Supporting Information

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SI Methods

Microarray Analyses. RNA was extracted from placentas or brain punches using TRIzol reagent (Invitrogen) and sample purity was assessed by an Agilent Bioanalyzer 2100. Two hundred fifty micrograms of total RNA was converted to first-strand cDNA using SuperScript II reverse transcriptase primed by a poly(T) oligomer that incorporated the T7 promoter. Second-strand cDNA synthesis was followed by in vitro transcription for linear amplification of each transcript and incorporation of biotinylated CTP and UTP. The cRNA products were fragmented to 200 nucleotides or less, heated at 99 °C for 5 min, and hybridized for 16 h at 45 °C to Affymetrix GeneChip Mouse Gene 1.0 ST Array microarrays. Microarrays were then washed at low (6× SSPE buffer, Affymetrix) and high (100 mM MES and 0.1 M NaCl) stringency and stained with streptavidin-phycoerythrin. Fluorescence was amplified by adding biotinylated anti-streptavidin and an additional aliquot of streptavidin–phycoerythrin stain. A confocal scanner was used to collect fluorescence signal at 3 μm resolution after excitation at 570 nm. The average signal from two sequential scans was calculated for each microarray feature. CEL files were generated using Affymetrix Microarray Suite 5.0, and subsequent analyses were performed using the affy (38) and limma (39) packages for R (version 2.14.2). Expression values were computed using the Robust Multiarray Averaging method (40), and linear models including gestational time points and embryo sex were fit to the data. Empirical Bayesian methods to shrink the probe-wise sample variances toward a common value and to augment the degrees of freedom for the individual differences were used, and genes were characterized as having differential expression between groups using a FDR (41) of 0.05 for placental analyses or 0.01 for hypothalamic analyses.

Taqman Quantitative RT-PCR Analyses. cDNA was synthesized from 4 μg of total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). mRNA levels were quantified by quantitative RT-PCR using Taqman probe Mm00507317_m1 (mouse samples; Invitrogen) and Hs00269228_m1 (human samples; Invitrogen) for Ogt and SYBR Green (Invitrogen) for all other transcripts, with primer sequences found in Table S3. Samples were run in technical triplicates, and calculated relative mRNA levels were normalized to β-actin mRNA levels [Taqman probe 4352341E (mouse) and Hs99999903_m1 (human) from Invitrogen for Ogt normalization and primers in Table S3 for all other transcript normalization] in the same samples. These relative values were then normalized to the average of male controls for rodent studies and to XY levels for human tissue. The final normalized values were used for statistical analyses.

Biochemical Analyses. Placental tissue was pestle-homogenized in RIPA buffer [150 mM NaCl, 0.1% (vol/vol) Triton X-100, 0.5% (wt/vol) sodium deoxycholate, 0.1% (wt/vol) SDS, and 50 mM Tris·HCl (pH 8.0)] with protease and phosphatase inhibitors (P0044 and P8340; Sigma) and kept under constant agitation for 2 h at 4 °C. Samples were then centrifuged at 13,300 × *g* at 4 °C for 20 min and the resulting supernatant was used for Western blot analysis. Protein concentration for each placental lysate was quantified using the Pierce BCA Protein Assay Kit (23227), and 30 μg of total protein for each sample was loaded into a 10% Bis-Tris gel (NP0301; Invitrogen) in MOPS SDS Running Buffer (NP0001; Invitrogen). Protein was then transferred to a nitrocellulose membrane (LC2000; Invitrogen) in transfer buffer (NP0006-1; Invitrogen) with 20% (vol/vol) methanol. Membranes were blocked in 5% (wt/vol) BSA in TBS, pH 7.4 (20 mM Tris and 150 mM NaCl) for 1 h at 23 °C and incubated with primary antibody (1:1,000) in 5% (wt/vol) BSA in TBS-Tween20 (TBST, 0.2%) at 4 °C overnight. Membranes were washed five times for 5 min in TBST and then incubated in secondary antibody (1:10,000) in 5% (wt/vol) BSA in TBST for 1 h at 23 °C; membranes were washed again five times for 5 min in TBST and one time for 5 min in TBS and then exposed to Western Lightning Plus-ECL (NEL104001EA ; PerkinElmer) to visualize protein quantities. All blots were then stripped of antibodies with two 5-min washes with a stripping buffer [1.5% (wt/vol) glycine, 0.1% (wt/vol) SDS, and 1% (vol/vol) Tween (pH 2.2)], two 10-min washes with PBS, and two 5-min washes with TBST and then reprobed for β-actin following the procedures as above. Antibodies were as follows: Ogt (ab96718; Abcam), O-GlcNAc (MMS-248R; Covance), P-threonine (9381s; Cell Signaling), P-MAPK/CDK substrate (2325; Cell Signaling), β-actin (04-1116; Millipore), anti-mouse HRP linked (NA931; GE Healthcare), and anti-rabbit HRP linked (NA934; GE Healthcare). Films were scanned and pixel density of each band or lane was quantified using ImageJ v1.44P. Optical densities were normalized to β-actin for each sample and then across groups to the male control and these normalized values were used for statistical analyses.

