskolin (in medium without G-418) for 48 hr. Cells were then washed with phosphate-buffered saline (PBS) twice, fixed with 4% paraformaldehyde (Aldrich, Steinheim, Germany) for 20 min and washed again with PBS three times. Fixed cells were permeabilized with PBS-0.2% Triton X100 (BioChemica Applichem, Darmstadt, Germany) for 10 min at room temperature and washed three times with PBS. Non-specific binding was blocked with PBS-3% bovine serum albumin (BSA, Albumin Fraction V, PanReac Applichem, Barcelona, Spain) for 30 min at room temperature. Cells were then incubated with mouse anti-γ-Catenin antibodies (1:200 dilution from ThermoFisher Scientific, Switzerland) diluted in PBS-3% BSA, overnight at 4°C. Cells were then washed with PBS three times and incubated with goat anti-mouse IgG Chromeo 642 (ab60318, dilution 1:500 from Abcam, Cambridge, UK) diluted in PBS-3% BSA, for 2 hr at room temperature. After three washes with PBS in the dark, cells were incubated with 300 nM DAPI solution (Panreac AppliChem, Barcelona, Spain) for 10 min at room temperature in the dark. Finally, cells were washed three times with PBS in the dark and images were acquired with an EVOS FL Cell Imaging System (ThermoFisher Scientific, Bothell, WA, USA). Images were processed by using ImageJ freeware. The fusion index expressed in percentage was calculated as follows: $[(N-S)/T]\times100$, where N equals the number of nuclei in syncytia, S the number of syncytia and T the total number of nuclei counted.

This index was calculated in three independent experiments, run in duplicate. xCELLIGENCE analysis was performed in triplicate with or without Forskolin with three starting concentrations of cells during 140 hr.
siRNA Knock-down of STOX1 was carried out on BeWo cells using the Mission® esiRNA (Sigma-Aldrich, ref HU-6582-1). Cells from two different experiments were cultivated in 12-well plates and the transfections were carried in 6-plicates, with control or siRNA (at 1nM final concentration), with or without forskolin (24µM final concentration). The siRNA was added to cells (50% confluency) on day1, the forskolin on day2, and the RNA were collected on day 5 in TriZol, using standard protocols.

3. Membrane repair assay

JEG-3 and BeWo cells were cultured in complete growth medium complemented with Geneticin at 500 µg/mL on µ-slide 8-well IbiTreat chamber slides (Biovalley). Cells were incubated for 5 min before acquisition with 5 µg/mL FM1-43 (Invitrogen) in D-PBS and maintained over ice. FM1-43 is a water-soluble dye that becomes fluorescent upon integration into lipid membranes but is unable to cross them. When the cell membrane is damaged, the molecule enters passively into the cytosol and incorporates into intracellular membranes, thus increasing the recorded fluorescence. To induce membrane damage, cells were irradiated at 820 nm with a tunable pulsed depletion laser Mai Tai HP (Spectra-Physics, Irvine, CA, USA) with a two-photon confocal scanning microscope (TCS SP5, Leica) equipped with an HCX PL APO CS 63.0 x 1.40 oil-objective lens. Irradiation consisted of 1 scan of a 1 x 1 µm area with power 110 (±5) mW. We acquired 512 x 512 images at 1.6-s intervals with pinhole set to 1 Airy unit. Membrane rupture and repair processes were monitored by measuring variations in fluorescence intensity of FM1-43. FM1-43 was excited by the 488-nm laser line (intensity set at 30% of maximal power) and fluorescence emission was measured between 520 and 650 nm. For each condition, at least 100 cells from three independent experiments were analyzed. For quantitative analysis, the fluorescence intensity was integrated over the whole cell surface and corrected for the fluorescence value recorded before irradiation by using ImageJ (Carmeille et al., 2017; Carmeille et al., 2015).

4. High-throughput studies

Microarray analysis on BeWo cell lines were performed with ClariomS human Microarrays (ThermoFisher Scientifics) at the Genom'IC platform of Cochin Institute (https://www.institutcochin.fr/core_facilities/genome-sequencing-studies?set_language=en). mRNAs were purified from the three cell lines treated or not with forskolin. The data were submitted to GEO Profiles under the accession number GSE148088, and analyzed using the Transcriptome Analysis Console from Affymetrix (Thermofisher ™). P value and FDR values were estimated and are accessible. The genes that are analyzed as modified in the present study have all a FDR-p value <0.05, and are presented as Supplementary Table 1.

