Nuclear Factor Erythroid-derived 2 (Nfe2) Regulates JunD DNA-binding Activity via Acetylation

A NOVEL MECHANISM REGULATING TROPHOBLAST DIFFERENTIATION  

From the Department of Internal Medicine I and Clinical Chemistry, University of Heidelberg, INF 410, 69120 Heidelberg, Germany, the Department of Neurobiology, Interdisciplinary Center for Neurosciences (IZN) University of Heidelberg, INF 364, 69120 Heidelberg, Germany, the Institute for Biochemistry, Emil-Fischer-Center, University of Erlangen-Nürnberg, Fahrstrasse 17, 91054 Erlangen, Germany, the Department of Cardiology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, 430030 Wuhan, China, and the Department of Clinical Chemistry and Pathobiochemistry, Otto-von-Guericke-University Magdeburg, Leipziger Strasse 44, 39120 Magdeburg, Germany

**Background:** Nfe2 restricts Gcm1 expression, placental vascularization, and embryonic growth.

**Results:** Nfe2 induces hypoacetylation of JunD, thus limiting JunD binding to the Gcm1 promoter (at −1441).

**Conclusion:** In trophoblast cells Nfe2 negatively controls Gcm1 expression and syncytiotrophoblast formation by repressing JunD-binding activity.

**Significance:** This identifies a novel, acetylation dependent interaction of bZip transcription factors regulating placental and embryonic development.

We recently demonstrated that the bZip transcription factor nuclear factor erythroid-derived 2 (Nfe2) represses protein acetylation and expression of the transcription factor glial cell missing 1 (Gcm1) in trophoblast cells, preventing excess syncytiotrophoblast formation and permitting normal placental vascularization and embryonic growth. However, the Gcm1 promoter lacks a Nfe2-binding site and hence the mechanisms linking Nfe2 and Gcm1 expression remained unknown. Here we show that Nfe2 represses JunD DNA-binding activity to the Gcm1 promoter during syncytiotrophoblast differentiation. Interventional studies using knockdown and knockin approaches show that enhanced JunD DNA-binding activity is required for increased expression of Gcm1 and syncytiotrophoblast formation as well as impaired placental vascularization and reduced growth of Nfe2−/− embryos. Induction of Gcm1 expression requires binding of JunD to the −1441 site within the Gcm1 promoter, which is distinct from the −1314 site previously shown to induce Gcm1 expression by other bZip transcription factors. Nfe2 modulates JunD binding to the Gcm1 promoter via acetylation, as reducing JunD acetylation using the histone acetyltransferase inhibitor curcumin reverses the increased JunD DNA-binding activity observed in the absence of Nfe2. This identifies a novel mechanism through which bZip transcription factors interact. Within the placenta this interaction regulates Gcm1 expression, syncytiotrophoblast formation, placental vascularization, and embryonic growth.

We recently identified a novel function of the bZip transcription factor Nfe2 in trophoblast cells (1). In trophoblast cells Nfe2 prevents excess syncytiotrophoblast formation during the late pregnancy stages in the mouse (after day 14.5 p.c.,2 corresponding to the 3rd trimester), thus ensuring sufficient vascularization of the placenta and normal embryonic growth (1). This new function of Nfe2 may provide novel insight into mechanisms underlying placental dysfunction and intrauterine growth restriction. These are frequent but incompletely understood pregnancy complications not only with increased peripartal morbidity and mortality of the newborn, but also with diseases such as diabetes mellitus or cardiovascular diseases later on in adult life (2, 3). Hence, deciphering the mechanism through which Nfe2 modulates trophoblast differentiation and placental development and function may reveal new diagnostic or therapeutic targets for placental dysfunction and intrauterine growth restriction.

Nfe2 is the larger (45 kDa) subunit of the heterodimeric transcription factor NF2. Until recently expression of Nfe2 was thought to be restricted to hematopoietic cells (cells of the megakaryocyte, erythrocyte, or mast cell lineage) (4). We identified a function of Nfe2 in nonhematopoietic cells (1). In trophoblast cells Nfe2 represses Gcm1 (also referred to as GCMa) activity (1). Gcm1 belongs to a unique family of transcription factors hallmarked by a highly conserved zinc-coordinated DNA-binding domain (5). Gcm1 is required for branching and intrauterine growth restriction, and both loss and gain of Gcm1 function disturb syncytiotropho-

---

1 To whom correspondence should be addressed. Tel.: 49-0-391-13900; Fax: 49-0-391-13902; E-mail: berend.isermann@med.ovgu.de or ikcp@med.ovgu.de.

2 The abbreviations used are: p.c., post coitus; Nfe2, nuclear factor erythroid-derived 2; CREB, cAMP-response element-binding protein; TSA, trichostatin A; HDAC, histone deacetylase; TS, trophoblast stem; HAT, histone acetyltransferase; kd, knockdown.
blast formation and placental development (1, 6). Loss of Nfe2 function increases acetylation of histone H4 within the Gcm1 promoter and of Gcm1 itself, enhancing Gcm1 expression in murine placenta and trophoblast cells (1). Although these studies identified a novel function of Nfe2 as a repressor during syncytiotrophoblast differentiation, the positive gene regulators repressed by Nfe2 during normal trophoblast and placental differentiation remain unknown. Based on in silico analyses of the Gcm1 promoter the 6000-bp 5’ of the ATG lack potential Nfe2-binding sites, indicating that Nfe2 must regulate Gcm1 expression through an indirect mechanism. Interaction of Nfe2 with CBP or other bZip transcription factors has been reported and may be mechanistically linked to the developmental function of Nfe2 (1, 7, 8).

Indeed, bZip transcription factors have established functions for Gcm1 expression and placental development. Thus, the bZip transcription factors CREB and OASIS stimulate Gcm1 expression in vitro and enhance expression of syncytiotrophoblast markers in vivo (9). The relevant cAMP response element (CRE)-binding site was located at −1337 within the mouse Gcm1 promoter. In vivo, expression of CREB was replaced by expression of OASIS around late midgestation (E13.5), at which time the hemochorial placenta is fully established. This demonstrates a dynamic regulation of bZip transcription factors during placental development.

Other bZip transcription factors with known function during placentation belong to the AP-1 proteins. Knockout studies in mice revealed embryonic lethal placental defects in JunB and Fra1-deficient embryos (10, 11), whereas placental development was normal in mice lacking other AP-1 proteins. Placental JunB or Fra1 deficiency impairs vascularization of the labyrinthine layer, causing early embryonic lethality between days 8.5 and 10.5 p.c. (10, 11).

Whether Nfe2 regulates syncytiotrophoblast formation and vascularization of the placental labyrinthine layer through an interaction with TORC1, OASIS, JunB, Fra1, or yet to be identified transcription factors remained hitherto unknown. Within the current study we explored the interaction partners of Nfe2 during placental development, identifying a so far unknown interaction of Nfe2 with JunD. During placental development Nfe2 regulates through an acetylation dependent mechanism JunD-binding activity to the −1441 site of the Gcm1 promoter, regulating syncytiotrophoblast formation, placental vascularization, and embryonic growth.

