Supplementary information for Cabral-Teixeira et al. "Glucocorticoids control early fate specification".

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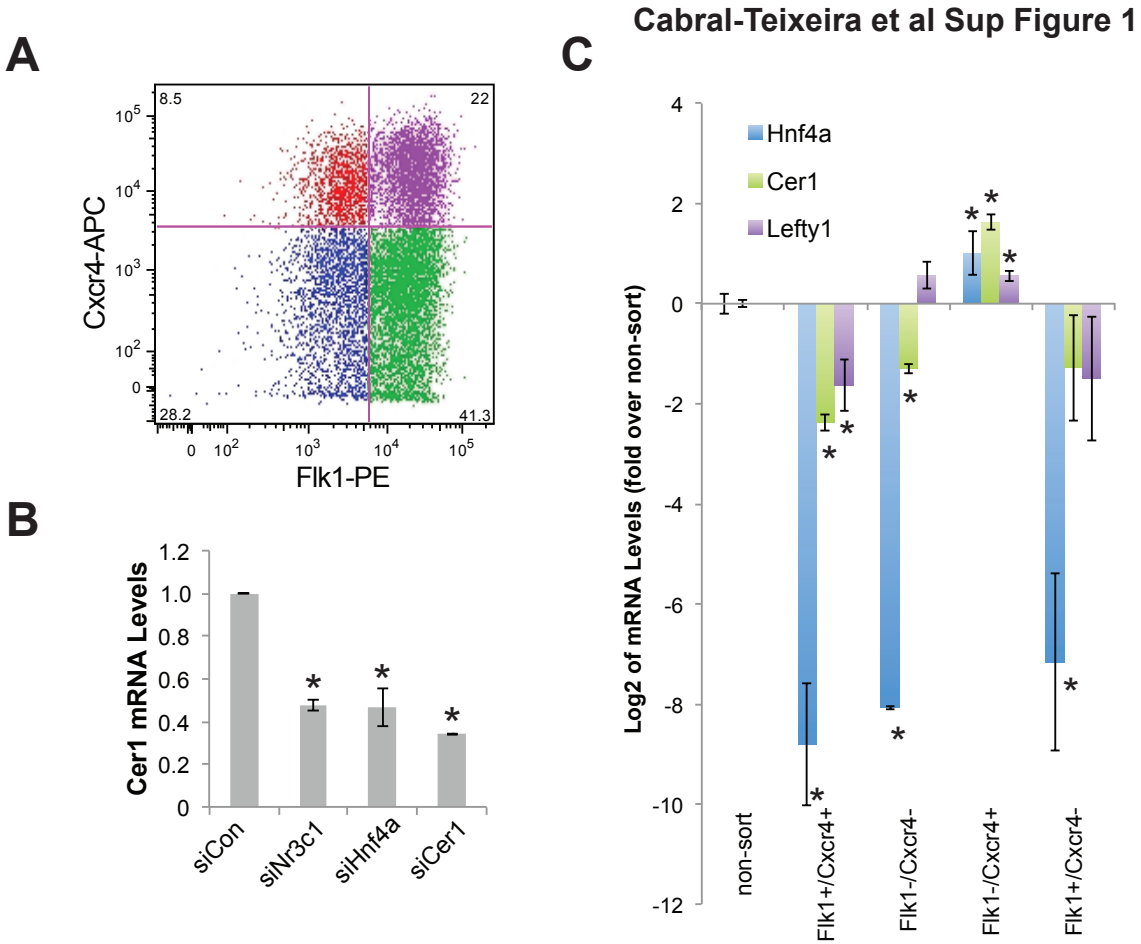
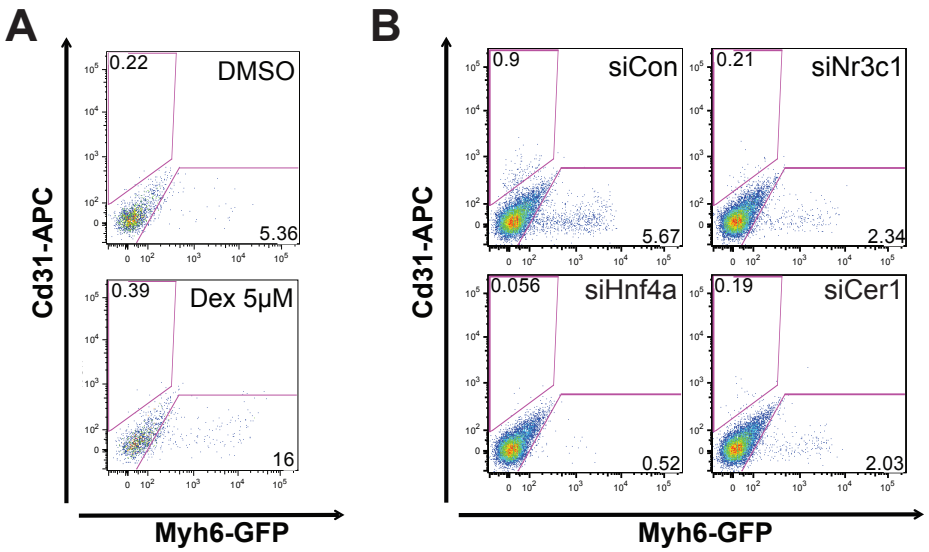
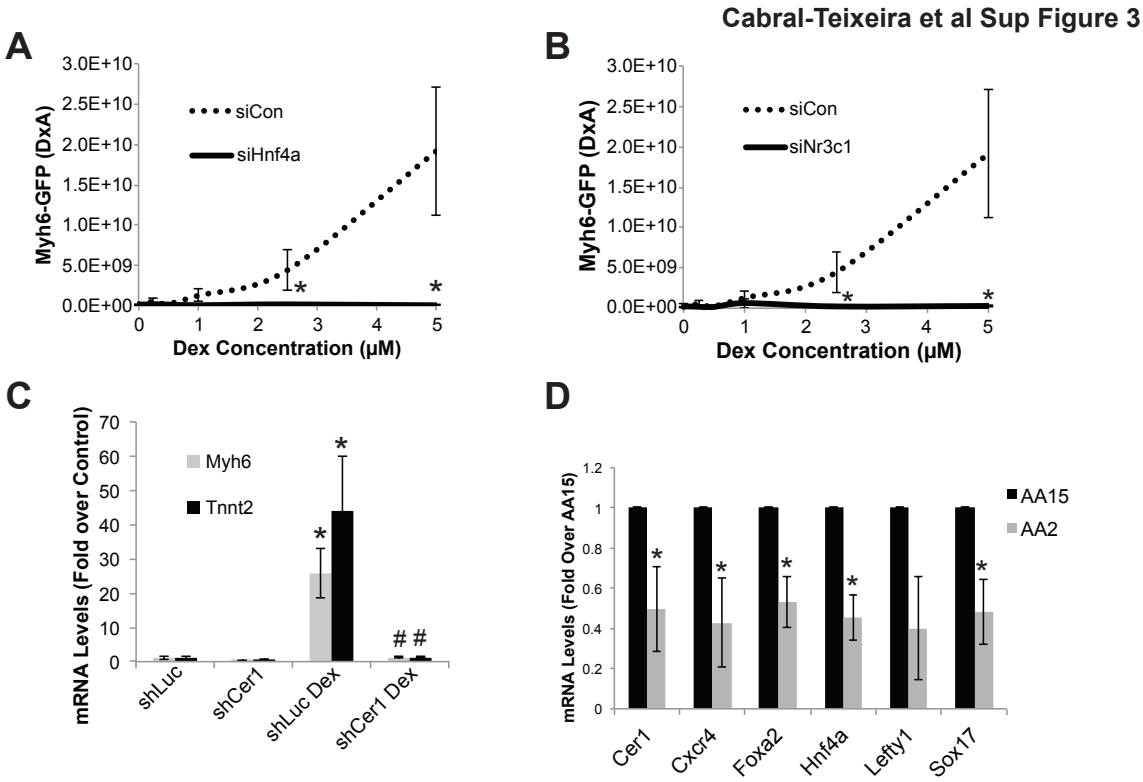


