

bZIP-Type transcription factors CREB and OASIS bind and stimulate the promoter of the mammalian transcription factor GCMa/Gcm1 in trophoblast cells

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ABSTRACT

One of the master regulators of placental cell fusion in mammals leading to multi-nucleated syncytiotrophoblasts is the transcription factor GCMa. Recently, we proved that the cAMP-driven protein kinase A signaling pathway is fundamental for up-regulation of GCMa transcript levels and protein stability. Here, we show that Transducer of Regulated CREB activity (TORC1), the human co-activator of cAMP response element-binding protein (CREB), but not a dominant-negative CREB mutant, significantly up-regulates the GCMa promoter. We identified potential cAMP response element (CRE)-binding sites within the GCMa promoter upstream of the transcriptional start site. Only the CRE site at -1337 interacted strongly with CREB in promoter mapping experiments. The characterization of GCMa promoter mutants and additional bZIP-type family members demonstrated that also old astrocyte specifically-induced substance (OASIS) is able to stimulate GCMa transcription. Knockdown of endogenous CREB or OASIS in BeWo cells decreased endogenous GCMa mRNA level and activity. Overexpression of TORC1 or OASIS in choriocarcinoma cells led to placental cell fusion, accompanied by placental expression of gap junction forming protein connexin-43. Further, we show that CREB expression is replaced by OASIS expression around E12.5 suggesting that a sequential order of bZIP-type family members ensures a high rate of GCMa transcription throughout placentation.

INTRODUCTION

Members of the GCM (glial cells missing; in flies also named glide = glial cell deficient) proteins constitute a

unique family of transcription factors (1–3). They share a common biochemical hallmark, a highly conserved zinc-coordinating DNA binding domain at the amino-terminus composed of two subdomains each largely consisting of β -sheets (1,4). Interaction between each GCM DNA binding domain and an octameric binding motif composed of 5'-ATGCGGGT-3' is mediated by contacts between amino acid residues of the β -sheets of GCM proteins and the major groove of double-stranded DNA (5–8).

GCM homologs have been identified in a wide range of species and always shown to be involved as master regulators in key steps of differentiation processes. As mammalian GCM members, GCMa/Gcm1 and GCMb/Gcm2 were reported (5,7,9). GCMa is necessary for the differentiation of trophoblasts to syncytiotrophoblasts, a process required for placental labyrinth formation (10,11). Accordingly, GCMa knockout mice lack a functional placental labyrinth layer and die at mid-embryogenesis. Remarkably, GCMa is the first transcription factor capable of initiating syncytiotrophoblast formation. In adult mice, additional GCMa expression sites have been reported in kidney, thymus and brain (12,13). GCMb is required for the generation of parathyroid glands (14). Consequently, GCMb knockout mice suffer from hypoparathyroidism characterized by affected calcium and phosphate homeostasis.

Recently, several studies reported about the important question of what mechanisms direct the regulation of GCMa itself. We and others showed that stimulation of GCMa transcriptional activity occurs by cAMP/protein kinase A (PKA) signaling (15,16). Further, we demonstrated that the rate of GCMa transcription is directly stimulated by the cAMP/protein kinase A pathway (15). Moreover, an increase of GCMa protein stability is attributed to cAMP response element-binding protein (CREB)-binding protein (CBP)-mediated acetylation (16). Histone deacetylase 3 turned out to bind and thereby regulate GCMa transcriptional activity (17). Additionally, small ubiquitin-like modifier modification was shown to

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down-regulate the DNA binding activity of GCMa (18). Having observed that the cAMP/PKA pathway stimulated the rate of GCMa transcription, we asked how this is achieved mechanistically. It is well known that transcription factors belonging to the bZIP-type family, like CREB, are modulated by the cAMP/PKA pathway (19). Members of the ATF/CREB transcription factor family comprise apart from CREB itself, transcription factors like, p.ex. CREM, ATF1, ATF2, ATF6, OASIS, Jun, Fos and C/EBP proteins (20). All CREB family members bind to conserved cAMP-responsive elements (CRE) composed of 5'-TGACGTCA-3', act upon phosphorylation and modulate as homo- or heterodimers cellular gene expression in response to growth factor signals (19). Worth mentioning is that some members of the ATF/CREB family like CREB, ATF6 or OASIS strongly favor homodimerization (21). Recently, three potent activators of CREB family members referred to here as Transducers of Regulated CREB activity (TORC) were identified by a high-throughput expression screen (22). TORC1 was shown to stimulate CREB-dependent transcription via a phosphorylation-independent interaction with CREB (22).

Here, we present data proving that GCMa transcription is increased by TORC1-dependent stimulation of the promoter of GCMa. We identified a number of potential CRE-sites within the mouse and human GCMa promoter. We detected TORC1-dependent stimulation of the transcription of GCMa mainly in the presence of the CRE-site at -1337 at the GCMa promoter in mice and the formation of a complex between a radiolabeled probe containing this site and CREB protein. Luciferase reporter studies led to the identification of OASIS, another bZIP-type protein, stimulating the transcription of GCMa in HEK293 and the choriocarcinoma cell line BeWo. Using chromatin-immunoprecipitation, we resolved the binding pattern of CREB and OASIS on the promoter of GCMa in human and mouse cells. Further, knockdown of endogenous CREB or OASIS in BeWo cells led to significant reduction of endogenous GCMa transcript amount and activity. In fact, we could confirm our data *in vivo* by detecting GCMa-dependent connexin-43 immunostaining after transfection of BeWo cells with TORC1 or OASIS. Finally, we showed that CREB and OASIS are expressed sequentially during murine placentogenesis.

MATERIALS AND METHODS

Construction of plasmids, mutagenesis of CRE-sites

The promoter of mouse GCMa or parts thereof were amplified by PCR using as template DNA extracted from mouse embryonic stem cells and cloned 5' to the luciferase gene into pGL2-Basic (Promega) using the restriction sites KpnI and XhoI to create reporter plasmids. The following primers were used: (1) GCM351 + GCM356 (GCMa promoter -4041 to +1959), (2) GCM361 + GCM356 (GCMa promoter -2892 to +1959), (3) GCM362 + GCM356 (GCMa promoter -1637 to +1959), (4) GCM363 + GCM356 (-396 to +1959), (5) GCM364 + GCM356 (GCMa promoter +1018 to +1959) (primer sequences are listed in

Supplementary Table 1; for a scheme of constructs see Figure 1C).

CRE-sites within the mouse GCMa promoter located at -1337, -1317, -809 and +1578 were mutated by replacing single bases within the respective luciferase reporter plasmids mentioned above using a commercial mutagenesis kit (QuikChange XL Site-Directed Mutagenesis Kit, Stratagene) with the following primers: (1) GCM390 + GCM391 (-1337; AC at -1333/-1332 were replaced by TG), (2) GCM392 + GCM393 (-1317; AC at -1313/-1312 were changed to TG), (3) GCM394 + GCM395 (-809; AC at -805/-804 were mutated to TG), (4) GCM450 + GCM451 (+1578; AC at +1582/-1583 were mutated to TG) (primer sequences are listed in Supplementary Table 1; see Figure 2B).

The promoter of human GCMa or parts thereof were amplified by PCR using as template DNA extracted from HeLa cells and cloned 5' to the luciferase gene into pGL2-Basic (Promega) using the restriction sites NheI and XhoI to create reporter plasmids. The following primers were used: (1) GCM434 + GCM437 (GCMa promoter -3966 to +3197), (2) GCM436 + GCM437 (GCMa promoter -300 to +3197), (3) GCM438 + GCM437 (GCMa promoter +2258 to +3197), (4) GCM443 + GCM437 (+2700 to +3197), (primer sequences are listed in Supplementary Table 1; for a scheme of constructs see Figure 5E).

Mouse bZIP-type transcription factors were amplified by RT-PCR from placental or renal 1st strand cDNA using the following primers: (1) ATF1-1 + ATF1-2, (2) ATF2-1 + ATF2-2, (3) ATF4-1 + ATF4-2, (4) ATF5-1 + ATF5-2, (5) ATF7-1 + ATF7-2, (6) MafK-3 + MafK-4, (7) CREB-H-1 + CREB-H-2, (8) CREB-3-1 + CREB-3-2, (9) OASIS-30 + OASIS-31, (10) OASIS-30 + OASIS-1, (11) Fos-5 + Fos-6, (12) Jun-1 + Jun-2, (13) C/EBP α -5 + C/EBP α -6, (14) C/EBP γ -1 + C/EBP γ -2 (primer sequences are listed in Supplementary Table 1).