**EXPERIMENTAL PROCEDURES**

**Materials**—Heparin sodium salt from porcine intestinal mucosa, human acidic fibroblast growth factor 4 (aFGF4), trichostatin A (TSA), Polybrene, and puromycin dihydrochloride from Streptomyces alboniger were obtained from Sigma. All cell culture media, FBS, glutamine, penicillin/streptomycin mixture, neurymycin, nonessential amino acids, and sodium pyruvate were from PAA, Cölbe, Germany. P24 tag antigen ELISA and the CHIP assay kit were from BioCat, Heidelberg, Germany. ECL reagent was from Amersham Biosciences. Murine Nfe2 and JunD shRNA constructs were from Open Biosystems, Huntsville, AL. GoTaq DNA polymerase, AP-1, and CREB consensus oligonucleotides for EMSA were obtained from Promega. Rabbit polyclonal IgG antibodies against Nfe2, c-Jun, JunD, JunB, FosB, and Fra1 were purchased from Santa Cruz Biotechnology. Rabbit polyclonal IgG antibody against acetylated lysine was purchased from Cell Signaling Technology, Boston, MA. CellMask Deep Red stain was bought from Invitrogen. QuikChange XL Site-directed Mutagenesis kit was purchased from Stratagene. All primers were synthesized by Thermo Scientific, Langenselbold, Germany.

**Plasmids**—Murine JunD expression (pCMV-JunD-SPORT6) and JunD knockdown (pLKO1-JunD) constructs were purchased from Open Biosystems. The JunD expression plasmid pLV-JunD was generated by replacing the EGFP fragment (EcoRI/Xhol) of the plasmid pLV-EGFP (kindly provided by Masahito Ikawa, Osaka, Japan) (12) with the EcoRI/Xhol fragment of pCMV-JunD-SPORT6. To demonstrate specificity of the knockdown experiment six nucleotides within the JunD shRNA hybridization sequence (5’-ACT CGA GTT GAG CAC CCC AAG ATC CCG CTT TTT TTG G-3’) were replaced in pLV-JunD (5’-ACT CGA GTT CAA CCG AAA ATC CGC CTG TTC G-3’) by site-directed mutagenesis. This resulted in silent mutations yielding a JunD resistant to the shRNA used (pLV-JunD-shRes).

Reporter constructs consisting of various fragments of the murine Gcm1 promoter (starting at −4041, −2892, −1637, and −396 relative to ATG) linked to the luciferase coding sequence (Promega, Mannheim, Germany) have been previously described (9). Gcm1 promoter constructs containing mutated AP-1-binding sites were generated by site-directed mutagenesis at positions −1572/−1571 (AC to TG), −1434/−1433 (TC to AG), and −1310/−1309 (AC to TG). The Gcm1 −1637 PGL2 luciferase reporter construct was used as the template. The following primers were used: gcm-1 −1572, AC to TG (5’-GGT TGT TGT TGG TGT TGT GTA CTT TAT TTA TCA-3’ and 5’-AGG GAA ATA AAA CTG TAC TAT ACA CAA GCA TCA CAG CAC ACC-3’); gcm-1 −1434, TC to AG (5’-CCC AGC GTG GCC TCT GAT AGA TTAG TAA GAA TTT ACT CAA-3’ and 5’-TGG ACT GAA TTA TTG TTA TTA CCA TCT ATG AGC GCC GTG GGG GGG-3’); gcm-1 −1310, AC to TG (5’-GCC TGG TCT ATA GGA GGT CTG TGC TCC CAT CTC AAA AAA CTA-3’ and 5’-TAG TCT TGA GAT GGA CAG ACC TTC TAT AGA CCA GGC-3’). All mutations were generated using the QuikChange® II Site-directed Mutagenesis kit (Stratagene) and identity of all mutations was confirmed by sequencing.

**Mice**—Nfe2-deficient mice were kindly provided by R. Shvidrasani (Harvard Medical School, Cambridge, MA). All animals were housed and the experiments were performed at the Interdisciplinary Biomedical Research institution (IBF) of the University of Heidelberg, Germany. For in vivo intervention studies we used TSA (15 μg/mouse (13)) or valproic acid (sodium salt, 155 mM, 25 ml/kg of body weight (14)) as HDAC (histone deacetylase) inhibitors and epigallocatechin-3-gallate (50 mg/kg in saline (15)) or curcumin (0.5 mg/kg (16)) as HAT inhibitors. Animal experiments were conducted following standards and procedures approved by the local Animal Care and Use Committee (Regierungspräsidium Karlsruhe, Germany).
Cell Culture—HEK293T and HEK293 cells were kindly provided by Dr. Peter Seeburg (Max-Planck-Institute for Medical Research, Heidelberg, Germany). They were used to generate lentiviral particles and perform the luciferase assay. These cells were maintained in DMEM supplemented with 4.5 g/ml of glucose, 10% FBS, 2 mM L-glutamine, 50 μg/ml of penicillin/streptomycin, and 4 μg/ml of neomycin at 37 °C in a humidified incubator with 5% CO₂. For transfection experiments medium without antibiotics was used.

Mouse trophoblast stem (TS) cells were obtained from J. Rossant (Hospital for Sick Children, Toronto, Ontario, Canada) and were induced to differentiate in plain TS cell medium (RPMI 1640 supplemented with 25 mM HEPES, 2.0 g/liter of NaHCO₃, 20% FBS, 1 mM sodium pyruvate, 100 μM β-mercaptoethanol, 2 mM L-glutamine, and 50 μg/ml of penicillin/streptomycin) or maintained as stem cells in complete TS cell medium (30% plain TS cell medium plus 70% fibroblast-conditioned TS cell medium, 25 ng/ml of FGF4, and 1 μg/ml of heparin) as described previously (1, 17–19). In some experiments TS cells were treated with 100 nM TSA or 2.5 mM sodium butyrate for HDAC inhibition (20) or 30 nM curcumin or 5 μM epi-galactosein-3-gallate for HAT inhibition (21–23).

In Vitro Syncytium Formation—Syncytiotrophoblast formation was monitored in nearly confluent TS cells differentiated for 6 days, washed with PBS, incubated with 2.5 μM CellMask Deep Red for 5 min at 37 °C, and then fixed for 3 min in 3.75% formaldehyde at RT. Cells were counterstained with 0.5 μg/ml of DAPI in PBS for 10 min at RT in the dark. For quantification of syncytiotrophoblasts at least 30 random images per slide were captured (University of Texas Health Science Center San Antonio ImageTool software) and a blinded investigator determined the frequency of syncytial cells in these random images (1).

Production of Lentiviral Particles—Highly purified plasmid DNA prepared by alkaline lysis preparation was used for lentiviral particle production. Vesicular stomatitis virus-G pseudotyped lentiviral particles were generated as described (1). Briefly, HEK 293T cells were transfected with pLKO1-JunD, pLV-JunD, or pLV-JunD-shRes plasmids together with the packaging plasmid (pCMV-dr8.91) and vesicular stomatitis virus-G-expressing plasmid using the standard calcium phosphate method. Lentiviral particles were harvested 2 and 3 days after transfection and concentrated by ultracentrifugation (50,000 × g, 2 h, 2 times). After resuspension in HBSS buffer the viral particle concentration was determined by measuring the p24 gag antigen by ELISA according to the manufacturer’s instruction (BioCat, Heidelberg, Germany). Viral particles were stored at −80 °C.

Luciferase Assay—The HEK293 cells were transfected in 60-mm dishes with 1 μg of luciferase reporter construct and 0.1 μg of CMV driven expression vectors using a standard calcium phosphate method. At 48 h post-transfection, cells were harvested for luciferase assays using the luciferase assay system according to the manufacturer’s instruction.