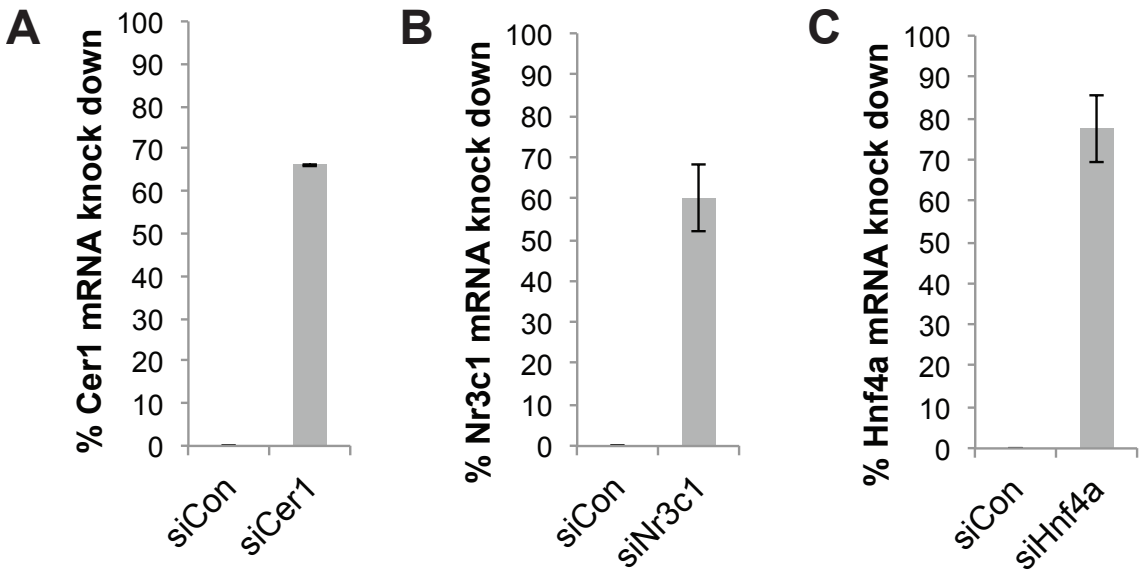
Supplementary Figure 1 (related to Figure 1G and 2E,G,H): Gcrt regulates Cer1 expression in the anterior visceral endoderm. (A) Gate selection for sorting populations of mesoderm ($Flk1^{+}/Cxcr4^{-}$), endoderm ($Flk1^{-}/Cxcr4^{+}$), ectoderm ($Flk1^{-}/Cxcr4^{-}$) and cardiogenic mesoderm ($Flk1^{+}/Cxcr4^{+}$) from day 5 ESC cultures differentiated in serum. **(B)** RT-qPCR analysis of *Cer1* expression on day 5 of serum differentiated ESC cultures, after transfection of siRNAs at day 3. Asterisk indicates $p<0.05$ relative to siControl treated cells. **(C)** RT-qPCR analysis of *Hnf4a*, *Cer1* and *Lefty1* expression in FACS sorted differentiation d5 populations as described in panel A. Asterisk indicates $p<0.05$ relative to unsorted cells.



Supplementary Figure 2 (related to Figure 1J,K and 3B): Gcrt signaling affects absolute numbers of cardiomyocytes and endothelial cells in differentiating ESC cultures. (A-B) Representative flow cytometry scatter plots for *Myh6*-GFP and Cd31 in day 15 cultures treated with Dex or siRNA respectively.



Supplementary Figure 3 (related to Figure 3): Dexamethasone regulates cardiac fate via Cer1. (A-B) Dexamethasone (Dex) dose response in a serum-containing mESC differentiation assay, using image analysis of *Myh6*-GFP to quantify cardiogenesis. Dex and indicated siRNAs were introduced at day 3. Asterisk indicates $p<0.05$ relative to corresponding siControl treated cells. **(C)** RT-qPCR analysis of the cardiac genes *Myh6* and *Tnnt2* in differentiated shRNA ESC lines for luciferase and *Cer1*, in absence or presence of 5μM Dex. Asterisk indicates $p<0.05$ relative to shLuc treated cells. # indicates $p<0.05$ relative to shLuc and Dex treated cells. **(D)** RT-qPCR analysis of day 4 cells that were transfected or treated as shown in Figure 2I,J. Asterisk indicates $p<0.05$ relative to 15ng/ml Activin A (AA15) treated cells.



