

Nucleolar retention of a translational C/EBP α isoform stimulates rDNA transcription and cell size

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The messenger RNA of the intronless *CEBPA* gene is translated into distinct protein isoforms through the usage of consecutive translation initiation sites. These translational isoforms have distinct functions in the regulation of differentiation and proliferation due to the presence of different N-terminal sequences. Here, we describe the function of an N-terminally extended protein isoform of CCAAT enhancer-binding protein α (C/EBP α) that is translated from an alternative non-AUG initiation codon. We show that a basic amino-acid motif within its N-terminus is required for nucleolar retention and for interaction with nucleophosmin (NPM). In the nucleoli, extended-C/EBP α occupies the ribosomal DNA (rDNA) promoter and associates with the Pol I-specific factors upstream-binding factor 1 (UBF-1) and SL1 to stimulate rRNA synthesis. Furthermore, during differentiation of HL-60 cells, endogenous expression of extended-C/EBP α is lost concomitantly with nucleolar C/EBP α immunostaining probably reflecting the reduced requirement for ribosome biogenesis in differentiated cells. Finally, overexpression of extended-C/EBP α induces an increase in cell size. Altogether, our results suggest that control of rRNA synthesis is a novel function of C/EBP α adding to its role as key regulator of cell growth and proliferation.

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Introduction

Increased cell proliferation, for example in cancerous growth, requires an increase in protein synthesis to fulfill the need for extra cell mass. Stimulation of RNA polymerase I (Pol I)-dependent ribosomal DNA (rDNA) transcription is a crucial event in ribosome biogenesis, which is prerequisite for

enhanced protein synthesis (Ruggero and Pandolfi, 2003). In recent years, a growing number of RNA polymerase II (Pol II)-dependent transcription factors have been found to regulate RNA Pol I-dependent rDNA transcription in addition. These transcription factors either stimulate rRNA synthesis during proliferation like c-Myc (Arabi *et al.*, 2005; Grandori *et al.*, 2005) or inhibit rRNA synthesis as has been shown for CTCF (Torrano *et al.*, 2006) and the lineage-specific transcription factors MyoD, myogenin and CCAAT enhancer-binding protein β (C/EBP β) that selectively interact with rRNA genes during differentiation (Ali *et al.*, 2008).

C/EBP α controls cell proliferation as well as transcription of genes involved in differentiation and energy homeostasis in various cell types through redundant as well as specific functions compared with C/EBP β and other C/EBP family members (Nerlov, 2007). In the haematopoietic system, C/EBP α is required for the differentiation of myelomonocytic cells, and mutations in the *CEBPA* gene are considered to be critically involved in the etiology of about 10% of human acute myeloid leukemia (AML) (Nerlov, 2004; Kirstetter *et al.*, 2008). A pivotal mechanism that controls C/EBP α expression in the cell is the regulated translation of its intronless messenger RNA into three functionally different protein isoforms from three successive translation initiation sites (Ossipow *et al.*, 1993; Calkhoven *et al.*, 2000) (Figure 1A). The full-length-C/EBP α isoform (p42) that is translated from the first AUG codon in the C/EBP α reading frame is a complete transcription factor possessing all sequences for transactivation of genes and for inhibition of proliferation (Ossipow *et al.*, 1993). In contrast, the truncated-C/EBP α isoform (p30), which is translated from an internal AUG codon, lacks the amino-terminal sequences for transactivation and is unable to restrict proliferation probably due to the absence of sequences required for the inhibition of E2F-dependent target genes (Lin *et al.*, 1993; Porse *et al.*, 2001). An evolutionary conserved upstream open reading frame (uORF) in the mRNA leader sequence controls the ratio between the full-length (p42) and truncated (p30) C/EBP α isoforms. Because of the *cis*-regulatory uORF, translation of the C/EBP α isoforms is under the control of the mTOR kinase-mediated regulation of eukaryotic initiation factor (eIF) 4E as well as the stress-induced eIF2 α -kinase family (Calkhoven *et al.*, 2000). Differential expression of the translational isoforms of C/EBP α provides a versatile mechanism to deliver functions on demand. However, it seems also to provide a critical target for deregulation and mutation during cancer development as is illustrated by a mouse model for the frequent mutation in human AML (Pabst *et al.*, 2001) that results in the synthesis of only the truncated-C/EBP α isoform and causes AML with complete penetrance (Kirstetter *et al.*, 2008).

The function of the extended-C/EBP α isoform, which is translated from an upstream non-AUG codon has never been

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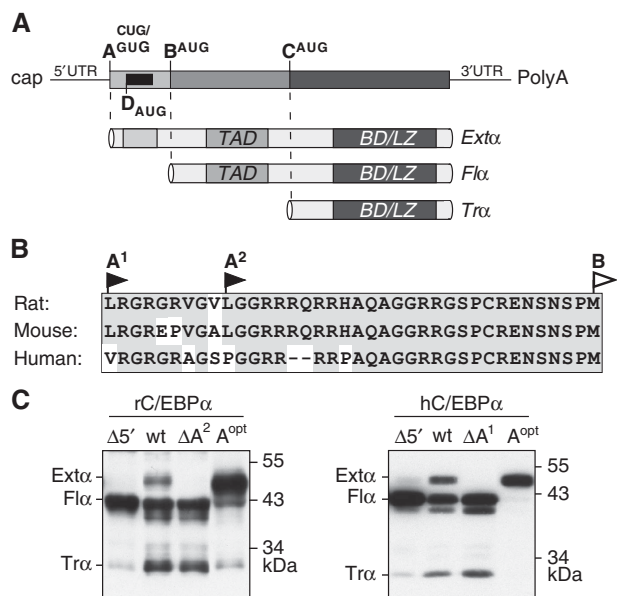


Figure 1 The extended-C/EBP α isoform is translated from a non-AUG codon. **(A)** Schematic representation of the C/EBP α messenger RNA with three alternative translation initiation sites, indicated as A (CUG or GUG), B (AUG) and C (AUG), and the initiation site for the *cis*-regulatory uORF, indicated as D (AUG), respectively. The mRNA can be translated into three separate protein isoforms extended- (Ext α), full-length- (Fl α) or truncated-C/EBP α (Tr α) that contain different sets of the functional domains TAD (Transactivation Domain), BD (Basic Domain) and LZ (Leucine Zipper). **(B)** Amino-acid sequence comparison of the extended domains of rat, mouse and human C/EBP α ; conserved amino-acid residues are shaded in grey. **(C)** Immunoblots comparing expression from wild-type and mutant rat (rC/EBP α) and human (hC/EBP α) C/EBP α constructs in COS-1 cells: Wild-types (wt), mutants lacking the translation initiation site A ($\Delta A^{1/2}$), constructs devoid of the 5' leader ($\Delta 5'$), and constructs with the CUG or GUG exchanged by an AUG codon (A^{opt}).

experimentally addressed. In this study, we reveal a novel function of C/EBP α that is specific for the extended isoform. We show that the extended-C/EBP α isoform can localize to nucleoli where it binds to the rDNA promoter. The extended-C/EBP α isoform interacts with and facilitates the recruitment of the Pol I-specific transacting factor upstream-binding factor 1 (UBF-1) and components of the TATA-binding protein (TBP)-TBP-associated factor (TAF α) complex SL1, and it enhances acetylation of histone H3 and H4 at the rDNA promoter. Furthermore, its nucleolar localization results in an increase in cell size. We identified a motif within the additional amino-terminal domain of the extended isoform that serves as a nucleolar localization signal (NoLS) and is required for interaction with nucleophosmin (NPM). In addition to the NoLS, a phosphorylation-mimicking mutation of a specific serine residue (S299D) strongly stimulates nucleolar retention, indicating that nucleolar retention of extended-C/EBP α may be further regulated through phosphorylation. We also show that downregulation of endogenous expression of extended-C/EBP α on phorbol ester-induced differentiation of HL-60 cells correlates with loss of C/EBP α -specific nucleolar staining. Our data further strengthens the concept that regulated translation of the C/EBP α mRNA into protein isoforms with distinct functions is a key-regulatory mechanism in the control of proliferation and differentiation in C/EBP α expressing cells.

Results

Extended-C/EBP α is translated from a non-AUG alternative initiation codon

Amino-acid sequence comparison reveals a high degree of conservation among mammals of the region upstream of the first AUG-codon in the C/EBP α coding frame as exemplified in Figure 1B for the murine and human C/EBP α cDNAs. For murine C/EBP α , these N-terminally extended sequences can be incorporated into the C/EBP α proteins through initiation from one of the two successive CUG alternative initiation codons (A-sites in Figure 1A) that lie -28 and -37 codons upstream of the first AUG (site B in Figure 1A) of the C/EBP α coding frame (Calkhoven *et al*, 2000) (and data not shown). In case of the human sequence, a GUG alternative initiation codon is found at position -35 . Mutation of the most prominently used CUG codon (ΔA^2) in the rat C/EBP α cDNA or the GUG codon (ΔA^1) in the human C/EBP α cDNA as well as removal of the complete sequence upstream of the first AUG ($\Delta 5'$) result in complete loss of extended-C/EBP α expression in COS-1 cells (Figure 1C). Conversely, converting the CUG or GUG codons into a proper AUG codon (A^{opt}) results in predominant expression of the extended isoform (Figure 1C). Hence, alternative CUG or GUG codons in the 5'-leader sequence of C/EBP α mRNAs can be used for initiation of translation.

