Dual Regulatory Role of Polyamines in Adipogenesis*

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Background: Polyamines are required for the process of adipogenesis.

Results: Polyamine depletion causes inactivation of C/EBPβ that is correlated with elevation of CHOP, and independently causes inhibition of mitotic clonal expansion.

Conclusion: Polyamines are required for both main hubs of adipogenesis; completion of MCE and activation of C/EBPβ.

Significance: Our results assist clarifying the mechanism by which polyamines support adipogenesis.

Adipogenesis is a complex process, accompanied by a chain of interdependent events. Disruption of key events in this cascade may interfere with the correct formation of adipose tissue. Polyamines were demonstrated necessary for adipogenesis; however, the underlying mechanism by which they act has not been established. Here, we examined the effect of polyamine depletion on the differentiation of 3T3-L1 preadipocytes. Our results demonstrate that polyamines are required early in the adipogenic process. Polyamine depletion inhibited the second division of the mitotic clonal expansion (MCE), and inhibited the expression of PPARγ and C/EBPα, the master regulators of adipogenesis. However, it did not affect the expression of their transcriptional activator, C/EBPβ. Additionally, polyamine depletion resulted in elevation of mRNA and protein levels of the stress-induced C/EBP homologous protein (CHOP), whose dominant negative function is known to inhibit C/EBPβ DNA binding activity. Conditional knockdown of CHOP in polyamine-depleted preadipocytes restored PPARγ and C/EBPα expression, but failed to recover MCE and differentiation. Thus, our results suggest that the need for MCE in the adipogenic process is independent from the requirement for PPARγ and C/EBPα expression. We conclude that de novo synthesis of polyamines during adipogenesis is required for down-regulation of CHOP to allow C/EBPβ activation, and for promoting MCE.

Consumption of unutilized calories induces a metabolic state that promotes the commitment of mesenchymal stem cells to become preadipocytes, followed by their differentiation into mature adipocytes (1). The molecular events driving this differentiation process are identified and characterized using model cell culture systems, most notably the 3T3-L1 preadipocytic cell line (2, 3). Stimulation of growth-arrested 3T3-L1 preadipocytes with a stimulating mixture (composed of fetal bovine serum, insulin, dexamethasone, and 3-isobutyl-1-methylxanthine), initiates a sequential program of molecular events, which leads to their differentiation into adipocytes (4, 5). This treatment elicits a mitogenic response, resulting in roughly two rounds of cell division, a process termed mitotic clonal expansion (MCE),2 which is necessary for successful differentiation (6). Shortly following induction, the stimulated preadipocytes express high levels of CCAAT/enhancer-binding protein β (C/EBPβ), which is required for MCE (7). C/EBPβ is also the transcription factor of peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT/enhancer-binding protein α (C/EBPα), the master transcriptional regulators of adipogenesis (8). Once expressed, these two transactivators induce the expression of downstream genes that execute differentiation and arrest the process of MCE (8–13). Although expressed early after stimulation, C/EBPβ acquires DNA binding activity only after a lag of about 16 h (14). This lag is required to delay the expression of PPARγ and C/EBPα, which display an antiproliferative effect that can negate the essential manifestation of MCE. The delay in C/EBPβ activation is achieved through its suppression by interaction with the dominant negative C/EBP homologous protein (CHOP) (15, 16).

The polyamines spermidine and spermine and their diamine precursor, putrescine, are ubiquitous polycations that were demonstrated essential for fundamental cellular processes, including cellular proliferation and differentiation (17). Ornithine decarboxylase (ODC) is the rate-limiting enzyme in the polyamine biosynthesis pathway. Treatment with the reversible, mechanism-based ODC inhibitor, α-difluoromethylornithine (DFMO), results in polyamine depletion and inhibition of cellular proliferation and differentiation (18, 19). It is frequently hypothesized that polyamines exert their pro-proliferative effect through their electrostatic interactions with various cellular components such as DNA, RNA, membranes, receptors and channels. However, none of these interactions was validated as the direct cause for the proliferative arrest of polyamine-depleted cells. In fact, our recent studies suggested that establishment of integrated stress response through activation of the PERK arm of the unfolded protein response (UPR) leads to the proliferative inhibition of polyamine-depleted cells (20, 21).
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C/EBPβ activation is required for both MCE and for the expression of PPARγ and CEBPα. Therefore, it was difficult to uncouple between the importance of MCE and the expression of PPARγ and C/EBPα. Consequently, the standalone significance of MCE is still unclear. Since polyamines are vital for cellular proliferation, and the need for MCE in adipogenesis is strongly suggested, the requirement for polyamines in adipogenesis is ought to be examined. In fact, early studies revealed that polyamines are indeed essential for adipogenesis (22–24). However, the exact mechanism, by which polyamine depletion inhibits adipogenesis, is still to be deciphered. A recent study suggested that elevated interaction of ANP32 with HuR and PP2A in polyamine-depleted cells may account for the inhibition of adipogenesis, by decreasing translation of C/EBPβ (25).