Silencing of CREB or OASIS was achieved by use of plasmid-derived shRNAs. shRNA primers were designed with the software Sfold (23). For knocking down gene expression of human CREB (accession no. NM_004379), complementary primers (CREB19 + CREB20, or CREB25 + CREB26; was not effective and therefore used as scramble) were hybridized and subcloned into pSUPERneoGFP (Oligoengine) using restriction sites HindIII and BglII. For knocking down gene expression of human OASIS (accession no. NM_052854) the following complementary primers were used the same way like for CREB (OASIS4 + OASIS5, or OASIS8 + OASIS9; was not effective and therefore used as scramble). The efficiency of knockdown of respective gene expression at mRNA level was quantified by a gene reporter assay. Therefore, a luciferase gene and either PCR-amplified CREB (primers CREB-35 + CREB-36; restriction sites PstI, NheI) or OASIS (primers OASIS-16 + OASIS-17; restriction sites XhoI, NheI) encoding cDNAs were subcloned as bicistronic messages into pCMX-PL1. At protein level, the efficiency of knockdown of the CREB or OASIS was confirmed by Western blot.

For the generation of *in situ* riboprobes, OASIS and CREB fragments were amplified using primers

OASIS-20 + OASIS 21 (24), or CREB-41 + CREB-42 and subcloned into pGEM-Teasy (Promega). For generation of antisense probes both plasmids were linearized with NdeI and antisense riboprobes were made using T7 polymerase. Sense riboprobes were generated restriction digesting the CREB-containing plasmid with SacII and the OASIS-containing plasmid with NcoI and using the SP6 polymerase. The strategy for generating mGCMa riboprobes was described (25).

The RFPnls plasmid was constructed in two parts. Primers RFP5 and RFPNLS_R were used to amplify by PCR RFP from plasmid pDS-Red N1 (Clontech). Then, primers S6F and hS6_NLS_R were used to amplify the nuclear localization signal from human S6 protein from pCMX-PL1-GFP-Nls (26). The RFP fragment was cloned into pCR2.1 (Invitrogen) using restriction sites NheI and EcoRI, while the nls was cloned into the same vector using NotI and EcoRI. Finally, both fragments were cloned into NheI/NotI digested pCMV5-T7b (primer sequences are listed in Supplementary Table 1).

A TORC1 expression construct was obtained from the mammalian genome collection (accession number BC028050), HA-CREB and A-CREB have been described before (27,28).

Tissue culture, transfection, luciferase assay

HEK293 and BeWo cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10 or 20% (v/v) fetal calf serum, respectively (FCS, Invitrogen). The cells were transfected in 60-mm dishes with 1 µg of luciferase reporter and 0.1 µg of CMV-driven expression vectors using DNA calcium phosphate precipitates or Superfect (Invitrogen). Where indicated, BeWo cells were incubated with forskolin (100 µM) or cAMP (150 µM). At 48 h post-transfection, cells were harvested for luciferase assays as described (29). All experiments were repeated independently at least three times.

Electrophoretic mobility shift assays and chromatin-immunoprecipitation

For electrophoretic mobility shift assays, 0.5 ng of 32P-labeled probe were incubated with HEK293 cell extract which were transiently transfected with an HA-tagged CREB expression plasmid for 20 min on ice in a 20 µl reaction mixture as described using poly[d(IC)] as unspecific competitor and 5 mM MgCl₂ (29). For supershift studies 0.5 µl of mouse anti-HA antibody (Cell Signaling Technology) recognizing HA-tagged CREB was added to the reaction mixture. For competition studies, 10- to 500-fold excess of competitor was used. Samples were loaded onto native 4% (w/v) polyacrylamide gels and electrophoresed in 0.5 × TBE (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.3) at 120 V for 1.5 h. Gels were dried and exposed for autoradiography. Band intensities were analyzed by Scion Image program (Scion Corporation).

Chromatin-immunoprecipitation was performed such that cellular protein and genomic DNA from freshly prepared mouse embryonic stem cell or BeWo cells transiently transfected with T7-tagged OASIS or HA-tagged

CREB by Lipofectamine (Invitrogen) were cross-linked in 1% formaldehyde before chromatin extraction and sonication to an average fragment length of 300–600 bp. Immunoprecipitations were performed overnight at 4°C with monoclonal IgG against HA- or T7-tag antibodies (Cell Signaling Technology). DNA was purified from precipitates after crosslink reversal and subjected to PCR. For detection of the mouse GCMa promoter the following primers were used in 37 or 40 cycles of standard PCR with an annealing temperature of 58°C: (1) GCM400 + GCM401 (–3547 refers to –3587 to –3378), (2) GCM402 + GCM403 (–2608 refers to –2646 to –2426), (3) GCM404 + GCM405 (–2066 refers to –2150 to –1872), (4) GCM406 + GCM407 (–1735 refers to –2040 to –1706), (5) GCM408 + GCM409 (–1337/–1317 refers to –1446 to –1241), (6) GCM410 + GCM411 (–809 refers to –925 to –637), (7) GCM412 + GCM413 (+1578 refers to +1498 to +1777), (8) GCM428 + GCM429 (negative control –9508 to –9256). For detection of the human GCMa promoter following primers were employed in 32 or 35 cycles of standard PCR for OASIS or CREB, respectively: (1) GCM414 + GCM415 (–3894 refers to –3948 to –3731), (2) GCM416 + GCM417 (–3080 refers to –3277 to –3009), (3) GCM418 + GCM419 (–2796 refers to –2862 to –2583), (4) GCM420 + GCM421 (+1004 refers to +832 to +1108), (5) GCM422 + GCM423 (+2375 refers to +2233–2459), (6) GCM424 + GCM425 (+3187 refers to +2986 to +3196), (7) GCM426 + GCM427 (negative control –10129 to –9867). All primer sequences are listed in Supplementary Table 1.

RNA extraction, reverse transcription and lightcycler PCR, western blot, in situ hybridization

Total RNA was extracted from mouse tissues or cells with TRIzol reagent (Invitrogen) as described previously (29). After reverse transcription, cDNA was used in RT-PCR experiments. Quantitative PCR reactions were performed using the LightCycler-FastStart DNA Master SYBR Green kit and the Light-Cycler Thermal Cycle system (Roche Applied Science) according to the manufacturer's instructions with the following primers for murine genes: (1) OASIS-32 + OASIS-33, (2) CREB-17 + CREB-18, (3) Tpbpa-1 + Tpbpa-2, (4) β-actin-111 + β-actin-112, and the following primers for human genes: (1) OASIS-24 + OASIS-25, (2) CREB-15 + CREB-16, (3) GCM315 + GCM319, (4) hβActin1 + hβActin2 (primer sequences are listed in Supplementary Table 1).

For detection of proteins on nitrocellulose membranes after Western blotting, the following primary antibodies were used: monoclonal antibodies directed against the T7-epitope (Novagen), or the hemagglutinin-epitope (Santa Cruz). Monoclonal antibodies were used at 1:10 000. Horseradish-peroxidase-coupled anti-mouse-Ig was used as secondary antibodies at 1:3000 dilution together with the ECL detection system (Amersham).

Extra-embryonic placental tissues from mice at E9.5 were dissected and fixed in 4% paraformaldehyde, deposited in 12-well plates and hybridized with sense or

antisense RNA specific for OASIS, CREB or GCMa according to protocols previously described (25).

Immunocytochemistry of BeWo cells

Configuration of gap junctions in BeWo cells was shown by immunolabeling for connexin43 (Cx43). For immunocytochemistry, 5×10^4 cells were seeded in chamber slides and transiently transfected with the indicated expression plasmids. For identification of transfected cells, a RFPnls expression plasmid generated in analogy to a previously created GFPnls was co-transfected (Becton Dickinson Biosciences) (26). Thereafter, cells were fixed with 4% paraformaldehyde and incubated with a rabbit polyclonal antibody reactive against Cx43 (Chemicon, final dilution 1:300), followed by an Alexa 488-conjugated goat anti-rabbit IgG treatment (Molecular Probes, final dilution 1:250). Cell nuclei were stained with DAPI (Boehringer Mannheim, final concentration 1 μ g/ml). Cells were analyzed and documented using a Leica inverted microscope (DMIRB) equipped with a cooled MicroMax CCD camera (Princeton Instruments).

