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Carbonic anhydrase III regulates peroxisome proliferator-activated receptor- γ 2

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Abstract

Carbonic anhydrase III (CAIII) is an isoenzyme of the CA family. Because of its low specific anhydrase activity, physiological functions in addition to hydrating CO₂ have been proposed. CAIII expression is highly induced in adipogenesis and CAIII is the most abundant protein in adipose tissues. The function of CAIII in both preadipocytes and adipocytes is however unknown. In the present study we demonstrate that adipogenesis is greatly increased in mouse embryonic fibroblasts (MEFs) from CAIII knockout (KO) mice, as demonstrated by a greater than 10-fold increase in the induction of fatty acid-binding protein-4 (FABP-4) and increased triglyceride formation in CAIII^{-/-} MEFs compared with CAIII^{+/+} cells. To address the underlying mechanism, we investigated the expression of the two adipogenic key regulators, peroxisome proliferator-activated receptor- γ 2 (PPAR γ 2) and CCAAT/enhancer binding protein- α . We found a considerable (approximately 1000-fold) increase in the PPAR γ 2 expression in the CAIII^{-/-} MEFs. Furthermore, RNAi-mediated knockdown of endogenous CAIII in NIH 3T3-L1 preadipocytes resulted in a significant increase in the induction of PPAR γ 2 and FABP-4. When both CAIII and PPAR γ 2 were knocked down, FABP-4 was not induced. We conclude that down-regulation of CAIII in preadipocytes enhances adipogenesis and that CAIII is a regulator of adipogenic differentiation which acts at the level of PPAR γ 2 gene expression.

Keywords

Adipocyte; Adipogenesis; Aging; Caloric restriction; Carbonic anhydrase III; oxidative stress; Preadipocyte; PPAR γ 2; FABP-4

Introduction

Carbonic anhydrase III (CAIII) belongs to a family of structurally related enzymes that catalyze the reversible hydration of carbon dioxide ($\text{H}_2\text{O} + \text{CO}_2 \leftrightarrow \text{HCO}_3^- + \text{H}^+$) [1, 2]. The CA isoenzymes are involved in physiological processes, such as acid-base balance,

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lipogenesis, and cell growth. The importance of CAI and CAII for the efficient transport and elimination of CO₂ from tissues and lungs has been well documented [1, 2]. However, the specific activity of CAIII as a CO₂ hydratase is only about 2% of CAI and CAII [3]. Thus, it has been hypothesized that CAIII has other functions in the cell. CAIII has two reactive sulfhydryl groups, which can reversibly conjugate to glutathione (GSH) through a disulfide bond [4, 5]. This S-glutathionylation reaction is likely one important component of cellular defense mechanisms that prevent the irreversible oxidation of proteins [6]. CAIII is rapidly glutathionylated when the cells are exposed to oxidative stress, and it is one of the most carbonylated proteins in rodent liver, suggesting that it is located in an oxidizing environment [7]. Moreover, the overexpression of CAIII in experimental cell lines has been found to protect them from H₂O₂-induced pro-apoptotic and anti-proliferative effects [8]. In aged rats, where glutathione levels are reduced, the amount of irreversibly oxidized CAIII increased [9–11]. Also, 4-hydroxynonenal-conjugated CAIII has been found to accumulate during muscle disuse [12]. These studies have further underlined a role for CAIII in oxidative stress situations, such as aging [13]. Indeed, these data suggest that CAIII might function to protect cells from oxidative damage.

CAIII is abundantly expressed in highly metabolically active tissues, such as fat, liver, and slow-twitch skeletal muscle fibers [1, 14]. In contrast, only trace amounts of CAIII have been detected in other tissues [15]. Adipose tissues serve as a fat storage depot and controls whole-body energy homeostasis [16]. Although evidence for an involvement of CAIII in fatty acid synthesis has been presented [14], it is also known that adipose tissues generate high levels of reactive oxygen species (ROS) and lipid peroxidation products [7, 14, 17, 18]. In addition, CAIII expression has been found to be very low in preadipocytes [19] and become substantial upon adipogenic differentiation [18]. In fact, CAIII has been shown to be one of the most abundant transcripts in both human [20] and rodent [21] adipose tissues, accounting for up to 2% of the total mRNA. Moreover, CAIII constitutes the most abundant protein in mature adipocytes, comprising up to 24% of the total soluble protein fraction [18]. These data suggest an important adipocyte-related function for CAIII, which could lie in the protection against oxidative stress. This function of CAIII could be of importance in obesity, which is characterized by fat accumulation and increased adipose tissue mass [22] that contribute to insulin resistance and metabolic syndrome [23]. Obesity leads to elevated oxidative stress caused by a hypoxia-induced increase in the production of ROS and lipotoxicity [24–27]. In keeping with these considerations, CAIII has been implicated in fatty acid metabolism [14] in adipocytes of both obese and lean mice [28, 29]. However, neither the function of CAIII in adipocytes nor the mechanism of adipogenesis-dependent up-regulation of CAIII is well understood.

Development of adipose tissue mass is a result of an increase in the number and size of the fat cells [30, 31]. New adipocytes arise from precursors referred to as adipose-derived stem cells and/or preadipocytes, which most likely reside at perivascular sites in adipose tissues [31]. The differentiation of preadipocytes into mature adipocytes, referred to as adipogenesis, is initiated by several extra- and intracellular factors. These factors include mitogens, growth factors, hormones and a cascade of activated signaling molecules (e.g., MAP kinases, AKT, protein kinase A, and glucocorticoid receptor), which activate a network of transcription factors that control the expression of many genes representing the adipocyte phenotype [32]. At the end of the intracellular signaling cascade, the transcription factors, CCAAT/enhancer binding protein- β (C/EBP β) and C/EBP δ are activated [33]. These factors induce the expression of nuclear hormone receptor peroxisome proliferator-activated receptor γ (PPAR γ 2) [34] and CCAAT/enhancer binding protein- α (C/EBP α) [33], which are the two major regulators of terminal adipogenic differentiation, shown in the murine preadipocyte cell line, NIH 3T3-L1 and primary preadipocytes [32]. Following these events, PPAR γ 2 and C/EBP α activate the expression of adipocyte-specific genes which are

involved in lipogenesis and necessary for the accumulation of lipid droplets and adipokines [32]. PPAR γ 2, the transcriptional activity of which is regulated by binding to the appropriate ligands, also plays a role in the regulation of oxidative stress. It has been shown that the ligand activation of PPAR γ 2 attenuated the production of ROS induced by different stimuli in NIH 3T3-L1 adipocytes and in the insulin-resistant leptin-deficient ob/ob mouse [35]. In contrast, adipose tissue-specific PPAR γ 2 deficiency has been found to increase resistance to oxidative stress in mice [36]. These data suggest that PPAR γ 2 activity can negatively and positively affect oxidative stress in adipose tissues depending on the conditions; however, these functions are not yet precisely understood.

In the present study, we investigated whether CAIII affects adipogenesis using MEFs from a mouse lacking CAIII and RNAi-mediated knockdowns of CAIII in NIH 3T3-L1 preadipocytes. We present evidence that the down-regulation of CAIII in preadipocytes dramatically activated PPAR γ 2 expression and adipogenesis, raising the possibility that CAIII can trigger the initiation of adipogenesis in preadipocytes by regulating PPAR γ 2 gene expression.

Materials and methods

Chemicals

3-Isobutyl-1-methylxanthin (IBMX), dexamethasone, troglitazone, Oil Red O, and DMEM were obtained from Sigma-Aldrich (Vienna, Austria), Insulin from Roche (Vienna, Austria), and all other supplements from Gibco (Vienna, Austria).

Plasmids

pMD2.G, psPAX2 and pLKO.1 TRC control vector (D. Trono # 12259, D. Trono #12260, Root #10879, Addgene, Cambridge, USA). A CAIII overexpressing lentivirus, pLentiCAIII, was constructed by inserting the full length mouse CAIII cDNA from pCMV-SPORT6-CA3, NM_007606 (Clone 4195712) MMM1013-65396 (Open Biosystems, Vienna, Austria) into pLenti6/DEST (Invitrogen, Lofer, Austria) using the Gateway Lentiviral Cloning System, Invitrogen, Lofer, Austria. The sequence of plentiCAIII was verified by automated sequencing. CAIII shRNA: sh#2 and sh#3 (TRCN0000114445 and TRCN0000114442 for NM_007606 [murine CAIII], #RMM4534, PPAR γ 2 shRNA: sh#1 and sh#4 (TRCN0000001657 and TRCN0000001659 for NM_011146 [murine PPAR γ 2], Open Biosystems, Vienna, Austria).

Cell culture

NIH 3T3-L1 preadipocytes (ATCC CL-173) were grown in DMEM with 1 g/L glucose, 1 g/L L-glutamine, 1 g/L sodium bicarbonate (Sigma, Vienna, Austria), 10% CS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin (Gibco, Vienna, Austria), without reaching confluence. For differentiation, 10,000 cells/cm² were sowed and grown for 4 days until density-mediated growth arrest. Differentiation was induced by the addition of 1 μ g/ml insulin, 0.5 mM IBMX, 0.25 μ M dexamethasone, 10% FCS and, where indicated, 5 μ M troglitazone. After two days, only insulin and 10% FCS were added. The medium was changed every second day. Mouse embryonic fibroblasts (MEFs) were isolated as described [37] from wild-type, CAIII^{+/-}, and CAIII^{-/-} mice developed in the Laboratory of Biochemistry, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland USA (1). MEFs were cultured in DMEM/10% FCS, 1% NEAA, 1% L-glutamine, 1% penicillin/streptomycin and passaged twice before seeding at a density of 40,000 cells/cm². Differentiation was induced in density-arrested MEFs as in the NIH 3T3-L1 cells.