In the present study we validated the essentiality of polyamines for adipogenesis. We show that polyamine depletion has a dual impact on adipogenesis: It induces expression of CHOP, which is correlated with loss of C/EBPβ activity. This results in inhibition of MCE and repression of PPARγ and C/EBPα expression. In parallel and independently of CHOP, polyamine depletion hinders MCE. Both mechanisms lead to loss of adipocytic differentiation.

Experimental Procedures

Cell Culture Conditions and Induction of Differentiation—3T3-L1 mouse preadipocytes were grown in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen), supplemented with 10% (v/v) adult bovine serum (Biological Industries), 100 units/ml penicillin, and 100 g/ml streptomycin (Pen-Strep, Biological Industries) at 37 °C in 7% CO2. Induction of differentiation was done as described before (14): Two-day post-confluent preadipocytes (marked day 0) were fed DMEM containing 10% (v/v) fetal bovine serum (FBS; Gibco), 1 μg/ml insulin (Biological Industries), 1 μM dexamethasone (Sigma), and 0.5 mM 3-isobutyl-1-methylxanthine (IBMX, Sigma) for 2 days. Then, the medium was replaced to DMEM supplemented with 10% FBS and insulin for two additional days, after which the medium was replaced every other day for 12 days. The differentiated cells were then washed with PBS, incubated for 1 h in 10% formalin, and washed with 60% isopropanol. The cells were incubated with Oil Red O solution (205 μM Oil Red O (SIGMA) in 60% isopropanol, filtered through a 0.22 μm membrane filter) for 10 min and washed four times with distilled water.

OCD Activity Assay—Cells were washed with PBS, suspended in OCD activity buffer (25 mM Tris-HCl, pH 7.5, 2.5 mM DTT, 200 μM PLP) and lysed by three cycles of freeze/thaw. Lysates were centrifuged, supernatant was collected and protein concentration was determined using the Bradford reagent. A portion of 300 μg of protein was added to a final volume of 90 μl of OCD activity buffer in a 96 well microtiter plate. C14-Orotate (0.1 μCi; Perkin Elmer) in OCD activity buffer was added to each well. Whatman 3-mm filter paper impregnated with saturated BaOH was used to cover the microtiter plate. The closed plate was incubated for 3 h at 37 °C. The filter was washed with acetone and dried. Liberated C14-CO2 that was trapped in the filter paper was monitored using a Fujifilm BAS-2500 phosphor-imaging scanner.

Polyamine Analysis—3T3-L1 mouse preadipocytes were harvested, pelleted, and suspended in 100 μl of PBS. The cells were lysed in 3% perchloric acid, and the precipitated material was removed by centrifugation (5 min at 13,000 rpm). The supernatant was collected for polyamine analysis, and the pellet was used for normalization by DNA quantification (DNA was quantified by suspending the pellet in 400 μl of 4% diphenylamine (Sigma) in acetic acid, 400 μl of 10% perchloric acid, and 20 μl of 1:500 acetaldehyde (Sigma), and by incubation for 16 h at 30 °C followed by absorbance determination at 595 and 700 nm). For polyamine analysis, 100 μl of the perchloric acid supernatant was mixed with 200 μl of 6 mg/ml dansyl-chloride (in acetone). The mixture was incubated for 16 h in the dark with 3 mg/ml sodium carbonate. To neutralize residual dansyl-chloride, 16.67 mg/ml l-proline solution was added and incubated for 1 h at room temperature. Dansylated derivatives were extracted into toluene by centrifugation. Samples were spotted on Silica 60 F254 TLC plate (Merck), and the dansylated derivatives were resolved by TLC using ethyl acetate/cyclohexane (1:1.5) as a solvent, in the dark. The plate was visualized by UV.

Immunoblot Analyses—Cells were induced to differentiate as described above, and cellular extracts were prepared at the indicated time points. Cellular extracts were prepared by lysing cells in RIPA buffer (50 mM Tris-HCl pH 8, 150 mM KCl, 1.0% Nonidet P-40 (IGEPAL), 0.5% sodium deoxycholate, 0.1% SDS) supplemented with protease and phosphatase inhibitors cocktails (Sigma). Equal portions of protein were resolved by electrophoresis in SDS-polyacrylamide gel, blotted to a nitrocellulose membrane and incubated with the indicated antibodies followed by horseradish peroxidase-conjugated anti-IgG antibodies. The antibodies used were: rabbit pAb anti-C/EBPβ, rabbit pAb anti-C/EBPα, mouse mAb anti-PPARγ, mouse mAb anti-Actin (Sigma-Aldrich), rabbit mAb anti-C/EBPα, rabbit pAb anti-P27 (Abcam), mouse mAb anti-cyclin D1 (Santa Cruz Biotechnology), mouse mAb anti-CyclinB1 (Cell Signaling), mouse mAb anti-Skp2 (ZYMED), mouse mAb anti-GAPDH (Sigma-Aldrich), rabbit pAb and mouse mAb anti-CHOP (Gadd153) (Santa Cruz Biotechnology), rabbit pAb anti-ETO (Calbiochem), rabbit pAb anti-p-eIF2α (Invitrogen), rabbit pAb anti-eIF2α (Santa Cruz Biotechnology), rabbit pAb anti-ATF4 (Santa Cruz Biotechnology). Signals were developed using “EZ-ECL” (Biological Industries), and the membranes were analyzed using ImageQuant LAS4000 luminescent image analyzer (General Electric).