RESULTS

The GCMa promoter is stimulated by cAMP-PKA-signaling through specific binding of the bZIP-type transcription factor CREB

Previously, we and others demonstrated that the PKA pathway acts upstream of GCMa by inducing the expression of GCMa and by acetylation-mediated stabilization of GCMa protein (15,16). Here, we investigated how mechanistically the cAMP-PKA pathway is able to stimulate the rate of GCMa transcription. As bZIP-type transcription factors like CREB are known to be stimulated by the cAMP-PKA pathway, we decided to examine if this holds true for the GCMa promoter (19). We transfected HEK293 cells with a reporter plasmid containing the sequence of mouse GCMa (mGCMa) from position -4041 relative to the transcriptional start site in front of a luciferase gene together with TORC1, a specific positive co-activator of CREB (Figure 1A and C). In fact, TORC1 was able to stimulate the mGCMa promoter by 12.9-fold, whereas A-CREB, a dominant-negative mutant of CREB, significantly inhibited TORC1-mediated transactivation [Figure 1A; (28)]. Next, we transfected a luciferase reporter plasmid containing six tandemly arranged GCM binding sites in front of a luciferase gene into BeWo choriocarcinoma cells which are known for endogenous human GCMa (hGCMa) expression (30). As shown before, concomittant treatment of these cells with either forskolin or cAMP was sufficient to stimulate endogenous hGCMa activity (15). By co-transfection of TORC1 we detected a significantly higher amount of endogenous hGCMa activity, while co-transfection of A-CREB abolished TORC1-mediated higher hGCMa activity (Figure 1B).

By computational analysis, we identified several potential CRE-binding elements within the mGCMa promoter (Figure 1C). To rule out those sites which are not stimutable by CREB, we cloned a number of 5' deletion

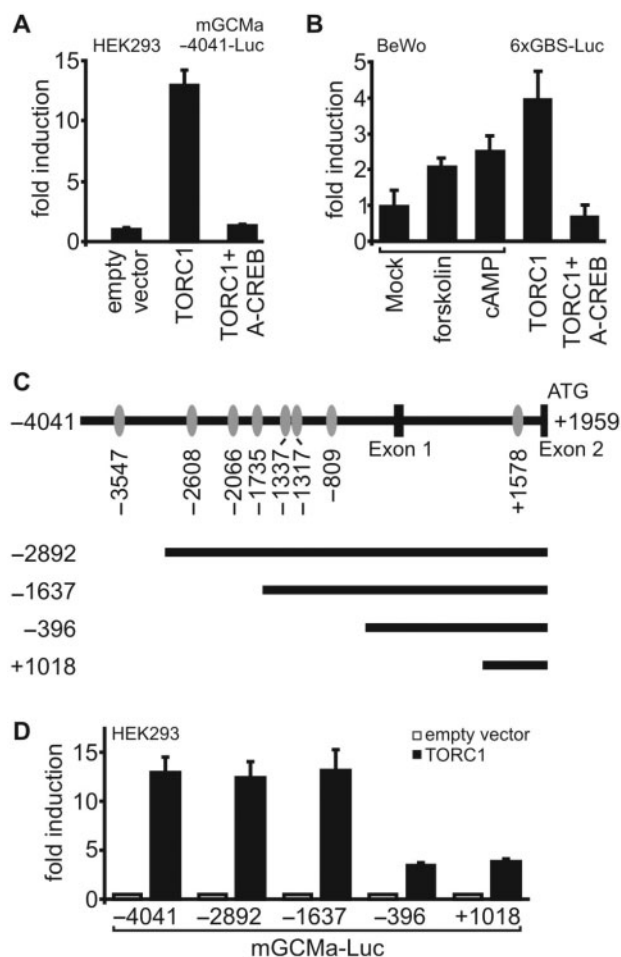


Figure 1. Forskolin, cAMP, and the transcriptional co-activator of the transcription factor CREB, TORC1, stimulate the promoter of GCMa. (A) HEK293 cells transfected in duplicate three independent times by a mGCMa promoter containing luciferase reporter construct (mGCMa-4041-Luc) together with either an empty expression plasmid or expressions plasmids containing TORC1, or TORC1 and A-CREB as indicated. Luciferase activities were measured after 48 h. (B) BeWo cells were transfected in duplicate three independent times with a six GCM-binding-sites containing luciferase reporter alone or together with TORC1, or TORC1 and A-CREB. Cells only transfected with the luciferase reporter construct were left untreated or further incubated with forskolin or cAMP. Luciferase activities were measured after 48 h. (C) Upper part of the scheme shows the area between -4041 to the ATG of the mGCMa promoter. Exons are indicated as black boxes. Grey circles point to potential CRE-binding sites. The lower part symbolizes 5' truncated deletion mutants of the mGCMa promoter which were used for construction of luciferase reporter plasmids. (D) HEK293 cells were transfected in duplicate three independent times with luciferase reporter constructs as described in (C).

mutants of the mGCMa promoter, fused them to a luciferase reporter and transfected them alone or together with TORC1 into HEK293 cells (Figure 1D). The deletion mutant reporter plasmids containing either -4041 , -2892 or -1637 of the mGCMa promoter were all stimulated by TORC1, while the mutants -396 and $+1018$ lack significant amounts of luciferase activity (Figure 1D). Deletion mutant -1637 contained inducible CRE-binding sites in the area needed for TORC1-dependent activation. These were located at -1337 ,

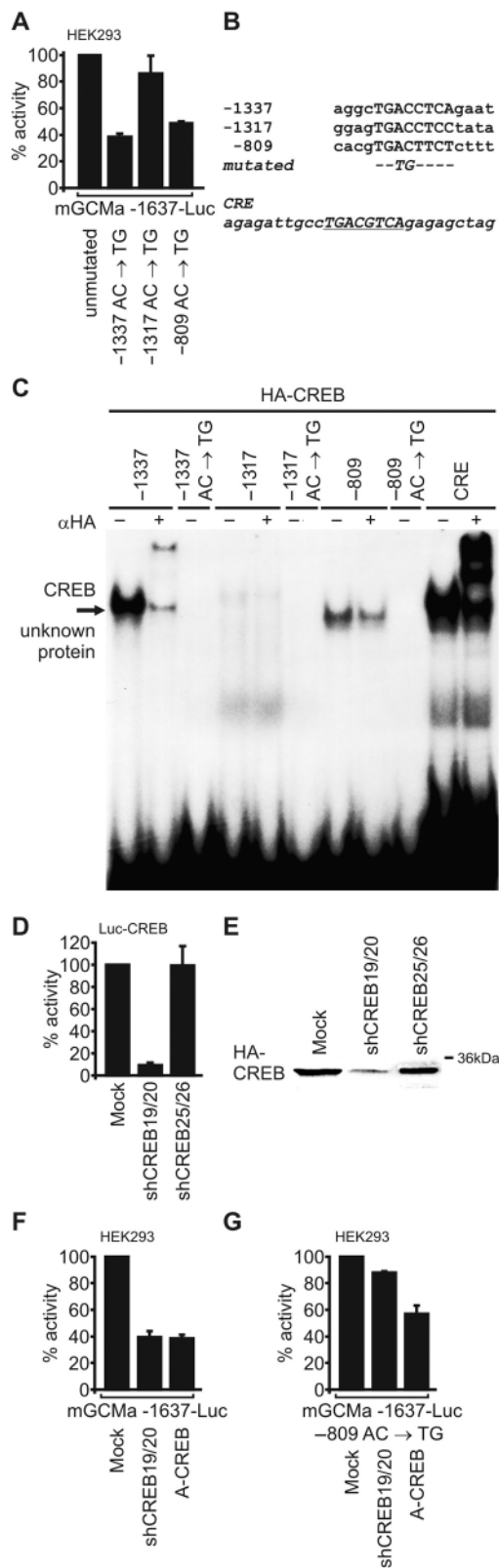


Figure 2. CREB binds to the GCMA promoter pre-dominantly at position -1337 . (A) HEK293 cells transfected in duplicate three independent times with TORC1 and the GCMA -1637 -luc reporter construct (as shown in Figure 1D) or mutations thereof as indicated. Luciferase activities were measured after 48 h. (B) Oligonucleotide sequences which were used in electrophoretic mobility shift assays