Retroviral gene expression system

NIH 3T3-L1 cells were transiently transfected with different pLKO.1 lentiviral target gene shRNA vectors for accession NM_007606 and NM_011146 (#RMM4534, Open Biosystems, Vienna, Austria), using the effectene method (Qiagen, Hilden, Germany). After selection with 2 µg/ml puromycin, the most efficient knockdown clones for CAIII (sh#2 and sh#3) and for PPAR γ 2 (sh#1 and sh#4), were identified by Western blotting and then used for lentiviral production employing the ViraPowerTMLentiviral Expression System (Invitrogen, Lofer, Austria) and a second-generation system provided by the Trono lab (Addgene, Cambridge, USA). The same procedure was used to generate virus particles of the CAIII overexpressing lentivirus plentiCAIII. The purified viral particles were stored at -80°C. For viral infection, 500,000 NIH 3T3-L1 cells were seeded in a 75-cm² bottle, with 5 ml medium, 6 µg/ml polybrene and virus particles at a multiplicity of infection (MOI) of 1:2. After 24 h, the medium was changed, and the cells were grown for another 48 h before they were trypsinized and seeded at 10,000 cells/cm² for subsequent differentiation.

Oil Red O staining

The differentiated cells were fixed with 4% paraformaldehyde in PBS for 1 h and stained with 0.3% Oil Red O (Sigma, Vienna, Austria) in isopropanol:water (60:40) for 1 h. The final washing was then performed two times with distilled water. Triglyceride determination was performed by analysis of Oil Red O stained cells with AlphaEase FC software, version 6.0.0, Alpha Innotec Corporation.

Western blot analysis

Cells were extracted in 1.5x Laemmli sample buffer, and the protein concentration was measured using the BCA Assay (Thermo-Scientific, Rockford, USA). The lysates were separated on either a 12.5% or 10% gel by SDS-PAGE. Western blotting was performed as described [38]. Primary antibodies: β -actin (Clone Ac-15) Sigma-Aldrich, Vienna, Austria; C/EBP α (C-18), FABP-4 (C-15), and PPAR γ 2 (E-8), Santa Cruz, California, USA; CAIII (Produced by the Bethesda laboratory). Horseradish peroxidase-conjugated secondary antibodies (Promega, Mannheim, Germany). Enhanced Chemiluminescence Western blotting Detection System (NEN, Vienna, Austria).

Quantitative RT-PCR

Total RNA was isolated with the RNeasy Micro Kit (Qiagen, Hilden, Germany), and cDNA synthesis was performed with the First Strand cDNA Synthesis Kit (Fermentas, St.Leon-Rot, Germany). Quantitative expression analysis was performed using the LightCycler® 480 Real-Time PCR System (Roche, Vienna, Austria). The mRNA quantification was performed using β -tubulin for normalization. Primers: FABP-4-forw: AAGAAGTGGGAGTGGGCTTT. FABP-4-rev: CTGTCGTCTGCGGTGATTT. CAIII-for: CTTGATGCCCTGGACAAAAT. CAIII-rev: AGCTCACAGTCATGGGCTCT. CEBP α -for: AGGTGCTGGAGTTGACCACT. CEBP α -rev: CAGCCTAGAGATCCAGCAAC. NRF2-for: AGGACATGGAGCAAGTTTGG. NRF2-rev: TTCTTTTCCAGCGAGGAGA. PPAR γ 2-forw: TGGGTGAAACTCTGGGAGAT. PPAR γ 2-rev: GCTGGAGAAATCAACTGTGG. β -Tubulin rev: GTATTCATGATACGGTCAGGA. β -Tubulinforw: CCTTCTTGTTTCGGTACCTACA. GST-A-forw: CGCCACCAAATATGACCTCT. GST-A-rev: CCTGTTGCCCAAGGTAGT.

Statistical analysis

Student's t-test was used to determine the significance of all data.

Results

CAIII regulates adipogenesis in mouse embryonic fibroblasts

Mouse embryonic fibroblasts (MEFs) were isolated from wild type (CAIII^{+/+}), heterozygous (CAIII^{+/-}) and homozygous CAIII knockout (CAIII^{-/-}) C57BL/6 mice [1]. Western blot analysis confirmed the deletion of CAIII in the homozygous KO cells and a reduction of CAIII expression in heterozygous KO cells, compared with wild type cells (Fig. 1A). To study whether CAIII has an impact on fat cell differentiation, the MEFs were grown to confluence, and adipogenesis was induced by adding a hormone cocktail (dexamethasone, IBMX, insulin, and FCS). After 8 days, strong differences in lipid accumulation were detected between the three cell types (Fig. 1B and 1C). Whereas the wild type MEFs differentiated only at a moderate rate, a strong increase in the size of the fat droplets (Fig. 1B) and amount of triglycerides (Fig. 1B and 1C) was observed in the MEF CAIII^{+/-} cells, and an even greater increase was seen in the MEF CAIII^{-/-} cells. The CAIII protein was detectable in the MEF CAIII^{+/-} cells at day 0 before adipogenesis, although at a lower level than in the MEF CAIII^{+/+} cells (Fig. 1B, lower panels). During adipogenesis, the CAIII protein levels were strongly increased in the MEF CAIII^{+/+} cells and, to a lesser extent, in the MEF CAIII^{+/-} cells (Fig. 1B, lower panels). In the CAIII^{-/-} negative MEFs, no CAIII protein was detected at day 0 nor during differentiation. These data indicate that CAIII negatively regulated adipogenesis in preadipocytes in a dose-dependent manner.

To investigate how CAIII regulates adipogenesis, we analyzed whether the CAIII KO affects the expression of the adipogenic key regulator PPAR γ 2, which is considered essential for the precursor cells to express the genes constituting the adipocyte phenotype [32]. When adipogenesis was induced by the hormone cocktail, we observed over an 1,100-fold induction of PPAR γ 2 gene expression in both CAIII^{+/-} and MEF CAIII^{-/-} MEF (Fig. 2A, left panel). The induction of PPAR γ 2 was considerably stronger than in the MEF CAIII^{+/+} cells which showed a similar induction of PPAR γ 2 as NIH 3T3-L1 preadipocytes committed to adipogenic differentiation (Fig. 2A, left panel). To corroborate these findings, we also stimulated adipogenesis with the strong PPAR γ 2 agonist, troglitazone [39], and analyzed whether this had a synergistic effect on PPAR γ 2 gene expression in the MEF CAIII^{+/-} as well as the MEF CAIII^{-/-} cells. Under these conditions, we detected more than a 7,000-fold induction of PPAR γ 2 gene expression in both CAIII^{+/-} MEF and CAIII^{-/-} MEF (Fig. 2A, right panel). Troglitazone by itself, without the differentiation cocktail, had no effect on PPAR γ 2 expression in the MEFs (Fig. 2B), and fat droplets did not form until 8 days after addition of the troglitazone. Thus, the differentiation cocktail is essential for induction of adipogenesis in the CAIII knockout MEFs. These data strongly suggest that the CAIII KO dramatically and specifically facilitated differentiation cocktail-induced expression of the PPAR γ 2 gene at the onset of terminal adipogenic differentiation and implies that CAIII can decrease PPAR γ 2 gene expression.

Next, we investigated whether the CAIII KO facilitates the induction of other adipogenic regulators and differentiation products. We monitored the expression of C/EBP α , which is considered to be another adipogenic regulator contributing to terminal adipogenic differentiation [32], and the terminal differentiation marker, fatty acid binding protein-4 (FABP-4), in the CAIII KO MEFs. We found a significant increase in the FABP-4 gene expression levels in the MEF CAIII^{-/-} cells (Fig. 2C, left panel), which was markedly increased in the troglitazone-treated cells (Fig. 2C, right panel). C/EBP α was only weakly induced, irrespective of the addition of troglitazone (Fig. 2D). In the presence of troglitazone, the CAIII KO cells showed a significantly stronger increase in C/EBP α induction than did the wild type cells. However, this was not true for differentiation without troglitazone, in which no significant differences in C/EBP α expression between the three MEF types was observed (Fig. 2D). Together, these findings suggest that the CAIII KO

strongly facilitated PPAR γ 2 gene expression and that this was sufficient to drive terminal adipogenic differentiation. C/EBP α was only weakly up-regulated by the CAIII KO. This is consistent with studies that have shown that C/EBP α -deficient cells are capable of adipocyte differentiation [40].

Using Western blot analysis, we investigated whether mRNA expression of adipogenic key factors was mirrored at the protein level (Fig. 2E). NIH 3T3-L1 differentiation served as the control. C/EBP α was only detected in CAIII $^{-/-}$ cells 8 days after differentiation cocktail treatment, indicating that the most potent induction of differentiation occurred in these cells. PPAR γ 2 and FABP-4 appeared in all three of the differentiation cocktail-treated cell lines, but the amount in the CAIII $^{+/-}$ and CAIII $^{-/-}$ cells was much higher than that in the CAIII $^{+/+}$ MEFs.

The intracellular redox set point becomes more oxidizing during adipogenesis in NIH 3T3-L1 preadipocytes [41]. Blocking the increase in oxidizing potential slows adipogenesis while further increasing the potential speeds adipogenesis [41, 42]. If CAIII functions as an antioxidant [7, 8], knocking out its gene would be expected to increase adipogenesis, as we observed. NF-E2-related factor 2 (NRF2) is a transcription factor activated by diverse oxidants that regulates gene expression by binding to antioxidant response elements (ARE) in the promoter regions of certain target genes [38, 43]. The PPAR γ 2 promoter has an ARE and NRF2 was shown to induce PPAR γ 2 in NIH 3T3-L1 preadipocytes [44]. We therefore quantitated NRF2 transcripts in the MEFs to determine whether they were increased in the CAIII KO cells. They were not; indeed, we found an approximately 50% repression of NRF2 transcripts in the CAIII $^{-/-}$ MEFs relative to the wild type MEFs (Fig. 3A). NRF2 mRNA levels were also lower in *in vitro*-differentiated adipocytes derived from the MEF CAIII $^{-/-}$ cells than from the MEF CAIII $^{+/+}$ cells (Fig. 3A). To address the question whether this led actually to decreased nuclear activity of NRF2, we measured the level of the transcript of a bona fide NRF2 antioxidant target gene, GSTA [45,46]. The mRNA level of GSTA was reduced by ~70% in the CAIII $^{-/-}$ MEFs compared to the wild type MEFs (Fig. 3B). GSTA mRNA levels were also lower in *in vitro*-differentiated adipocytes derived from the CAIII $^{-/-}$ cells than from the CAIII $^{+/+}$ cells (Fig. 3B). Moreover, the protein level of GST decreased in the CAIII $^{-/-}$ cells relative to the CAIII $^{+/+}$ cells (Fig. 3C). Together these data suggest that loss of CAIII decreases the expression level of NRF2 and the nuclear activity of NRF2 and indicate that PPAR γ 2 is not stimulated by NRF2. Intriguingly, a study by Shin et al., [47] showed that adipogenesis is accelerated in NRF2 $^{-/-}$ MEFs which have lower expression level of the NRF2 target gene aryl hydrocarbon receptor (AHR), a negative regulator of adipogenesis which is downregulated following adipogenesis in MEFs. We found that the AHR mRNA levels were also considerably lower in CAIII $^{-/-}$ MEFs relative to CAIII $^{+/+}$ MEFs (Fig. 3D). Thus, downregulation of NRF2 could indirectly contribute to increasing adipogenesis in our MEF CAIII $^{-/-}$ cells. The elucidation of the precise underlying mechanism will require additional studies.