Nuclear Extraction—To analyze GSK3β and ETO proteins, we used Thermo Scientific NE-PER nuclear extraction reagents, and followed the manufacturer’s protocol.

Immunofluorescence Microscopy—3T3-L1 preadipocytes were plated onto chamber-slides and grown to confluency. Two days postconfluence the cells were induced to differentiate as mentioned above. At the indicated times, the cell were washed with cold PBS, fixed with 4% para-formaldehyde in PBS at room temperature for 20 min, permeabilized with 0.5% Triton X-100 in PBS for 20 min and blocked with 90% fetal bovine serum, 10%
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**Results**

**Polyamines Are Required at Early Stages of Adipogenesis**—To validate the requirement for polyamines in the process of adipogenesis, DFMO was added to 1 day confluent culture of 3T3-L1 preadipocytes followed by their adipogenic stimulation 1 day thereafter (indicated as day 0). As shown in Fig. 1A, treatment with DFMO completely inhibited adipogenesis, which was clearly observed in control cells. This inhibition was fully reversed by the addition of spermidine (10 μM) together with the stimulating mixture. To gain better insight regarding the stage of the differentiation process at which polyamines are required, we added spermidine on consecutive days following the adipogenic stimulation. Addition of spermidine on days 0 and 1 reversed the inhibitory effect of DFMO, but was less effective when added on day 2 and almost ineffective from day 3 onwards (Fig. 1B). These results indicate that polyamines are necessary at early stages of the adipogenic process.

**ODC Activity and Polyamine Synthesis Are Induced Early in the Adipogenic Process**—Since the above experiments suggested that polyamines are required at an early stage of the adipogenic process, we next set out to determine whether the adipogenic process utilizes pre-existing or newly synthesized polyamines. For this purpose, two-day post-confluent preadipocytes (day 0) were stimulated to differentiate in the absence or presence of DFMO. Cellular extracts were prepared on the first 4 days following stimulation, and were subjected to ODC activity assay and polyamine analysis. ODC activity was sharply induced, rising already 1 day following stimulation, and was completely inhibited by the addition of DFMO to the stimulated cells (Fig. 2A). The level of spermidine and spermine rose sharply from day 2 and on. DFMO completely prevented the induction of spermidine which dropped from day 1 and on, probably due to its consumption in the differentiation process. The level of spermine remained unchanged in the DFMO-treated cells (Fig. 2B). Our results therefore agree with previous studies, showing that spermidine is the polyamine that supports adipogenesis (23, 27).

**Polyamine Depletion Inhibits the Second Division of the Mitotic Clonal Expansion**—When 3T3-L1 preadipocytes are induced to differentiate, the cells undergo two sequential rounds of mitosis (MCE). The first round of mitosis is completed 24–36 h following induction, and the second round is completed 48–60 h post induction (28, 29). Since MCE is necessary for adipogenesis (6) and polyamines are necessary for cellular proliferation (17, 30), we next tested whether polyamine depletion interferes with adipogenesis. To do so, preadipocytes, induced in the presence or absence of DFMO, were harvested at the indicated times and counted. While two rounds of replication were noted in the untreated cells, the...
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Polyamine Depletion Inhibits PPARγ and C/EBPα Induction, without Affecting the Expression of C/EBPβ—Induction of two-day post-confluent 3T3-L1 preadipocytes initiates a cascade of molecular events, essential for the manifestation of the adipogenic phenotype. This cascade starts with rapid expression of C/EBPβ, and is followed by the induction of its target genes PPARγ and C/EBPα, which in turn trans-activate a set of adipogenic genes (8, 9, 12). To determine whether polyamine depletion affects the expression of these key regulators, their level was determined in cellular extracts prepared at successive days following stimulation of preadipocytes in the presence or absence of DFMO. While the expression of C/EBPβ remained unaffected, the induction of C/EBPα and PPARγ was greatly inhibited by the DFMO treatment (Fig. 4).

Polyamine Depletion Interferes with C/EBPβ DNA Binding Activity—Expression of C/EBPβ, the transcriptional activator of PPARγ and C/EBPα, is rapidly induced following adipogenic stimulation. However, it is synthesized as an inactive monomeric transcription factor, lacking DNA binding activity, which is acquired only at a later stage. As a result, there is a lag between the stage at which expression of C/EBPβ is initiated and the time at which it transcriptionally activates PPARγ and C/EBPα (14). The unaffected expression of C/EBPβ and inhibited expression of PPARγ and C/EBPα may therefore suggest that polyamine depletion inhibits the activation of C/EBPβ. It has been demonstrated that as C/EBPβ acquires DNA binding activity, it assumes centromeric localization, resulting in punctate immunostaining. This staining is known to co-localize with DAPI staining, which strongly interacts with the centromeric heterochromatin of mouse chromosomes, yielding the same typical punctate staining. This co-localization was shown to

FIGURE 2. ODC activity and polyamine levels increase early during adipogenesis. 3T3-L1 preadipocytes were induced to differentiate, and cellular extracts were prepared at the indicated times. DFMO (5 mM) was added to the cells 1 day before induction. Lysates were subjected to ODC activity assay (A) and polyamine analysis (B) as described under “Experimental Procedures.” The positions of polyamine markers are indicated. Put, putrescine; Spd, spermidine; Spm, spermine.