The extended-C/EBP α isoform is retained in the nucleolus

C/EBP α is a transcription factor that predominantly localizes to the nucleus. It contains a bipartite nuclear localization signal (NLS) within its carboxy-terminal basic region that is present in all three translational isoforms (Williams *et al*, 1997). To compare the subcellular distribution between the three translational isoforms of C/EBP α , we examined their cellular localization by fluorescence microscopic analysis using the Zeiss ApoTome system. For this purpose, we transfected C33A cells with expression constructs containing sequences for the separate C/EBP α isoforms or constructs with the C/EBP α isoforms fused at their carboxy-termini to enhanced green fluorescent protein (EGFP). We found that in a consistent percentage (10%) of transfected cells, the extended-C/EBP α isoform localized to the nucleoli as was detected by either direct (Figure 2A) or indirect fluorescence (see Supplementary Figure S1). Nucleolar localization was verified by co-immunostaining of the nucleolar marker protein Fibrillarlin, which is a component of the rRNA processing machinery. In contrast, nucleolar localization was never observed with transient expression of the full-length- or truncated-C/EBP α isoforms, indicating that nucleolar localization is a specific feature of the extended-C/EBP α isoform. Interestingly, during the establishment of C33A cell lines that stably express extended-C/EBP α , the percentage of cells with nucleolar retention continuously raises up to 100%, in long-term cultures, suggesting that these cells have a selective proliferation or survival advantage (data not shown).

NoLSs are often characterized by stretches of basic amino acids (Carmo-Fonseca *et al*, 2000; Emmott and Hiscox, 2009). The extended-C/EBP α domain contains a conserved RRRR motif that is also found in proven NoLSs of other proteins like Parp2, p14/19Arf, ING1b, Rpp29 or HIV Tat (Kubota *et al*, 1989; Jarrous *et al*, 1999; Weber *et al*, 1999; Scott *et al*, 2001;

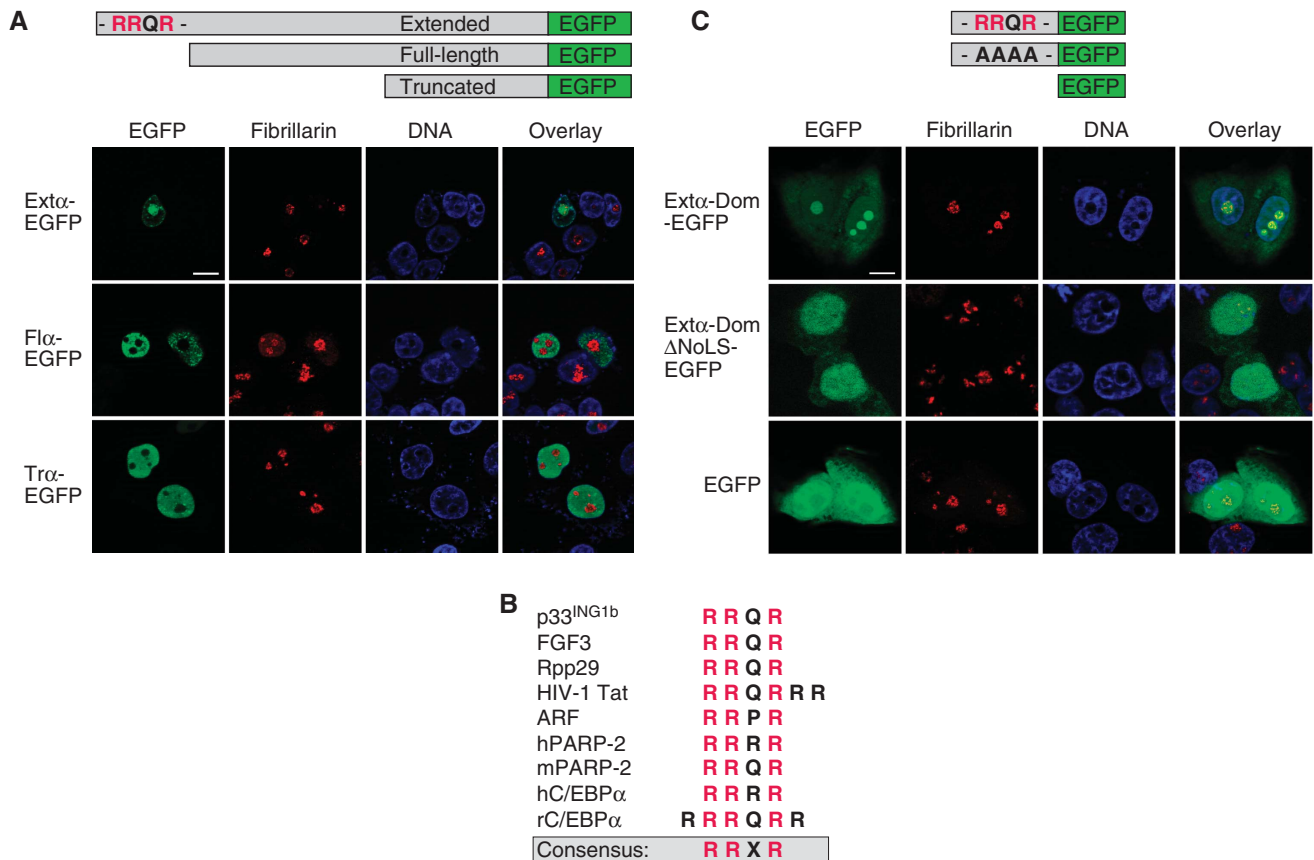


Figure 2 Nucleolar localization of the extended-C/EBP α isoform. (A) Subcellular localization of extended- (Ext α), full-length- (Fl α) or truncated- (Tr α) C/EBP α proteins fused to EGFP after transient transfection in C33A cells. DNA was stained with DAPI to visualize the nucleus and immunostaining with the Fibrillarin-specific antibody served to locate the nucleolus. (B) Alignment of the RRXR motifs within the domains mediating nucleolar localization of extended-C/EBP α and other proteins with known nucleolar localization. (C) Subcellular localization of the isolated extended domain of C/EBP α either harbouring the wt sequence (Ext α -Dom) or a mutation of its RRQR motif (Ext α -Dom Δ NoLS) fused to EGFP. Bars, 10 μ m.

Meder *et al*, 2005) (Figure 2B). To examine whether the extended domain is required and sufficient for nucleolar retention, we fused the 28 amino acids of the extended domain to EGFP and determined cellular localization by microscopic analysis. Figure 2C shows that the sequences of the extended C/EBP α domain alone are very efficient in retaining the attached EGFP in the nucleolus in almost 100% of transfected cells. In contrast, the mutation of the RRQR motif into AAAA abolished nucleolar localization of the extended domain EGFP fusion construct and resulted in even distribution over the cell similar to the EGFP control. Hence, nucleolar localization is a unique feature of the extended-C/EBP α isoform and the RRXR NoLS-motif within the extended domain of C/EBP α is required for its nucleolar retention.

Pseudo-phosphorylation of serine 299 of extended-C/EBP α stimulates nucleolar retention

The observation that transiently expressed extended-C/EBP α is retained in the nucleoli in a portion of cells, yet unrestrained nucleolar retention can be achieved with the sole EGFP-fused extended domain indicates that nucleolar retention of the whole protein may be subject of further regulation. It has been shown for other proteins with partial nucleolar localization like Parp2 or ING1b that in addition to the RRXR

Table I Number of cells with nucleolar staining per 100 EGFP-positive cells

	Ext α ^{wt} -EGFP		Ext α ^{S299D} -EGFP		Ext α ^{S299D} Δ NoLS-EGFP				
C33A	11	9	11	99	100	99	0	0	0
HEK293	13	12	11	99	99	98	0	0	0
COS-1	11	11	10	100	99	100	0	0	0
HeLa	7	7	9	94	94	93	0	0	0

Counts were obtained from three independent transfection experiments.

motif also their NLSs contributed to the efficiency of nucleolar retention independent from their function for nuclear localization (Scott *et al*, 2001; Meder *et al*, 2005). We, therefore, examined the effect of different mutations within the bipartite NLS of C/EBP α and identified a serine residue within the NLS (S299 in full-length-C/EBP α) whose mutation into a phosphorylation-mimicking aspartate causes efficient nucleolar retention. Table I presents data from triplicate transfection experiments in four cell lines (C33A, HEK293, COS-1, HeLa) showing that in all cell lines, the S299D mutation rendered nucleolar retention to virtually 100% of transfected cells, compared with around 10% nucleolar versus 90% nucleoplasmic staining for the wt extended-C/EBP α -EGFP. Mutation of the RRQR NoLS-motif in the extended-C/EBP α ^{S299D}-EGFP

construct completely abolished nucleolar retention in all cases. Notably, we did not score a single cell with nucleolar localization of full-length- or truncated-C/EBP α ^{S299D}-EGFP fusion proteins either with or without the S299D mutation during the examination of all the cell lines. Figure 3A shows a typical example for all C/EBP α ^{S299D}-EGFP isoforms in C33A cells and Figure 3B shows nucleolar retention for extended-C/EBP α ^{S299D}-EGFP in five additional cell lines. Mutation of

the serine 299 into alanine did not stimulate nucleolar retention of the extended-C/EBP α -EGFP fusion protein or the extended-C/EBP α isoform (data not shown). Similar results were obtained by immunostaining of cells transfected with expression constructs for native C/EBP α isoforms without EGFP (Supplementary Figure S2A). Although, a similar extended isoform of the related C/EBP β exists that is translated from an upstream AUG-codon, it does not have a NoLS, and