Chromatin Immunoprecipitation Analyses. Placental tissue was pestle-homogenized in ice-cold PBS + protease inhibitors and centrifuged at 13,300 × *g* for 10 s. The resulting tissue pellet was resuspended in a 1.11% (vol/vol) formaldehyde/PBS solution and under constant agitation at 23 °C for 10 min to cross-link the chromatin structure. Cross-linking was terminated by adding glycine to a final concentration of 0.14 M. Cross-linked tissue was washed in ice-cold PBS, and the resulting pellet was Dounce-homogenized in ChIP Cell Lysis buffer [10 mM Tris·HCl (pH 8.0), 10 mM NaCl, 3 mM $MgCl_2$, and 1% (vol/vol) Nonidet P-40]. Isolated nuclei were disrupted in ChIP Nuclear Lysis buffer [50 mM Tris·HCl (pH 8.0), 5 mM EDTA, and 1% SDS] and chromatin was sheared in a bath sonicator (BioRuptor UCD-200; Diagenode) for five 5-min cycles of 30 s on high setting and 30 s off. Ice was replenished between each 5-min cycle to maintain 4 °C in the bath. The resulting chromatin was stored at −80C. For immunoprecipitation, 10 μg of chromatin was diluted in 1 mL of ChIP dilution buffer [16.7 mM Tris·HCl (pH 8.1), 167 mM NaCl, 0.01% (wt/vol) SDS, and 1.1% (vol/vol) Triton-X 100] and incubated with 2 μg of an H3K4me3 antibody (39159; Active Motif) at 4 °C overnight. One hundred microliters of blocked protein A beads (Dynabeads, 100-02D; Invitrogen) was added to each sample and incubated at 4 °C for 1 h. Conjugated beads and chromatin were washed one time each in the following buffers: Tris-salt-EDTA (TSE) I [20 mM Tris·HCl (pH 8.0), 150 mM NaCl, 2 mM EDTA, 0.1% (wt/vol) SDS, and 1% (wt/vol) Triton-X 100], TSE II [20 mM Tris·HCl (pH 8.0), 500 mM NaCl, 2 mM EDTA, 0.1% (wt/vol) SDS, and 1% (vol/vol) Triton X-100]), ChIP buffer III [10 mM Tris·HCl (pH 8.0), 0.25M LiCl, 1 mM EDTA, 1% (vol/vol) Nonidet P-40, and 1% (wt/vol) deoxycholate], and Tris-EDTA (TE) [10 mM Tris·HCl (pH 8.0) and 1 mM EDTA], and chromatin was eluted in 1% (wt/vol) SDS and 0.1 M $NaHCO_3$. Cross-links were reversed by adding NaCl to a final concentration of 192 mM and incubated at 65 °C overnight. Proteins were digested at 45 °C for 1 h using 10 μg of proteinase K and adding Tris·HCl (pH 7.5) and EDTA to final concentrations of 36 mM and 9 mM, respectively. DNA was purified using a standard phenol extraction and ethanol precipitation; DNA pellet was eluted in

20 μL of TE buffer. One hundred nanograms of immunoprecipitated and input (nonprecipitated) DNA were quantified for levels of the Ogt promoter region, 188 bp upstream of the transcription start site, using quantitative RT-PCR with SYBR green; the primer sequences used are in Table S3. Levels of the amplified sequence for each sample were normalized to the amount of input DNA to account for interassay variations in starting material and extraction efficiencies, and this value was used for statistical analyses.