Influence of CAIII on adipogenesis in NIH 3T3-L1 preadipocytes

To confirm the results from the CAIII-deficient MEFs, we studied NIH 3T3-L1 cells, a well-accepted tissue culture system for studying fat cell differentiation. CAIII was knocked down in NIH 3T3-L1 cells using two different shRNA constructs for CAIII mRNA, sh#2 and sh#3 (Fig. 4A). In undifferentiated NIH 3T3-L1 preadipocytes, qRT-PCR analysis demonstrated that CAIII was transcribed at low levels, similar to that described in preadipocytes [19]. Nevertheless, a significant reduction of CAIII mRNA was found in sh#2- and sh#3-containing NIH 3T3-L1 preadipocytes. NIH 3T3-L1 preadipocytes were differentiated according to the same protocol as that used for the MEFs. During differentiation, when CAIII was induced in the wild type NIH 3T3-L1 cells, a strong down-regulation of CAIII gene expression was detected in both the sh#2- and sh#3-containing NIH 3T3-L1 cells

relative to the control shRNA-expressing cells. This relative down regulation became stronger with the progression of adipocyte differentiation (Fig. 4A). In keeping with the very low endogenous CAIII gene expression, the CAIII protein was barely detectable in the undifferentiated NIH 3T3-L1 preadipocytes in western blot experiments (Fig. 4B). Traces of CAIII protein were detectable only after a very long exposure (Fig. 4B, right panel), consistent with the qRT-PCR observations. After 4 days of induction of adipogenesis, CAIII protein was significantly reduced by both sh#2 and sh#3, although sh#2 was more effective than sh#3 (Fig. 4B, lower panel). The induction and progression of differentiation were monitored by assessing the expression of key adipogenic markers. Because the CAIII knockdown did not have a strong effect until day 4 of differentiation (Fig. 4B), the increase in induction of PPAR γ 2 and FABP-4 was less pronounced at day 4 than in the MEF CAIII^{-/-}. However, both transcripts, PPAR γ 2 and FABP-4, were more strongly induced in the CAIII knockdown cells compared with the control cells (Fig. 4C). To demonstrate that the CAIII knockdown led to a stronger up-regulation of the PPAR γ 2 protein levels during differentiation, Western blot analysis at days 0, 2 and 4 of differentiation was conducted. Sh#2- and sh#3-transfected NIH 3T3-L1 cells had significantly higher PPAR γ levels at day 4 of differentiation than wild-type cells. Because of the relatively strong induction of the basal PPAR γ 2 protein levels during adipogenic differentiation in the NIH 3T3-L1 cells, the increases due to CAIII knockdown were moderate but significantly different from the control (Fig. 4D). Moreover, the PPAR γ 2 protein levels correlated with the CAIII knockdown efficiency obtained in the sh#2 and sh#3 NIH 3T3-L1 cells (compare Fig. 4A, 4B and 4D). To test whether the activation of adipogenesis (p.e. FABP4) in the CAIII knockdown cells is in fact through PPAR γ 2, we analyzed whether FABP4 was still induced if PPAR γ 2 was silenced. We employed two different PPAR γ 2 shRNAs (Fig. 4E) in conducting CAIII/PPAR γ 2 double knockdown experiments. We treated cells with two PPAR γ 2 shRNAs, shPPAR γ 2#1 and PPAR γ 2#4, and with two CAIII shRNAs, shCAIII#2 and shCAIII#3 (Fig. 4E). We found that FABP4 induction was abrogated when PPAR γ 2 was silenced. This was detected at the FABP4 mRNA (Fig 4E) as well as protein level (Fig. 4F). We conclude that PPAR γ 2 is necessary for the induction of FABP4 in the CAIII knockdown cells.

To analyse whether the overexpression of CAIII has also effects on adipogenesis, NIH 3T3-L1 preadipocytes were infected with a CAIII overexpressing lentivirus, plentiCAIII, or a control lentivirus. Adipogenesis was induced, and the expression of CAIII, PPAR γ 2, C/EBP α and FABP4 was monitored. On day 0 and day 2, CAIII mRNA levels were considerably higher in NIH 3T3-L1/plentiCAIII relative to NIH 3T3-L1/plentiEmpty (Fig. 5A, upper panel). These differences narrowed on later days (Fig. 5A, lower panel), most likely due to the strong induction of endogenous CAIII during adipogenesis. Western blot experiments showed that the CAIII protein levels were also higher in the NIH 3T3-L1/plentiCAIII cells through day 4 of differentiation (Fig. 5B). Q-RT-PCR analysis demonstrated that the induction of the adipogenic factors PPAR γ 2 and C/EBP α and of the adipogenic differentiation marker FABP4 were similar in CAIII overexpressing cells and control cells (Fig. 5C). We conclude that overexpression of CAIII in NIH 3T3-L1 preadipocytes has no substantive effect on adipogenic differentiation.

Discussion

Several studies have suggested that CAIII plays an important role as an anti-oxidant in the protection against oxidative stress and aging [4–12]. CAIII is the most abundant protein in murine fat tissues [21], and CAIII levels are specifically fat-regulated in obesity [28]. For these reasons, it is conceivable that CAIII may be involved in adipocyte metabolism and function, probably playing a role in the protection against ROS generated in the highly metabolically active fat tissues [48]. However, mouse CAIII deletion mutants have normal

development and life span; no phenotypic changes were detected [1]. While no difference in the function, distribution, and amount of fat tissue [1] was observed in the CAIII KO mice, we have now shown a clear effect of CAIII in adipocyte differentiation of cultured MEFs from those mice. CAIII was involved in the regulation of adipogenesis in preadipocytes. Cells lacking CAIII exhibited increased fat cell differentiation in response to hormone cocktail. Despite the fact that CAIII has been shown to be strongly induced during adipogenesis and is highly abundant in mature adipocytes [20], the protein does not seem to be essential for the course of the normal terminal adipogenic differentiation process. Rather, our data suggest that CAIII regulates the initiation of adipogenesis during an early step in preadipocytes. This conclusion is supported by the finding that the expression of PPAR γ 2 was strongly enhanced after the CAIII deletion, indicating that CAIII negatively regulates adipogenesis at the level of PPAR γ 2 expression. With the addition of troglitazone, the enhancing effect of CAIII deletion on PPAR γ 2 became even more striking, possibly because of the positive feedback loop of PPAR γ 2 on its own expression [49]. For these reasons, we suggest that CAIII is a negative regulator of PPAR γ 2 expression and hence, adipogenesis.

As noted above, CAIII may participate in defense against oxidative stresses [7, 8]. If so, deletion of CAIII would increase oxidative stress which would be expected to increase the level of NRF2, one of the master regulators of the antioxidant response. Interestingly, we found that NRF2 levels actually decreased in MEF CAIII^{-/-} cells. The mechanism by which a decrease in CAIII causes a decrease in NRF2 levels is unknown and will be of interest to study. Regardless of the mechanism affecting NRF2, PPAR γ 2 and adipogenesis are increased in the knockout and knockdown cells, and these increases must be mediated by factors other than NRF2.

In conclusion our data suggest that CAIII modulates the adipogenesis through two mechanisms, its antioxidant function [4–12] and its ability to regulate gene expression.

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Abbreviations

ARE	antioxidant response elements
CAIII	Carbonic anhydrase III
C/EBPα	CCAAT/enhancer binding protein- α
FABP-4	fatty acid-binding protein-4
GSH	glutathione
IBMX	3-Isobutyl-1-methylxanthin
KO	knockout
MEF	mouse embryonic fibroblasts
NRF2	NF-E2-related factor 2
PPARγ2	peroxisome proliferator-activated receptor- γ 2
ROS	reactive oxygen species
Tgl	troglitazone

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Highlights

- We discover a novel function of Carbonic anhydrase III (CAIII).
- We show that CAIII is a regulator of adipogenesis.
- We demonstrate that CAIII acts at the level of PPAR γ 2 gene expression.
- Our data contribute to a better understanding of the role of CAIII in fat tissue.

