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Functional interaction of CCAAT/enhancer-binding-protein-α basic region mutants with E2F transcription factors and DNA

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ABSTRACT

The transcription factor CCAAT/enhancer-binding protein α (C/EBPα) regulates cell cycle arrest and terminal differentiation of neutrophils and adipocytes. Mutations in the basic leucine zipper domain (bZip) of C/EBPα are associated with acute myeloid leukemia. A widely used murine transforming C/EBPα basic region mutant (BRM2) entails two bZip point mutations (I294A/R297A). BRM2 has been discordantly described as defective for DNA binding or defective for interaction with E2F. We have separated the two BRM2 mutations to shed light on the intertwined reciprocity between C/EBPα-E2F-DNA interactions. Both, C/EBPα I294A and R297A retain transactivation capacity and interaction with E2F-DP. The C/EBPα R297A mutation destabilized DNA binding, whereas the C/EBPα I294A mutation enhanced binding to DNA. The C/EBPα R297A mutant, like BRM2, displayed enhanced interaction with E2F-DP but failed to repress E2F-dependent transactivation although both mutants were readily suppressed by E2F1 for transcription through C/EBP cis-regulatory sites. In contrast, the DNA binding enhanced C/EBPα I294A mutant displayed increased repression of E2F-DP mediated transactivation and resisted E2F-DP mediated repression. Thus, the efficient repression of E2F dependent S-phase genes and the activation of differentiation genes reside in the balanced DNA binding capacity of C/EBPα.

INTRODUCTION

CCAAT/enhancer-binding protein α (C/EBPα) is a basic region leucine zipper (bZip) transcription factor that consists of an N-terminal tripartite transactivation domain, a C-terminal DNA-binding basic region and a coiled-coil leucine zipper dimerization domain (bZip) [1-4]. C/EBPα plays important roles in liver metabolism, hematopoietic lineage specification, neutrophil-, skin-, and adipocyte differentiation [5-9]. Furthermore, C/EBPα mediates cell cycle arrest and resistance to tumorigenic transformation in many cell types, suggesting a tumor suppressor function [10, 11]. Suppression of cell proliferation by C/EBPα involves interactions with E2F-DP-Rb and SWI/SNF complexes, stabilization of $p21^{WAF}$, and inhibition of CDK2/4 [12-16].

Alternative translation initiation generates long (p42) and truncated (p30) C/EBPα protein isoforms that differentially affect proliferation, differentiation, and tumorigenesis [17, 18]. Mutations that abrogate expression of the long isoform, often in conjunction with bZip mutations of the second C/EBPα allele, are salient features of approximately 10-15% of human acute myeloid leukemia (AML). The synergy of bi-allelic mutations in leukemogenesis has been experimentally demonstrated in rodents to faithfully reflect development of AML [19-28]. Mechanistically, the truncated p30 isoform lacking the amino terminal transactivation region is sufficient to induce a myeloproliferative disease and may exert dominant inhibitory effects by heterodimerization with other C/EBPs and/or compatible leucine zipper factors [21, 28]. The function of C/EBPα bZip mutations are less clear but may involve compromised DNA-binding, intramolecular crosstalk with the N-terminus, and altered protein interactions [29].

Murine C/EBPα BRM2 serves as a paradigm bZip mutant that contains two point mutations in the bZip domain (I294A, R297A). BRM2 knock-in mice display defective adipogenesis and develop a myeloproliferative disorder with a partial block of granulocyte differentiation [30, 31]. Critical effects of BRM2 entail failure to repress E2F-DP mediated S-phase gene transcription. Many E2F target gene promoters, including *E2F1, DHFR, PCNA, Rbl1, CcnA2, CcnE1, Cdc2, Mcm3, Mcm6,* have C/EBP binding sites in the vicinity of E2F binding sites [32, 33]. Expression profiling in combination with chromatin immunoprecipitation demonstrated extensive cross-regulation between C/EBP and E2F target genes in cell cycle entry during liver regeneration [34]. Disparate interpretations, however, exist about the importance of C/EBPα DNA binding versus binding to E2F-DP complexes, respectively [35-37], leaving the mechanism unresolved,

as to how C/EBPα BRM2 interferes with cell proliferation and differentiation[35-37].

To shed light on the mechanism, we set out to examine the contribution of the individual BRM2 mutations. Using isothermal titration calorimetry (ITC), reporter assays, differentiation/proliferation and protein interaction tests we found that the positive charge at amino acid position 297 in C/EBPα is pivotal for stabilization of DNA binding but not for the interaction with the E2F-DP complex. Surprisingly, the C/EBPα I294A mutation leads to enhanced DNA binding and inhibition of E2F-DP mediated transactivation, although interaction with E2F-DP remains unchanged. In contrast, C/EBPα R297A enhanced interaction with E2F-DP but reduced DNA binding and repression of E2F-DP driven transcription. Our results show that BRM2 exhibits a multi-factorial phenotype and that proliferation control by C/EBPα and E2F-DP requires balanced and simultaneous interaction between C/EBPα-E2F-DP complexes and DNA.