Supplementary Figure 4 (related to Figure 2E and 3): Knockdown efficiency of siRNAs used. (A-C) RT-qPCR analysis of the indicated mRNA in day 5 differentiated mESC and 48hrs after transfection with a selective siRNA for Cer1 (A), Nr3c1 (B) and Hnf4a (C).

Gene name	Species	Accession #	Marker for	RTPD ID #
Actb	Mm	NM_007393	Reference	3574
T/Brach	Mm	NM_009309	Mesoderm	3633
Gsc	Mm	NM_010351	Mesendoderm	3636
Gata4	Mm	NM_008092	Cardiac mesoderm	8758
Sox17	Mm	NM_011441	Definitive endoderm	3638
Foxa2	Mm	NM_010446	Definitive endoderm	3727
Myh6	Mm	NM_010856	Cardiomyocyte	8281
Cdh5	Mm	NM_009868	Endothelium	8282
Acta2	Mm	NM_007392	Smooth muscle	8283
Cer1	Mm	NM_009887	Anterior visceral endoderm	3642
Hhex	Mm	NM_008245	Anterior visceral endoderm	3641
Sfrp1	Mm	NM_013834	Anterior visceral endoderm	3409
Nr3c1	Mm	NM_008173	Nuclear receptor	8657
Cd31	Mm	NM_008816	Endothelium	8288
Runx1	Mm	NM_009821	Blood	8659
Cxcr4	Mm	NM_009911	Definitive endoderm	8668
Lefty1	Mm	NM_010094	Visceral endoderm	3643
Hnf4a	Mm	NM_008261	Visceral endoderm	8660
Tspan7	Mm	NM_019634	Definitive endoderm	8661
Bmp6	Mm	NM_007556	Visceral endoderm	8662
Emp2	Mm	NM_007929	Visceral endoderm	8663
Rhox5	Mm	NM_008818	Visceral endoderm	8664
Tnnt2	Mm	NM_011619	Cardiomyocytes	8658
Evx1	Mm	NM_007966	Posterior mesoderm	3635

Supplementary Table 1: RT-qPCR primers used. Overview of genes, accession numbers, marker specificity and RTprimerDB ID numbers for the primers used.

Supplementary Materials and Methods

Mouse ESC culture and differentiation

CGR8 mouse ESC were maintained on gelatin-coated dishes in presence of 1000U/ml of Leukemia inhibitory factor (LIF, Millipore). For differentiation in presence of serum, cells were then plated in black 384 well µClear plates (Greiner) to allow differentiation in DMEM with high glucose containing 10% FBS (GIBCO) as described previously (Willems et al., 2012). Dexamethasone (Sigma) or Etomidate (Sigma) treatment was performed at day 3 or as indicated. siRNAs were forward transfected at day 3 of differentiation using Lipofectamine RNAiMax (Life Technologies). siRNAs used were all pre-validated (Ambion) and tested for efficiency before use: Cyp21a1 (s64681), Cyp17a1 (s64664), Cer1 (s63883), Hnf4a (s67633) and Nr3c1 (s67065).

Serum-free differentiation was performed through an embryoid body (EB) step. Cells were seeded at 50000/ml in a non-tissue culture treated 10 cm dish and were allowed to aggregate in a serum-free medium described previously (Gadue et al., 2006). At day 2 EB were dissociated and reaggregated in the presence of growth factors to specify particular lineages. Activin A (R&D Systems) at 15ng/ml and ~0.3ng/ml BMP4 (R&D Systems)(usually between 0.2 and 1 ng/ml depending on the lot) was used to stimulate formation of endoderm, and Activin A at 2ng/ml and ~0.3ng/ml BMP4 (usually between 0.2 and 1 ng/ml depending on the lot) was used to optimally drive mesoderm. At day 3 EB were dissociated again, and, as indicated, plated in the presence of siRNA and Lipofectamine RNAiMax (Life Technologies). Endoderm assays were then continued with 15ng/ml Activin A and ~0.3ng/ml BMP4 (usually between 0.2 and 1 ng/ml depending on the lot), while mesoderm cultures were continued with 2ng/ml Activin A and ~0.3ng/ml BMP4 (usually between 0.2 and 1 ng/ml depending on the lot). At day 5 the media was replaced with media containing the Wnt inhibitor 53AH at 1µM (Lanier et al., 2012; Willems et al., 2011).