Proteomics Identification of O-GlcNAcylated Proteins. Placental lysates were prepared and underwent SDS/PAGE in the same manner as for Western blot analysis. Gels were stained using a colloidal Coomassie blue stain (SimplyBlue SafeStain; Invitrogen), and the bands corresponding to those identified as differential immunoblotting for O-GlcNAcylation in male placentas (~28 and 37 kDa in size) were excised. The proteomics core at the University of Pennsylvania performed in-gel trypsin digestion and nano-liquid chromatography (LC)-MS/MS protein identification. Identified peptide sequences were analyzed using Scaffold v3 software and determined which proteins were present. These identified proteins were then cross-checked to known targets of OGT for O-GlcNAcylation using dbOGAP (29). The resulting list represented the most likely candidates responsible for the differential O-GlcNAcylation observed.

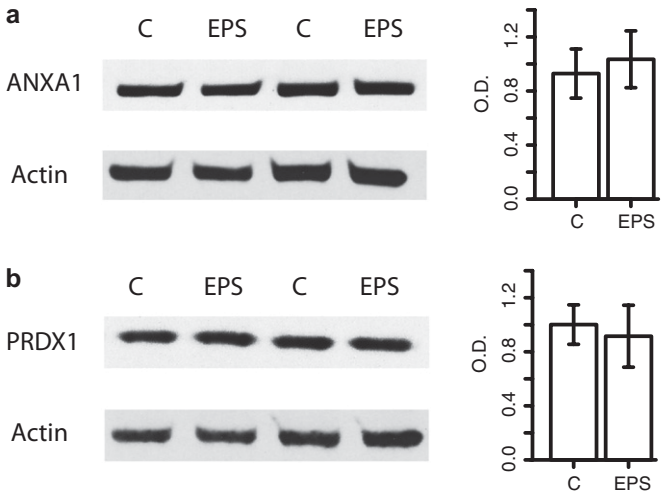

Fig. S1. Total annexin A1 (ANXA1) and peroxiredoxin 1 (PRDX1) protein levels are not affected by early prenatal stress (EPS). (*A*) Representative Western blot images of total ANXA1 from male control and EPS mouse placentas. Histogram is the maximum likelihood estimate for the normalized optical densities of each group (control, $n = 7$; EPS, $n = 8$) ± the 95% confidence interval for that estimate. (*B*) Representative Western blot image of PRDX1 levels from both male control and EPS mouse placentas. Histogram is derived as in *A*. Unlike the apparent reduction in O-GlcNAcylated ANXA1 and PRDX1, total protein levels for these proteins were not affected in male placentas by EPS.

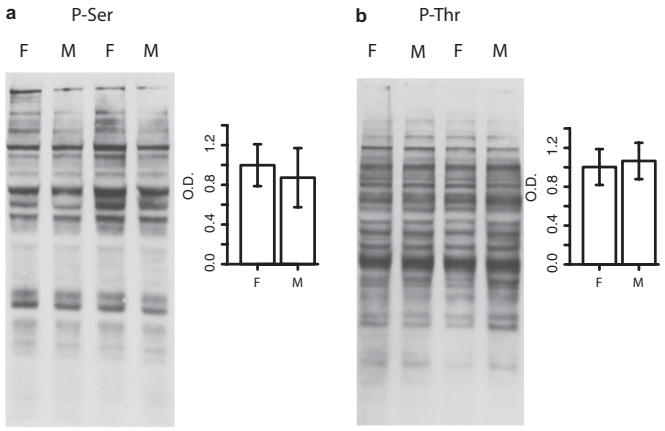

Fig. S2. Total phosphorylation patterns in mouse placentas do not seem sex-biased. (*A*) Representative Western blot images of total phosphoserine (P-Ser) levels from male and female mouse placentas. Histogram is the maximum likelihood estimate for the normalized optical densities of each group ($n = 8$) ± the 95% confidence interval for that estimate. (*B*) Representative Western blot images of total phosphothreonine (P-Thr) levels from male and female mouse placentas. Histogram is derived as in *A*. M, male; F, female.