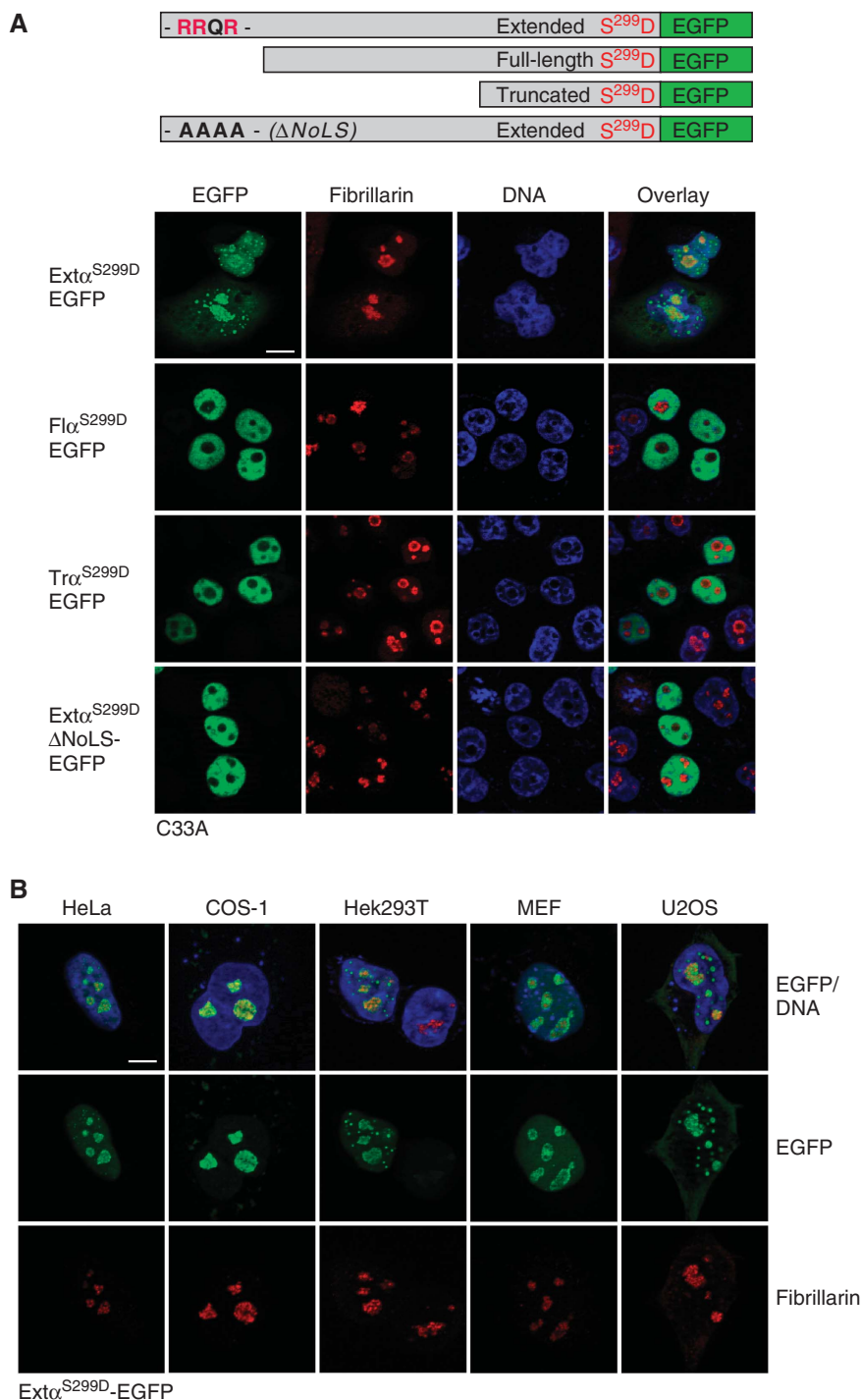


Figure 3 Pseudo-phosphorylation of serine 299 in extended-C/EBP α stimulates its nucleolar retention. **(A)** Subcellular distribution of S299D mutants of extended- (Ext α ^{S299D}), extended Δ NoLS (Ext α ^{S299D} Δ NoLS), full-length- (Fl α ^{S299D}) or truncated-C/EBP α (Tr α ^{S299D}) proteins fused to EGFP. **(B)** Nucleolar retention of extended-C/EBP α ^{S299D} in HeLa, COS-1, Hek293T, MEF and U2OS cells. C/EBP α ^{S299D}-EGFP constructs were transiently transfected into the different cell lines and subcellular localization was analysed as described in Figure 2. Bars, 10 μ m.

we did not observe localization of extended-C/EBP β in the nucleolus whether it harbours an S239D mutation that corresponds to the S299D mutation in C/EBP α or not (data not shown). Hence, our data suggest that nucleolar retention of extended-C/EBP α is cell-type independent, requires the RRQR NoLS-motif in the extended domain and is enhanced by phosphorylation of serine 299 within the NLS.

Endogenous C/EBP α localizes to the nucleolus in myelomonocytic cells

The promyelocytic human leukemia cell line HL-60 has retained the capacity to differentiate in cell culture and is one of the few cell lines that express endogenous C/EBP α . Immunoblot analysis confirmed that proliferating and undifferentiated HL-60 cells express all three C/EBP α translational isoforms, depicted extended-, full-length- and truncated-C/EBP α (Figure 4A). Intriguingly, indirect fluorescent immunostaining of C/EBP α in HL-60 cells using four different anti-C/EBP α antibodies revealed a prominent staining of the nucleoli in addition to the expected nuclear staining (Figure 4B; Supplementary Figure S3A). Induction of macrophage differentiation by phorbol ester (TPA) treatment resulted in complete disappearance of the C/EBP α -specific staining of the nucleoli although staining of the remaining part of the nucleus remained unchanged (Figure 4B). Immunoblotting revealed that the expression of the extended- and truncated-C/EBP α isoforms were specifically downregulated in the differentiated HL-60 cells (Figure 4A). Hence, loss of expression of the extended-C/EBP α isoform correlates with loss of nucleolar staining. C/EBP α -specific nucleolar staining

was also found in another human leukemia cell line, the promonocytic U937 cell line (Supplementary Figure S3B and C).

Extended-C/EBP α interacts with Pol I-specific factors and its nucleolar retention depends on ongoing rDNA transcription

The nucleolus is the place where rDNA transcription is performed through the activity of RNA Pol I. The nucleolar transcription factor UBF-1 is a crucial component of the RNA Pol I preinitiation complex (Grummt, 2003). Immunofluorescence microscopy revealed that ectopically expressed extended-C/EBP α ^{S299D} but not the corresponding Δ NoLS mutant localizes with endogenous UBF-1 in the nucleoli (Supplementary Figure S4A). To examine whether C/EBP α interacts with UBF-1, HEK293 cells were transfected with expression plasmids for extended-, full-length or truncated-C/EBP α , respectively, followed by co-immunoprecipitation (Co-IP) experiments. We found that both wt and the S299D mutant of extended-C/EBP α (Figure 5A) as well as full-length-C/EBP α (Figure 5B) can be co-precipitated together with endogenous UBF-1 using anti-UBF-1 antibodies. In contrast, we could not detect a clear co-precipitation of truncated-C/EBP α .

RNA Pol I transcription requires the SL1 complex, which is composed of the TBP and three Pol I-specific TAFs, TAF₁₁₀, TAF₆₃ and TAF₄₈. SL1 was shown to be essential for UBF-1 recruitment and pre-initiation complex formation (Friedrich *et al*, 2005). As full-length C/EBP α is known to bind to and recruit TBP to Pol II promoters (Nerlov and Ziff, 1995), we examined whether extended-C/EBP α interacts with TBP and TAF₄₈. Co-IP using anti-C/EBP α antibodies resulted in precipitation of TBP by extended-C/EBP α , as well as the S299D and the S299D- Δ NoLS mutant thereof (Figure 5C). Hence, interaction with TBP is a genuine property of both full-length and extended-C/EBP α . In addition, we could co-precipitate extended-C/EBP α ^{S299D} with anti-TAF₄₈ antibodies; however, co-precipitation was not seen with the Δ NoLS mutant (Figure 5D, upper panel). Furthermore, TAF₄₈ can be precipitated with anti-TBP antibodies more efficiently in cells that express extended-C/EBP α , in particular the S299D mutant, compared with Δ NoLS or empty vector controls (Figure 5D, lower panel). Overall these results indicate that extended-C/EBP α , UBF-1, and TBP and TAF₄₈ as part of the SL1 complex reside as a complex in the nucleolus.

It has been shown that the RRQR motif in Parp2 is required for nucleolar retention and for interaction with NPM (also known as B23) (Meder *et al*, 2005), a ubiquitously expressed phosphoprotein with multiple functions that constantly shuttles between the nucleolus, nucleoplasm and cytoplasm (Grisendi *et al*, 2006). To examine whether a similar interaction between NPM and C/EBP α exists, Co-IP experiments were set up and as presented in Figure 5E, NPM could only be co-immunoprecipitated with wt and the S299D mutant of extended-C/EBP α while co-immunoprecipitation failed with the Δ NoLS mutant or full-length isoform that both lack RRQR motif.