FIGURE 3. Polyamine depletion inhibits the second division of the mitotic clonal expansion. 3T3-L1 preadipocytes were induced to differentiate. DFMO (5 mM) was added to the cells 1 day before induction. A, cells were counted at the indicated times. Data are presented as the mean of three independent biological repeats. Standard deviations are represented by error bars. Statistically significant counts of DFMO-treated cells are marked with asterisks (**, p < 0.001; ****, p < 0.0001). B, cellular extracts were prepared at the indicated times, and the protein level was determined by Western blot analysis with the indicated antibodies. Representative blots of at least three independent experiments are shown. CycD1, Cyclin D1; CycB1, Cyclin B1; *, nonspecific band.

FIGURE 4. Polyamine depletion inhibits PPARγ and C/EBPα induction, without affecting the expression of C/EBPβ. 3T3-L1 preadipocytes were induced to differentiate. DFMO (5 mM) was added to the cells 1 day before induction. Cellular extracts were prepared at the indicated times, and the protein level was determined by Western blot analysis with the indicated antibodies. Representative blots of at least five independent experiments are shown for C/EBPβ and at least three independent experiments for PPARγ and C/EBPα are shown.

second round of cell division was completely prevented in the DFMO treated cells. The addition of spermidine to the induction mixture fully reversed the inhibitory effect of DFMO (Fig. 3A). Complete inhibition of MCE causes an alteration in the expression of cell cycle related proteins (7). Inhibition of polyamine synthesis during adipogenic differentiation, which inhibited only one division of the MCE, did not alter the expression of cyclin D1, cyclin B1, P27, and CDK2 (Fig. 3B).
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Polyamine Depletion Interferes with the Regulation of CHOP mRNA Levels—We have previously demonstrated that polyamine depletion in NIH-3T3 cells provokes an integrated stress response (ISR) via activation of the PERK arm of the UPR, resulting in phosphorylation of eIF2α and induction of ATF4 and CHOP (20). The demonstrated induction of CHOP raises the possibility that a similar mechanism is activated in stimulated preadipocytes that were depleted of polyamines. To test whether polyamine depletion provokes ISR in the stimulated preadipocytes, we tested for the status of eIF2α and ATF4. The phosphorylation pattern of eIF2α was not affected by polyamine depletion. Interestingly, eIF2α was already phosphorylated in the growth-arrested preadipocytes, and became dephosphorylated at the first day following stimulation. Likewise, the expression pattern of ATF4 was similar in control and DFMO treated cells (Fig. 6C). This finding indicates that once stimulated, the infrastructure for transcription and translation of CHOP is laid both in control and in DFMO-treated preadipocytes, but CHOP protein accumulates to high levels only in the polyamine-depleted cells.

Since differences in factors of the PERK arm that are upstream to CHOP were not noted, we next determined whether the increase in the level of CHOP in polyamine-depleted cells is a result of a transcriptional activation. For this purpose we analyzed CHOP mRNA levels by real time PCR. In agreement with a previous study (16), we have noticed a basal level of CHOP mRNA in the growth arrested preadipocytes, which is dramatically reduced in the first 2 days following stimulation. From the third day onwards, CHOP mRNA rises gradually, until it regains its basal level (Fig. 6D, control). We measured the relative levels of CHOP mRNA on consecutive days following stimulation, and compared them to its level in non-stimulated two-day post-confluent cells (day 0), in control and in DFMO-treated cells. Inhibition of newly synthesized polyamines interfered significantly with the early decrease of CHOP mRNA. Furthermore, CHOP mRNA was elevated to levels higher than the basal level from day 3 and on (Fig. 6D), being compatible with elevated CHOP protein levels on compatible days. Comparison of the relative levels of CHOP mRNA in each day between control and polyamine-depleted cells revealed that polyamine depletion increased the relative level of CHOP mRNA by up to more than 4-fold (Fig. 6E).

Induction of CHOP Correlates with C/EBPβ Inactivation—Several mechanisms were described previously as potentially regulating C/EBPβ activity. These include phosphorylation by GSK3β, which regulates its nuclear translocation (31, 32), inhibition by ETO, a protein highly expressed in preadipocytes (33), and heterodimerization with CHOP, a dominant negative member of the C/EBP family of proteins, which suppresses C/EBPβ DNA binding activity (15, 16, 34). We have therefore tested whether polyamine depletion affects the expression pattern or behavior of any of these regulators. Both GSK3β and ETO responded similarly to the adipogenic stimulation both in control and in the DFMO treated cells (Fig. 6A). In contrast, CHOP expression was induced to a significantly higher level in the DFMO treated cells (Fig. 6B). Interestingly, the rise in the level of CHOP in DFMO-treated cells was noted from day 3 post induction, in correlation with the timing of the loss of C/EBPβ activity.