EXPERIMENTAL PROCEDURES

Plasmids

The pBabePuro-based retroviral C/EBPα basic region point mutants (BRM2: I294A and R297A) were obtained from Claus Nerlov [31] and cloned into pcDNA3 plasmid as described [37]. WT C/EBPα fused to a C-terminal FLAG-tag was cloned into pcDNA3. Individual C/EBPα mutations (I294A, R297A, R297Q and R297K) were introduced by Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's protocol and confirmed by sequencing. The C/EBP responsive -82 cMGF-luciferase reporter as well as HA-tagged DP1, HA-tagged E2F1, and pE2Fx6-TATA-luciferase reporter were previously described [37].

Cell culture and transfections

HEK-293T and C/EBPα-/-MEFs were cultivated in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) containing 10% FCS. Transient transfection with HEK-293T cells and C/EBPα-/-MEFs were performed by calcium phosphate method or with Metafectene reagent according to the manufacturer's protocol (Biontex). All experiments were repeated at least 3 times.

GST pull-down and immunoblotting

N-terminal GST-fusion proteins of E2F1 or DP1 were expressed in *E.coli* BL21 (DE3) bacteria, lysed and purified with glutathione sepharose 4B beads (GE Healthcare) following the instruction of manufacturer. In pulldown assays, GST-fusion proteins were bound to glutathione sepharose 4B beads and incubated with an equal volume of lysates of HEK-293T cells transfected with expression plasmids. Bound proteins were detected by immunoblotting and quantified by Odyssey Infrared Imaging System. Western blots were incubated with appropriate antibodies and developed using the enhanced chemiluminescence method (GE Healthcare). The following antibodies were used: anti-C/EBPα (14AA, Santa Cruz), anti-rabbit IRDye 800 (Invitrogen), anti-mouse IgG Alexa-Fluor®680 (Invitrogen), or secondary antibodies conjugated to horseradish peroxidase.

Adipogenesis and cell proliferation assay

Vector control, WT I294A, R297A, R297Q, R297K, or BRM2 variants of C/EBPα were transfected in C/EBPα-/- MEFs and selected by 3mg/ml puromycin. After selection, stably transfected cells were seeded in duplicates in tissue culture dishes and grown to confluency.

Cells were differentiated with MEM-AlphaMedium supplied with 10% serum, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 10µg/ml insulin and 1µM dexamethasone for 2 days. From day 3 onwards, cells were cultured in alpha-MEM, 10% serum and 10µg/ml insulin. Medium was changed every 2 days. After 10 days adipocytes were fixed with 4% paraformaldehyd and stained by oil red O. Cellular morphology was documented using bright-field microscopy and protein expression was determined by protein blotting. For cell growth assays or colony assays, $0{,}5 \times 10^5$ or 1×10^5 C/EBPα-/- MEFs were stably transfected with WT or mutant C/EBPα constructs, seeded in triplicates, and cell numbers determined over a period of 6 days as indicated. Colonies were stained with crystal violet (Sigma) after 6 days. Adipogenesis and shRNA experiments were performed as described [37].

Chromatin immunoprecipitation (ChIP) and quantitative PCR (qPCR)-

ChIP and qPCR from differentiated C/EBPα-/-MEFs were performed as described [37]. pPCR primers for the ACSL1 promotor (appr. -450 bp) were described in [38] and http://genome.ucsc.edu/. Mouse negative control primer Set1 was used according to the manufacturer protocol (Active motif #71011).

Electrophoretic mobility shift assay

Gel shift experiments with C/EBP oligonucleotides derived from the cMGF promoter were described [39]. P^{32} labeled oligonucleotides were incubated for 5 minutes with nuclear extract from HEKT cells transfected with WT or C/EBPα variants. After certain time intervals (0-80 minutes), 100-fold excess of unlabeled oligonucleotides was added to competition binding reactions. Reactions were separated by EMSA gel electrophoresis.

Protein expression, purification and isothermal titration calorimetry (ITC)

DNA encoding the bZip-domain of C/EBPα (amino-acids 280-340) was subcloned into the pQLinkH vector [39]. The WT and mutant genes were expressed as N-terminal His7-tagged proteins at 20°C in *E. coli* Rosetta® (DE3) (Novagen). The purification procedure comprises mechanical cell lysis by sonication (SONOPULS HD 2200, Bandelin), affinity chromatography on a 5 ml HisTrap FF crude column (GE Healthcare) charged with Ni^{2+}, and a size exclusion chromatography on a Superdex 75 prep grade column (XK 26/60, GE Healthcare). The His7-tag was cleaved with tobacco etch virus protease prior separation by gel filtration, followed by a reapplication of the cleaved protein on a Ni^{2+}-affinity column. Isothermal titration calorimetric (ITC) measurements were performed in 20 mM HEPES (pH 7.5) and 0.15 M KCl at 25°C using