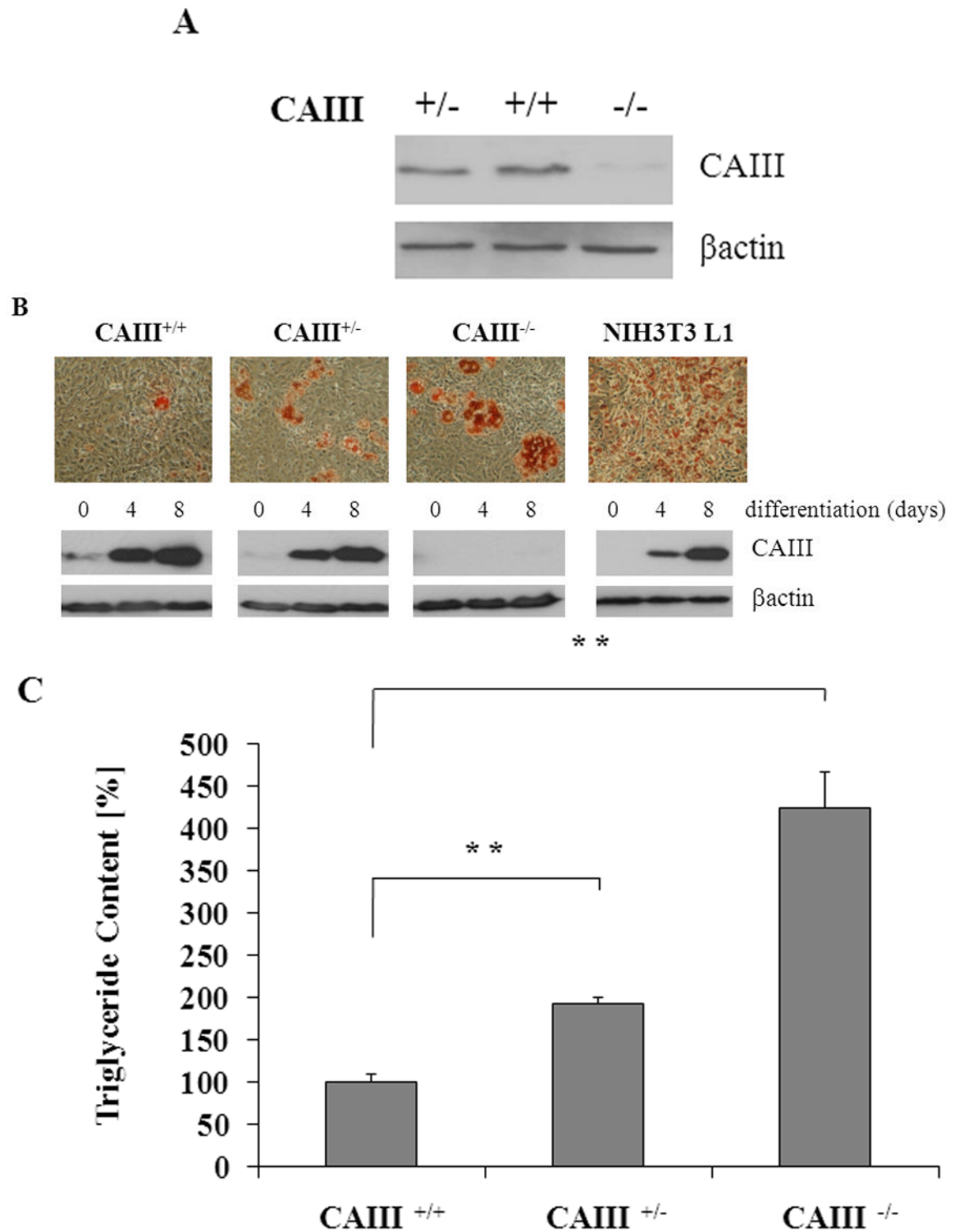
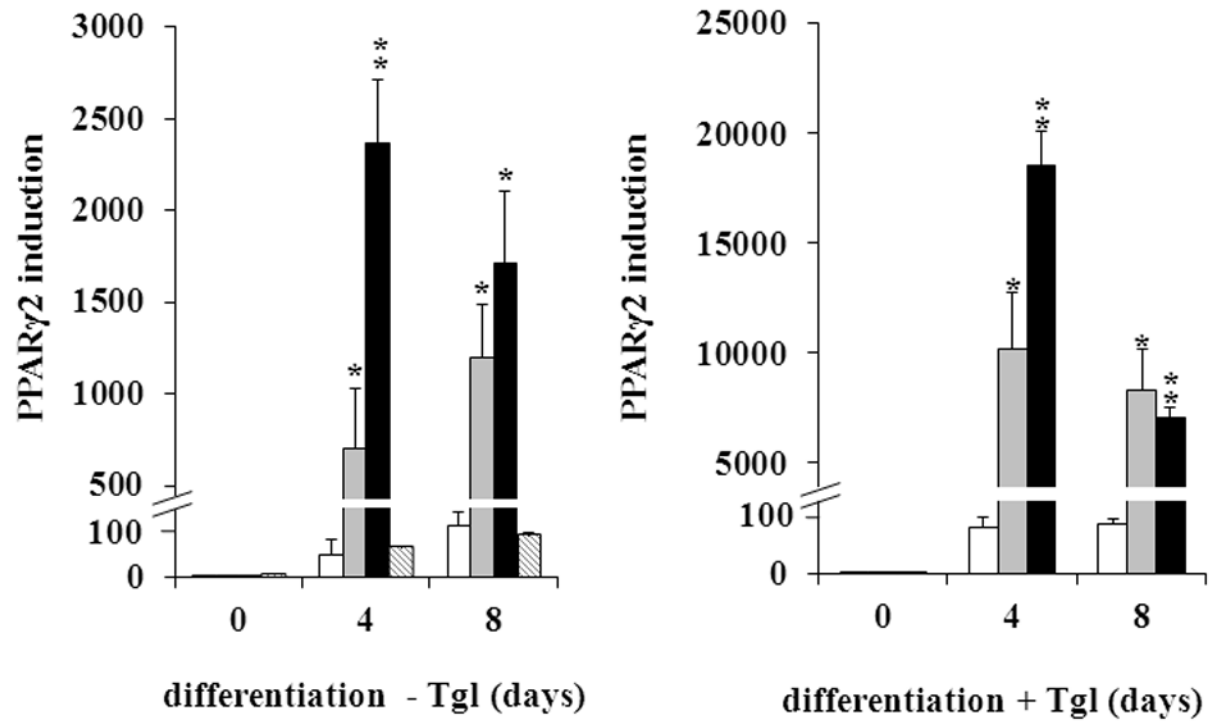
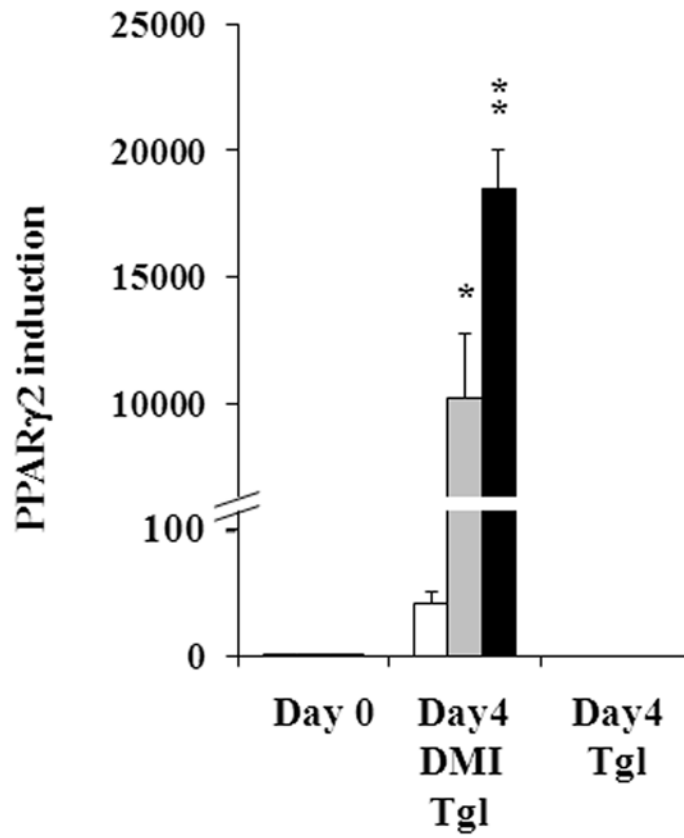
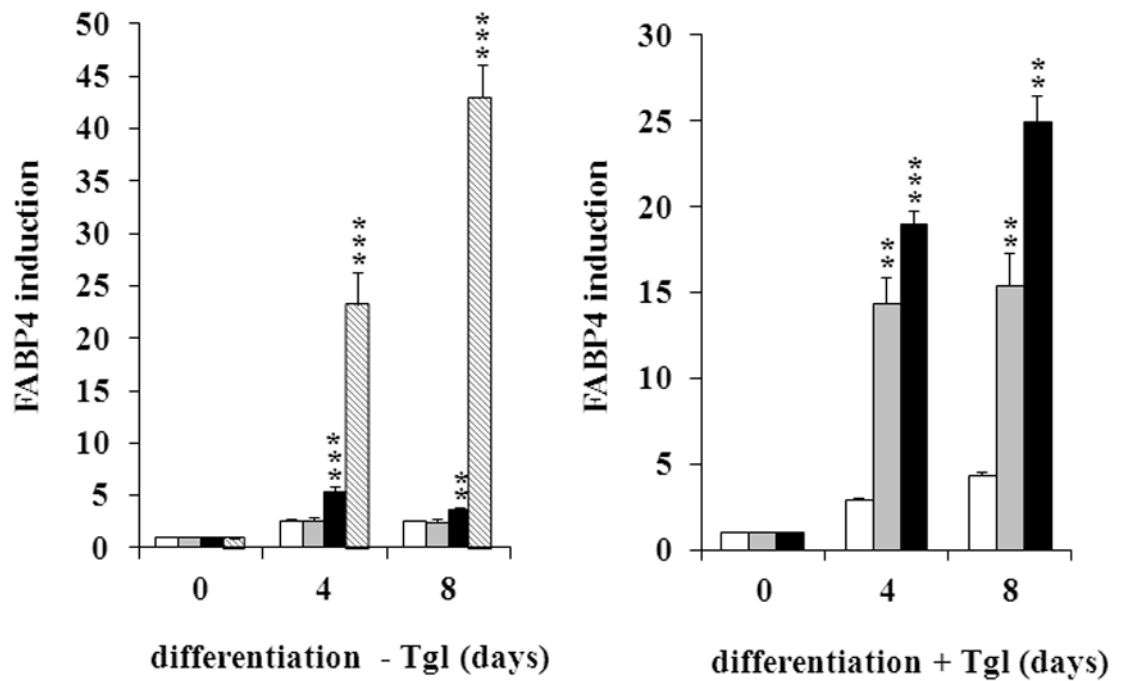