Electrophoretic Mobility Shift Assay (EMSA)—Nuclear proteins were isolated and binding activity of CREB and AP-1 was determined as described previously (1, 25). Briefly, for isolation of nuclear proteins cells were washed in PBS and resuspended in 200 μl of ice-cold buffer A (10 mM HEPES-KOH, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF). For tissue extracts the tissue samples were homogenized in 400 μl of ice-cold buffer B (10 mM HEPES-KOH, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF, 1 mM EDTA, 0.6% Nonidet P-40), vortexed for 10 s, and incubated on ice for 10 min. Samples were centrifuged at 12,000 × g for 2 min and the supernatant was discarded. The pellet was either resuspended in 100 μl of buffer C (cell lyses: 20 mM HEPES-KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF) or 200 μl of buffer D (tissue lyses: 20 mM HEPES-KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, 0.2 mM benzamidine, 5 μg/ml of leupeptin) and incubated on ice for 20 min. After centrifugation (12,000 × g, 5 min, 4 °C) the supernatant was transferred to a new tube, protein concentration was measured by colorimetric assay (BSA), and samples were quick-frozen at −80 °C. Binding of transcription factors to 1 ng of radiolabeled oligonucleotides (AP-1 consensus: 5'–CGC TTG ATG ACT CAG CCG GAA–3', JunD-binding site at −1441 derived from Gcm1: 5’–G GTG GCC TTC TGA TTT ATT GTA AG–3', JunD mutated (TC to AG), derived from Gcm1 at −1441: 5’–G GTG GCC TTC TGA TAG ATT GTA AG–3', CREB consensus: 5’–AGA GAT TGC CTG ACG TCA GAG AGC TAG–3') was performed for 20 min at RT in 10 mM HEPES, pH 7.5, 0.5 mM EDTA, 100 mM KCl, 2 mM DTT, 2% glycerol, 4% Ficoll, 0.25% Nonidet P-40, 1 mg/ml of BSA, and 0.1 mg/ml of poly(dI/dC). Protein-DNA complexes were separated on 5% native polyacrylamide gels containing 3.25% glycerol and 0.5× Tris/boric acid/EDTA buffer. Identity of AP-1 subunits was determined by supershift experiments. To this end 4 μg of antibody against AP-1 subunits (Fra1, JunB, c-Jun, JunD, and FosB) were preincubated with 10 μg of protein extract for 20 min at RT before adding radiolabeled nucleotides.

Transduction of Preimplantation Embryos—Female Nfe2−/− or wild type (WT) mice were superovulated by intraperitoneal injection of pregnant mare’s serum gonadotropin (5 units) followed by human chorionic gonadotropin (5 units) 48 h later and then mated with Nfe2−/− or WT males, respectively. Two-cell stage embryos were collected from the females at day 1.5 p.c. and incubated for 2 days to obtain blastocysts. The zona pellucida was removed with acidic Tyrode solution. Zona pelucida-free embryos were incubated individually in 50 μl of KSOM medium containing JunD with either shRNA or JunD expressing lentiviral particles (1 × 10⁵ ng of p24/ml) for 4 h (1, 12). Transduced blastocysts were transferred into pseudo-pregnant females. The transfer day was counted as day 3.5 p.c. and analyses were performed at day 14.5 or 16.5 p.c. Embryos generated from blastocysts transduced with lentiviral particles generated from nonspecific shRNA or empty vector served as controls.

Morphological Analyses of Placenta—Placental tissues were obtained from Nfe2−/− or WT females after lentiviral transduction at day 14.5 p.c. Half of the placental tissue was fixed in 4% buffered formalin, whereas the other half was further divided and stored either in RNAlater or quick frozen in liquid nitrogen for protein isolation. The fixed tissue was embedded in paraffin, sectioned, and stained with hematoxylin and eosin.
For vascular space quantification randomly selected microscopic fields (magnification ×10) of the labyrinthine region from 3 nonconsecutive placental sections (each section ≥10 images) were acquired at 1280 × 960 resolution using a digital camera (Nikon digital sight DS-U1) connected to a light microscope (Nikon Eclipse TE2000). We implemented an image analysis routine using NIS Elements AR2.30 imaging software.

Briefly, after acquisition, the images underwent an automated analysis procedure and were given a color threshold to cover the area corresponding to the lumen of blood spaces. The coverage percentage was calculated as the ratio between the number of pixels covered by the area defined by the threshold and the overall number of pixels in the image (1). A blinded investigator performed analyses.

**Transmission Electron Microscopic Analyses of Placenta**—Placenta tissues obtained at day 16.5 p.c. were fixed with 2.5% glutaraldehyde, 2.5% polyvinylalcohol 25, 0.1 M sodium cacodylate, pH 7.4. After washing with 0.1 M sodium cacodylate buffer, pH 7.4, samples were post-fixed with 2% osmium tetroxide, 1.5% potassium ferrocyanide in 0.1 M sodium cacodylate buffer, pH 7.4, for 1 h, washed, contrasted en bloc with uranyl acetate, dehydrated with an ascending series of ethanol, and embedded in glycid ether 100-based resin. Ultrathin sections were cut with a Reichert ULTRACUT S ultramicrotome (Leica Microsystems, Wetzlar, Germany), contrasted with uranyl acetate and lead citrate, and viewed with an EM 10 CR electron microscope (Carl Zeiss NTS, Oberkochen, Germany).

**Gene Knockdown and Knockin in Mouse Trophoblast Cells**—Generation of stably transfected Nfe2 knockdown mouse trophoblast cells (Nfe2kd) has been previously described (1). Transient knockdown or knockin of JunD in control, Nfe2kd, or Nfe2kd JunDkd TS cells was performed by lentiviral transduction. Briefly, pLKO1-JunD, pLV-JunD-shRes, or pLV-JunD viral particles (5 × 10⁵ TU/ml) were generated as described above and used to infect Nfe2kd (yielding Nfe2kd JunDkd cells), Nfe2kd JunDkd (yielding Nfe2kdJunDkdRes cells), or control (yielding Nfe2kd JunD cells) TS cells, respectively, in the presence of 8 μg/ml of Polybrene in TS cell medium. The efficiency of the JunD knockdown or knockin was determined after 3 days of differentiation by RT-PCR and EMSA. In all experiments cells transduced with viral particles generated from nonspecific shRNA or empty pLV-CAG1.1 plasmid served as controls.

**Quantitative Real Time PCR (qRT-PCR)**—RNA was isolated from mouse placental or TS cells. Placental tissues were stored initially in RNAlater and placed into TRIzol for RNA isolation, whereas TRIzol was added directly to TS cells. Following incubating with TRIzol (5 min at RT), 0.2 ml of chloroform/1 ml of TRIzol was added, mixed, and centrifuged (12,000 × g, 15 min, 4 °C). The aqueous phase containing the RNA was transferred to a fresh tube and RNA was precipitated by adding 0.5 ml of 2-propanol/1 ml of TRIzol reagent initially used. Samples were incubated at RT (10 min) and centrifuged (12,000 × g, 10 min, 4 °C). Supernatant was removed and RNA was washed by adding 1 ml of 75% ethanol/1 ml of TRIzol. Samples were vortexed, centrifuged (7,500 × g, 5 min, 4 °C), the RNA pellet was air dried (5 min), dissolved in 20 μl of diethyl pyrocarbonate/water, and incubated for 10 min at 55 °C. The RNA concentration was measured in a photometer, and RNA purity and integrity were verified on a 1.8% agarose gel. cDNA was synthesized according to the manufacturer’s protocol (SuperScript First-Strand Synthesis System). All primers were custom synthesized by Thermo Fisher Scientific and quantitative RT-PCR was performed as described previously (1, 26).