Typically, nucleolar retention of regulatory factors depends on ongoing rDNA transcription. To examine whether nucleolar localization of the extended-C/EBP α ^{S299D} mutant depends on ongoing rDNA transcription, we analysed its localization in the presence of a low concentration of actinomycin D

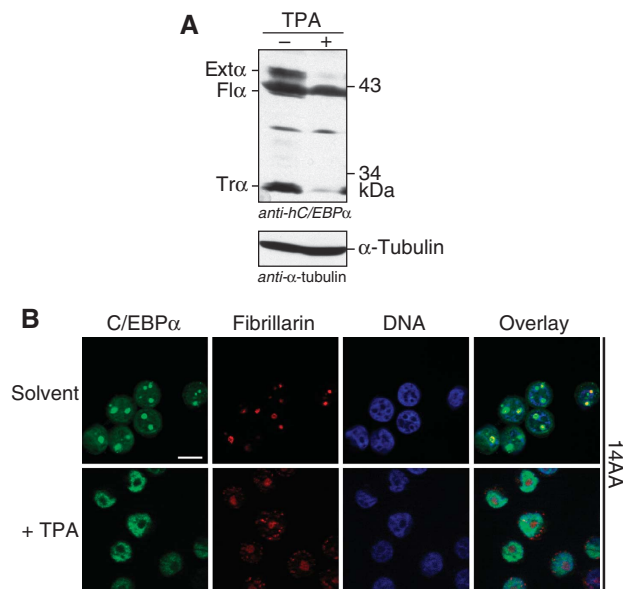


Figure 4 C/EBP α isoform expression and subcellular localization on phorbol ester (TPA)-induced monocytic differentiation of HL-60 cells. **(A)** Immunoblot showing endogenous expression of the extended- (Ext α), full-length- (Fl α) and truncated-C/EBP α (Tr α) hC/EBP α isoforms in untreated proliferating HL-60 cells and after treatment with 200 nM phorbol ester (TPA) for 12 h. α -tubulin staining in the lower blot serves as loading control. **(B)** Immunostainings of TPA-treated cells (+ TPA) and control cells (Solvent) were performed with an anti-C/EBP α antibody against a C-terminal epitope (14AA). DNA was stained with DAPI to visualize the nucleus and immunostaining with a Fibrillarlin-specific antibody served to locate the nucleolus. Bars, 10 μ m.

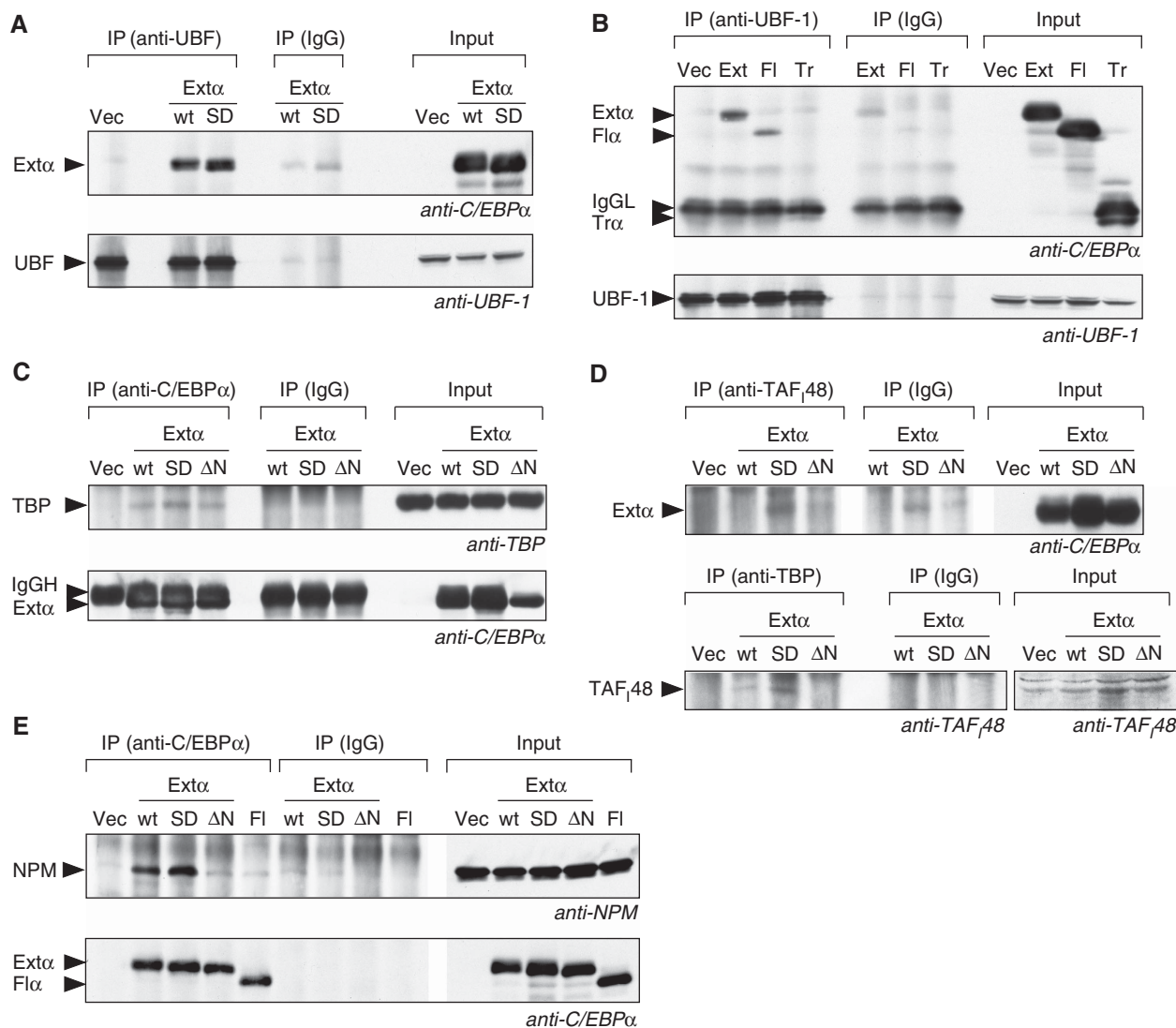


Figure 5 Extended-C/EBP α interacts with UBF-1, SL1 and NPM. Immunoprecipitations (IPs) were performed with specific antibodies or with mouse IgG as control from total lysates of HEK293 cells expressing one of the C/EBP α isoforms encoded: wild type (wt) extended-C/EBP α (Ext α or Ext), extended-C/EBP α^{S299D} (SD), extended-C/EBP $\alpha^{S299D}\Delta$ NoLS (Δ N), full-length-C/EBP α (FI), truncated-C/EBP α (Tr). Immunoblots of immunoprecipitates (IP) and total lysates (Input) were stained as indicated. (A) Extended-C/EBP α co-precipitates with UBF-1 using anti-UBF-1 antibodies (B) extended- and full-length-C/EBP α but not truncated-C/EBP α co-precipitate with UBF-1 using anti-UBF-1 antibodies. Immunoglobulin light chain is marked by IgGL. (C) TBP co-precipitates with extended-C/EBP α using anti-C/EBP α antibodies. Immunoglobulin heavy chain is marked by IgGH. (D) Extended-C/EBP α^{S299D} but not the Δ NoLS mutation co-precipitates with TAF $_{48}$ using anti-TAF $_{48}$ antibodies (upper panel). TAF $_{48}$ co-precipitates more efficiently with TBP using anti-TBP antibodies only in cells expressing extended-C/EBP α^{S299D} (lower panel). (E) NPM co-precipitates with extended-C/EBP α^{S299D} but not with the extended-C/EBP $\alpha^{S299D}\Delta$ NoLS using anti-C/EBP α antibodies.

(50 ng/ml), which specifically inhibits RNA Pol I transcription (Perry and Kelley, 1970). As shown in Figure 6 and Supplementary Figure S4B, nucleolar localization of extended-C/EBP α^{S299D} was lost in the presence of actinomycin D, whereas inhibition of RNA Pol II driven transcription with α -amanitin had no effect on the localization of the extended isoform. Taken together, these results show that extended-C/EBP α interacts with functional nucleolar proteins, UBF-1 and NPM, and the SL1 components TBP and TAF $_{48}$, and that its nucleolar retention depends on ongoing rDNA transcription.

Extended-C/EBP α stimulates RNA Pol I-dependent rRNA synthesis

As C/EBP α is a transcription factor, we examined whether extended-C/EBP α was able to regulate RNA Pol I-mediated

rRNA synthesis as has been shown also for other RNA Pol II-dependent transcription factors, including c-Myc (Arabi *et al*, 2005; Grandori *et al*, 2005). To address this question, we co-transfected a human rRNA minigene reporter construct (pMr1930-BH) (Mayer *et al*, 2005) together with expression vectors for extended-C/EBP α in HEK293T cells. We observed that overexpression of both extended-C/EBP α and the S299D mutant thereof strongly induced RNA Pol I-mediated transcription of the reporter RNA to a similar extent as c-Myc, which was used as a positive control (Figure 7A). To examine whether extended-C/EBP α is able to stimulate endogenous rDNA transcription, we analysed the expression levels of 45S pre-rRNA by Northern blotting in long-term C33A cell lines that stably express extended-, full-length- or truncated-C/EBP α wt proteins, the S299D mutants thereof and Δ NoLS