Induction of ShCHOP expression in polyamine-depleted cells, resulted in significant inhibition of CHOP induction, and an increased expression of PPARγ and C/EBPα, versus ShNT (Fig. 7A), indicating that the inhibition of CHOP expression relieved the inhibition of C/EBPβ activity. Interestingly, despite

reflect the ability of C/EBPβ to bind DNA (14). Hence, we used in situ immunofluorescence analysis to determine whether polyamine depletion affects the activity of C/EBPβ. As shown in Fig. 5, in control cells C/EBPβ was already expressed four hours post induction and was diffusely distributed in the nuclei. Next, this pattern was changed into a punctate centromeric pattern at 24 h, which remained stable up to 96 h. In the DFMO treated cells, punctate centromeric pattern appeared as in control cells at 24 h, was maintained up to 48 h, but became diffused at 72 h. This result suggests that C/EBPβ activity was established normally in the polyamine-depleted cells at the beginning of the differentiation process (24 and 48 h post stimulation), but was inhibited at later stages (from 72 h and on).

FIGURE 5. Polyamine depletion interferes with C/EBPβ DNA binding activity. 3T3-L1 preadipocytes were induced to differentiate on chamber-slides. DFMO (5 mM) was added to the cells 1 day before induction. Preadipocytes were fixed at the indicated times and immunostained with anti-C/EBPβ antibody and RRX-labeled secondary antibody. The cells were mounted by ProLong Gold antifade reagent, which contains DAPI.

Inactivation of CHOP Correlates with C/EBPβ Activity—Inactivation—Interaction with CHOP inactivates C/EBPβ, resulting in inhibition of MCE and the expression of PPARγ and C/EBPα (16, 34). It is possible however, that increased CHOP expression is not the only cause for inhibition of adipogenesis, as polyamine depletion may inhibit MCE due to other reasons (20, 21, 35). To address this possibility we have established a 3T3-L1-derived cell line stably transduced by a pLKO-Tet-On vector, expressing a Tet-inducible ShRNA for CHOP (ShCHOP) and ShRNA for a non-targeting sequence (ShNT) as a control.

Induction of ShCHOP expression in polyamine-depleted cells, resulted in significant inhibition of CHOP induction, and an increased expression of PPARγ and C/EBPα, versus ShNT (Fig. 7A), indicating that the inhibition of CHOP expression relieved the inhibition of C/EBPβ activity. Interestingly, despite
these changes, the inhibition of CHOP expression was not sufficient to allow full differentiation of polyamine-depleted preadipocytes, similarly to ShNT (Fig. 7B). This is most likely due to the inability of CHOP knockdown to rescue polyamine-depleted cells from the halted MCE similarly to ShNT (Fig. 7C).

This observation suggests an independent requirement of MCE in the adipogenic differentiation process.

Discussion

In the present study we show that polyamine depletion inhibits adipogenesis because of induction of CHOP, a stress-related protein, which inhibits the activity of C/EBPβ, a key regulator of adipogenesis, and independently due to the requirement of polyamines for the process of MCE.

Polyamines are essential for proliferation and differentiation of mammalian cells (17, 22, 23, 30, 36, 37). Here we validate that adipogenic stimulation is accompanied by the induction of ODC and de novo synthesis of polyamines. Polyamine depletion by DFMO treatment halted differentiation and inhibited the second division of the MCE, suggesting that one division is not sufficient for successful progression throughout the differentiation process. In agreement with previous studies (23, 27), DFMO treatment prevented the induction of spermidine but not of spermine. Thus, we conclude that spermidine is the active polyamine in this differentiation program.

C/EBPβ is an early induced transcription factor essential for adipogenesis. C/EBPβ is required for the execution of the process of MCE and for the induction of PPARγ and C/EBPα, the master regulators of adipogenesis (7, 14, 38). As shown here, both MCE and the induction of PPARγ and C/EBPα are impaired in DFMO-treated cells, but the expression of C/EBPβ is unaffected. It has been established that while C/EBPβ expression is rapidly induced upon adipogenic stimulation, it acquires DNA binding activity only at a later stage (14). We show here that C/EBPβ expression is rapidly induced both in control and DFMO-treated cells and is maintained at high levels throughout the process of adipogenesis. Moreover, C/EBPβ gains DNA binding activity 24 h post induction, both in control and DFMO-treated cells. However, DNA binding activity is lost 2 days later in the polyamine-depleted cells. A recent study reported a decrease in the level of C/EBPβ protein due to polyamine depletion (25). The reason for the discrepancy between this and our present findings is not clear.