Samples were taken and processed as indicated for RT-qPCR, flow cytometry or high content imaging. For high content imaging, plates were loaded onto an

automated microscope (Incell 1000, GE Healthcare) and images were processed using Cyteseer (Vala Sciences Inc.). GFP reads are represented as the GFP area (A) multiplied by the GFP intensity (D).

Immunostaining on embryos and cells

Decidua containing embryos at day E6.5 through E7.5 were dissected and flash frozen prior to cryosectioning at 10 µm thick. Slides were washed, permeated for 30 min in 1% Triton X100 and blocked in superblock for 2h. Samples were incubated overnight at 4°C with primary antibodies: goat anti-Cer-1 (AF1075, R&D Systems), mouse anti-Hnf4a (clone K9218, Abcam), rabbit anti-Nr3c1 (clone M-20, Santa Cruz Biotechnology). Alexa-conjugated secondary antibodies (Life Technologies) were used, with samples incubated at room temperature for 1h, followed by DAPI staining and mounting. Images were acquired in a LSM510 Zeiss confocal microscope. Cells from differentiation cultures were stained similarly after being fixed with 4% paraformaldehyde for 5 min.

Flow cytometry and cell sorting

Cells were dissociated with trypsin to single cell suspensions and analyzed on a FACSCanto or LSRFortessa (BD Biosciences) for eGFP under control of the *Myh6* promoter. For extracellular staining, cells were dissociated with enzyme free cell dissociation buffer (GIBCO). Cells were incubated for 30 minutes with Cd31-APC (clone 390, eBioscience), Flk1-PE (clone Avas12a1 eBioscience) and Cxcr4-Alexa Fluor 647 (clone 2B11, eBioscience). Prior to analysis, cells were incubated with propidium iodide (PI) to label non-viable cells. Data analysis was performed using FlowJo (Treestar). Measured events were gated for PI negative populations (exclusion of dead cells) and/or forward/side scatter (exclusion of debris and aggregates) before producing histograms or dot plots. Samples for sorting where processed similarly and were run on a FACS Vantage-Diva sorter (BD Biosciences).

RNA extraction and RT-qPCR

RT-qPCR was performed following MIQE guidelines (Bustin et al., 2009). Samples for RNA were collected and immediately frozen until processed. Trizol was used to isolate total RNA using the manufacturer's instructions. RNA integrity analyses were performed on randomly selected samples to ensure overall RNA quality. RNA was quantified using a Nanodrop and 500ng of total RNA was used for reverse transcription, which was performed with the Quantitect RT kit (Qiagen) according to the included instructions, and the kit includes a genomic DNA removal step. cDNA was diluted 5 times to avoid PCR inhibition by contaminants. 7µl qPCR reactions were run in 384 well plates on an ABI 7900 (Life Technologies) using iTaq Universal Sybr Green Master mix (Bio-Rad). Amplification was performed by 40 cycles of denaturation at 95°C for 10 seconds and annealing extension at 60°C for 30 seconds, after which a melt curve cycle was performed. Primers were developed over intron-exon boundaries and were evaluated for efficiency and assay performance by standard curve analyses and were redesigned when necessary to achieve assays of high quality. Reference gene stability was investigated in representative samples as described before and the most stable gene was used for normalization (Willems et al., 2006). Primer sequences are made available in the RTPrimerDB (http://www.rtprimerdb.org/)(Pattyn et al., 2003) and primer ID numbers are listed in Supplementary Table 1. RT-qPCR data was standardized and analyzed before statistical analysis as described previously (Willems et al., 2008).

Statistical analysis

All samples were analyzed in at least three biological replicates and were tested for significance by a Student's t-test. Data in the figure is represented as mean with error bars indicating SEM, with asterisks indicating a p-value < 0.05.

Supplementary References

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