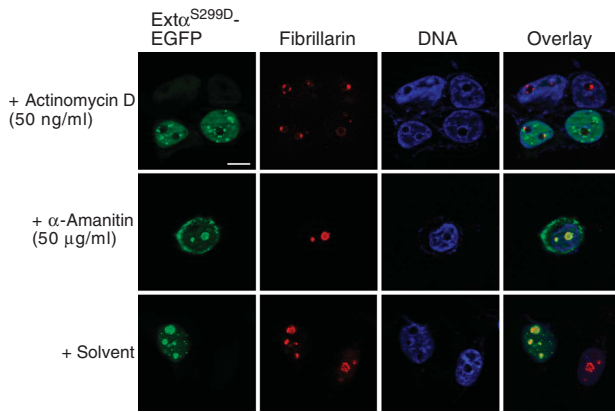


Figure 6 Nucleolar retention of extended-C/EBP α depends on ongoing rDNA synthesis. Blocking RNA polymerase I activity with actinomycin D (50 ng/ml) abrogates nucleolar retention of extended-C/EBP α^{S299D} , whereas blocking RNA polymerase II with α -amanitin (50 μ g/ml) or solvent treatment has no effect. C33A cells transiently transfected with extended-C/EBP α^{S299D} -EGFP were treated for 2 h as indicated and subcellular localization was analysed as described in Figure 2. Bars, 10 μ m.

mutants. Figure 7B shows that expression of extended-C/EBP α and extended-C/EBP α^{S299D} results in increased levels of endogenous 45S pre-rRNA compared with the empty vector control. In contrast, expression of the full-length- or truncated-C/EBP α isoforms either with or without the S299D mutation was not able to enhance 45S pre-rRNA levels. The Δ NoLS mutation prevents induction of 45S pre-rRNA (Figure 7C). To examine a possible indirect Pol II-dependent regulation of rDNA transcription by extended-C/EBP α , 45S pre-rRNA levels were analysed under inhibition of Pol II by α -amanitin (50 μ g/ml). The results in Figure 7D show that inhibition of Pol II only slightly affected induction of 45S pre-rRNA, suggesting a direct regulation of Pol I by extended-C/EBP α . To examine whether selective elimination of extended-C/EBP α impedes rRNA synthesis, we knocked-down expression of endogenous C/EBP α in HL-60 cells by introduction of lentiviral human C/EBP α -specific shRNA (Figure 8A). Subsequently, in the C/EBP α knock-down cells, expression was restored from retrovirally transduced constructs coding for all three C/EBP α -FLAG isoforms, or from a construct with a Δ CUG mutation avoiding expression of the extended-C/EBP α isoform (Figure 8B). As demonstrated by Northern analysis, 45S pre-rRNA levels were only significantly stimulated in the presence of extended-C/EBP α and not when full-length- and truncated-C/EBP α are expressed alone from the Δ CUG α construct. Once more, the requirement of the N-terminal NoLS was demonstrated as stimulation of 45S pre-rRNA levels by overexpression of extended-C/EBP α^{S299D} alone is abrogated when NoLS is mutated (Figure 8C). Taken together, extended-C/EBP α directly and specifically enhances rDNA transcription, which depends on nucleolar retention mediated by the N-terminally located NoLS.

Bioinformatic analysis revealed two C/EBP-binding motifs in the intergenic spacer of the human rDNA repeats (Figure 9A). One of the C/EBP-binding motifs lies 25 bp upstream of the transcription start, in the same proximal promoter sequences that are crucial for Pol I regulation by c-Myc (Figure 9A; region Cp, same as region 42.9 in Grandori

et al, 2005). Chromatin immunoprecipitation (ChIP) experiments using anti-C/EBP α antibodies revealed that extended-C/EBP α occupies this rDNA promoter segment (Figure 9B; upper chart, region Cp) in C33A cells. Notably, the more efficient nucleolar retention of extended-C/EBP α^{S299D} results in recovery of more Cp DNA fragments whereas the Δ NoLS mutation abolishes DNA recovery (Figure 9B and C). In addition, extended-C/EBP α binds to a more distal region containing the second C/EBP-binding motif (Figure 9A and B; upper chart, region Cd). Extended-C/EBP α does not interact with region M that does not contain a C/EBP-binding motif, which, however, was shown to be implicated in c-Myc-dependent regulation (Figure 9A and B; region M, same as region 42 in Grandori *et al*, 2005).

Region Cp covers the Pol I promoter and is also heavily occupied by UBF-1, as was also observed by others (Figure 9B, lower chart, region Cp) (Grandori *et al*, 2005; here region 42.9). Interestingly, the enhanced interaction of extended-C/EBP α^{S299D} with the regions Cp and Cd improves recruitment of UBF-1 to these regions (Figure 9B and C). To investigate whether extended-C/EBP α also facilitates the recruitment of components of the SL1 complex to the rDNA promoter, we performed a ChIP assay using an anti-TBP antibody. Figure 9D shows that indeed recruitment of TBP is enhanced on extended-C/EBP α binding to region Cp. c-Myc like extended-C/EBP α binds most strongly to region Cp and stimulates histone H3 and H4 acetylation especially at the neighbouring region M (Grandori *et al*, 2005). C/EBP α is known to activate transcription from Pol II promoters through recruitment of histone acetylases (Erickson *et al*, 2001; Bararia *et al*, 2008). ChIP analysis using anti-histone H3 or H4 antibodies revealed that histone acetylation is enhanced particularly at site M and to a lesser extent to Cp when extended-C/EBP α binds to the rDNA promoter (Figure 9E). Hence, our results suggest that the occupation of the rDNA promoter region by extended-C/EBP α facilitates recruitment of UBF-1 and SL1, and induces histone acetylation.

Overexpression of extended-C/EBP α results in an increase in cell size

Increase in cell size is a well-known consequence of enhanced ribosome biogenesis (Baserga, 2007), and the stimulation of rRNA synthesis by c-Myc has been associated with increased cell size in different systems (Oskarsson and Trumpp, 2005). Cell volume analysis of the stably transfected cells revealed that expression of extended-C/EBP α^{wt} - and in particular the extended-C/EBP α^{S299D} isoform results in a profound increase in the mean cell volume, whereas the other isoforms show no effect (Figure 10). C33A cells lack a functional Brahma protein as part of the SWI/SNF chromatin-remodelling complex, which is required for C/EBP α -induced proliferation arrest (Müller *et al*, 2004). To study whether the absence of Brahma is instrumental in the pronounced increase in cell volume, we analysed C33A cells in which Brahma function was restored by expression from a retroviral construct compared with dominant negative mutant of Brahma and empty vector. In none of the situations, however, cell size was affected (Supplementary Figure S5A). Hence, Brahma function has no effect on cell size. In addition, overexpression of extended-C/EBP α^{wt} - and the extended-C/EBP α^{S299D} isoform in HEK293 cells that have a functional