Activation of C/EBPβ is regulated in several ways, including its phosphorylation by GSK3β, inhibition by ETO, and heterodimerization with CHOP. While excluding the involvement of GSK3β and ETO, we noted a significant induction of CHOP in DFMO-treated cells. All proteins in the C/EBP family contain a C-terminal leucine-zipper dimerization domain, which allows formation of homo or heterodimers with other C/EBP.
family members. The dimerization is obligatory for the DNA binding activity of these proteins. The DNA binding domain of CHOP is nonfunctional and therefore its dimerization with C/EBP blocks DNA binding activity, and as a result blocks both MCE and adipogenesis (15, 16, 34). When expressed ectopically in 3T3-L1 cells, CHOP blocks adipogenesis (34). Furthermore, when growth arrested by contact inhibition, preadipocytes normally express CHOP (16, 34). Apparently, CHOP expression is required to keep C/EBP in an inactive state, until a differentiation signal is given, resulting in rapid down-regulation of CHOP. This way, C/EBP receives the appropriate time window to fulfill its role in supporting MCE prior to the manifestation of the anti-mitotic effect of PPARγ and C/EBPα (16). As shown here, polyamine depletion interferes with this program by forcing CHOP expression when it should be repressed, causing the inactivation of C/EBPβ.

CHOP is mostly known as a stress-induced protein, and is generally up-regulated by the PERK arm of the UPR. PERK activation leads to the phosphorylation of eIF2α, resulting in general inhibition of protein synthesis and preferential translation of a set of stress responsive mRNA including that of ATF4, which in turn induces the expression of CHOP (39). We have recently demonstrated that polyamine depletion in NIH-3T3 fibroblasts provokes ISR, activating only the PERK arm of the UPR (20). We show here that eIF2α is phosphorylated and

FIGURE 7. Polyamine depletion inhibits MCE also in a CHOP independent manner. 3T3-L1 preadipocytes were infected with a lentivirus expressing Tet-induced CHOP shRNA (ShCHOP) and non-targeting shRNA (ShNT), and then were induced to differentiate. Where indicated, DFMO (5 mM) and doxycycline (Dox, 1 μM) were added to the cells 1 day before induction. A, cellular extracts were prepared at the indicated times, and protein levels were determined by Western blot analysis. Representative blot of at least three independent experiments is shown. B, cells were fixed and stained with Oil red O on day 12 (a) Control (b) DFMO (c) Dox (d) DFMO + Dox. C, cells were counted at the indicated times. DFMO and/or Dox were added as specified. Data are presented as the mean of three independent biological repeats. Standard deviations are represented by error bars. Statistically significant counts are marked with asterisks (**, p < 0.01; ***, p < 0.001; ****, p < 0.0001).
ATF4 is expressed to the same level both in control and in DFMO-treated preadipocytes.

When stimulated to differentiate, preadipocytes down-regulate CHOP expression, in order to acquire C/EBPβ DNA binding activity (16). In accordance, we show here that phosphorylation of eIF2α and CHOP mRNA levels are down-regulated 24 h post induction, and that both are regained gradually thereafter. This down-regulation of CHOP is critical for efficient progression of the process of adipogenesis (16). In contrast, while the pattern of eIF2α phosphorylation remains the same in polyamine-depleted cells, CHOP mRNA was not down regulated to the same level as in control cells, and it rose to much higher levels at later time points. The difference in CHOP mRNA levels in control and polyamine-depleted cells was compatible with the difference observed for the CHOP protein.

We provide evidence that increased level of CHOP in polyamine-depleted preadipocytes inhibits their differentiation by inhibiting C/EBPβ, which is required both for MCE and for the expression of PPARγ and C/EBPα. In parallel, we observed that differentiation was still inhibited even when the increase in the level of CHOP was prevented by inducible expression of CHOP shRNA. Under these conditions the expression of PPARγ and C/EBPα was restored, while MCE remained inhibited. Hence, our results demonstrate that depletion from polyamines inhib- its the second division of MCE also via CHOP-independent mechanism(s). Moreover, we support the notion that MCE is essential for successful adipogenic differentiation, independently from its requirement for the expression of PPARγ and C/EBPα.

When polyamine synthesis is inhibited by DFMO, preadipocytes manage to start differentiating by consuming their pre-existing polyamines, but without de novo synthesis they are unable to complete the process. Taken together, our present findings demonstrate that de novo synthesis of polyamines is required both for enabling acquisition of C/EBPβ DNA binding activity by releasing it from inhibition by CHOP, and independently for successful completion of MCE (Fig. 8).

Author Contributions—S. B. conducted most of the experiments, analyzed the results, and wrote most of the manuscript. Z. B. conducted some of the cell counts and Western blots. Y. F. helped with the real-time PCR experiment. R. K. contributed to the interpretation of some data. C. K. initiated the project and together with S. B. wrote the manuscript.

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FIGURE 8. Proposed role of polyamines in adipogenesis. Polyamines are required for adipogenesis in their absence this differentiation process is inhibited. Polyamines play dual role in this process; they are required for efficient and uninterrupted activation of C/EBPβ that is required for the manifestation of MCE and for the expression of PPARγ and C/EBPα, the master regulators of adipogenesis. In addition and independently of C/EBPβ activation, they are directly required for MCE. In their absence, the reduction in the level of CHOP, a dominant negative regulator of C/EBPβ, is inhibited. CHOP is greatly stimulated thereafter, repressing C/EBPβ activity. Polyamine depletion inhibits MCE and adipogenesis also when the expression of PPARγ and C/EBPα is uninterrupted, indicating the importance of polyamines for MCE and adipogenesis independently of C/EBPβ activity.