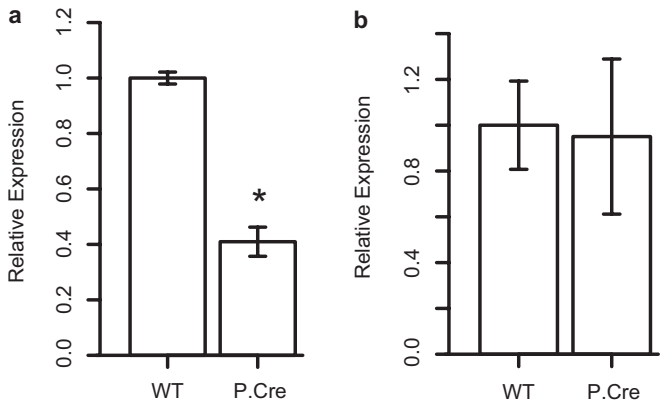

Fig. S3. Hemizygous expression of O-linked-*N*-acetylglucosamine transferase (OGT) is specific to placental tissue in a cross between Cyp19-Cre × X^{Ogt}/ X^{WT} mice. (*A*) OGT gene expression in the embryonic day 12.5 (E12.5) placenta associated with female (X^{Ogt}/ X^{WT}) embryos with or without placental Cre (P.Cre) expression [$P.Cre^{+/-}$ (n = 5) or $P.Cre^{-/-}$ (n = 6)]. Data were normalized to $P.Cre^{-/-}$ levels. Bars are the maximum likelihood estimate for each group ± the 95% confidence interval for that estimate. Asterisk indicates a main effect with a confidence interval that does not bound zero as determined by the linear model ($y \sim$ P.Cre). (*B*) OGT gene expression in the E12.5 liver tissue from the embryos used in *A*; data were normalized and analyzed as in *A*. WT, wild-type or no P.Cre expression.

Table S1. Genes with sex-biased placental expression

Gene ID	E12.5	E15.5	E18.5	Protein function
Ddx3y	M	M	M	RNA helicase
Uty	M	M	M	H3K27me3 demethylase
Eif2s3y	M	M	M	Initiation of translation
Kdm5d	M	M	M	H3K4me3 demethylase
Ogt	F	F	F	Glycotransferase
Eif2s3x	F	F	F	Initiation of translation
Kdm5c	F	F	F	H3k4me3 demethylase
Xist	F	F	F	X inactivation
Rpsa	F	—	—	Ribosomal protein
Pramel3	F	—	—	Unknown
Taf1	—	—	F	Initiation of transcription

F, gene expressed at higher levels in females; M, gene expressed at higher levels in males; —, gene expressed at comparable levels in males and females. Ddx3y: DEAD box polypeptide 3, Y-linked; Uty: ubiquitously transcribed tetratricopeptide repeat containing, Y-linked; Eif2s3y: eukaryotic translation initiation factor 2, subunit 3, structural gene Y-linked; Kdm5d: lysine-specific demethylase 5d; Eif2s3x: eukaryotic translation initiation factor 2, subunit 3, structural gene X-linked; Kdm5c: lysine-specific demethylase 5c; Xist: X-inactive specific transcript; Rpsa: ribosomal protein SA; Pramel3: preferentially expressed antigen in melanoma-like 3; Taf1: TATA box binding protein-associated factor 1; H3K27me3: histone H3 trimethyl lysine 27; H3K4me3: histone H3 trimethyl lysine 4.