The following primer sequences were used for amplification: actin, 5'-CTA GAC TTC GAG CAG GAG ATG G-3' and 5'-GCT AGG AGC GAG AGC AGT AAT C-3'; Cebpa, 5'-CGC TGG TGA TCA AAC AAG AG-3' and 5'-GTC ACT GGT CAA CTC CAG CA-3'; Gcm1, 5'-GAC TGC TTC CCA ACA GAG GAA GG-3' and 5'-GGG GAG CCA TAG GTG AGC AG-3'; Synb, 5'-TGA CCT TGA AGT GGG TAG GG-3' and 5'-TGA CCT TGA AGT GGG TAG GG-3'; Syna, 5'-GGT TGG TCC TTG TTT TCT-3' and 5'-GGA GTG GGG TGG ATT CAA TGG T-3'; and Cstq, 5'-TTC CTG TAG GTG CCA CCA TAG CAT TGT GCA AA-3')

**Chromatin Immunoprecipitation**—Chromatin immunoprecipitation (ChIP) was performed on placental tissue extracts using the Epiquik ChIP assay kit for tissue following the manufacturer’s instructions. Briefly, tissue samples were minced into small fragments using a scalpel and exposed to 1% formaldehyde in TS cell medium for cross-linking (20 min at RT on a rocking platform). Tissue extracts were washed in 125 mM glycine in TS cell medium, centrifuged, and the pellet was digested using a Dounce homogenizer in 1 ml of homogenization buffer/200 μg of tissue. Following centrifugation the pellet was resuspended in lysis buffer CP3 containing protease inhibitors and incubated on ice for 10 min. DNA was sheared by sonication (5 intervals, each 7 s with 5-s breaks). Following centrifugation (8,000 × g, 10 min, 4 °C) the supernatant was diluted with buffer CP4, an aliquot was removed as “input” control, and from the remaining solution proteins were precipitated using rabbit polyclonal anti-JunD IgG antibody. Following incubation (60 min at RT) supernatants were removed and the precipitates were washed 6 times (buffer CP1). DNA was released using proteinase K in buffer CP5 (15 min at 65 °C) and then captured, washed, and finally eluted using spin columns. Purified DNA was stored at −20 °C until final analyses. Recovered DNA was analyzed by PCR using the following primers for the various mouse Gcm1 promoter regions: −3150 to −2950 (5'-GAA GAA ACC ACC TAA AGC TA-3' and 5'-GGG AAG ACT CAC CCA ACT CA-3'), −2930 to −2720 (5'-AGG ATG CTG TAT AAG CCC TCC TTC-3' and 5'-CCT CAG GCA TGC TAT GGG TGA-3'), −2140 to −1930 (5'-TGG GAA AAT AGT ATG AGA TTC ATC ACC CAG CAT ACT-3' and 5'-GAA ACC ACC TCT GCA ATC-3'), −1910 to −1720 (5'-CAG AAT GTA ATA TCT AGG GA-3' and 5'-GGG GTA GCT GAC CCT ACC TG-3'), −1630 to −1460 (5'-ACG GAG GCG ACA AAC AC-3' and 5'-CTA TAT TAC GCT TGA GGC GC-3'), −1450 to −1300 (5'-CAG CGT GGC CTC TGA TTC AT-3' and 5'-GAG ATG GGA GTG ACC TCC TTC TA-3'), and −1100 to −900 (5'-AAA AAC CCT TCG ACT CAG AG-3' and 5'-TTA TCA TTT TGG CTA ATC CA-3'). Cycling conditions were 35 cycles with an annealing temperature of 58 °C.

**Statistical Analysis**—All in vitro experiments were performed at least three times in duplicates. All data were expressed as mean ± S.E. Statistical analyses were performed using Student’s t test or analysis of variance as appropriate. Post
Nfe2 Regulates JunD Binding Activity via Acetylation

RESULTS

Nfe2 Modulates AP-1 Binding in Murine Placenta—We have recently shown that Nfe2 suppresses Gcm1 expression and syncytiotrophoblast formation in trophoblast cells. Considering the negative gene regulatory function of Nfe2 in regard to Gcm1 and the absence of a Nfe2-binding site within the first 6000 bp of the Gcm1 promoter (both in humans and mice) we hypothesized that bZip transcription factor Nfe2 regulates Gcm1 indirectly, potentially by interacting with other bZip transcription factors. To this end we analyzed binding activity to two bZip transcription factor-binding sites, which have been previously shown to regulate placental development and/or Gcm1 expression (CREB and AP-1) (9–11, 27). We observed a marked induction of AP-1 DNA-binding activity (251 versus 100% in Nfe2+/− embryos, p < 0.001), but not of CREB DNA-binding activity (121 versus 100% in Nfe2+/− embryos, p = 0.8, Fig. 1, A and B), in placental tissue nuclear extracts obtained from day 14.5 p.c. Nfe2−/− murine placenta. Next, we performed EMSA using cellular nuclear extracts obtained from control (Nfe2+/+) and Nfe2 knockdown (Nfe2−/−) trophoblast cells differentiated for 3 days. Again, AP-1 DNA-binding activity (206 versus 100% in Nfe2+/+, p < 0.01), but not CREB DNA-binding activity (117 versus 100% in Nfe2+/+, p = 0.12, Fig. 1, C and D), was markedly enhanced in Nfe2−/− samples. These results suggest that in the absence of Nfe2 expression of Gcm1 is induced through an AP-1-dependent mechanism.

Nfe2 Modulates JunD Binding in Murine Placenta and Trophoblast Cells—Given the strong induction of AP-1-binding activity in the absence of Nfe2 and the established role of some AP-1 family members (JunB and Fra1) during placental development (10, 11, 27) we next performed supershift analyses. These revealed enhanced binding activity particularly of the JunD subunit in the absence of Nfe2 both ex vivo (Nfe2−/−, Fig. 1E) and in vitro (Nfe2−/−, Fig. 1F). No relevant contribution of c-Jun, JunB, Fra1, or FosB to the enhanced AP-1-binding activity could be detected. Thus, in the absence of Nfe2, binding activity of JunD is markedly increased. This indicates that Nfe2 may reduce Gcm1 expression and trophoblast syncytium formation by repressing JunD, an AP-1 protein not previously known to be relevant for placental development.

JunD Is Required for Nfe2-dependent Syncytiotrophoblast Formation in Vitro—To explore whether enhanced syncytiotrophoblast formation in the absence of Nfe2 depends on JunD we reduced expression of JunD in trophoblast cells in vitro. Consistent with our previous observation, syncytiotrophoblast formation and expression of Gcm1, Cebpa, and Synb were increased in Nfe2−/− trophoblast cells (Fig. 2, A–C) (1). Following inhibition of JunD using shRNA (supplemental Fig. S1) the frequency of syncytiotrophoblast cells in 6-day differentiated TS cells was normalized (2.7 syncytiotrophoblast cells/random image in Nfe2−/− versus 0.8 in Nfe2−/−JunD−/−, p < 0.01, Fig. 2, A and B). Likewise, expression of Gcm1 (109 versus 183%, p < 0.001), Cebpa (103 versus 138%, p < 0.001), and Synb (108 versus 162%, p < 0.001, Fig. 2C) were normalized in Nfe2 and JunD double knockdown trophoblast cells as compared with Nfe2−/− cells after 3 days of differentiation. In agree-
The interaction of JunD and Nfe2 regulates syncytiotrophoblast formation in vitro. A–C, reduction of JunD expression in Nfe2-deficient trophoblast cells (Nfe2<sup>−/−</sup>JunD<sup>−/−</sup>, gray bars) reduces syncytiotrophoblast formation and expression of syncytiotrophoblast markers, which are characteristically increased in Nfe2-deficient (Nfe2<sup>−/−</sup>, white bars) trophoblast cells, to levels observed in control trophoblast cells (control, black bars). Overexpression of a shRNA-resistant JunD in Nfe2<sup>−/−</sup>JunD<sup>−/−</sup> trophoblast cells reverses the phenotype to that observed in Nfe2<sup>−/−</sup>c cells (Nfe2<sup>−/−</sup>JunD<sup>+/−</sup>res cells; increased syncytiotrophoblast formation and increased expression of Gcm1, Cebpa, and Synb, striped bars) and isolated gain of JunD function (Nfe2<sup>−/−</sup>JunD<sup>+/+</sup>) is sufficient to increase syncytiotrophoblast formation and expression of Gcm1, Cebpa, and Synb (dotted bars). Representative images of 6-day differentiated trophoblast cells stained with CellMask (red) and counterstained with DAPI (blue) (A, scale bar: 20 μm) and the bar graph summarizing results (B, C, bar graph summarizing results of quantitative RT-PCR analyses of the syncytiotrophoblast markers Gcm1, Cebpa, Synb, Syna, and Cstq. Mean value ± S.E., **, * p < 0.01 (analysis of variance).