References

1. Shepherd, P. R., Gnudi, L., Tozzo, E., Yang, H., Leach, F., and Kahn, B. B. (1993) Adipose cell hyperplasia and enhanced glucose disposal in transgenic mice overexpressing GLUT4 selectively in adipose tissue. J. Biol. Chem. 268, 22243–22246
2. Green, H., and Kehinde, O. (1974) Sublines of mouse 3T3 cells that accumulate lipid. Cell 1, 113–116
3. Green, H., and Meuth, M. (1974) An established pre-adipose cell line and its differentiation in culture. Cell 3, 127–133
4. Mackall, J. C., Student, A. K., Polakis, S. E., and Lane, M. D. (1976) Induction of lipogenesis during differentiation in a ‘preadipocyte’ cell line. J. Biol. Chem. 251, 6662–6664
5. Coleman, R. A., Reed, B. C., Mackall, J. C., Student, A. K., Lane, M. D., and Bell, R. M. (1978) Selective changes in microsomal enzymes of triacylglycerol phosphatidylcholine, and phosphatidylethanolamine biosynthesis during differentiation of 3T3-L1 preadipocytes. J. Biol. Chem. 253, 7256–7261
6. Tang, Q.-Q., Otto, T. C., and Lane, M. D. (2003) Mitotic clonal expansion: a synchronous process required for adipogenesis. Proc. Natl. Acad. Sci. U.S.A. 100, 44–49
7. Zhang, J., Tang, Q., Vinson, C., and Lane, M. D. (2004) Dominant-negative C/EBP disrupts mitotic clonal expansion and differentiation of 3T3-L1 preadipocytes. Proc. Natl. Acad. Sci. U.S.A. 101, 43–47
8. Tang, Q.-Q., Zhang, J.-W., and Daniel Lane, M. (2004) Sequential gene promoter interactions by C/EBPbeta, C/EBPalpha, and PPARgamma during adipogenesis. Biochem. Biophys. Res. Commun. 318, 213–218
9. Lane, M. D., Tang, Q.-Q., and Liang, M.-S. (1999) Role of the CCAAT Enhancer Binding Proteins (C/EBPs) in Adipocyte Differentiation. Biochem. Biophys. Res. Commun. 266, 677–683
10. Tontonoz, P., Hu, E., and Spiegelman, B. M. (1994) Stimulation of adipogenesis in fibroblasts by PPARgamma: a lipid-activated transcription factor. Cell 79, 1147–1156
11. Rosen, E. D., Sarraf, P., Troy, A. E., Bradwin, G., Moore, K., Milstone, D. S., Spiegelman, B. M., and Mortensen, R. M. (1999) PPAR gamma is required for the differentiation of adipose tissue in vivo and in vitro. Mol. Cell 4, 611–617
12. Rosen, E. D., Hsu, C. H., Wang, X., Sakai, S., Freeman, M. W., Gonzalez, F. J., and Spiegelman, B. M. (2002) C/EBPalpha induces adipogenesis through PPARgamma: a unified pathway. Genes Dev. 16, 22–26
13. Christy, R. J., Yang, V. W., Ntambi, J. M., Geiman, D. E., Landschulz, W. H., Friedman, A. D., Nakabeppu, Y., Kelly, T. J., and Lane, M. D. (1989) Differentiation-induced gene expression in 3T3-L1 preadipocytes: CCAAT/enhancer binding protein interacts with and activates the pro-
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14. Tang, Q., and Lane, M. D. (1999) Activation and centromeric localization of CCAAT/enhancer-binding proteins during the mitotic clonal expansion of adipocyte differentiation. *Genes Dev.* **13**, 2231–2241.

15. Ron, D., and Habener, J. F. (1992) CHOP, a novel developmentally regulated nuclear protein that dimerizes with transcription factors C/EBP and LAP and functions as a dominant-negative inhibitor of gene transcription. *Genes Dev.* **6**, 439–453.

16. Tang, Q., and Lane, M. D. (2000) Role of C/EBP homologous protein (CHOP-10) in the programmed activation of CCAAT/enhancer-binding protein-β during adipogenesis. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 12446–12450.

17. Pegg, A. E. (2009) Mammalian polyamine metabolism and function. *IUBMB Life* **61**, 880–894.

18. Metcalf, B. W., Bey, P., Danzin, C., Jung, M. J., Casara, P., and Vevert, J. P. (1978) Catalytic irreversible inhibition of mammalian ornithine decarboxylase (E.C.4.1.1.17) by substrate and product analogs. *J. Am. Chem. Soc.* **100**, 2551–2553.

19. Poulin, R., Lu, L., Ackermann, B., Bey, P., and Pegg, A. E. (1992) Mechanism of the irreversible inactivation of mouse ornithine decarboxylase by α-difluoromethylornithine. Characterization of sequences at the inhibitor and coenzyme binding sites. *J. Biol. Chem.* **267**, 150–158.

20. Landau, G., Ran, A., Bercovich, Z., Feldmeser, E., Horn-Saban, S., Korkotian, E., Jacob-Hirsh, J., Rechavi, G., Ron, D., and Kahana, C. (2012) Expression profiling and biochemical analysis suggest stress response as a potential mechanism inhibiting proliferation of polyamine-depleted cells. *J. Biol. Chem.* **287**, 35825–35837.