5. Western blot and Dot-Blot analyses

Cells were trypsinized, pelleted, washed twice in PBS, and resuspended for 1 hr at 4°C in RIPA (5 mM TrisHCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) with a cocktail of protease inhibitors (100X Thermofisher), DTT 50 mM 1%, PMSF 50 mM 1%. Then, after centrifugation (20000 g, 4°C), the supernatant was kept at -70°C after quantification by absorbance evaluation at 280 nm, against a BSA reference scale. Oxidized proteins were measured by using the Oxyblot kit (Merck) following the manufacturer's advice. For eNOS and CAV1, 30 µg denatured proteins (in Nupage LDS sample buffer, Invitrogen, heated 5 min at 100°C) were loaded on 12% (CAV1), 8% (eNOS) or 10% (oxidized proteins) acrylamide-bis acrylamide denaturing gels. Runs were performed at 130 volts for 2 hr in Tris-

Glycine, 2% SDS buffer, at room temperature, then proteins were transferred to nylon or ethanol-activated PVDF membranes at 70 volts in Tris-Glycine buffer at 4°C for 2 hr. The transfer was evaluated by Ponceau red visualization. Membranes were blocked in PBS 1X-Tween 0.1% and 5% defatted milk (Regilait) for 1 hr, then rinsed thrice in PBS 1X-Tween 0.1%. Dot blots were prepared on a grid with serial ½ PBS dilutions by spotting protein extracts on a nitrocellulose gridded membrane.

Antibodies were used at 1 µg/ml (1/1000) for eNOS (BDscience) and 1/500 for caveolin 1 (PA1-064, Thermofisher), and those provided for the Oxyblot kit (Merck, diluted 1/500 in PBS 1%, Tween 0.1% 20% and 5% BSA, 2 µg/ml = 1/500) for nitrated proteins on dot blots (A-21285, Thermofisher). Following incubations and washings, horseradish peroxidase-coupled secondary antibodies were incubated, and signals were revealed by autoradiography, followed by Scion Image or ImageJ analyses for quantification. In the paper, blots were cropped and reorganized for consistency.

6. Quantitative RT-PCR

RNA preparation was carried out in cells by direct lysis in Trizol. For tissues, a metal bead was added, and the tissue was homogenized using violent back and forth agitation (1 min, 30 Hz). Chloroform (1/5 of the Trizol volume) was then added to the tube. After centrifugation at 5000g the upper phase (aqueous) was collected in fresh tubes, precipitated with isopropanol; the pellet was resuspended in 100 µl of water, and reprecipitated using 250µl of a NaAc-Etoh mix (300 mM NaAc final). Reverse transcription was carried out using the MMLTV reverse transcriptase kit of Invitrogen (Thermofisher). By using the geometric average of the SDHA and Cyclophilin Cts as a calibrator and the Sybergreen SYBR Hi-ROX qPCR kit from Bioline. cDNA quantity was estimated by the $2^{-\Delta\Delta c\tau}$ method (Livak and Schmittgen, 2001). Primers are given as supplementary Table 1.

7. PCR-selection and EMSA

PCR selection was performed by incubating 50 µg protein extracts from transfected cells in RIPA (25 mM NaCl, 10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 0.1% NP40 with 1X Sigma protein inhibitor cocktail) with 5.5 µg PoldI-dC and 3 ng of 76-bp oligonucleotides including a central random sequence of 26 bp in 50 µl distilled water. In parallel, 1.5 mg G protein-Dynabeads (Life technologies) were washed twice in PBS (400 µl), after incubation and coupling with 1 µg of the anti-Flag M2 antibody (the flag being the nucleotide sequence encoding the peptide DYKDDDDK). The two were mixed and incubated for 20 min. with agitation, rinsed three times in PBS (400 µl), then beads were resuspended in 30 µl, denatured for 10 min at 100°C and the supernatant was amplified by PCR by using the primers framing the 26 random base pairs. After 3% agarose gel purification of 76-bp bands, the procedure was performed again five times. A control experiment was carried out in parallel with non-flagged STOX1B. The proteins were purified after transfection and STOX1 complexes were enriched by using the anti-flag M2 antibody coupled with magnetic beads before PCR amplification. After cloning and sequencing, analysis was preformed using MEME(Bailey et al., 2009) (http://meme.ncbr.net)Then the products were cloned in TOPO-TA, colonies were isolated after bacteria transformation, and DNA was miniprepped and sequenced before searching for common motifs using MEME (http://meme-suite.org/tools/meme).