Nfe2 Regulates JunD Binding Activity via Acetylation

FIGURE 2. The interaction of JunD and Nfe2 regulates syncytiotrophoblast formation in vivo—We have recently shown that Nfe2 restricts syncytiotrophoblast formation, thus regulating vascularization of the labyrinthine layer of the placenta and embryonic growth (1). Given the before mentioned results we next explored whether cross-coupling of Nfe2 with JunD in trophoblast cells represses Gcm1 in vivo. We reduced JunD expression in cells of the trophectodermal lineage by transducing Nfe2<sup>−/−</sup> blastocysts with JunD shRNA expressing lentiviral particles. This resulted in a marked reduction of JunD expression and of JunD DNA-binding activity within the placenta of Nfe2<sup>−/−</sup>/JunD<sup>−/−</sup> embryos as compared with JunD<sup>−/−</sup> embryos (see supplemental Fig. S2). Reduction of JunD expression normalizes placental vascularization within the labyrinthine layer of day 14.5 p.c. placental tissues (93% in Nfe2<sup>−/−</sup>/JunD<sup>−/−</sup> versus 75% in Nfe2<sup>−/−</sup> embryos, p < 0.001, Fig. 3, A and B). Restoration of placental vascularization was associated with a normalization of growth in Nfe2<sup>−/−</sup>JunD<sup>−/−</sup> embryos (weight 97% and length 95% of that of control and Fig. 3C). Furthermore, electron microscopic analyses revealed a normalization of the total width of the feto-maternal placental barrier (108% in Nfe2<sup>−/−</sup>/JunD<sup>−/−</sup> versus 122% in Nfe2<sup>−/−</sup> embryos, p < 0.001, Fig. 3, D and E) and syncytiotrophoblast layer II (110 versus 138%, p < 0.001, Fig. 3, D and E). Using quantitative RT-PCR we observed a normalization of Gcm1 (112 versus 192%, p < 0.001), Cebpa (107 versus 142%, p < 0.001), and Synb (110 versus 172%, p < 0.001, Fig. 3F) expression in Nfe2<sup>−/−</sup> placenta following JunD knockdown in cells of the trophectodermal lineage. In agreement with our previous observations we did not observe any morphological change in syncytiotrophoblast layers I and III, in endothelial cells, or in the expression of Syna or Cstq (Fig. 3F and data not shown) (1).

Nfe2 and JunD Coordinately Control Placental Vascularization and Syncytiotrophoblast Formation in Vivo—We have recently shown that Nfe2 restricts syncytiotrophoblast formation, thus regulating vascularization of the labyrinthine layer of the placenta and embryonic growth (1). Given the before mentioned results we next explored whether cross-coupling of Nfe2 with JunD in trophoblast cells represses Gcm1 in vivo. We reduced JunD expression in cells of the trophectodermal lineage by transducing Nfe2<sup>−/−</sup> blastocysts with JunD shRNA expressing lentiviral particles. This resulted in a marked reduction of JunD expression and of JunD DNA-binding activity within the placenta of Nfe2<sup>−/−</sup>/JunD<sup>−/−</sup> embryos as compared with JunD<sup>−/−</sup> embryos (see supplemental Fig. S2). Reduction of JunD expression normalizes placental vascularization within the labyrinthine layer of day 14.5 p.c. placental tissues (93% in Nfe2<sup>−/−</sup>/JunD<sup>−/−</sup> versus 75% in Nfe2<sup>−/−</sup> embryos, p < 0.001, Fig. 3, A and B). Restoration of placental vascularization was associated with a normalization of growth in Nfe2<sup>−/−</sup>JunD<sup>−/−</sup> embryos (weight 97% and length 95% of that of control and Fig. 3C). Furthermore, electron microscopic analyses revealed a normalization of the total width of the feto-maternal placental barrier (108% in Nfe2<sup>−/−</sup>/JunD<sup>−/−</sup> versus 122% in Nfe2<sup>−/−</sup> embryos, p < 0.001, Fig. 3, D and E) and syncytiotrophoblast layer II (110 versus 138%, p < 0.001, Fig. 3, D and E). Using quantitative RT-PCR we observed a normalization of Gcm1 (112 versus 192%, p < 0.001), Cebpa (107 versus 142%, p < 0.001), and Synb (110 versus 172%, p < 0.001, Fig. 3F) expression in Nfe2<sup>−/−</sup> placenta following JunD knockdown in cells of the trophectodermal lineage. In agreement with our previous observations we did not observe any morphological change in syncytiotrophoblast layers I and III, in endothelial cells, or in the expression of Syna or Cstq (Fig. 3F and data not shown) (1).
Nfe2 Regulates JunD Binding Activity via Acetylation

**FIGURE 3.** The interaction of Nfe2 and JunD regulates placental vascularization and syncytiotrophoblast formation in vivo. A and B, impaired placental vascularization, which is observed in the absence of embryonic Nfe2 (Nfe2+/−; cshRNA, white bars), is normalized following suppression of JunD expression in cells of the trophodermal cell lineage (compare Nfe2+/−; JunDkd, gray bars, with control Nfe2+/−; cshRNA, black bars). Isolated overexpression of JunD is sufficient to impair placental vascularization (Nfe2−/− JunD+, striped bars, compare with Nfe2−/− cshRNA, black bars). Representative hematoxylin and eosin-stained sections of placental tissue (A) and bar graph summarizing results of vascularization analyses (B, n = 6 per group). C, embryonic length of Nfe2−/− embryos is normalized following reduction of JunD expression in cells of the trophodermal cell lineage, whereas isolated gain of JunD function (Nfe2−/− JunD+, striped bars) is sufficient to improve embryonic length. D and E, the characteristic enhanced width of syncytiotrophoblast layer II (SynT-II, marked with a white star) in the absence of Nfe2 (Nfe2+/−; cshRNA), which is associated with an increased width of the total embryonic-maternal barrier (total), is reversed following reduction of JunD expression in Nfe2−/− embryos (Nfe2−/− JunDkd, gray bars). Isolated gain of JunD function is sufficient to increase the total width of the embryonic-maternal barrier and the width of syncytiotrophoblast layer II (Nfe2−/− JunD+, striped bars). For control Nfe2−/− embryos were infected with lentiviral particles expressing control shRNA (Nfe2−/− cshRNA). EM images of the maternal embryonic barrier (D) and bar graph summarizing results of EM analyses (E, 6 embryos per group, for each embryo at least 50 images were analyzed) are shown. F, reduction of JunD normalizes expression of syncytiotrophoblast markers in Nfe2−/− placenta (gray bars), whereas isolated overexpression of JunD is sufficient to increase expression of these markers (striped bars). Bar graph summarizing results of qRT-PCR analyses of Gcm1, Cebpα, Synba, Syna, and Cstq expression in placenta tissue samples (n = 6 embryos each) is shown. Mean value ± S.E., size bars are 50 μm (A) and 1 μm (D); *, p < 0.05, **, p < 0.01 (compared with Nfe2+/− cshRNA, analysis of variance).
expression construct (JunD), or both JunD and Nfe2 expression constructs (JunD + Nfe2). The −4041, −2982, and −1637 reporter constructs, but not the −396 reporter construct, contained inducible JunD-binding site(s). Hence, the functional relevant JunD-binding site must be within the −1637 to −396 promoter fragment. Of note, co-transfection with Nfe2 suppressed JunD-binding activity, directly demonstrating the repressive effect of Nfe2 in regard to JunD driven Gcm1 promoter activation.