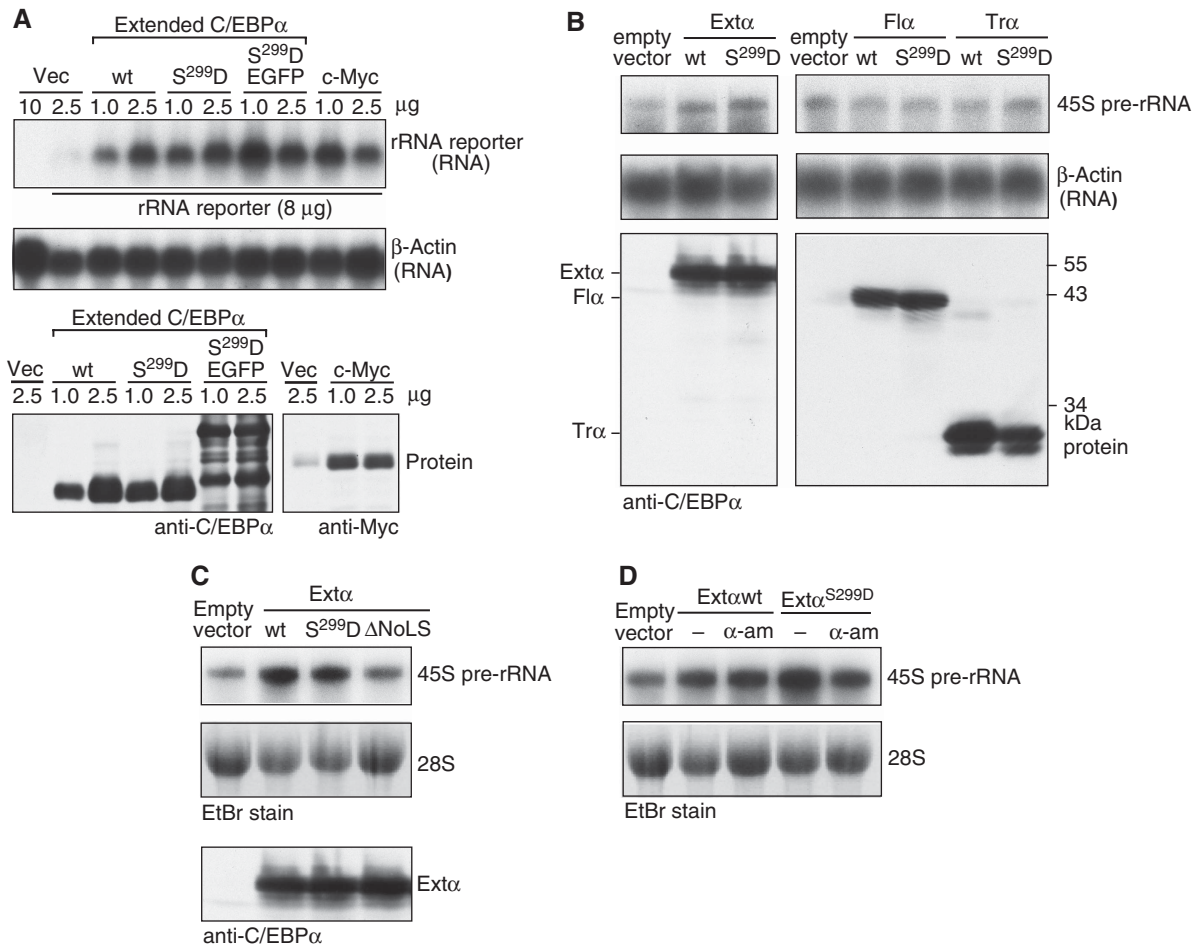


Figure 7 Extended-C/EBP α stimulates RNA Pol I-dependent rDNA transcription. **(A)** Hek293T cells were transiently transfected with the human rRNA minigene reporter construct pHrP2-BH together with expression constructs for wild type extended-C/EBP α (wt), the extended-C/EBP α ^{S299D} (S²⁹⁹D) mutant, the latter fused to EGFP (S²⁹⁹D EGFP), c-Myc or the empty vector (Vec) as control as indicated. Half of the cells were analysed for transcription from the rRNA minigene reporter by Northern blotting using 10 μ g of total RNA and a construct-specific probe. The other half of the cells were used for protein analysis. **(B)** The levels of the endogenous 45S rRNA precursor in C33A cells stably expressing wild type (wt) extended-, full-length-, truncated-C/EBP α or the S229D mutants thereof (S²⁹⁹D), or the empty vector as control was analysed by Northern blotting from 30 μ g of total RNA. **(C)** The levels 45S rRNA precursor in C33A cells stably expressing wild type (wt) extended-C/EBP α or the S229D or Δ NoLS mutants thereof, or the empty vector as control was analysed by Northern blotting from 30 μ g of total RNA. **(D)** The levels 45S rRNA precursor in C33A cells stably expressing wild type (wt) extended-C/EBP α , the S229D mutant or the empty vector as control untreated or treated with 50 μ g/ml α -amanitin (α -am) was analysed by Northern blotting from 30 μ g of total RNA. A human β -actin probe or ethidium bromide (EtBr) inverted staining of the gel was used for loading control. Total protein extracts of the cells were blotted for detection of expression of C/EBP α with anti-C/EBP antibodies or expression of c-Myc, with anti-Myc antibodies as indicated.

SWI/SNF complex resulted in a clear although less pronounced increase in cell size (Supplementary Figure S5B).

Discussion

In this study, we reveal a novel function of the transcription factor C/EBP α that is specific for the translational protein isoform called extended-C/EBP α . Results presented in this manuscript provide evidence that extended-C/EBP α localizes in the nucleoli and stimulates RNA Pol I-dependent rDNA transcription, and concomitantly increases cell size.

Regulation of Pol I activity and rRNA synthesis by C/EBP α

We show an increase in rRNA levels from endogenous loci and increased Pol I transcription from an rRNA minigene reporter in response to extended-C/EBP α expression. These findings are supported by CHIP assays that revealed interac-

tion of extended-C/EBP α with rDNA promoter sequences. Furthermore, expression of extended-C/EBP α results in increased acetylation of histones H3 and H4 within the rDNA promoter, reflecting enhanced promoter activation through recruitment of acetyltransferases. Finally, our results suggest that extended-C/EBP α stimulates Pol I pre-initiation complex formation as extended-C/EBP α interacts with UBF-1 and components of the SL1 complex, and facilitates their recruitment to rDNA promoter sites. Remarkably, similar functions have also been described for c-Myc (Arabi *et al*, 2005; Grandori *et al*, 2005). Indeed, both proteins can bind to the proximal promoter region (region Cp), recruit the same set of factors and induce histone acetylation in the same region M. Other RNA Pol II transcription factors like Runx2, MyoD, Myogenin and C/EBP β interact with rRNA genes, yet repress rRNA synthesis during differentiation (Grandori *et al*, 2005; Young *et al*, 2007; Ali *et al*, 2008). These inhibitory factors contact the rRNA promoter during mitosis when the nucleolar

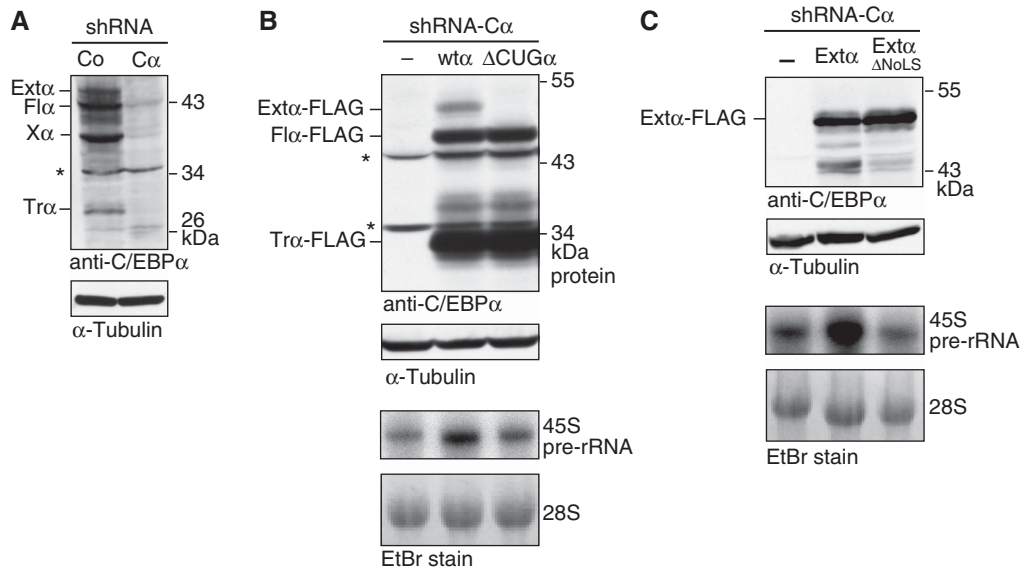


Figure 8 Selective elimination of extended-C/EBP α impedes rRNA synthesis. (A) Lentiviral shRNA-mediated knock-down of endogenous human C/EBP α (C α) compared with control scrambled shRNA (Co) in HL-60 cells. (B) Retroviral-mediated expression in C/EBP α knock-down HL-60 (shRNA-C α) cells from rat C/EBP α (wt α) or the Δ CUG α mutant cDNAs. (C) Retroviral-mediated expression of extended-C/EBP α and the Δ NoLS mutant thereof in C/EBP α knock-down HL-60 (shRNA-C α) cells. Immunoblots were probed with anti-C/EBP α antibodies (14A), and with anti- α -tubulin antibodies for loading control. The X α -labelled band is of indefinite C/EBP α origin; possibly sumoylated truncated-C/EBP α (data not shown). Asterisk (*) indicates non-specific bands. Levels of 45S pre-rRNA were measured by Northern blotting of 15 μ g of total RNA. Ethidium bromide (EtBr) inverted staining of the gel was used for loading control.

structures are dissolved (Young *et al*, 2007; Ali *et al*, 2008), making nucleolar retention signals dispensable. This stands in contrast to the activating function of extended-C/EBP α that requires the NoLS for rDNA promoter binding and stimulation of rRNA synthesis.

We show that besides extended- also full-length-C/EBP α interacts with UBF-1. In contrast to the extended isoform, full-length-C/EBP α is not retained in the nucleoli and rather seems to inhibit rRNA synthesis (Figures 2A and 7B; data not shown). Interestingly, the Pol I inhibitory factors Runx2, MyoD and Myogenin also interact with UBF-1 (Young *et al*, 2007; Ali *et al*, 2008). Although the observed repression by full-length-C/EBP α could be caused indirectly by its anti-proliferative activities, or through sequestration of UBF-1 within the nucleoplasm, it may inhibit rRNA synthesis involving direct rDNA promoter interaction similarly to Runx2, MyoD or Myogenin. Such a direct interaction of full-length C/EBP α with the rDNA promoter is likely as we recovered promoter rDNA (region Cp) in ChIP analysis using both anti-UBF-1 as well as anti-C/EBP α antibodies from proliferating HL-60 cells expressing all translational C/EBP α isoforms as well as from differentiating HL-60 cells, which express only the full-length C/EBP α isoform (data not shown).

Extended-C/EBP α expression results in an increased cell size

Overexpression of extended-C/EBP α , in particular of the S299D mutant that shows augmented nucleolar retention and Pol I activation, results in a pronounced increase in cell volume. Obviously, cell growth (increase in cell size) requires an enhanced protein biosynthesis rate, which directly depends on ribosome biogenesis with rRNA synthesis as the rate-limiting process. Accordingly, other positive regulators of PolI-dependent rRNA transcription like c-Myc (Oskarsson

and Trumpp, 2005) and IRS-1 (Valentinis *et al*, 2000; Sun *et al*, 2003) are able to increase cell size in specific systems.

Specific nucleolar retention of the extended-C/EBP α isoform

Extended-C/EBP α is the only translational C/EBP α isoform that can be retained in the nucleoli. This can be explained by the presence of a conserved RRXR nucleolar localization (NoLS) motif in the N-terminal part of the protein that is also found in other nucleolar-retained factors (Kubota *et al*, 1989; Jarrous *et al*, 1999; Weber *et al*, 1999; Zhang and Xiong, 1999; Scott *et al*, 2001; Meder *et al*, 2005). For both Parp2 (Li, 1997; Meder *et al*, 2005) and extended-C/EBP α (this study), the RRXR motif is required for interaction with NPM (also known as B23), which could mean that either these factors need to be nucleolar to interact with NPM or that they are recruited to the nucleolus through binding to NPM. NPM is implicated in cancer pathogenesis as it is over-expressed in a diversity of solid tumours, and in several haematologic malignancies the *NPM1* gene is found mutated or rearranged (Falini *et al*, 2007). Therefore, it would be interesting to examine whether cancerous mutations of NPM have an effect on functions of C/EBP α in the control of cell proliferation and growth.