EMSA involved using the double-stranded biotinylated probes 3X STRE1: biotin-AGAGC**CATYTCACGG**AGAGC**CATYTCACGG**AGAGC**CATYTCACGG**AGAGC-biotin, 3XSTRE2: biotin-GCTAT**GGTGYGAMA**GCTAT**GGTGYGAMA**GCTAT**GGTGYGAMA**GCTAT-biotin and the ANXA1-like probe in Figure 7C. The competitors were obtained by using the same

sequences but non-biotinylated for the specific competitors and a mutated double-strand STRE1 (3XmutSTRE1):
AGAGC**CACYTCATGG**AGAGC**CACYTCATGG**AGAGC**CACYTCATGG**AGAGC.
The experiments were performed with an EMSA kit and the Chemiluminescent Nucleic Acid Detection Module of Thermo Scientific, following the provider's advice.

8. Cloning of fragments in luciferase vectors and luciferase assays

Relevant DNA fragments (HMGN1 promoter, STRE1 and STRE2 polymers) were synthesized with linkers or amplified with linkers by using KAPA hifi Polymerase (KAPA Biosystems, Boston) and cloned in pGL3 basic in the HindIII restriction site. HMGN1 promoter primers were cgataagcttCGCTCACCTTCCTCTTGGGCAT and cgataagcttAGGAAGGAAGGAAGTTACACAGA (amplification product 831 bp, from 788 bp 5' of HMGN1), cgataagcttTATCTCCATCCCTGCCACTTTAA and cgataagcttCTCGCTTTACTTACAGCTGACAA (amplification product of 2192 bp, from 1661 bp 5' of HMGN1). The plasmids were transfected in JEG-3 cells in 24-well plates by using a combination of the pCMX plasmids overexpressing STOX1A, STOX1B, or the empty pCMX (400 ng), 10 ng Renilla vector and 590 ng promoter-reporter vector by using Lipofectamin 2000 (Thermo Fisher Scientific, Montigny-Le-Bretonneux, France), following the manufacturer's advice. After 48 hr, the proteins were collected in 100 µL Passive Lysis Buffer, and the Renilla and Luciferase activity were measured at 20 µl with an automated plate reader for each sample, to calculate the ratio of fluorescence and normalize the luciferase activity with renilla activity. The inductions were normalized again relative to the level of modification of pcDNA3. For in vitro methylation of cloned promoters such as HMGN1 the plasmid was treated with the CpG Methyltransferase M.SssI (New England Biolabs) following the manufacturer's protocol.

9. Chromatin immunoprecipitation

ChIP involved JEG-3 cells transfected with pCMX-6flag-STOX1A, pCMX-4flag-STOX1B or empty pCMX as described previously. Between 10^6 to 3.10^6 cells were used at 24 hr post-transfection, rinsed in PBS, and cross linked with 1% paraformaldehyde (Sigma-Aldrich), 10 min at room temperature. Then the crosslink was interrupted by incubation of glycine 125 mmol/L for 5 min at room temperature. The cells were then scraped in PBS, rinsed twice in PBS by centrifugation at 1000g at 4°C. The pellets were used or dropped in liquid nitrogen and stored at -80°C. Nuclei were recovered after resuspension in 50 mmol/L HEPES/KOH, pH 7.5, 0,14 mol/L NaCl, 5 mmol/L EDTA, pH 7.5, 0.1% NaDeoxycholate) with a SIGMA proteases inhibitor cocktail with PMSF 1 mmol/L and Aprotinine 50 µg/mL (Euromedex). The samples were centrifuged at 20000g (4°C, 10 min) and the supernatant was eliminated. The nuclei-enriched pellet was resuspended in the same buffer with 1% Triton X-100, with passages through a G26 syringe, then agitated for 30 min at 4°C. Sonication involved using a Bioruptor Pico (Diagenode) for 15-40 cycles for 30 s ON – 30 s OFF. To obtain fragments ranging from 150 to 300 bp. q-PCR was performed on the immunoprecipitate and supernatant with the primers WNT2Prom1, CAGCAAACCCATGGAGTTCT, and WNT2BProm2 CCCTCCATCTCAGCATCAGT; F3Prom1, TGAGGGTCAGTTGG, and F3Prom2 CACAGAGCTGCAGATGTCAC; HMGN1Prom1, CTTAATTGATCCCGGACCCC, and HMGNProm2, CGGCTTCAAACTACCGTGA. Two microsatellites were controls (D7S820 and D8S1179), with average qPCR Ct used as a calibrator.

10. Statistics

In the different experiment, statistics were based on parametric tests, mostly ANOVA followed by post-hoc Student Neuman-Keuls tests using the StatistiXL add-in of Excel.

References:

GascoinLachambre, G., Buffat, C., Rebourcet, R., Chelbi, S.T., Rigourd, V., Mondon, F., Mignot, T.M., Legras, E., Simeoni, U., Vaiman, D., et al., 2010. Cullins in human intra-uterine growth restriction: expressional and epigenetic alterations. Placenta 31, 151–157.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25, 402–408.