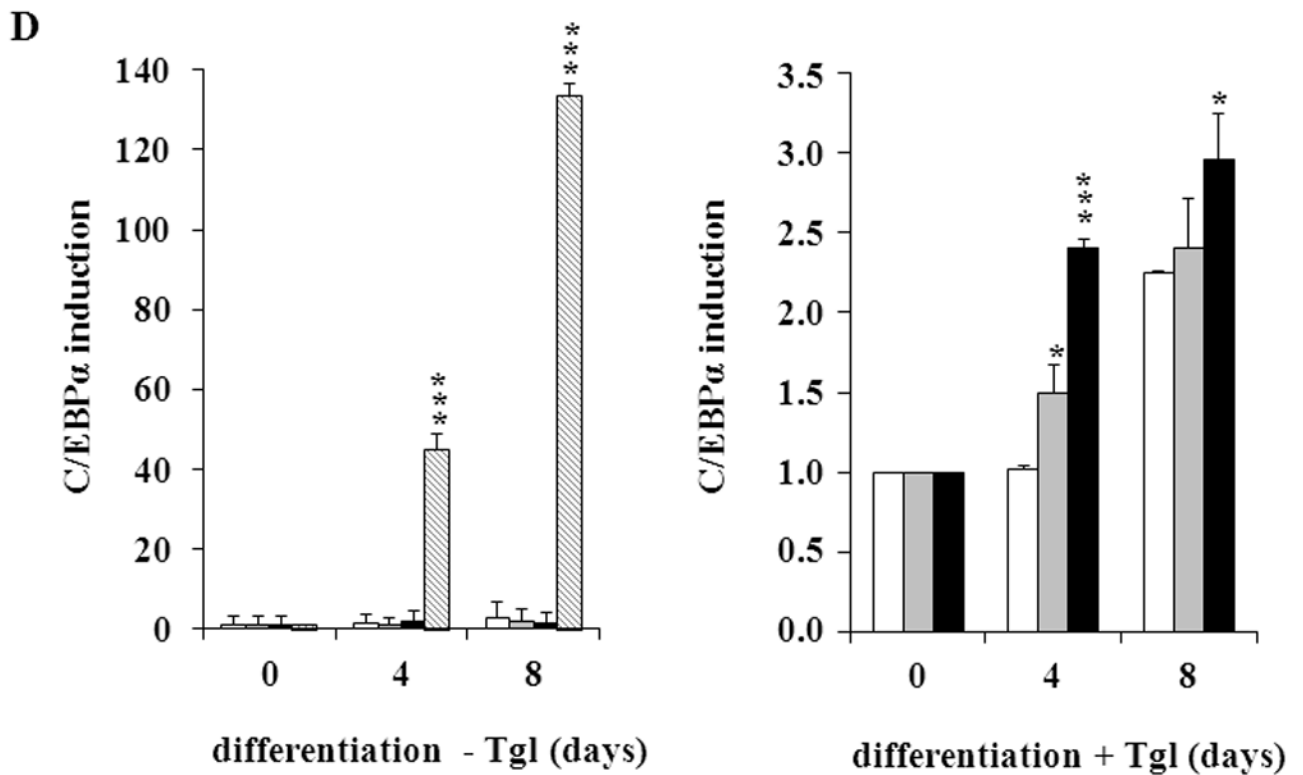
Fig. 1. CAIII knockout enhances fat droplet formation in MEFs
 (A) CAIII protein levels in MEFCaIII^{+/+}, MEFCaIII^{+/-} and MEFCaIII^{-/-} analyzed by Western blotting. β -Actin served as a loading control. (B and C) MEFs were exposed to hormone-cocktail to induce adipogenesis for 8 days. (B) At day 8 of differentiation, the

MEFs were fixed, and the fat droplets were stained with Oil Red O (upper panels). Protein samples were taken for western blot analysis at days 0, 4 and 8 (lower panels). Differentiated NIH 3T3-L1 adipocytes are shown as the positive control. (C) The triglyceride content in the different MEFs was quantified by densitometric analysis. Data are presented as the mean \pm SD of 4 independent experiments, (***) $p \leq 0.01$.

A



B**C**



E

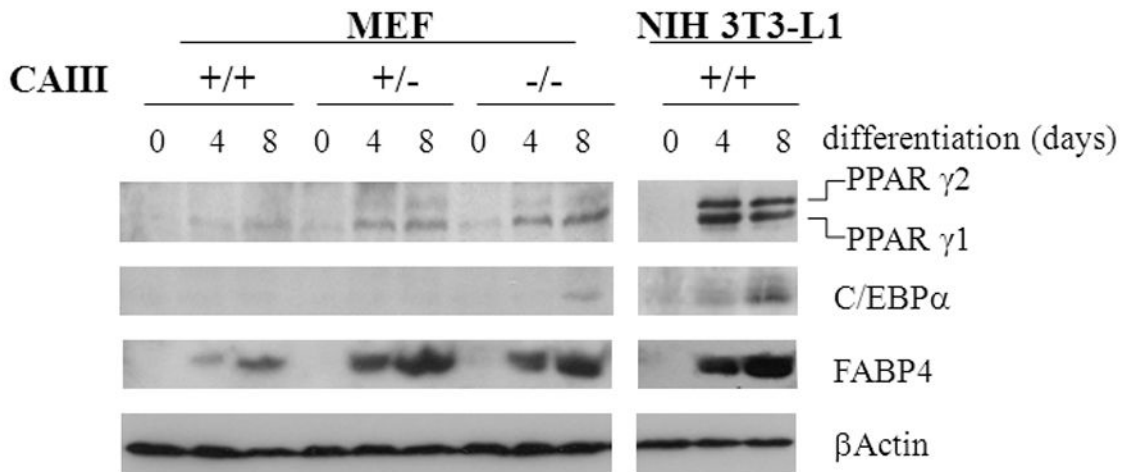
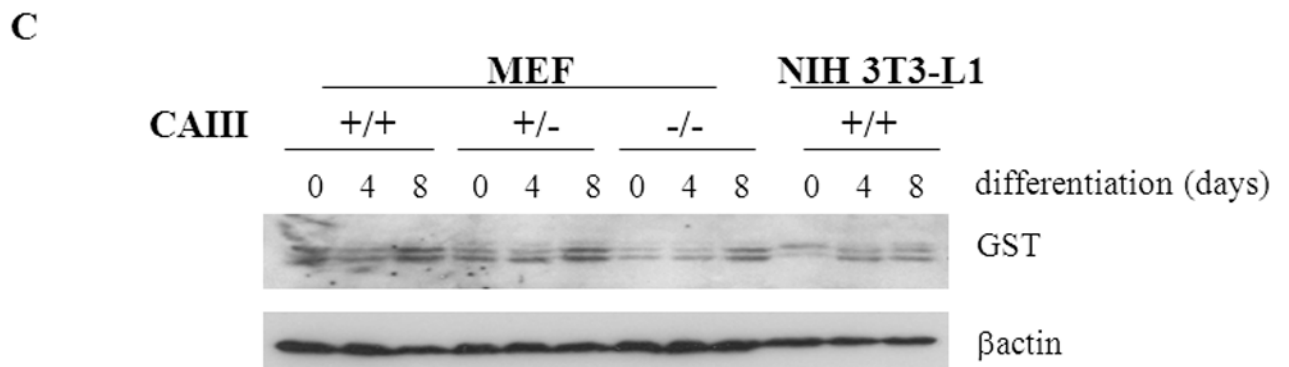
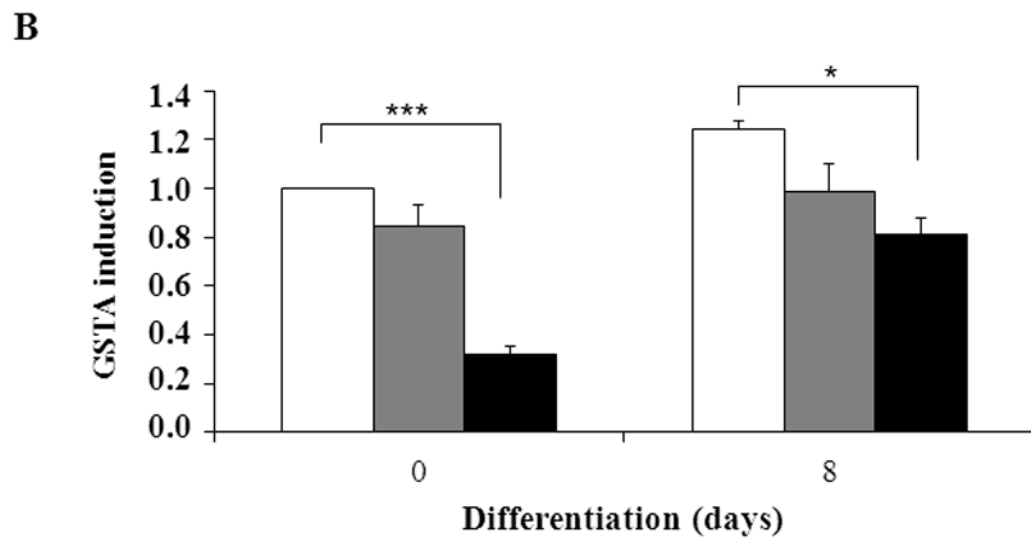
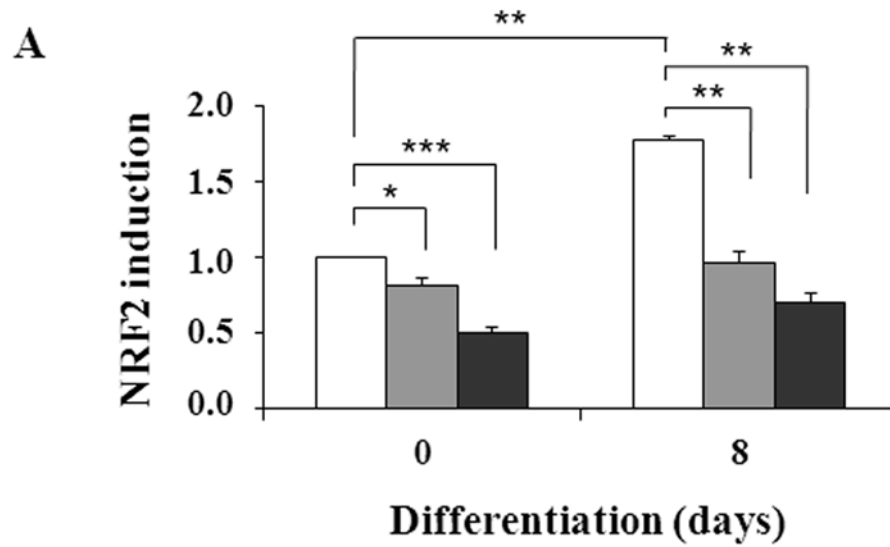


Fig. 2. Expression of adipogenic key markers in MEFCaIII^{+/+}, MEFCaIII^{+/-} and MEFCaIII^{-/-}