-1317 and -809 (Figure 1C). To investigate the contribution of these sites to CREB-mediated mGCMA stimulation, we mutated each site separately within the mGCMA -1637 -luc reporter plasmid and performed luciferase and electrophoretic mobility shift assays (Figure 2A–C). The reporter plasmid containing a mutated CRE-binding site at -1317 was still inducible with TORC1 (Figure 2A). Only if CRE-binding sites -1337 (-1337 AC-TG) or -809 (-809 AC-TG) within the mGCMA promoter were mutated in respective reporter plasmids and transfected with TORC1 in HEK293 cells a significant decrease of luciferase activity was observed which was even more distinct for -1337 AC-TG (Figure 2A). To find out if CREB binds to the sites at -1337 , -1317 or -809 within the mGCMA promoter, we incubated radiolabeled oligonucleotides with HEK293 cell extract transiently transfected with HA-tagged CREB expression plasmid (Figure 2B). CREB turned out to bind to the oligonucleotide comprised of the CRE-binding site at -1337 of the mGCMA promoter (Figure 2B and C). If the HA-CREB-containing lysate was incubated with the mutated radiolabeled oligonucleotide (-1337 AC-TG) we failed to detect any band (Figure 2C). Accordingly, we were able to super-shift the complex composed of radiolabeled -1337 oligonucleotide with HA-tagged CREB if we added an HA-specific antibody to the reaction mixture (Figure 2C). In agreement with the reporter assay, a mutated -1317 oligonucleotide failed to be shifted after incubation with HA-CREB-containing HEK293 cell lysate (Figure 2C). Interestingly, by using radiolabeled oligonucleotide -809 with a HA-tagged CREB-containing HEK293 cell lysate in electrophoretic mobility shift assays, we detected a band running close to the complex between CREB and the radiolabeled oligonucleotide; but the same band was detected regardless of transient transfection of HEK293 with an empty CMV or an HA-CREB containing expression plasmid (Figure 2C). This complex was only to a very minor part super-shifted by additional incubation with HA-specific antibody

are depicted. Sequences correspond to parts of the mGCMA promoter which contain potential CRE binding sites and point substitutions thereof as used in (C) for electrophoretic mobility shift assays. Additionally, the sequence of an oligonucleotide used a positive control is shown containing a typical CRE-binding motif. (C) Electrophoretic mobility shift assay using oligonucleotides as mentioned in (B) and cell lysate of HEK293 cells transfected with HA-tagged CREB expression plasmid. The specificity of the detected bands were confirmed by super-shifts using HA-specific antibodies. Note, CREB binds strongly to -1337 and to a minor extent to -809 . (D) Different shRNAs were examined regarding their ability to knockdown the mRNA of CREB by a luciferase-reporter assay. For that, bicistronic mRNAs bearing a full-length luciferase cDNA and the full-length cDNA encoding CREB were created. shCREB19/20 knocked down CREB by more than 90% on mRNA level. shCREB25/26 did not affect CREB transcription and served as scramble control. (E) The lack of CREB at protein level was demonstrated after transient transfection in HEK293 cells by Western blot. (F, G) HEK293 cells transfected in duplicate three independent times with the mGCMA -1637 -luc reporter construct (as shown in Figure 1D) or mutations thereof as indicated and empty pSuper plasmid, CREB-specific shRNA (shCREB19/20), or dominant-negative A-CREB. Note, if CRE-binding site -809 was mutated the measured luciferase activity decreased only down to 90%, while without mutation it decreased down to 40% (as shown in A).

(Figure 2C). These findings suggest both binding of an unknown endogenous protein and, to a minor amount, binding of CREB to the radiolabeled oligonucleotide -809. By using a mutant variant of the radiolabeled oligonucleotide -809 (-809 AC-TG) we failed to detect a respective band (Figure 2C). As control, a radiolabeled oligonucleotide containing a CRE consensus site complexed HA-tagged CREB and was further super-shifted by a HA-specific antibody (Figure 2C). We decided to establish shRNA constructs to substantiate that CREB is affecting mGCMa gene regulation by binding to CRE-binding sites at -1337, and weakly at -809. To this end, we investigated several shRNA constructs and found one which significantly knocks down CREB on mRNA and protein level (Figure 2D and E). Now, we transfected TORC1, CREB-specific shRNA or the dominant-negative A-CREB and mGCMa -1637-luc reporter plasmid or its mutant variant (-809 AC-TG) (Figure 2F and G). Both, CREB-specific shRNA and A-CREB were able to impair TORC1-specific stimulation of the mGCMa -1637-luc reporter plasmid, but less pronounced the stimulation of mGCMa -1637-luc (-809 AC-TG) (Figure 2A and F, G).

To elucidate the binding characteristics of CREB to the CRE-containing site at -1337 and to confirm that CREB does not significantly interact with the CRE-containing site -809 within the GCMa promoter we decided to

perform comparative electrophoretic mobility shift assays. To this end, we investigated the binding abilities of each oligonucleotide -1337, -809 and consensus CRE by incubating them with HEK293 cell lysate containing HA-tagged CREB together with increasing amounts of unlabeled oligonucleotide competitors (Figure 3A-F). If we used radiolabeled consensus CRE oligonucleotide, only increasing amounts of unlabeled consensus CRE oligonucleotide was able to disturb binding of CREB to the radiolabeled consensus CRE site indicating that CREB binds to -1337 with lower affinity (Figure 3A and B). However, binding of the radiolabeled oligonucleotide -1337 was only significantly competed by increasing amounts of either unlabeled -1337 or consensus CRE oligonucleotide (Figure 3C and D). Unlabeled oligonucleotide -809 was not able to compete binding of radiolabeled -1337 (Figure 3C and D). Finally, radiolabeled oligonucleotide -809 was demonstrated to bind a yet unknown protein and little amounts of CREB as unlabeled -1337 was not able to suppress the formation of a complex composed of protein and oligonucleotide (Figure 3E and F, see also Discussion). The consensus CRE oligonucleotides competed only slightly the binding of radiolabeled -809 (Figure 3F). On the other hand, increasing amounts of unlabeled oligonucleotide -809 significantly abolished binding of radiolabeled -809 to the unknown protein (Figure 3E and F).