Considering that the −1637 to −396 region contained inducible JunD-binding site(s) and that, based on the above ChIP analyses, JunD binds to the −1630 to −1460 and the −1450 to −1300 promoter regions, but not to the −1100 to −900 promoter fragment (see above, Fig. 4B), we hypothesized that the functional relevant JunD-binding site(s) must reside within the −1637 to −1300 region of the Gcm1 promoter. This region contains three potential JunD-binding sites (at −1576, −1441 and −1314). To determine their functional relevance for Gcm1 expression these sites were inactivated by targeted point mutations. JunD-mediated induction of the reporter construct containing the mutated JunD-binding site at −1441 was reduced to 45% (p = 0.02, Fig. 4D), whereas induction of the reporter construct containing the mutated JunD-binding site at −1576 or −1314 was not significantly reduced (94 and 88% compared with the control construct, p = 0.48 and 0.26, respectively, Fig. 4D).

To establish whether JunD binds to the endogenous Gcm1 promoter sequence we next performed EMSA using an oligonucleotide derived from the Gcm1 sequence (−1426 to −1448). Binding of nuclear extracts to this oligonucleotide was enhanced in the absence of Nfe2 (Nfe2<sup>kd</sup>, Fig. 4E). Using a mutated oligonucleotide (CT → AG) no binding activity could be detected (Fig. 4E). Supershift analyses confirmed binding of JunD to the Gcm1-derived oligonucleotide (Fig. 4E). Taken
Nfe2 Regulates JunD Binding Activity via Acetylation

**FIGURE 5. Modulation of JunD-binding activity by Nfe2 is acetylation dependent.** A and B, acetylation of JunD is enhanced in the absence of Nfe2 (ex vivo, −/−; in vitro, kd). Enhanced acetylation is reversed following intervention with the HAT inhibitor curcumin (Cu), both in placental samples (ex vivo) and in trophoblast cells (in vitro). Treatment with the HDAC inhibitor TSA (TS) is sufficient to increase JunD acetylation both ex vivo and in vitro. Immunoprecipitation using an antibody against acetylated lysine and immunoblotting for JunD (JunDinput, A, bottom). B, bar graph summarizing results. C and D, enhanced JunD DNA-binding activity (supershift analyses, C) and JunD binding to the mGcm1 promoter (analyses of the −1450 to −1300 mGcm1 promoter region by ChIP assay, D) in the absence of Nfe2 is acetylation dependent. Enhanced JunD DNA-binding activity and binding to the mGcm1 promoter in the absence of Nfe2 (ex vivo, −/−; in vitro, kd) is reversed following treatment with the HAT inhibitor curcumin in placental samples (C and D, ex vivo) and trophoblast cells (C and D, in vitro). Conversely, increasing acetylation using the HDAC inhibitor TSA (TS) is sufficient to increase JunD DNA-binding activity and JunD binding to the mGcm1 promoter both ex vivo (C and D) and in vitro (C and D). Representative images of JunD supershifts (C) and ChIP assay using an anti-JunD antibody for DNA precipitation (D) are shown.

together, JunD binds to the Gcm1 promoter at −1441, which is required for JunD-mediated Gcm1 promoter activation.

**Regulation of JunD-binding Activity by Nfe2 Is Acetylation Dependent**—We have previously shown that the regulation of Gcm1 by Nfe2 is acetylation dependent (1). Given the current finding that JunD is crucially involved in the regulation of Gcm1 we next evaluated the role of acetylation for the Nfe2-dependent regulation of JunD.

Using immunoprecipitation we detected a marked enhancement of JunD acetylation in Nfe2-deficient placenta (ex vivo: Nfe2−/−, c, Fig. 5, A and B) or trophoblast cells (in vitro: Nfe2kd, c, Fig. 5, A and B). Inhibition of acetylation using the HAT inhibitors curcumin or epigallocatechin-3-gallate reduced acetylation of JunD (103 versus 175% in Nfe2−/− placenta, p < 0.001, 98 versus 165% in Nfe2−/− trophoblast cells, p < 0.001, Fig. 5, A and B, and data not shown) and JunD-binding activity in Nfe2-deficient placenta (Nfe2−/−) and trophoblast (Nfe2kd) cells (Fig. 5C). Conversely, enhancement of acetylation by inhibition of HDAC activity using trichostatin A (TSA, TS), valproic acid (in vitro, data not shown), or sodium butyrate (in vitro, data not shown) increased acetylation of JunD (182 versus 100% in Nfe2−/− placenta, p < 0.001, 158 versus 100% in Nfe2−/− trophoblast cells, p < 0.001, Fig. 5, B and C) and JunD-binding activity in Nfe2 expressing placenta (Nfe2+/+/) and trophoblast cells (Nfe2+) (Fig. 5C). Modulation of JunD acetylation was associated with corresponding changes of JunD binding to the Gcm1 promoter. Thus, inhibition of acetylation using curcumin reduced JunD binding to the −1450 to −1300 Gcm1 promoter region in Nfe2-deficient placenta (Nfe2−/−, ex vivo) and in Nfe2kd trophoblast cells (in vitro), whereas enhancing acetylation in Nfe2 expressing trophoblast cells using TSA was sufficient to enhance JunD binding to the −1450 to −1300 Gcm1 promoter region both in Nfe2 expressing placenta (Nfe2−/−, ex vivo) and trophoblast cells (Nfe2kd, in vitro, Fig. 5D). These data provide experimental evidence that Nfe2 regulates JunD DNA-binding activity to the Gcm1 promoter via acetylation.

**DISCUSSION**

Previously we identified a novel function of the bZIP transcription factor Nfe2 during trophoblast differentiation. We demonstrated that Nfe2 represses Gcm1 expression, a key regulator of syncytiotrophoblast differentiation and placental vascularization, in trophoblast cells through an unknown modus operandi. In the present study we establish that the AP-1 protein JunD is a positive gene regulator during syncytiotrophoblast differentiation. Nfe2 prevents excess syncytiotrophoblast formation, which would impair placental vascularization and embryonic growth (1) by limiting JunD DNA-binding activity. The Nfe2-dependent repression of JunD DNA-binding activity is mediated via acetylation.

The acetylation dependent cross-coupling of Nfe2 with JunD during trophoblast differentiation provides a novel mechanism...
controlling Gcm1 expression, syncytiotrophoblast formation, placental vascularization, and embryonic growth. A function of other members of the AP-1 protein family, namely JunB and Fra1, for placental development has been previously described (10, 11). The function of JunD must be different from JunB or Fra1. Thus, loss of JunB or Fra1 function results in embryonically lethal defects, whereas in mice with a gain of JunB or Fra1 function no embryonic phenotype has been reported (28). Contrary, loss of JunD function has no impact on embryonic development (29), whereas gain of function impairs placental vascularization and embryonic growth (this study).

In contrast to the nonlethal consequences of increased JunD-binding activity in Nfe2−/− embryos after day 14.5 p.c. the absence of JunB or Fra1 results in a lethal placental defect associated with defective yolk sac vascularization during establishment of the hemochorial placenta (day 8.5 p.c. in JunB−/− and 10.5 p.c. in Fra1−/− embryos) (10, 11). Thus, whereas loss of function of JunB and Fra1 results in early fetal loss, bZip transcription factors Nfe2 and JunD coordinately regulate placental development and embryonic growth at later developmental stages. Such diverse functions of distinct Jun family proteins likely reflect their ability to form various heterodimers with other bZip family proteins resulting in either positive or negative regulation of target genes (28, 30). The newly identified interaction of JunD and Nfe2 expands our knowledge about the role of bZip transcription factors during placental development and demonstrates that this expanding network conveys crucial roles for placentation at various developmental stages.