(A) MEFs were exposed to hormone-cocktail to induce adipogenesis for 8 days, either in the presence or absence of the PPAR γ 2 activator troglitazone (Tgl), as indicated. Q-RT-PCR analysis was performed at days 0, 4 and 8 of differentiation in MEFCaIII^{+/+} (white columns), MEFCaIII^{+/-} (grey columns), MEFCaIII^{-/-} cells (black columns), and NIH 3T3-L1 (diagonal-striped columns) for the adipogenic key markers PPAR γ 2. (B) MEFs were exposed to troglitazone (Tgl) without hormone-cocktail (DMI). Q-RT-PCR analysis of PPAR γ 2 was performed at day 4 after addition of Tgl in MEFCaIII^{+/+} (white columns), MEFCaIII^{+/-} (grey columns), and MEFCaIII^{-/-} cells (black columns). PPAR γ 2 expression

in MEFCAlII^{+/+} (white columns), MEFCAlII^{+/-} (grey columns), and MEFCAlII^{-/-} cells (black columns) exposed to hormone-cocktail (DMI) and Tgl at day 4 of differentiation is shown for comparison. (C and D) The MEFs were exposed to hormone-cocktail to induce adipogenesis for 8 days, either in the presence or absence of troglitazone (Tgl), as indicated. Q-RT-PCR analysis was performed on days 0, 4 and 8 of differentiation in MEFCAlII^{+/+} (white columns), MEFCAlII^{+/-} (grey columns), MEFCAlII^{-/-} cells (black columns), and NIH 3T3-L1 (diagonal-striped columns) for FABP-4 (C), and C/EBP α (D). Data are presented as the mean \pm SD of 3 measurements, (*) $p \leq 0.05$; (**) $p \leq 0.01$; (***) $p \leq 0.001$. (E) Western blot of differentiated MEFCAlII^{+/+}, MEFCAlII^{+/-}, MEFCAlII^{-/-}, and NIH 3T3-L1 cells (days 0, 4 and 8) was performed as indicated, using antibodies against the adipogenic markers, PPAR γ 1 and 2, C/EBP α , and FABP-4. β -Actin served as a loading control. One representative set of western blots of three independent western blot experiments is shown.



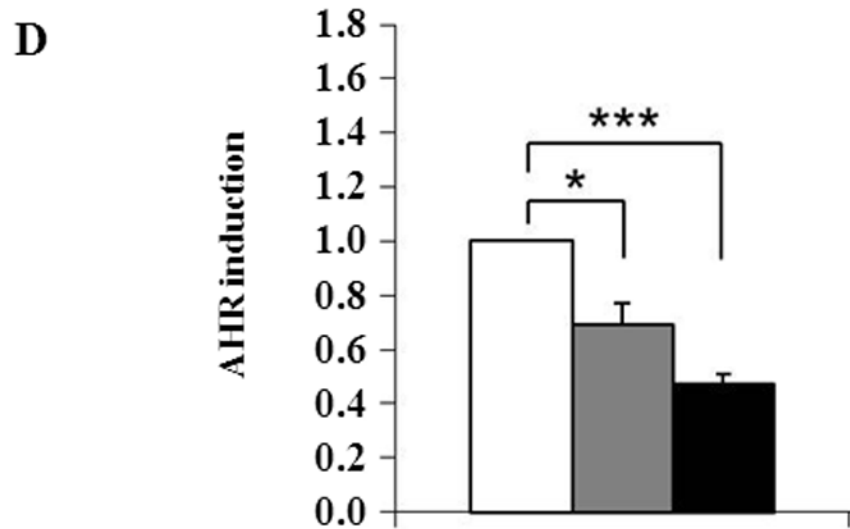
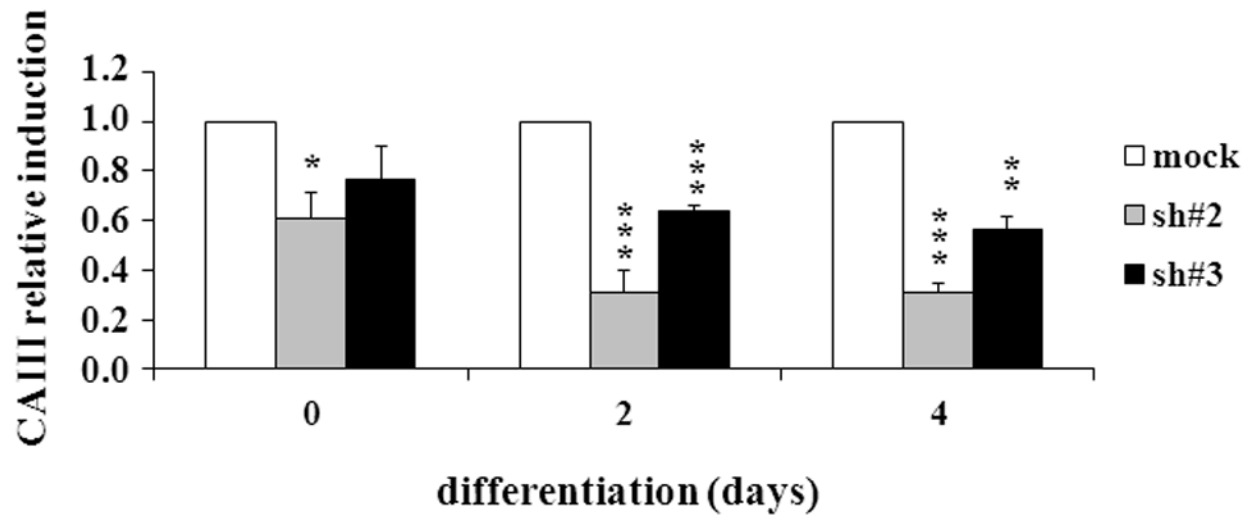
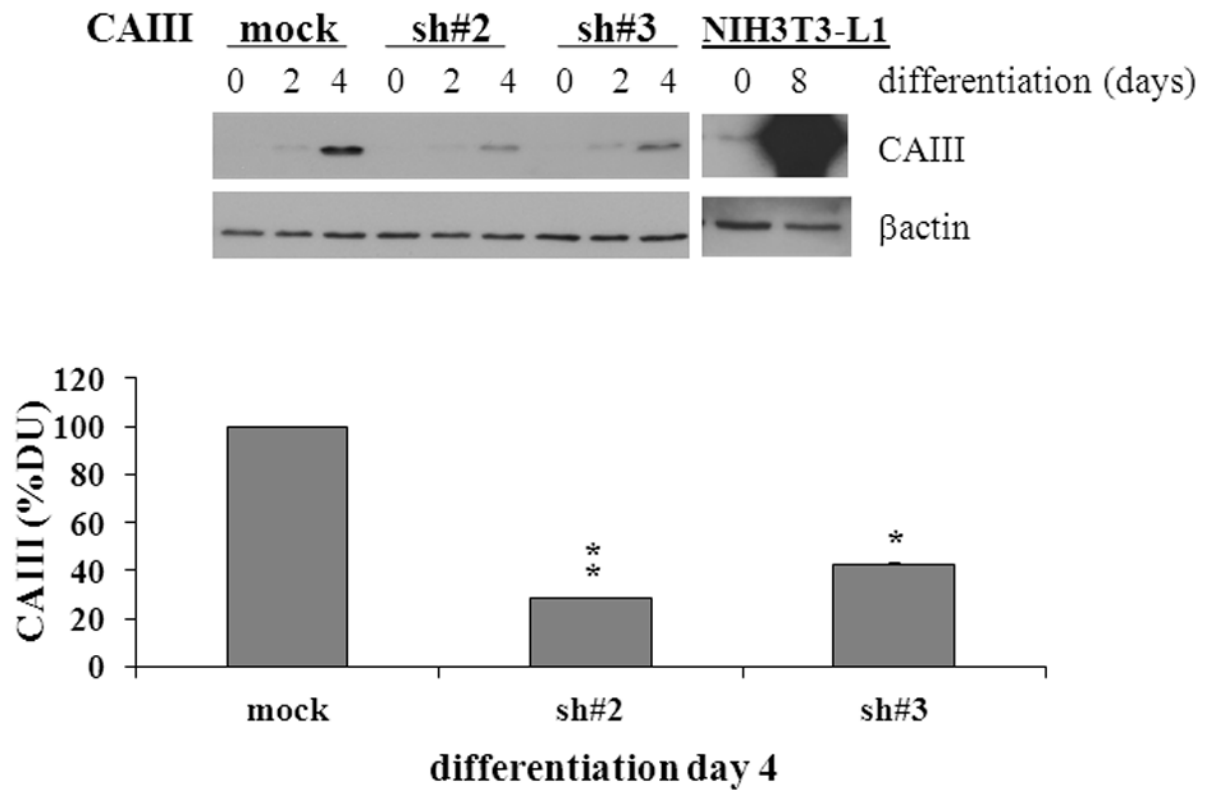
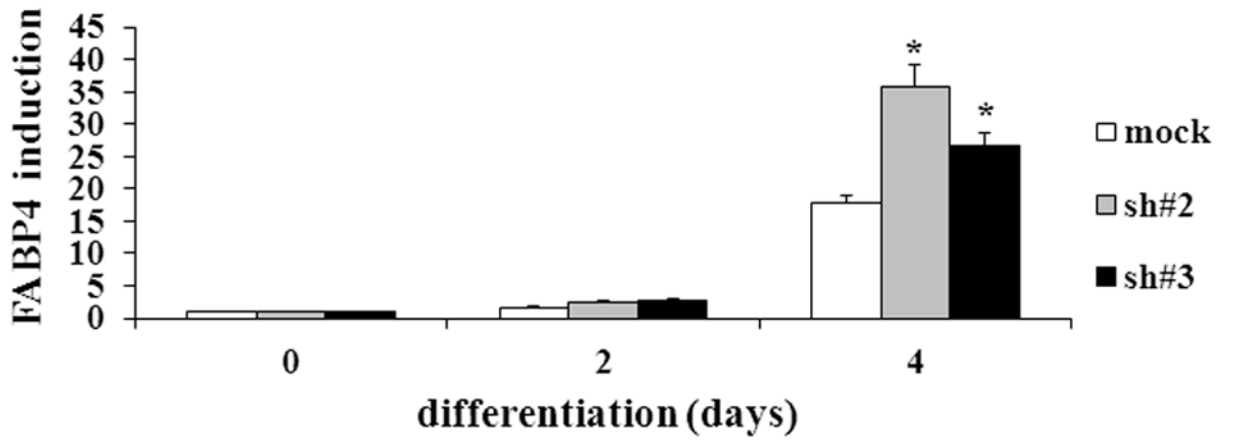
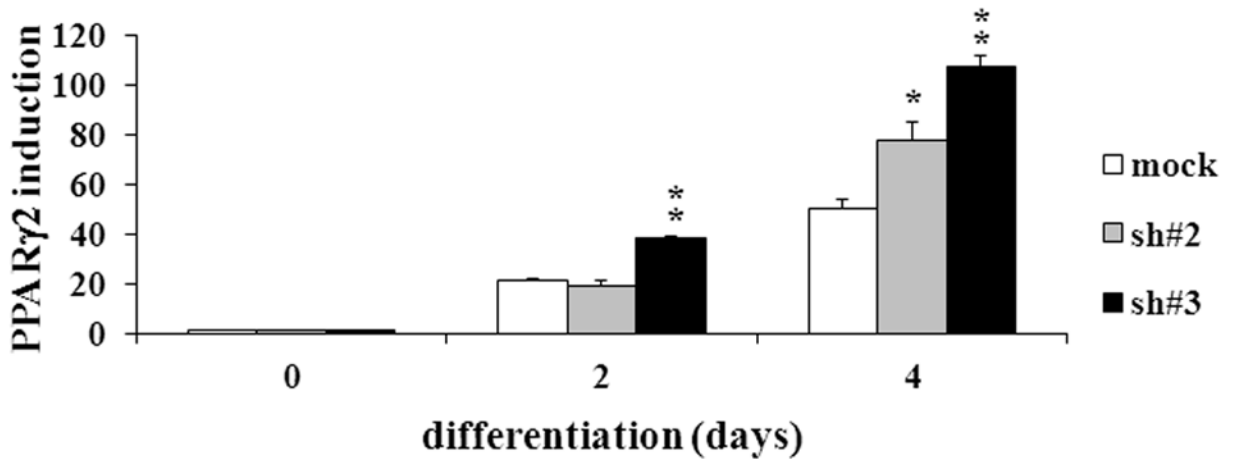