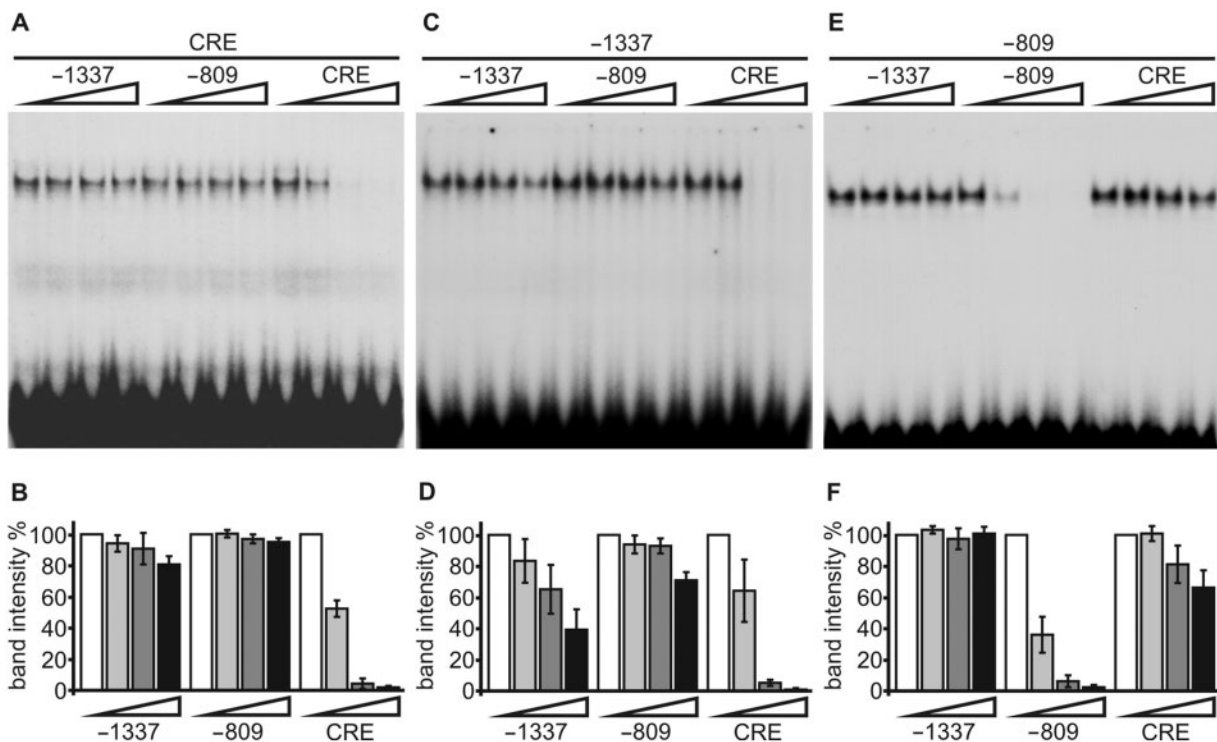
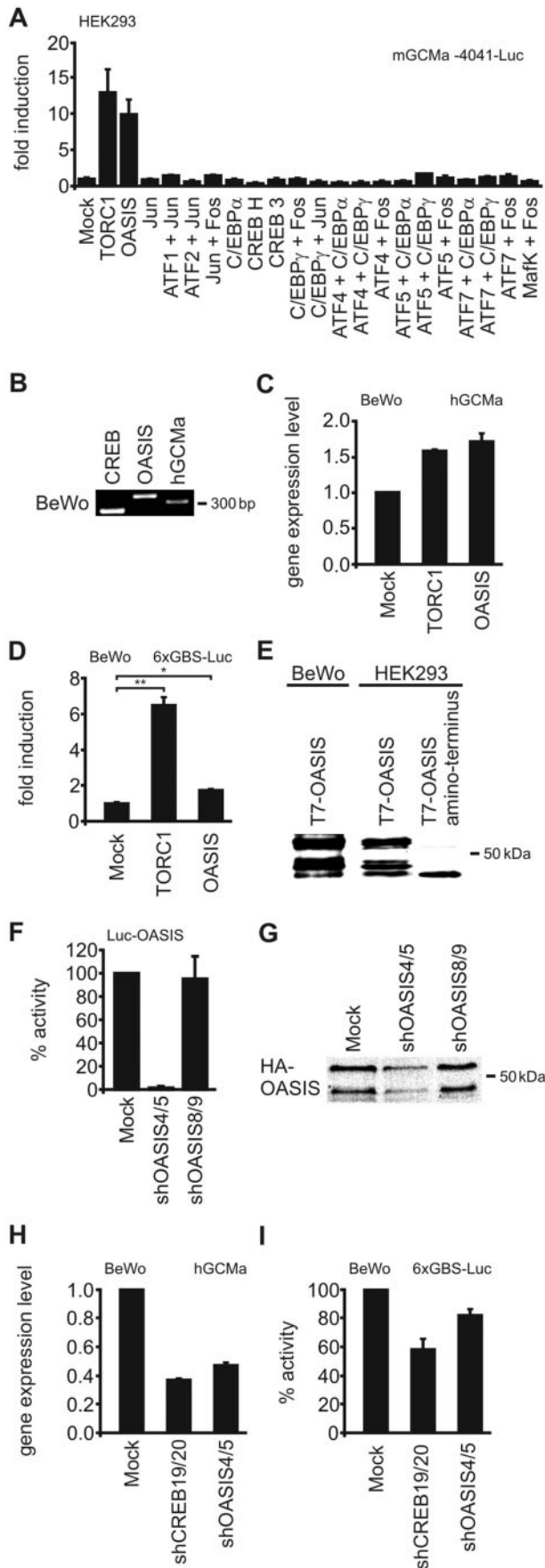


Figure 3. DNA-binding properties of oligonucleotides containing CRE-binding motifs and representing parts of the mGCMa promoter. Comparative DNA-binding analysis with HA-tagged CREB containing lysate from transiently transfected HEK293 cells and conditions such that oligonucleotides containing the typical consensus CRE binding site or the CRE binding sites within the mGCMa promoter were used. As competitors the same or one of the other oligonucleotides were used in a dose-dependent fashion. Conditions were such that in the absence of competitor, 20 ± 30% of the radioactively labeled probe was in complex with CREB. The competitor-dependent reduction of probe in the complex was determined by phosphorimager analysis. The graphs summarize the relative level of competition obtained with increasing amounts of each competitor. (A, B) CREB protein binding to the consensus CRE motif. As competitors increasing amounts (10- to 500-fold) of -1337, -809 or CREB oligonucleotides were used. (C-F) The same like in (A, B) but with other labeled oligonucleotides as indicated.



The murine and human GCma promoter are regulated by bZIP-type transcription factors CREB and OASIS

Being intrigued by the identification of an additional CRE-binding site at -809 which pre-dominantly binds another protein than CREB but is important for up-regulation of the mGCma promoter we investigated whether other bZIP-type transcription factors could additionally be involved in mGCma promoter stimulation. Therefore, we transiently transfected HEK293 cells with a reporter plasmid containing -4041 of the mGCma promoter 5' to a luciferase gene together with each of several bZIP-type family members alone or in combination, and measured promoter transactivation (Figure 4A). In addition to TORC1, only OASIS was able to induce the mGCma -4041-luc reporter plasmid but none of the other CREB family members, like Jun, Fos, CREB3, CREB-H, ATF1, ATF2, ATF4, ATF5, ATF7, C/EBP α , C/EBP γ and MafK (Figure 4A). To further substantiate these finding, we first explored if BeWo cells endogenously express OASIS. Using RT-PCR studies we detected significant amounts of endogenously transcribed mRNAs of hGCma, CREB and OASIS (Figure 4B). Next, endogenously hGCma-expressing BeWo cells were transfected by either a TORC1 or OASIS encoding expression plasmids, total RNA extracted and realtime RT-PCR experiments performed (Figure 4C). GCma mRNA level were increased in all cases (Figure 4C). If the BeWo cells were additionally co-transfected with a reporter plasmid

Figure 4. Identification of OASIS as an additional CREB family member binding to the mGCma promoter. (A) To investigate if other CREB family members bind to the mGCma promoter HEK293 cells were transfected in duplicate three independent times by the mGCma-4041-Luc reporter construct (Figure 1A) together with either an empty expression plasmid or expressions plasmids containing TORC1, OASIS, Jun, Fos, ATF1, ATF2, ATF4, ATF5, ATF7, CREB H, CREB 3, MafK, C/EBP α and C/EBP γ , as indicated. Luciferase activities were measured after 48 h. Note, only TORC1 and OASIS are able to stimulate the mGCma promoter-containing reporter construct. (B) Total RNA was extracted from BeWo cells, reverse transcribed, and used with specific primers for PCR reactions (30 cycles) to prove that these cells express endogenously CREB, OASIS, and hGCma. (C) BeWo cells were transfected with TORC1, or OASIS expression plasmids, total RNA extracted and realtime RT-PCR performed. Note, endogenous hGCma transcription increased after transfection of CREB or OASIS. (D) By transiently transfecting a plasmid containing six GCM binding sites arranged in tandem in front of the luciferase gene (6xgbs-Luc) it was shown that both TORC1 and OASIS are significantly inducing endogenous hGCma activity (students *t*-test, **P*-value <0.05, ***P*-value <0.01). (E) BeWo or HEK293 cells were transiently transfected with an expression plasmid encoding full length T7-tagged OASIS. Further, HEK293 cells were transfected with an expression plasmid encoding only the amino-terminal part of T7-tagged OASIS (control). By Western blot of the respective extracts showed that over-expressed full length OASIS is cleaved and its transactivation capacity containing amino-terminal part liberated in both cell types. (F, G) shRNAs which knock down OASIS were examined as described (Figure 2D) and their efficacy on the mRNA and protein levels was ascertained. (H, I) BeWo cells were transiently transfected by an empty plasmid (Mock) or plasmids encoding shRNAs as indicated. Total RNAs were extracted and subjected to realtime RT-PCR to prove that knockdown of endogenous CREB or OASIS ends up in lowered GCma transcript amounts. Additional transfection of the same cells with a six GCM-binding-sites containing luciferase reporter demonstrated less GCma activity after knockdown of CREB or OASIS (I).