Transcriptional activation of AP-1 proteins is regulated by MAPKs, in particular by the c-Jun NH2-terminal kinases (JNKs) and the extracellular signal-regulated kinases (ERKs), which phosphorylate AP-1 proteins (31, 32). The molecular mechanism of transcriptional activation of AP-1 proteins by MAPKs remained unknown until recently. Aguilera and colleagues (27) demonstrated that transcriptional activation of the AP-1 protein c-Jun is linked to increased histone acetylation within AP-1-dependent promoters. Nonphosphorylated c-Jun recruits the Mbd3/nucleosome remodeling and histone deacetylase (NurRD) repressor complex through an interaction with its transactivation domain, resulting in gene repression of AP-1 target genes. This repression is relieved by JNK-mediated c-Jun NH2-terminal phosphorylation. In the current study we demonstrate that DNA-binding and transcriptional activity of JunD is likewise modulated by acetylation, indicating that the mechanism identified by Aguilera and colleagues (27) is not specific for c-Jun. We previously demonstrated that acetylation of histone H4 within the Gcm1 promoter is modulated by Nfe2 in trophoblast cells and placenta (1), which is entirely consistent with the observation made by Aguilera et al. (27). In addition, we demonstrate now that the AP-1 protein JunD itself is modulated by acetylation. Whether other AP-1 proteins are directly modulated by acetylation and the relative importance of either histone or AP-1 protein acetylation for transcriptional activation needs to be explored in future studies.

Whether Nfe2-dependent acetylation of JunD depends on MAPK activity remains currently unknown. Unlike c-Jun, JunD lacks a functional JNK docking site within the NH2-terminal region (28). However, following heterodimerization with c-Jun, the serine residues of JunD can be phosphorylated (33), indicating that MAPK-mediated JunD phosphorylation may be involved in the regulation of acetylation. However, whereas regulation of Nfe2 by JNK has been demonstrated (34), the reverse, e.g. regulation of MAPK activity by Nfe2, has to the best of our knowledge not been reported. Hence the mechanism underlying Nfe2-dependent regulation of acetylation remains presently unknown. Based on previous and the current findings we propose a MAPK independent mechanism through which Nfe2 may inhibit acetylation. Interaction of Nfe2 with CBP in hematopoietic and trophoblast cells is established (1, 7, 35). CBP/p300 are coactivators generally influencing gene expression by functioning as scaffolding proteins facilitating protein-protein interactions and/or through their intrinsic acetyltransferase activity toward histone proteins and other nearby nuclear factors (36). Considering that Nfe2 acts as a repressor during trophoblast differentiation we speculate that Nfe2 may inhibit the intrinsic acetylation activity of CBP in trophoblast cells, thus inducing hypoacetylation of JunD and histones within the Gcm1 promoter (1) (and current results). The Nfe2-dependent repression of acetylation may result from a competition of bZip transcription factors for a limited CBP pool (37). Indeed, a competitive interaction between CBP and bZip proteins, including Nfe2 and c-Jun, has been previously reported (38). In addition, we observed a markedly increased interaction between CBP and Gcm1 in the absence of Nfe2 (1), which is in agreement with a competitive interaction of CBP with various transcription factors during trophoblast differentiation. Such competitive interaction with CBP may be the mechanism underlying the functional antagonism between bZip transcription factors like Nfe2 and JunD during trophoblast differentiation and may constitute an alternative, MAPK independent pathway through which AP-1 proteins regulate acetylation and gene expression. The existence of such a competitive mechanism, as proposed, needs to be explored in future studies.

Given the current and previous findings Nfe2 acts as an epigenetic and post-translational repressor during placental development, providing a mechanism limiting syncytiotrophoblast formation. It appears that all mechanisms through which Nfe2 modulates placental vascularization converge at the regulatory level of Gcm1. Thus, the current finding underscores a crucial function of Gcm1 in regulating placental labyrinthine formation (6). This novel, Nfe2-dependent pathway controlling Gcm1 activity is important to ensure sufficient placental vascularization and embryonic growth during later pregnancy stages (1) (and current findings).

Future studies are required to determine whether the same pathway is of functional relevance in the human placenta. We observed expression of Nfe2 in human placenta, specifically in human syncytiotrophoblast cells, and expression of Nfe2 within human syncytiotrophoblast is depicted on the human protein atlas webpage. Interestingly, JunD, but not other AP-1 proteins, is specifically expressed in human syncytiotrophoblast (39). Hence it appears possible, but remains to be shown,
that cross-coupling of Nfe2 and JunD via acetylation constitutes a novel mechanism regulating placental development in humans as well. Providing that the relevance of this pathway can be confirmed in humans this may identify new diagnostic and therapeutic targets for placental diseases associated with altered expression of Gcm1, such as pre-eclampsia.

Acknowledgments—Nfe2-deficient mice were kindly provided by R. Shivdasani (Harvard Medical School, Cambridge, MA). We thank S. Shivdasani (Harvard Medical School, Cambridge, MA). We thank S. Shivdasani (Harvard Medical School, Cambridge, MA). We thank S. Shivdasani (Harvard Medical School, Cambridge, MA). We thank S. Shivdasani (Harvard Medical School, Cambridge, MA)