Fig. 3. Expression of the NRF2 gene and of its target gene, GST, in MEFCAlII^{+/+}, MEFCAlII^{+/-} and MEFCAlII^{-/-}

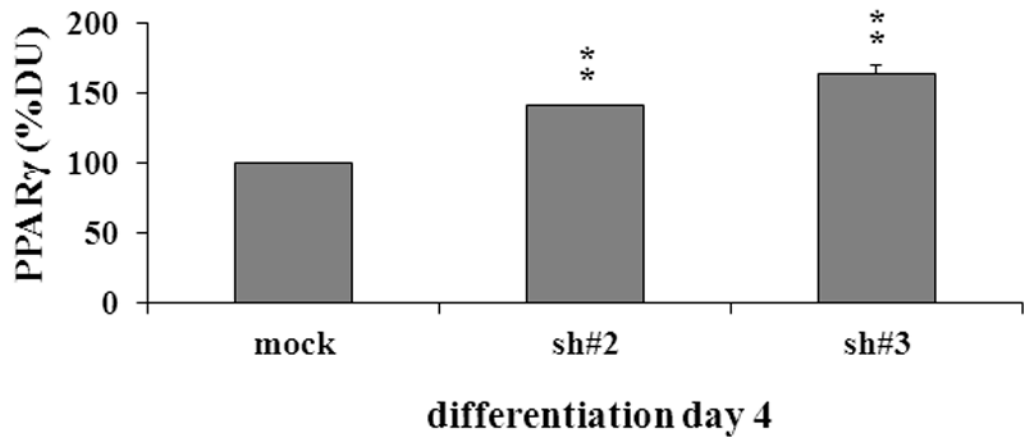
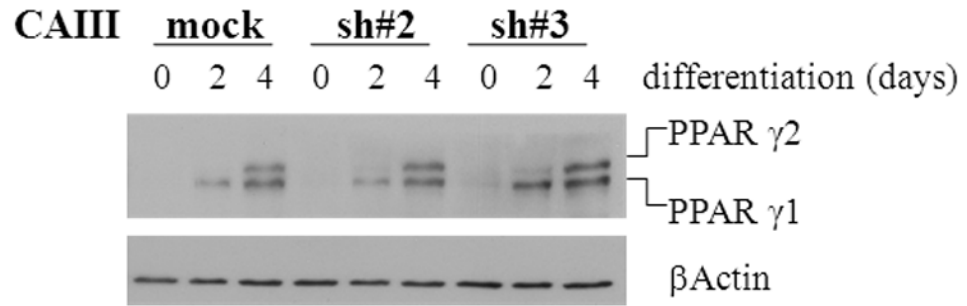
(A) NRF2 mRNA levels were monitored with q-RT-PCR analysis in MEFCAlII^{+/+} (white columns), MEFCAlII^{+/-} (grey columns), and MEFCAlII^{-/-} cells (black columns), before and 8 days after the induction of adipogenesis, as indicated. Data are presented as the mean \pm SD of 3 measurements, (*) $p \leq 0.05$; (**) $p \leq 0.01$; (***) $p \leq 0.001$. (B) GSTA mRNA levels were monitored with q-RT-PCR analysis in MEFCAlII^{+/+} (white columns), MEFCAlII^{+/-} (grey columns), and MEFCAlII^{-/-} cells (black columns), before and 8 days after the induction of adipogenesis. Data are presented as the mean \pm SD of 3 measurements, (*) $p \leq 0.05$; (***) $p \leq 0.001$. (C) A GST western blot analysis in MEFCAlII^{+/+}, MEFCAlII^{+/-}, MEFCAlII^{-/-}, and NIH 3T3-L1 cells before and 4 and 8 days after the induction of adipogenesis, as indicated, is shown. β -Actin served as a loading control. One representative western blot of three independent western blot experiments is shown. (D) AHR mRNA level were monitored with q-RT-PCR analysis in MEFCAlII^{+/+} (white columns), MEFCAlII^{+/-} (grey columns), and MEFCAlII^{-/-} cells (black columns). Data are presented as the mean \pm SD of 3 measurements, (*) $p \leq 0.05$; (***) $p \leq 0.001$.

A**B**

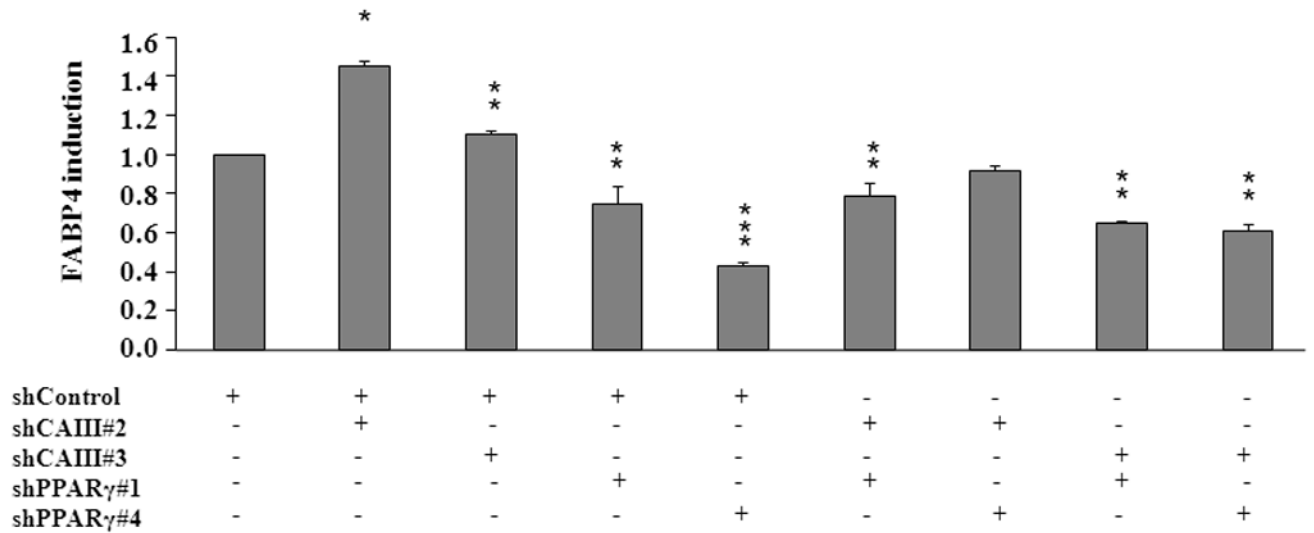
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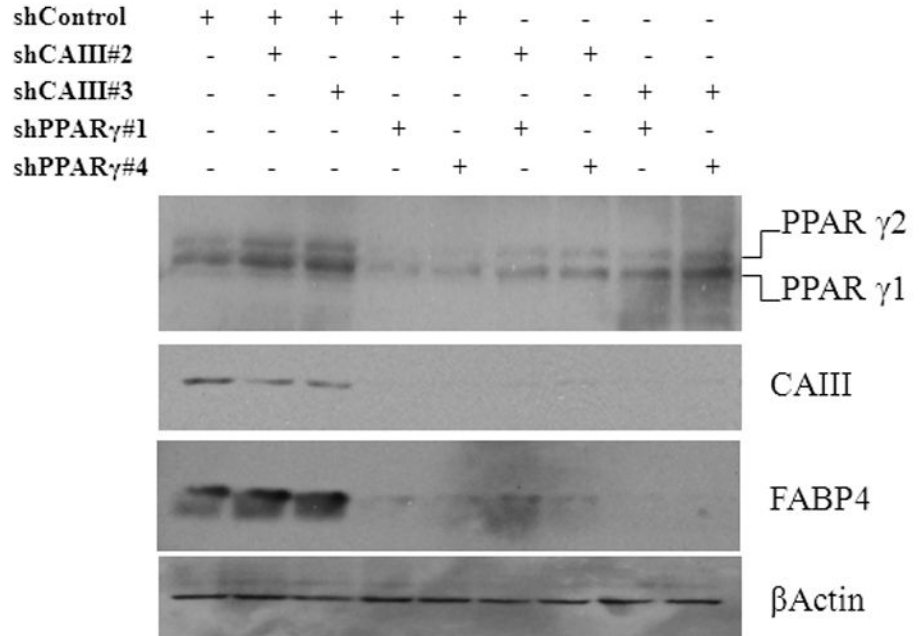
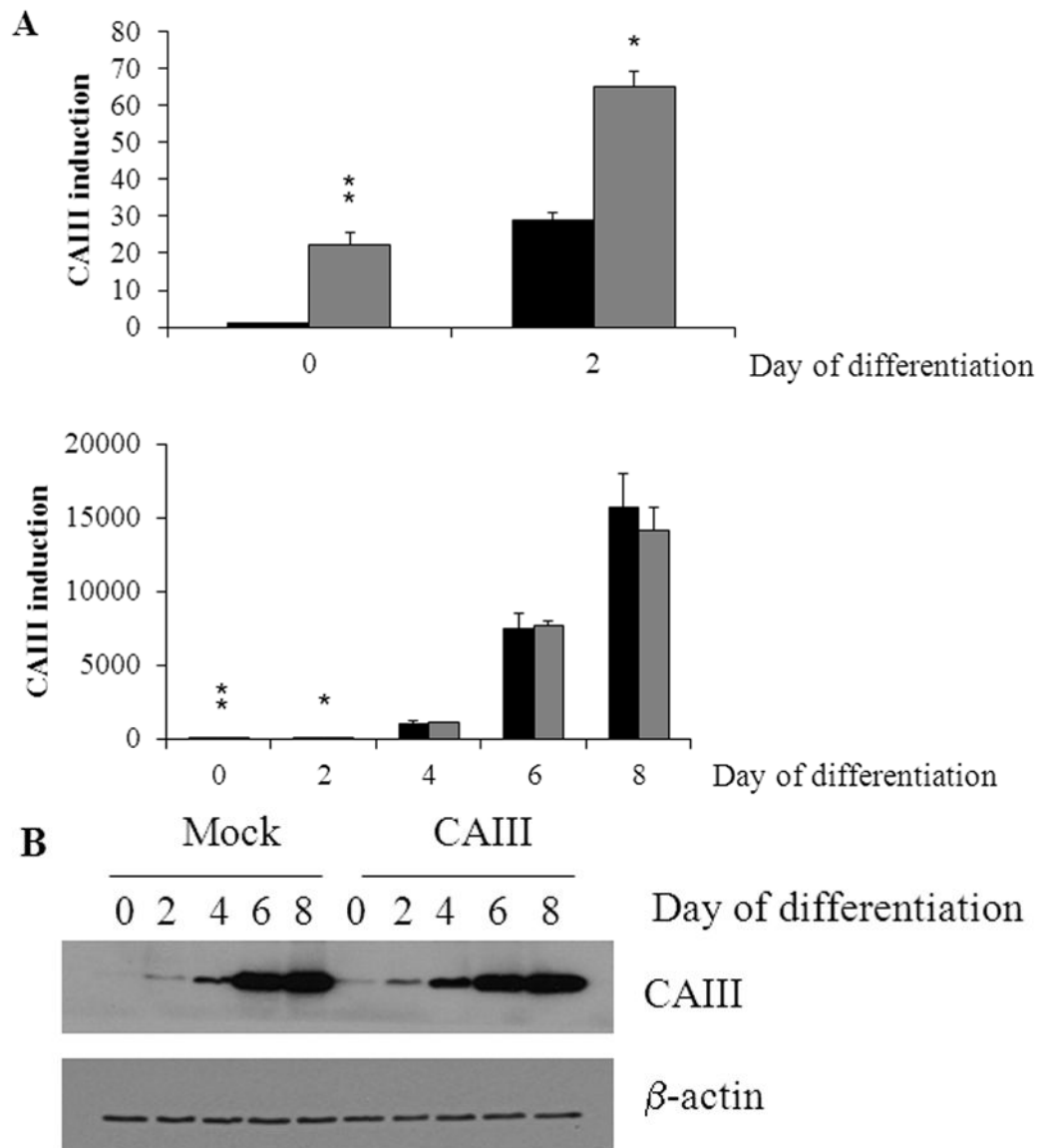
F