Apart from the RRXR motif, we observed that a phosphorylation-mimicking mutation of a Serine residue within the NLS (S299 as numbered for the full-length isoform) strongly enhances nucleolar retention of extended-C/EBP α . This observation is reminiscent to the contribution of NLSs of Parp2 or ING1b that contribute to the efficiency of nucleolar retention (Scott *et al*, 2001; Meder *et al*, 2005). Serine 299 lies in a region that is conserved between all C/EBPs and its phosphorylation has been associated with opposite effects on DNA binding and/or subcellular distribution (Mahoney *et al*, 1992; Yin *et al*, 1996; Buck *et al*, 2001). As we observed

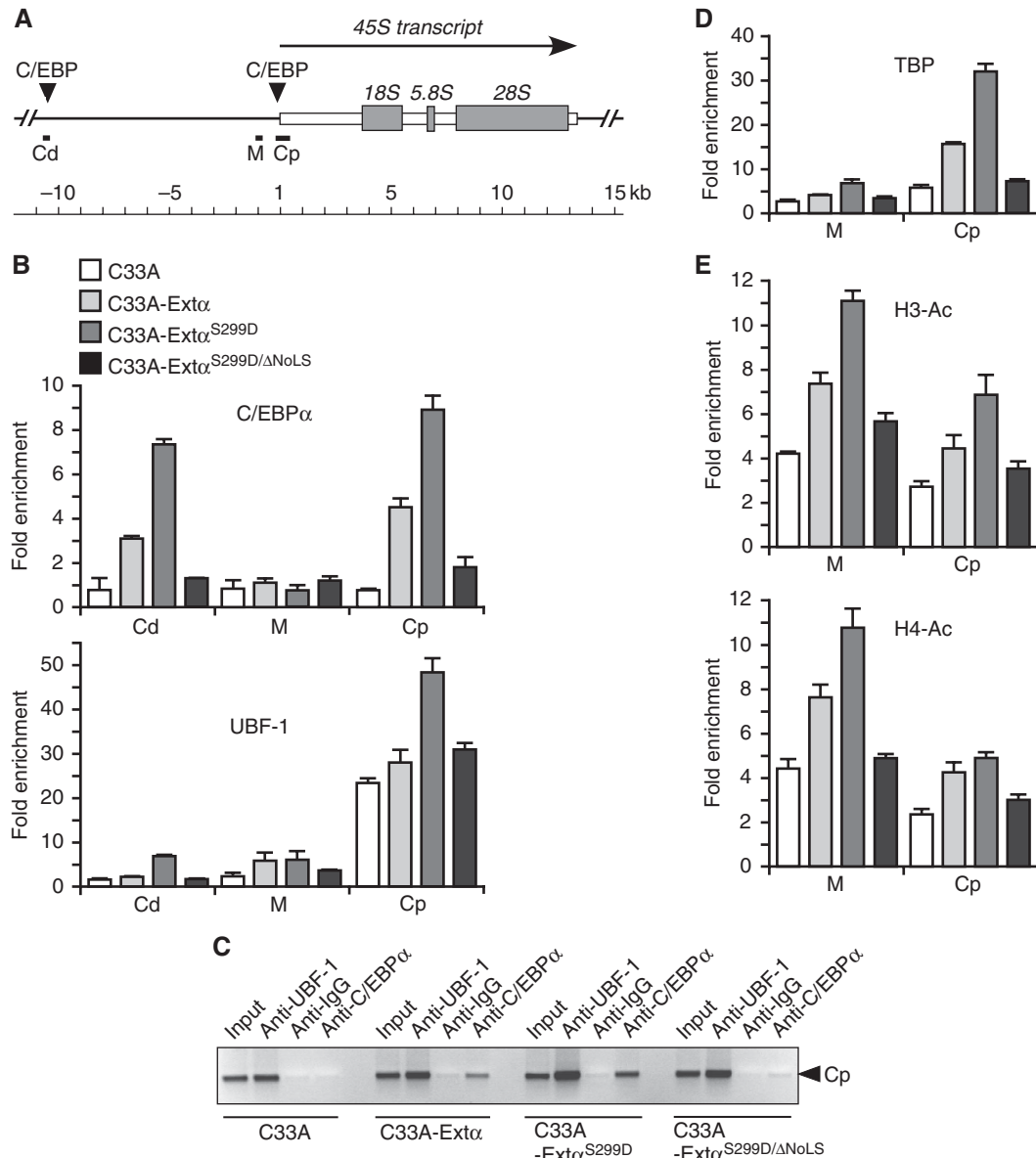


Figure 9 Occupation of rDNA sequences by extended-C/EBP α facilitates UBF-1 and TBP recruitment and induces histone acetylation. (A) Schematic representation of part of the rDNA repeat with the transcribed rRNA and C/EBP consensus recognition sequences as indicated. The rDNA sequences analysed in the ChIP assays are designated Cd (C/EBP distal), Cp (C/EBP promoter) and M (c-Myc). (B) Fold enrichment of rDNA obtained with chromatin cross-linking and immunoprecipitation (ChIP) analysis for the regions Cd, M and Cp with anti-C/EBP α or anti-UBF-1 as indicated versus non-specific rabbit IgG. ChIP assays were done with C33A cells expressing empty vector, extended-C/EBP α -wt, -S299D or -S299D- Δ NoLS mutant as indicated. (C) DNA gel of semi-quantitative PCR of region Cp with input, and ChIP assays with the antibodies as indicated for C33A cells expressing empty vector, extended-C/EBP α -wt, -S299D or -S299D- Δ NoLS mutant. (D) Fold enrichment of rDNA obtained with ChIP analysis for the regions M and Cp with anti-TBP versus non-specific rabbit IgG. (E) Fold enrichment of rDNA obtained with ChIP analysis for the regions M and Cp with anti-H3-AC or anti-H4-AC as indicated versus non-specific rabbit IgG. DNA was quantitated by real-time PCR and data are presented as means and standard deviation from two independent ChIP experiments each analysed by two independent PCR reactions.

that in long-term cultures of cells that stably express the wild-type extended-C/EBP α isoform, the percentage of cells with nucleolar localization of extended-C/EBP α continuously increases, a physiological mechanism must exist that stimulates nucleolar retention and thereby provides a proliferation or survival advantage for the cells. To adequately address which signalling pathways could be involved, a more comprehensive experimental setup is required.

Translation from non-AUG initiation codons

Expression of the extended translational isoform of C/EBP α depends on initiation from non-AUG codons, CUGs in murine

and GUG in human C/EBP α transcripts, respectively (Figure 1). The molecular mechanisms of selection of alternative non-AUG codons for initiation are still largely unknown; however, a study by Schwab *et al* (2004) indicates that a subset of ribosomes is scanning specifically for CUG initiation codons. Several studies point to a physiological role of selection of non-AUG codons in transcripts of other regulatory proteins, including c-Myc (Hann *et al*, 1992; Kim *et al*, 2003), JunD (Short and Pfarr, 2002), FGF2 (Kevil *et al*, 1995) and VEGF (Huez *et al*, 2001) (reviewed in (Touriol *et al*, 2003)). The underlying mechanism of translational upregulation of the extended C/EBP α isoform is currently under investigation.

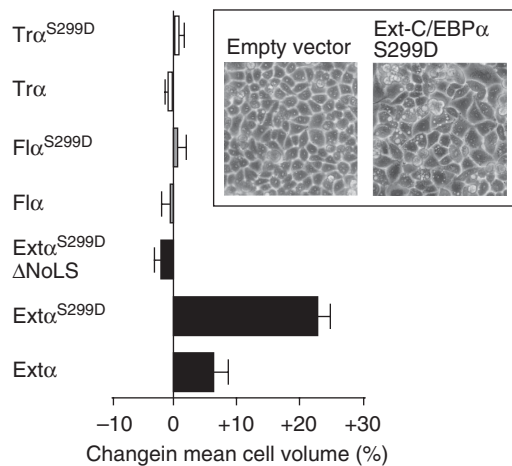


Figure 10 Overexpression of extended-C/EBP α results in an increase in cell volume. C33A cells expressing empty vector, extended-C/EBP α -wt, -S299D or -S299D- Δ NoLS mutants were analysed for cell volume. The percentage of change in the mean cell volume compared with empty vector was calculated from two independent cultures, each measured in triplicate. The error bars represent the data variation.

Regulation of extended-C/EBP α expression and function *in vivo*

The lack of antibodies that specifically recognize extended-C/EBP α and the impossibility to specifically target the endogenous protein by knockdown strategies (all protein isoforms can be translated from a single mRNA molecule) makes it technically difficult to address its physiological functions and sites of action in depth. Nevertheless, we could detect C/EBP α -specific nucleolar localization and expression of extended-C/EBP α in the leukemia cell line HL-60. The concomitant loss of both, C/EBP α -specific nucleolar localization and extended isoform expression during HL-60 differentiation provides a good indication that extended-C/EBP α is responsible for the nucleolar staining in the undifferentiated cells. We presume that expression of extended-C/EBP α is likely to be timely and spatially restricted only to those few cells that proliferate in a tissue. Preliminary analysis of primary bone marrow cells revealed weak but consistent staining of C/EBP α in the nucleoli as well as detection of an extended isoform by immunoblotting (data not shown). To uncover extended-C/EBP α physiology *in vivo* in more detail, antibodies have to be generated that specifically recognize this isoform. In addition, a mouse model in which either expression of extended-C/EBP α is abrogated by mutation of its initiation codon by a knock-in approach, or in which extended-C/EBP α can be induced from a transgene would be instrumental.