REFERENCES

1. Kashiﬁ, M., Hellwig, A., Kolleker, A., Shahzad, K., Wang, H., Lang, S., Wolter, J., Thati, M., Vinnikov, I., Bierhaus, A., Nawroth, P. P., and Isermann, B. (2011) p53NF-E2 represses Gcm1 in trophoblast cells to regulate syncytium formation, placental vascularization, and embryonic growth. Development 138, 2235–2247
2. McIntyre, D. D., Bloom, S. L., Carey, B. M., and Leveno, K. J. (1999) Birth weight in relationship to morbidity and mortality among newborn infants. N. Engl. J. Med. 340, 1234–1238
3. Gluckman, P. D., Hanson, M. A., Cooper, C., and Thornburg, K. L. (2008) Effect of in utero and early-life conditions on adult health and disease. N. Engl. J. Med. 359, 61–73
4. Chen, Z., Hu, M., and Shivdasani, R. A. (2007) Expression analysis of Nfe2 Regulates JunD Binding Activity via Acetylation 12. Okada, Y., Ueshin, Y., Isotani, A., Saito-Fujita, T., Nakashima, H., Kimura, S., Otsuka, K., Mochizuki, K., and Nakajima, A. (2000) Promotion of trophoblast stem cell proliferation by FGF4. Science 282, 2072–2075
5. Hashemolhosseini, S., Schmidt, K., Kilian, K., Rodriguez, E., and Wegner, M. (2004) Conservation and variation of structure and function in a newly identiﬁed GCM homolog from chicken. J. Mol. Biol. 336, 441–451
6. Anson-Cartwright, L., Dawson, K., Holmnyard, D., Fisher, S. J., Lazzarini, R. A., and Cross, J. C. (2000) The glial cells missing-1 protein is essential for branching morphogenesis in the chorionallantoic placenta. Nat. Genet. 25, 311–314
7. Hung, H. L., Kim, A. Y., Hong, W., Rakowski, C., and Blobel, G. A. (2001) Stimulation of NF-E2 DNA binding by CREB-binding protein (CBP)-mediated acetylation. J. Biol. Chem. 276, 10715–10721
8. Johnson, K. D., Grass, J. A., Boyer, M. E., Kiekhaefer, C. M., Blobel, G. A., Weiss, M. J., and Bresnick, E. H. (2002) Cooperative activities of hematopoietic regulators recruit RNA polymerase II to a tissue-speciﬁc chromatin domain. Proc. Natl. Acad. Sci. U.S.A. 99, 11760–11765
9. Schubert, S. W., Abendroth, A., Kilian, K., Vogler, T., Mayr, B., Knerr, I., and Hashemolhosseini, S. (2008) bZIP-type transcription factors CREB and OASIS bind and stimulate the promoter of the mammalian transcription factor GcmA/Gcm1 in trophoblast cells. Nucleic Acids Res. 36, 3834–3846
10. Schorpp-Kistner, M., Wang, Z. Q., Angel, P., and Wagner, E. F. (1999) JUNb is essential for mammalian placenta tion. EMBO J. 18, 934–948
11. Schreiber, M., Wang, Z. Q., Choum, W., Fetka, I., Elliott, C., and Wagner, E. F. (2000) Placental vascularization requires the AP-1 component fra1. Development 127, 4937–4948
12. Okada, Y., Ueshin, Y., Isotani, A., Saito-Fujita, T., Nakashima, H., Kimura, K., Mizoguchi, A., Oh-Hora, M., Mori, Y., Ogata, M., Oshima, R. G., Okabe, M., and Ikawa, M. (2007) Complementation of placental defects and mdx mice. Brain Res. 1123, 216–225
13. Pan, Y., Chen, C., Shen, Y., Zhu, C. H., Wang, G., Wang, X. C., Chen, H. Q., and Zhu, M. S. (2008) Curcumin alleviates dystrophic muscle pathology in mdx mice. Mol. Cells 25, 531–537
14. Tanaka, S., Kunath, T., Hadjantonakis, A. K., Nagy, A., and Rossant, J. (1998) Promotion of trophoblast stem cell proliferation by FGF4. Science 282, 2072–2075
15. Isermann, B., Sood, R., Pawliński, R., Zogg, M., Kalloway, S., Degen, J. L., Mackman, N., and Weiler, H. (2003) The thrombomodulin–protein C system is essential for the maintenance of pregnancy. Nat. Med. 9, 331–337
16. Himeno, S., Tanaka, S., and Kunath, T. (2008) Curr. Protoc. Stem Cell Biol. Chapter 1, Unit 1E.4
17. Okazaki, K. M., Red-Horse, K., Mak, W., Simon, M. C., and Fisher, S. J. (2005) Hypoxia-inducible factor-dependent histone deacetylation activity determines stem cell fate in the placenta. Development 132, 3393–3403
18. Balasubramanyam, K., Varier, R. A., Altﬁn, M., Swaminathan, V., Siddappa, N. B., Ranga, U., and Kundu, T. K. (2004) Curcumin, a novel p300/CREB-binding protein-speciﬁc inhibitor of acetyltransferase, represses the acetylation of histone/nonhistone proteins and histone acetyltransferase-dependent chromatin transcription. J. Biol. Chem. 279, 51163–51171
19. Tachibana, H., Fujimura, Y., and Yamada, K. (2004) Tea polyphenol epigallocatechin-3-gallate associates with plasma membrane lipid rafts. Lipid rafts mediate anti-allergic action of the catechin. Biofactors 21, 383–385
20. Tachibana, H., Koga, K., Fujimura, Y., and Yamada, K. (2004) A receptor for green tea polyphenol EGCG. Nat. Struct. Mol. Biol. 11, 380–381
21. Follenzi, A., Bakovic, S., Gual, P., Stella, M. C., Longati, P., and Comoglio, P. M. (2000) Cross-talk between the proto-oncogenes Met and Ron. Oncogene 19, 3041–3049
22. Bierhaus, A., Schiekofe, S., Schwanger, M., Andrassy, M., Humphert, P. M., Chen, J., Hong, M., Luther, T., Henle, T., Klötig, I., Morcos, M., Hofmann, M., Tritschler, H., Weigle, B., Kasper, M., Smith, M., Perry, G., Schmidt, A. M., Stern, D. M., Häring, H. U., Schielecher, E., and Nawroth, P. P. (2001) Diabetes-associated sustained activation of the transcription factor nuclear factor-kB. Diabetes 50, 2792–2808
23. Rudovsky, G. J., Reismann, P., Grafe, I. A., Konrade, I., Djurić, Z., Tafel, I., Buchbinder, S., Zorn, M., Humphert, P. M., Hamann, A., Morcos, M., Nawroth, P. P., and Bierhaus, A. (2007) Improved vascular function upon pioglitazone treatment in type 2 diabetes is not associated with changes in mononuclear NF-kB binding activity. Horm. Metab. Res. 39, 665–671
24. Aguiler, C., Nakagawa, K., Sancho, R., Chakraborty, A., Hendrich, B., and Behrens, A. (2011) c-Jun N-terminal phosphorylation antagonises recruitment of the Mbd3/NuRD repressor complex. Nature 469, 231–235
25. Reddy, S. P., and Mossman, B. T. (2002) Role and regulation of activator protein-1 in toxicant-induced responses of the lung. Am. J. Physiol. Lung Cell Mol. Physiol. 283, L1161–L1178
26. Thépot, D., Weitzman, J. B., Barra, J., Segretain, D., Stinnakre, M. G., Babinet, C., and Yaniv, M. (2000) Targeted disruption of the murine JunD gene results in multiple defects in male reproductive function. Development 127, 143–153
27. Franchet, C., Augery-Bourget, Y., Prenant, M., Walters, M., Martin, D. I., and Robert-Lézénes, J. (1997) c-Jun inhibits NF-E2 transcriptional activity in association with p18/maf in Friend erythroleukemia cells. Oncogene 14, 873–877
28. Dhanasekaran, N., and Premkumar Reddy, E. (1998) Signaling by dual specificity kinases. Oncogene 17, 1447–1455
29. Davis, R. J. (2000) Signal transduction by the JNK group of MAP kinases. Cell 103, 239–252
30. Kallunki, T., Deng, T., Hibi, M., and Karin, M. (1996) c-Jun can recruit phosphorylation dimerization partners via specific docking interactions.
34. Lee, T. L., Shyu, Y. C., Hsu, P. H., Chang, C. W., Wen, S. C., Hsiao, W. Y.,
Tsai, M. D., and Shen, C. K. (2010) JNK-mediated turnover and stabilization
of the transcription factor p45/NF-E2 during differentiation of murine erythroleukemia cells. Proc. Natl. Acad. Sci. U.S.A. 107, 52–57
35. Johnson, K. D., Norton, J. E., and Bresnick, E. H. (2002) Requirements for
utilization of CREB-binding protein by hypersensitive site two of the
β-globin locus control region. Nucleic Acids Res. 30, 1522–1530
36. Nandiwada, S. L., Li, W., Zhang, R., and Mueller, D. L. (2006) p300/cyclic
AMP-responsive element-binding protein mediates transcriptional co-
activation by the CD28 T cell costimulatory receptor. J. Immunol. 177, 401–413
37. Kamei, Y., Xu, L., Heinzle, T., Torchia, J., Kurokawa, R., Gloss, B., Lin, S. C.,
Heyman, R. A., Rose, D. W., Glass, C. K., and Rosenfeld, M. G. (1996) A
CBP integrator complex mediates transcriptional activation and AP-1 in-
hibition by nuclear receptors. Cell 85, 403–414
38. Cheng, X., Reginato, M. J., Andrews, N. C., and Lazar, M. A. (1997) The
transcriptional integrator CREB-binding protein mediates positive cross-
talk between nuclear hormone receptors and the hematopoietic bZip pro-
tein p45/NF-E2. Mol. Cell. Biol. 17, 1407–1416
39. Bamberger, A. M., Bamberger, C. M., Aupers, S., Milde-Langosch, K.,
Löning, T., and Makrigiannakis, A. (2004) Expression pattern of the acti-
vating protein-1 family of transcription factors in the human placenta.
Mol. Hum. Reprod. 10, 223–228