Fig. 4. Influence of CAIII knockdown on the induction of adipogenesis in NIH 3T3-L1 preadipocytes

NIH 3T3-L1 cells were infected with two different lentivirus constructs expressing shRNA against CAIII, sh#2 and sh#3, and a mock construct as a control. Differentiation was induced, and samples were taken at days 0, 2 and 4. (A) CAIII knockdown was measured by qRT-PCR. Normalization was calculated relative to the mock construct. (B) Upper panel: The CAIII protein level was investigated by western blot analysis. β -Actin served as the loading control. Lower panel: The CAIII protein level at day 4 was measured densitometrically (DU = densitometry units). (C) The qRT-PCR expression analysis of adipogenic key regulatory genes, PPAR γ 2 (upper panel) and FABP-4 (lower panel) is shown. (D) The PPAR γ protein levels were detected by western blotting. β -actin served as a loading control (upper panel). A densitometric evaluation of the PPAR γ protein levels at day 4 of differentiation was performed (lower panel). All data are presented as the mean \pm SD of 3 measurements, (*) $p \leq 0.05$; (**) $p \leq 0.01$; (***) $p \leq 0.001$. (E and F) NIH 3T3-L1 cells were infected with different combinations of lentivirus constructs expressing shRNA against CAIII (sh#2 and sh#3), PPAR γ 2 (sh#1 and sh#4) and/or a mock construct (sh#Control) as indicated. (E) Differentiation was induced and FABP4 mRNA level measured at day 4 by q-RT PCR. (F) Differentiation was induced and CAIII, FABP4 and PPAR γ protein level measured at day 4 by western blotting. β -actin served as a loading control.



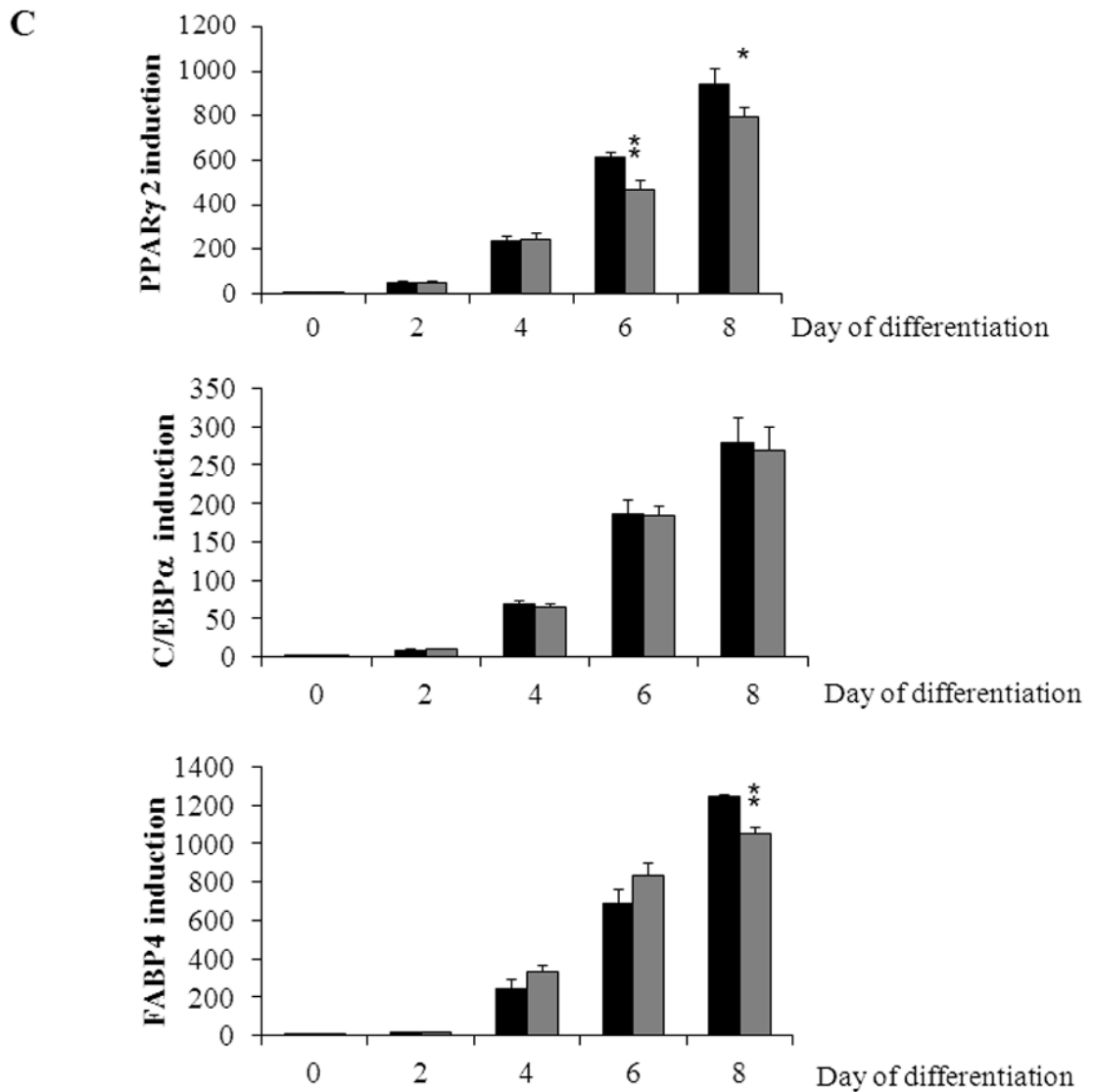


Fig. 5. Influence of overexpression of CAIII on the induction of adipogenesis in NIH 3T3-L1 preadipocytes

NIH 3T3-L1 cells were infected with plentiCAIII overexpressing CAIII or a mock construct (plentiEmpty). Differentiation was induced, and samples were taken on days 0, 2, 4, 6 and 8. (A) CAIII overexpression was measured by qRT-PCR in NIH 3T3-L1/plentiEmpty (black columns) and in NIH 3T3-L1/plentiCAIII (grey columns). Normalization was calculated relative to the mock construct. (B) The CAIII protein level was measured by Western blot. β -actin served as the loading control. (C) The qRT-PCR expression analysis of the key adipogenic regulatory genes, PPAR γ 2 (upper panel) and C/EBP α (middle panel) and of the differentiation marker FABP-4 (lower panel) is shown in NIH 3T3-L1/plentiEmpty (black columns) and in NIH 3T3-L1/plentiCAIII (grey columns). All data are presented as the mean \pm SD of 3 measurements, (*) $p \leq 0.05$; (**) $p \leq 0.01$.