A hypothetical model of a C/EBP α -based translational switch

During our study, we did not observe that overexpression of extended-C/EBP α stimulates cell proliferation in the cell lines tested. The truncated-C/EBP α isoform that is co-regulated with the extended isoform in HL-60 cells (Figure 4), however, drives proliferation in several cell types (Lin *et al*, 1993; Calkhoven *et al*, 2000). We would like to propose a hypothetical translational switch model explaining how controlled

translation into extended- and truncated-C/EBP α contributes to coordination of cell growth and proliferation: the truncated isoform blocks the anti-proliferative activity of the full-length isoform in the nucleus; the extended isoform escapes from this inhibition by relocating to the nucleolus where it stimulates rRNA synthesis as prerequisite for cell growth and proliferation (Supplementary Figure S6).

By revealing a function of the extended-C/EBP α isoform in ribosome biogenesis, a new and fascinating aspect of C/EBP α -mediated control of cell growth and proliferation emerges. Whether increased extended-C/EBP α expression or its nucleolar retention might be involved in cancer development has to be investigated.

Materials and methods

DNA constructs

The pSG5- and pcDNA3-based rC/EBP α wt and mutant constructs have been described earlier (Calkhoven *et al*, 2000). The Δ NoLS, S299D and S299A mutations were introduced by site-directed mutagenesis (Kunkel *et al*, 1991). Mutations of the hC/EBP α -cDNA-pcDNA3 were generated by PCR: GTG into ATG (A^{pp}) or CCC (ΔA^1) and removal of the 5'UTR ($\Delta 5'$). All EGFP constructs were generated using EGFP sequences from the pEGFP-N3 plasmid (BD Clontech). The extended-C/EBP α domain and the corresponding Δ NoLS-EGFP-constructs were generated by PCR. The CMV-Myc vector was kindly provided by Martin Eilers (University of Würzburg). The pHrP2-BH, pT β uc- and pGem3HR plasmids (Mayer *et al*, 2005) were kindly provided by Ingrid Grummt (DKFZ, Heidelberg).

Cell culture, transfection, immunofluorescence and cell volume analysis

HL-60 and U937 cells were propagated in RPMI, all other cells in DMEM, plus 10% FCS (Invitrogen) at 5% CO $_2$ and 37°C, and G-418 (0.8 mg/ml) whenever required. HL-60 differentiation was induced by 200 nM phorbol-12-myristate-13-acetate (TPA) for 12 h. Cells were transfected in 5-cm dishes with 5 μ g plasmid using FUGENE (Roche) for MEFs, DEAE-dextran for COS-1 (Calkhoven *et al*, 2000) or calcium phosphate. Treatments with actinomycin D (50 ng/ml; Sigma) or α -amanitin (50 μ g/ml; Sigma) were done for 2 h before fixation. For immunostaining, cells were washed twice with PBS, fixed with 3.7% paraformaldehyde for 10 min at 37°C, washed three times with PBS for 5 min at RT, permeabilized with 0.3% triton X-100 for 5 min at RT, blocked in 3% BSA in PBS and incubated with primary antibody (anti-C/EBP α 14AA, C18, N19 or anti-UBF-1 F-9: 1 μ g/ml, Santa Cruz Biotechnology Inc.); anti-C/EBP α MA1-825 (1 μ g/ml, ABR Affinity BioReagents); anti-Fibrillarin AFB01 (2.5 μ g/ml, Cytoskeleton Inc.) for 45 min at RT. After triple washing for 10 min with PBS, cells were incubated with Alexa Fluor 488 or 568 conjugated secondary antibodies (Invitrogen) for 45 min at RT, then triple washed again and incubated with DAPI (1 mg/ml MeOH) for 15 min at RT. After triple washing for 10 min with PBS and single rinsing with H $_2$ O at RT, coverslips were mounted with Moviol (Sigma) onto glass slides. Microscopic analysis was performed with the ApoTome deconvolution system (Zeiss). Cell volume in femtoliter was determined using the CASY Model TTC (Innovatis).

Lentiviral and retroviral transduction

HL-60 cells were infected with pLKO.1-puro retroviral constructs containing shRNAs against human C/EBP α (5'-CCG GGC TGG AGC TGA CCA GTG ACA ACT CGA GTT GTC ACT GGT CAG CTC CAG CTT TTT-3') or non-target shRNA control (Sigma-Aldrich) and propagated under puromycin selection (1.5 μ g/ml). Subsequently, HL-60 cells containing the human C/EBP α -shRNA were retrovirally transduced using pMSCV-neo-based vectors containing either wt rat C/EBP α -FLAG cDNA, cDNA containing the Δ CUG mutation, extended-C/EBP α^{S299D} -FLAG, extended-C/EBP α^{S299D} - Δ NoLS-FLAG or empty vector control, and propagated on G418 (1.8 mg/ml) in addition.

Co-immunoprecipitation

Hek293T cells (7×10^5) were transfected with 5 μ g of expression vectors as indicated. Cells were lysed in 50 mM Tris/HCl pH 7.4, 300 mM NaCl, 5 mM EDTA, 1% Triton X-100 supplemented with

1 mM PMSF and protease inhibitor cocktail (Roche). Lysates were pre-cleared for 30 min with Protein G-Sepharose beads (Amersham) and incubated for 4 h with 1 μ g of primary antibody (anti-UBF-1 (F-9), anti-C/EBP α (14AA), anti-TBP (N-12), anti-TAF₄₈ (M19) or normal mouse, rabbit or goat IgG, respectively, as negative control (all Santa Cruz Biotechnology), which had been coupled to Protein G-Sepharose beads before (1 h incubation in 1 \times PBS). Beads were then washed five times for 5 min with 50 mM Tris/HCl pH 7.4, 300 mM NaCl, 5 mM EDTA 0.1% TritonX-100 supplemented with 1 mM PMSF and protease inhibitor cocktail and once with 1 \times PBS. Proteins were eluted by boiling the beads in SDS sample buffer and analysed by western blotting.

Western blotting

Western blotting was performed as described earlier (Calkhoven *et al*, 2000) with the following antibodies: anti-C/EBP α antibodies (14AA, C-18), anti-Myc antibody (9E10), anti-UBF-1 (F-9), antibody anti-TBP (N-12), anti-TAF₄₈ (M19), anti- α -Tubulin (TU-02) all from Santa Cruz Biotechnology, anti-NPM (clone FC-61991) from Zymed Laboratories), HRP-conjugated secondary antibodies (Amersham Life Technologies or Santa Cruz Biotechnology). Bands were visualized by chemiluminescence (ECL, Amersham Life Technologies).

Chromatin immunoprecipitation

C33A cells expressing extended-C/EBP α , extended-C/EBP α ^{S299D}, extended-C/EBP α ^{S299D} Δ NoLS or control cells containing the empty pcDNA3 vector were cultured in DMEM with 10% FBS. ChIP assay was performed with $\sim 5 \times 10^6$ cells essentially as described in Puig *et al* (2003) using a Bioruptor (Diagenode, Inc.) for sonication (details on request). ChIP antibodies were against C/EBP α (14AA), UBF-1 (F-9), TBP (N-12) and non-specific rabbit IgG from Santa Cruz Biotechnology, acetylated histone H3 (#06-599) and acetylated histone H4 (#06-866) from Upstate Biotechnologies. The fold enrichment was calculated relative to the background detected with non-specific rabbit IgG. For the semi-quantitative PCR, 1/50 (1 μ l) of DNA obtained from the ChIP assay was used as template in a PCR reaction with 28 cycles. Primer pairs were for Cp (89 bp) 5'-ccc ggg gga ggt ata tct tt-3' and 5'-cca acc tct cag acg aca-3', for Cd (114 bp) 5'-aaa tag gac gga gaa cgt agc-3' and 5'-gtt aca ttg ccc gaa

aga tgg-3' for M (155 bp) 5'-aga ggg gct gcg ttt tcg gcc-3' and 5'-cga gac aga tcc ggc tgg cag-3'.

rRNA minigene reporter assay and Northern blotting

The rRNA minigene reporter assay was performed as described in Mayer *et al* (2005) using transfection of Hek293T cells with 8 μ g reporter plasmid (pHrP2-BH) and 1 or 2.5 μ g of pcDNA3-based C/EBP α expression vectors, supplemented with empty pcDNA3 plasmid to a total amount of 10.5 μ g. For analysis by Northern blotting, 10 μ g of total RNA was isolated 40 h later using the guanidine isothiocyanate method. For detection of the 45S rRNA transcript, cells were serum starved for 24 h, replenished with serum for 4 h in the absence or presence of 50 μ g α -amanitin following total RNA isolation. A measure of 30 μ g of total RNA was analysed by Northern blotting using a probe from the pGem3HR plasmid (Mayer *et al*, 2005) and Northern with a β -actin probe or ethidium bromide staining was used as loading control.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

Acknowledgements

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Conflict of interest

The authors declare that they have no conflict of interest.

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