Table S2. Peptides used for identification of Anxa1 and Prdx1

Sequence	Peptide identification probability, %	SEQUEST XCorr	SEQUEST deltaCn	Observed *m/z*	Z
(K) AAYLqEnGKPLDEVLR (K)	95	3.4553766	0.31300986	606.9937	3
(K) AAYLQENGKPLDEVLRK (A)	95	3.7940016	0.40932816	648.9756	3
(K) cATSTPAFFAEK (L)	95	3.1046205	0.36186966	666.0105	2
(K) cLTTIVK (C)	95	1.4342005	0.25853837	834.4546	1
(K) DITSDTSGDFR (K)	95	2.9742296	0.5770407	607.2842	2
(K) GDRcqDLSVNQDLADTDAR (A)	95	5.695049	6.76E-04	717.3694	3
(K) GGPGSAVSPYPSFnVSSDVAALHK (A)	95	4.4263906	0.52306485	782.7949	3
(K) GLGTDEDTLIEILTTR (S)	95	5.5465636	0.4826124	874.1169	2
(K) GTDVNVFTTILTSR (S)	95	4.9189854	0.54137176	762.9042	2
(K) GVDEATIIDILTK (R)	95	4.3583965	0.36278012	694.5239	2
(K) GVDEATIIDILTKR (T)	95	4.541132	0.47720218	772.6362	2
(K) ILVALcGGN (-)	95	2.006329	0.45245683	916.501	1
(K) TPAQFDADELR (G)	95	3.9030404	0.4087423	631.9116	2
(K) TPAQFDADELRGAmK (G)	95	4.401271	0.5122945	833.4669	2
(K) YGISLcqAILDETKGDYEK (I)	95	3.8477437	0.40719873	735.5937	3
(R) cQDLSVNqDLADTDAR (A)	95	6.101489	0.002838877	911.2661	2
(R) DLAKDITSDTSGDFR (K)	95	4.6686	0.57407	821.3329	2
(R) DLAKDITSDTSGDFRK (A)	95	4.8404903	0.49856132	590.1405	3
(R) FLEnQEqEYVQAVK (S)	95	5.689539	0.030226573	864.2731	2
(R) GAmKGLGTDEDTLIEILTTR (S)	95	2.9794672	0.299936	717.7614	3
(R) KGTDVNVFTTILTSR (S)	95	3.951606	0.5534965	826.8051	2
(R) RKGTDVNVFTTILTSR (S)	95	4.1287613	0.44915113	603.5828	3
(R) RVFQNYGK (Y)	95	2.9273252	0.2541856	506.6298	2
(R) SNEQIREINR (V)	95	2.517658	0.16831058	630.1634	2
(R) VFQNYGK (Y)	95	2.2905068	0.20341961	428.5896	2

SEQUEST XCorr, cross-correlation score; SEQUEST deltaCn, cross-correlation difference to top ranked peptide; *m/z*, mass-to-charge ratio; Z, charge.

Table S3. Primer sequences used for quantitative real-time PCR analyses

Primer ID	Sequence (5′ to 3′)
Ddx3y-F	GGTCTGGAAAAACTGCTGC
Ddx3y-R	TTGGTGGCATTGTGTCCTGC
Uty-F	ATATCTGTGTTAATGCAAAGAAG
Uty-R	ATGGTCCACATTGTCGATAGT
Eif2s3y-F	GGTGCTGTTGGAGCATTACC
Eif2s3y-R	TCAACTCGTCGGCTTAGAGC
Kdm5d-F	CTCTCGTGGGGATGAAGTCGATA
Kdm5d-R	AAGTATACTCCTGTGTAGCCTG
Eif2s3x-F	TGGAATTTCTTTTACTAGCCTAGGGGT
Eif2s3x-R	TTTGTTCTGTTGTTGGTGTGCTACTT
Taf1-F	AGCATGTGGTGCCATCGGGC
Taf1-R	CGGCGCCGGTGGATGGATTT
Xist-F	TTGCGGGGTTGTGCTAGCCG
Xist-R	CGGGCAAAGCCCGCCAAGTA
Eif2s3x-F	GGTCAGCCCCATCTTTCCCGA
Eif2s3x-R	ACAGAAGGGCCGCATCCATCAC
Rpsa-F	CCTGAGGTGGCCGACTGGTCT
Rpsa-R	GGTACAGTCTGCAGTGAGGACCA
Kdm5c-F	AACCTGCCGCGACTGGGACTTA
Kdm5c-R	GCGGCGGTAGGAAATCGTCGG
β-actin-F	ACCCACACTGTGCCCATCTA
β-actin-R	ATCGGAACCGCTCGTTGC
Ogt_Promoter-F	TCGCAATGGACCTTGGGCGG
Ogt_Promoter-R	GAAATGGCGGCGAGGGCTCC

Primer IDs are defined as gene_name-direction or genomic_location-direction in the case of Ogt_Promoter. F, 5′–3′; R, 3′–5′.

Other Supporting Information Files

Dataset S1 (XLSX)
Dataset S2 (XLSX)