containing six tandemly arranged GCM binding sites 5' to a luciferase gene (6 × GBS-Luc; Figure 4D), we detected elevated luciferase activities confirming that both, TORC1 and OASIS were able to stimulate endogenous hGCMa activity (Figure 4D). OASIS was shown to be a bZIP-type transcription factor with a transmembrane domain that allows it to associate with the endoplasmic reticulum (ER) (31). After cleavage at the membrane in response to ER stress, its cleaved amino-terminal cytoplasmic domain, which contains the bZip domain, translocates into the nucleus where it activates the transcription of target genes that are mediated by ER stress- and cyclic AMP-responsive elements (31). To find out if OASIS is also cleaved in HEK293 or BeWo cells, we transiently transfected a full length Oasis containing expression plasmid into the cells, prepared lysates and investigated if cleaved bZIP-domain in these lysates is detectable (Figure 4E). In fact, in the lysates of both cell types the full length OASIS and the cleaved bZIP-domain were present. To interrogate endogenous transcription factor action, we used a shRNA approach to specifically attribute endogenous hGCMa gene regulation by CREB and OASIS. We generated a number of shRNA constructs and found one which was able to significantly knockdown the expression of OASIS on mRNA and protein level (Figure 4F and G). Now, we transiently transfected the CREB- or OASIS-specific shRNAs into BeWo cells and showed by quantitative RT-PCR or luciferase assays as a consequence a subsequent down-regulation of both endogenous GCMa transcript level and activity (Figure 4H and I).

CREB and OASIS bind at several CRE sites within the murine and human GCMa promoter

To reveal the profile of CRE-binding sites used by CREB and OASIS under physiological conditions in human and mice, a number of chromatin-immunoprecipitations were performed. By computational analysis, potential CRE-binding sites were also identified within the human GCMa promoter (Figure 5A). T7-tagged OASIS or HA-tagged CREB expression plasmids were transiently transfected into either mouse embryonic stem cells or choriocarcinoma BeWo cells. Both cell lines are known to express GCMa endogenously (30,32). These experiments demonstrated that in mice OASIS binds to +1578, while CREB binds to -2066, -1735, -1337/-1317 and very weakly to -809 (Figure 5B). To demonstrate the impact of the newly identified OASIS-binding site at +1578 we transfected OASIS and mGCMa +1078-luc reporter plasmid or its mutant variant (-1578 AC-TG) into HEK293 cells and measured reporter activity. In fact, the mutant variant of the mGCMa +1078-luc reporter plasmid was almost 40% less active supporting that OASIS binds at +1578 thereby stimulating mGCMa promoter activity (Figure 5C). In human choriocarcinoma BeWo cells, CREB and OASIS interacted with different sites within the hGCMa promoter (Figure 5D). Here, OASIS binds to -3894, -2796, +1004 and +3187 (Figure 5C), whereas CREB only binds at +3187 and weakly to -2796 (Figure 5D). To further substantiate that CREB and OASIS binding sites on the hGCMa promoter are functional, we generated a series of

5' deletions, fused them to a luciferase gene and investigated their potential to be induced by CREB or OASIS. We observed that only the reporter plasmid carrying the human GCMa promoter from -3966 to +3197 was able to increase significantly luciferase activity (Figure 5E). This promoter fragment carries CRE-binding sites -3894 and -2796 which according to the ChIP analysis bind OASIS and CREB (weak band, see Figure 5D), respectively. These data prove that OASIS is an additional bZIP-type family member stimulating the expression of GCMa, but it is not the unknown endogenous CREB family member which interacts with -809 at the mGCMa promoter (see also Discussion).

TORC1 and OASIS stimulate endogenous GCMa expression in BeWo cells followed by connexin-43 expression and functional cell differentiation

To further confirm the role of OASIS and CREB in GCMa promoter stimulation *in vivo*, we decided to transfect endogenously hGCMa-expressing BeWo cells and follow functional cell differentiation by immunocytological stainings for connexin-43 (Cx43). Cx43 is known to be stimulated after GCMa-mediated activation of syncytin, a fusogenic protein necessary for syncytiotrophoblast formation during placentogenesis (15). To detect transfected BeWo cells, we co-transfected a RFPnls expression plasmid. We observed strong Cx43 immunostaining in RFPnls-positive BeWo cells only after transfection of either OASIS or TORC1 (Figure 6). If OASIS or TORC1 were additionally co-transfected with the dominant-negative variants of GCMa (GCMa220) or CREB (A-CREB), or respective shRNA plasmids which strongly knockdown OASIS or CREB (Figures 2D-E and 4F-G), respectively, we failed to detect strong Cx43 immunostaining [Figure 6; (15)]. Additionally, we show that the amino-terminal part of OASIS carrying its transactivation capacity is causing by its own stronger Cx43 immunostaining. This finding additionally demonstrates that OASIS is cleaved the proper way in BeWo cells (see also Figure 4D).

bZIP-Type transcription factors CREB and OASIS are induced consecutively during placentogenesis in mice

To understand the role of the bZIP-type transcription factors CREB and OASIS in mediating GCMa gene expression during placentogenesis, we analyzed their temporal and spatial expression profile in the placenta. First, we extracted total RNA from the fetal part of the placenta at different embryonic stages, reverse transcribed them into 1st strand cDNA and used them for quantitative RT-PCR studies. We found highest level of CREB transcripts at E6.5 to E12.5 (Figure 7A and B). While CREB transcription significantly decreases after E12.5, we observed elevated transcript amounts for OASIS starting from E12.5 (Figure 7A and B). To ensure that the same trophoblast cells express GCMa, CREB and OASIS, we performed whole mount *in situ* hybridization experiments with murine placentae at E9.5 (25). Treatment of the placentae with CREB- or OASIS-specific antisense