21. Landau, G., Bercovich, Z., Park, M., and Kahana, C. (2010) The role of polyamines in supporting growth of mammalian cells is mediated through their requirement for translation initiation and elongation. *J. Biol. Chem.* **285**, 12474–12481.

22. Bethell, D. R., and Pegg, A. E. (1981) Polyamines are needed for the differentiation of 3T3-L1 fibroblasts into adipocyte cells. *Biochem. Biophys. Res. Commun.* **102**, 272–278.

23. Erwin, B. G., Bethell, D. R., Pegg, A. E., and Pegg, E. (1984) Role of polyamines in differentiation of 3T3-L1 fibroblasts into adipocytes. *Am. J. Physiol.* **Cell Physiol.* **246**, C293-C300.

24. Ishii, I., Ikuchi, Y., Mano, H., Wada, M., Pegg, A. E., and Shirahata, A. (2012) Polyamine metabolism is involved in adipogenesis of 3T3-L1 cells. *Amino Acids* **42**, 619–626.

25. Hyvönen, M. T., Koponen, T., Weisell, J., Pietilä, M., Khotmutov, A. R., Vepsäläinen, J., Alhonen, L., and Keinänen, T. A. (2013) Spermidine promotes adipogenesis of 3T3-L1 cells by preventing interaction of ANP32 with HuR and PP2A. *Biochem. J.* **453**, 467–474.

26. Zhang, J., Tang, H., Zhang, Y., Deng, R., Shao, L., Liu, Y., Li, F., Wang, X., and Zhou, L. (2014) Identification of suitable reference genes for quantitative RT-PCR during 3T3-L1 adipocyte differentiation. *Int. J. Mol. Med.* **33**, 1209–1218.

27. Vuohelainen, S., Pirinen, E., Cerrada-Gimenez, M., Keinänen, T. A., Uimari, A., Pietilä, M., Khotmutov, A. R., Jänne, J., and Alhonen, L. (2010) Spermidine is indispensable in differentiation of 3T3-L1 fibroblasts to adipocytes. *J. Cell. Mol. Med.* **14**, 1683–1692.

28. Cornelius, P., MacDougald, O., and Lane, M. D. (1994) Regulation of adipocyte development. *Annu. Rev. Nutr.* **14**, 99–129.

29. MacDougald, O., and Lane, M. (1995) Transcriptional regulation of gene expression during adipocyte differentiation. *Annu. Rev. Biochem.* **64**, 345–373.

30. Pegg, A. E. (1988) Polyamine Metabolism and Its Importance in Neoplastic Growth and as a Target for Chemotherapy. *Cancer Res.* **48**, 759–774.

31. Tang, Q.-Q., Grenborg, M., Huang, H., Kim, J.-W., Otto, T. C., Pandey, A., and Lane, M. D. (2005) Sequential phosphorylation of CCAAT enhancer-binding protein β by MAPK and glycogen synthase kinase 3β is required for adipogenesis. *Proc. Natl. Acad. Sci. U.S.A.* **102**, 9766–9771.

32. Kim, I., Tang, Q.-Q., Li, X., and Lane, M. D. (2007) Effect of phosphorylation and S–S bond-induced dimerization on DNA binding and transcriptional activation by C/EBP. *Proc. Natl. Acad. Sci. U.S.A.* **104**, 1800–1804.

33. Rochford, J. J., Semple, R. K., Laudes, M., Boyle, K. B., Christodoulides, C., Mulligan, C., Lelliott, C. J., Schinner, S., Hadaschik, D., Mahadevan, M., Sethi, J. K., Vidal-Puig, A., and O’Rahilly, S. (2004) ETO/MTG8 Is an Inhibitor of C/EBPβ Activity and a Regulator of Early Adipogenesis. *Mol. Cell. Biol.* **24**, 9863–9872.

34. Batchvarova, N., Wang, X., and Ron, D. (1995) Inhibition of adipogenesis by the stress-induced protein CHOP (Gadd153). *EMBO J.* **14**, 4654–4661.

35. Park, M. H., Lee, Y. B., and Joe, Y. A. (1997) Hypusine Is Essential for Protein Citrullination and Functions as a Dominant-negative Inhibitor of Gene Transcription. *EMBO J.* **16**, 481–487.

36. Canellakis, Z. N., Marsh, L. L., and Bondy, P. K. (1989) Polyamines and their derivatives as modulators in growth and differentiation. *Yale J. Biol. Med.* **62**, 481–491.

37. Tabor, C. W., and Tabor, H. (1984) Polyamines. *Annu. Rev. Biochem.* **53**, 749–790.

38. Tanaka, T., Yoshida, N., Kishimoto, T., and Akira, S. (1997) Defective adipocyte differentiation in mice lacking the C/EBPβ and/or C/EBPδ gene. *EMBO J.* **16**, 7432–7443.

39. Lai, E., Teodoro, T., and Volchuk, A. (2007) Endoplasmic reticulum stress: signaling the unfolded protein response. *Physiology* **22**, 193–201.