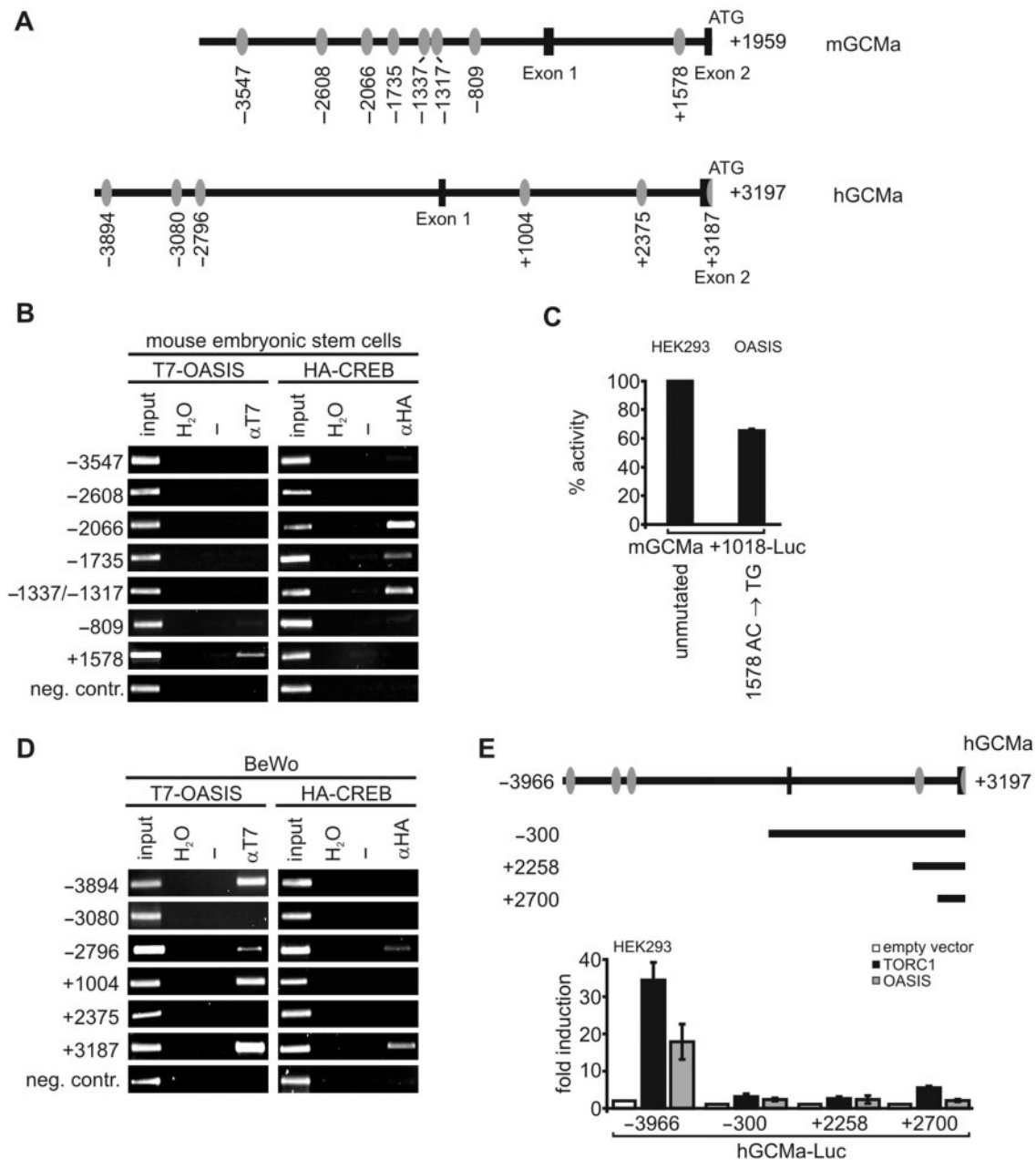


Figure 5. Chromatin-immunoprecipitation analysis revealed the binding profile of CREB and OASIS on the human and mouse GCMA promoters. (A) Scheme of the mouse and human GCMA promoter regions. Potential CRE-binding sites (grey circles) and exons (black boxes) are indicated. Numbers relate to the respective transcriptional start sites. (B) Images from agarose gels after chromatin-immunoprecipitation and PCR analysis demonstrate that both OASIS and CREB bind to several of the potential CRE-binding sites within the mGCMA promoter. (C) HEK293 cells transfected in duplicate three independent times with OASIS and the mGCMA +1018-luc reporter construct or mutations thereof as indicated. Luciferase activities were measured after 48 h. (D) The same like in (B) for the hGCMA promoter. (E) Upper parts, scheme symbolizes 5' truncated deletion mutants of the hGCMA promoter which were used for construction of luciferase reporter plasmids. Lower part, HEK293 cells were transfected in duplicate three independent times with luciferase reporter constructs as indicated.

riboprobes stained the same area like using a GCMA-specific antisense riboprobe (Figure 7D). In accordance with the previous PCR data shown, the intensity of the OASIS-specific staining located in the placental labyrinth was weaker (Figure 7A–D). Finally, murine placentae at E18.5 were dissected in the placental labyrinth layer containing mainly syncytiotrophoblast and the layer composed mostly of spongio- and giant trophoblast parts, total RNA was extracted and realtime RT-PCR

analysis performed. We proved the quality of this approach by performing PCR analysis using a syncytiotrophoblast specific marker (GCMA) or a spongiotrophoblast marker (Tpbpa: trophoblast specific protein alpha) and detecting bands corresponding to the respective tissue areas (Figure 7E). We then investigated these RNA fractions for the amount of CREB or OASIS transcript by realtime PCR analysis. In fact, we detected 90% more OASIS than CREB encoding transcripts (Figure 7F).

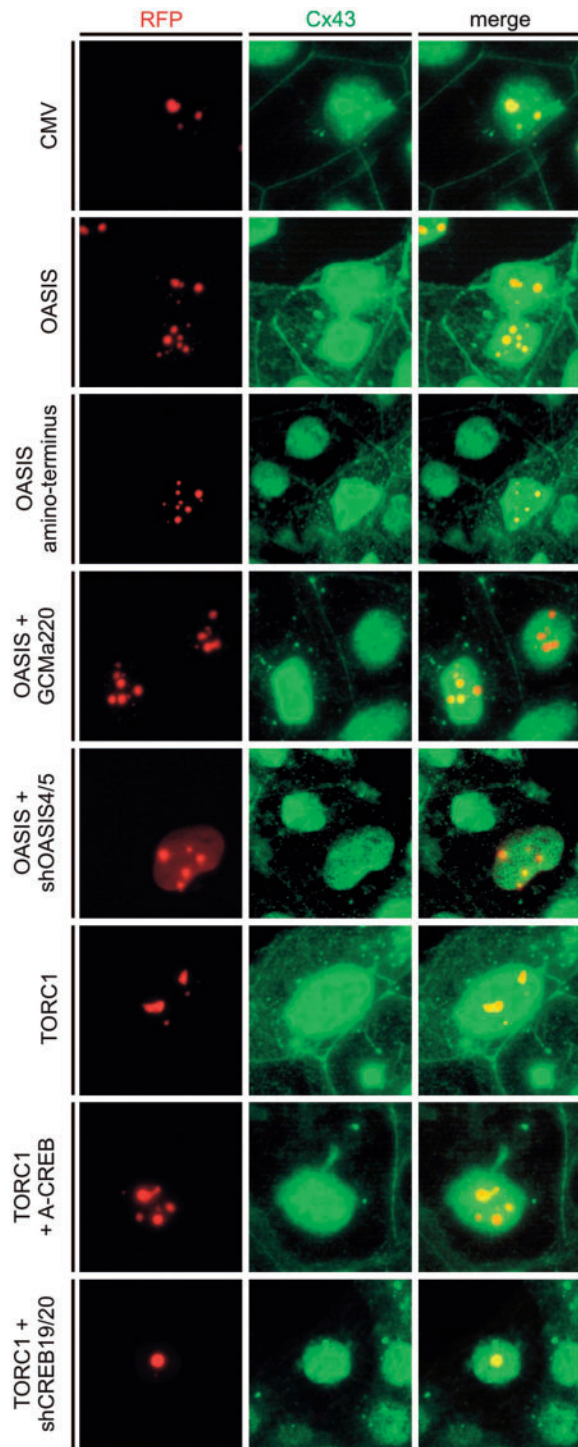


Figure 6. OASIS and TORC1 are able to stimulate endogenous GCMA transcriptional activity in BeWo cells indicated by expression of the marker of differentiation Connexin 43 as determined by immunostaining. Transfection of BeWo cells with either OASIS, the amino-terminal part of OASIS or TORC1 expression plasmids induced functional differentiation as depicted by immunocytochemical detection of gap junctional protein Connexin 43 (Cx43). Co-transfection of either GCMA220, a dominant-negative type of GCMA, together with OASIS or A-CREB together with TORC1 inhibits expression of Connexin 43. The same way, co-transfection of OASIS or CREB-specific shRNAs lead to a lowered Connexin 43 immunostaining. Note, a RFPnls expression plasmid was always co-transfected to enable identification of transfected cells.

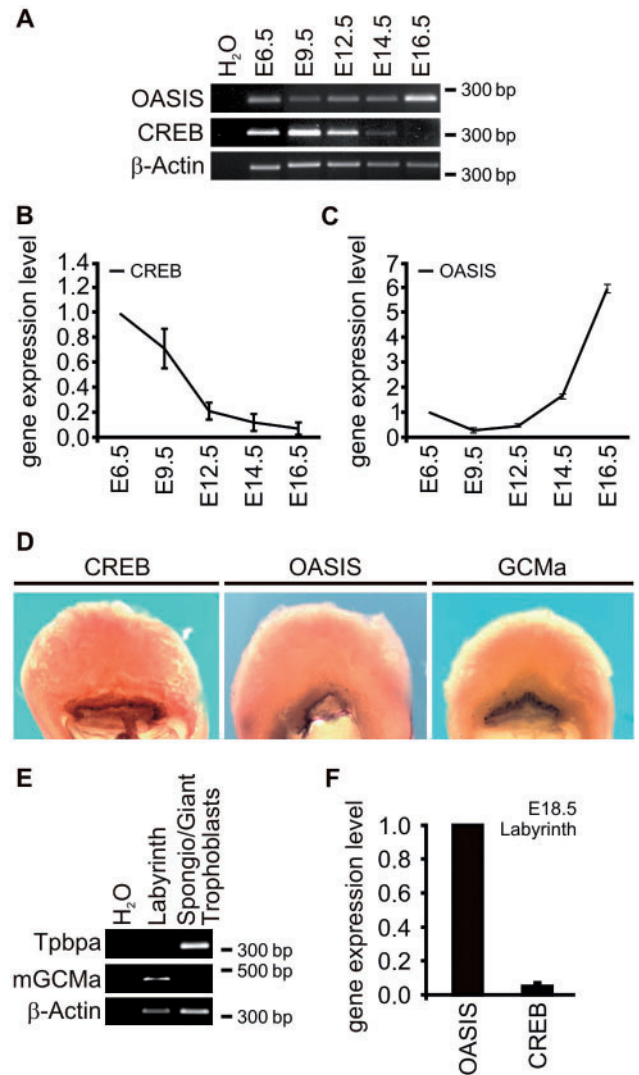


Figure 7. Temporal and spatial expression profile of CREB and OASIS in murine placenta. (A) Agarose gel electrophoresis of amplified transcripts of OASIS, CREB and β -actin after RT-PCR using total RNA extracted from placenta of the indicated age (E6.5-E16.5). (B, C) Realtime PCR analysis of transcriptional profiles of CREB and OASIS at different developmental stages of murine placenta. Values in graph show gene expression levels of the indicated genes after normalization with β -actin. Note, the transcript amount of CREB is highest from E6.5 to E12.5, while OASIS increases starting E12.5. (D) Whole mount *in situ* hybridization of placenta at E9.5 labeled with antisense riboprobes specific for CREB, OASIS and GCMA. No staining was detected by sense riboprobes (data not shown). (E) Placenta at E18.5 were dissected, total RNA extracted from the labyrinth or the spongio- and giant trophoblast cells containing layer and used for realtime RT-PCR experiments. Note, the markers (mGCMA, Tpbpa) are only detectable in RNA from the respective placental layers. (F) Placental labyrinth layer was used to extract total RNA for realtime RT-PCR experiments with OASIS- and CREB-specific primers.

DISCUSSION

Our data demonstrate for the first time the involvement of bZIP-type transcription factors in the control of the GCMA promoter in mice. Previous reports already stated an increase of GCMA activity (i) after incubation of BeWo

cells with either cAMP or forskolin, and (ii) upon transfection of HEK293 cells with mGCMA and a constitutive active protein kinase A due to an elevated GCMA protein stability (15,16). Additionally, CREB binding to the GCMA promoter was identified by comprehensive scanning of the human genome (33). Additionally, it was shown that incubation of BeWo cells or primary human trophoblasts with cAMP or forskolin led to an increase in GCMA transcript amounts. But the question how this mechanistically happens was remained unsolved (15). Here, we show that TORC1 upregulates the endogenous hGCMA promoter even stronger than forskolin or cAMP itself (Figure 1B). This up-regulation of the promoter activity of hGCMA is specifically mediated through CREB binding to the hGCMA promoter because (i) TORC1 is specifically upregulating CREB and not any other bZIP-type transcription factor, (ii) the TORC1-mediated transactivation of a GCMA reporter plasmid can be abolished by a dominant-negative CREB mutant (A-CREB) which is known to specifically inhibit only CREB and (iii) a CREB-specific shRNA transfected in BeWo cells decreased endogenous hGCMA mRNA level and activity. Furthermore, OASIS, another bZIP-type family member was, able to stimulate the rate of endogenous hGCMA transcription and specificity was verified knocking down endogenous OASIS expression by shRNAs which led to decreased endogenous GCMA mRNA level and activity (Figure 4). Overexpression of OASIS or CREB failed to increase the stability of GCMA in HEK293 cells arguing that higher GCMA activity is solely caused by stronger GCMA expression (data not shown). Remarkably, we detected a number of CRE-binding sites within the GCMA promoter of humans and mice and succeeded in demonstrating that some of them indeed interact with CREB or OASIS (Figures 2A–C and 5B–E). The algorithm according to which these CRE-sites were searched are based on structural data about the interaction of CREB with its binding motif (19). The predicted site at –1317 indeed functioned by CREB binding. The site at –809 was also functional, but bound mainly another bZIP-type family member of unknown identity. In trying to identify its identity we were, however, able to show that OASIS also stimulates the GCMA promoter (Figure 4). All our effort failed to detect any interaction between OASIS and the GCMA promoter at –809 (data not shown). Further, we failed to observe a stimulation of ATF1, another CREB family member, and ATF1 also did not interact with –809 at the GCMA promoter (data not shown).

Although we identified several CRE-binding sites within the human and mouse GCMA promoter only some of them turned out to be functional. This is not surprising given that not all computationally identified DNA sequence motifs have necessarily to be used in gene regulation. Furthermore, we cannot rule out that some of these sites are functional in trophoblast cells of living mice. It is very likely that the repertoire of additional co-activators of bZIP-type proteins in cultured HEK293 or BeWo cells does not match with trophoblast cells in human and mice. For example, CREB is thought to be an important factor for the expression of genes in

trophoblasts and during differentiation (34). Similar to nonclassical HLA class I molecule, like HLA-G, the alpha-subunit of glycoprotein hormone is regulated by multiple sites including CRE-binding sites (35–37). However, the tissue-specific expression of the alpha-subunit of glycoprotein hormone was controlled by an additional regulatory element mediating the trophoblast-specific expression that is not found in the HLA-G promoter. This example is evidence for the fact that not all transcription factor binding sites are necessarily functional. But functional sites on the genome are often maintained through evolution consistent with our finding that GCMA is regulated by bZIP-type transcription factors in both species, mouse and human (33).

TORC1 might act in a manner independent of cAMP as it was shown to be also regulatable by salt inducible kinase 2 (SIK2; a member of the AMPK family) signaling (38). It might be worth to investigate if SIK2 signaling plays a role in inducing GCMA expression.

The envelope gene of Human Endogenous RetroVirus W (HERV-W) syncytin is one of the placental targets of GCMA (30). It is known that GCMA-mediated stimulation of syncytin gene expression leads to higher amounts of trophoblast cell-associated connexin 43 immunoreactivity (15). Therefore, in order to gain *in vivo* support for the biological role of CREB and OASIS we examined if a higher Cx43 immunoreactivity is detectable in BeWo cells which were transfected with CREB- or OASIS-encoding expression plasmids. This was indeed the case. We proved specificity by demonstrating failure to increase Cx43 immunoreactivity by co-transfection of dominant-negative GCMA220 or A-CREB, or by CREB- or OASIS-specific shRNA into the same BeWo cells (Figure 6).

OASIS was also shown to be expressed in the placenta but its role there is unsettled (39). In astrocytes overexpression of OASIS is associated with the suppression of ER stress-induced cell death. Dysregulation of ER stress genes such as OASIS is followed by a loss of protective cellular responses and eventually cell death (40). Until now, there are no data available whether the transcription factor OASIS is involved in such processes in the placenta. Considering that placental trophoblasts at various stages of differentiation exhibit morphological changes, such as swelling of the mitochondrial intracristal space and ER, which are related to trophoblastic stress (41), it might well be that, conversely, the GCMA-dependent expression of placental fusogenic glycoprotein syncytin-1 serves anti-apoptotic function under certain conditions, *p.ex.* lower activation of caspase 3 in the apoptotic cascade (42).

In fact, the involvement of Cx43 is intriguing, since Cx43 was reported to contribute via cAMP signaling to organogenesis and differentiation of a variety of cellular systems (43). This might implicate that the stimulation of GCMA expression in trophoblast cells occurs as an autoregulation loop such that cAMP signaling stimulates GCMA expression which in response induces via Cx43 gene expression and cAMP signaling GCMA itself.

Finally, by summarizing these results and reported data it can be concluded that GCMA activity is regulated at different levels. First, GCMA activity is controlled by its transcriptional rate through bZIP-type mediated gene

regulation (this study). Second, GCMA activity is increased by stabilizing GCMA protein (15,16). Third, GCMA activity is modulated through sumoylation (18). Fourth, GCMA activity is decreased by protein degradation (44). In addition, GCMA activity might be regulated by subcellular localization (45).

In total, our data demonstrate that an intricate network of bZIP-type transcription factors regulates the GCMA promoter. Further experiments are required to understand the temporal and sequential pattern of bZIP-type transcription factor action to ensure required GCMA expression for functional placentogenesis.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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