a VP-ITC microcalorimeter (MicroCal, LLC, Northampton, IL). In an experiment 5 μl of 200 μM DNA solution was injected into the sample cell containing 20 μM protein solution (monomeric WT, I294A, R297A, R297Q, and R297K variant of C/EBPα-bZip domain, respectively). The DNA sequence of the half-palindromic C/EBP site is 5'-TCGACACAATGAGGCAAT-3' (forward) and 5'-TCGATTGCCTCATTGTG-3' (reverse). A total of 50 injections were performed with a spacing of 240 s and a reference power of 18 μcal/s. Integration of injection peaks and construction of binding isotherms was performed by using the high-precision automated peak shape analysis software Nitpic 1.0.3 [40]. Data analysis was performed with the Origin 7.0 software (MicroCal, U.S.A) using a one-site binding model.

RESULTS

The positive charge of C/EBPα R297 is critical for protein-DNA complex stability

C/EBPα interacts with its consensus target DNA via the basic leucine zipper region (bZip). The bZip residue R297 was of particular interest, as a R297P mutation has been identified in a human patient diagnosed with AML M2 subtype [42] and because the experimental C/EBPα BRM2 mutant contained a R297A mutation and induced a myeloproliferative disease in mice (Fig. 1A) [30]. Structural considerations suggested an important role of the guanidinium group of R297 that interacts with the phosphate group of G1 in its cognate DNA binding site. In addition, R297 also points towards the solvent and may thus interact with accessory proteins (Fig. 1B) [43, 44]. The situation in BRM2, however, is complicated by the fact that it contains a second mutation in the DNA binding domain, namely I294A. Our previous results suggested that BRM2 showed diminished DNA binding but retained interaction with E2F-DP in the absence of DNA, supporting the notion that binding to DNA is a prerequisite for suppression of E2F functions [37].

Here, both mutations were separated to distinguish between individual effects of I294 and R297 BRM2 mutations. WT and mutant C/EBPα were bacterially expressed and purified, and DNA binding was examined by isothermal titration calorimetry (ITC). The results showed diminished DNA binding for the bZip variants BRM2, R297A and R297Q, whereas the R297K variant displayed WT-like DNA binding compared to WT. This is consistent with published structural data [43] and demonstrates the importance of the positive charge of residue 297 for the interaction with the DNA backbone (Fig 1B-C and Table 1). Surprisingly, the I294A variant exhibits stronger DNA binding (Table 1). The fact that a bZip dimer binds to one DNA molecule, as indicated by the calculated stoichiometry value of approximately 0.5 (Table 1) holds true for WT and the I294A, R297A and R297K bZip variants. Unexpectedly, a stoichiometry value of around 1 was determined for the BRM2 and R297Q variants, indicating that these DNA-binding deficient variants bind to DNA in a monomeric fashion. Thus, bZip dimerization seems to be linked to sufficient DNA binding.

Electrophoretic mobility gel shift assays (EMSA) were performed to assess off-rates in the presence of a 100-fold excess of unlabeled oligonucleotide. As shown in Fig. 1D, both C/EBPα WT and R297K variant formed stable complexes, whereas the R297A variant readily dissociated from its binding site, suggesting that the protein-DNA complex formed by C/EBPα

R297A is unstable and substantiating the requirement of a positive charge at position 297 for stable interaction with DNA.

C/EBPα R297A substitution alters proliferation without affecting interaction with E2F-DP

The C/EBPα-bZip residues I294, R297 have been suggested to be involved in the physical association with E2F1 [31]. We wondered how individual substitutions of both residues affect the interaction with the E2F1-DP1 complex. In GST pull-down assays, I294A, R297Q, and R297K interacted with GST-E2F1 and GST-DP1 indistinguishable from WT C/EBPα (Fig. 2A). Previously, we had shown that BRM2 displays enhanced interaction with DP1 and now attribute this observation to the R297A and not to the I294A mutation (Fig. 2A and Fig. 1C) [37]. Next, we examined whether mutations of residues I294, R297 affect cell proliferation. WT C/EBPα, I294A, and R297K inhibited colony formation (Figure 2B) and retarded cell multiplication (Figure 2C) in C/EBPα-/-MEF cells, whereas BRM2, R297A, and R297Q largely failed to compromise colony growth and cell proliferation (Fig. 2B,C). Next, WT and mutant C/EBPα constructs were examined by chromatin immunoprecipitation for association with the E2F-DP target gene dihydrofolate reductase (*DHFR*) promoter. As shown in Figure 2D, the DNA binding deficient BRM2, R297A, and R297Q mutants displayed impaired binding to the DHFR promoter, whereas WT, I294A, and R297K mutants bound to the endogenous DHFR promoter, in accordance with the ITC results of DNA binding [35, 37]. Thus, although BRM2, R297A, and R297Q may still interact with E2F-DP in solution, efficient binding to DNA is a prerequisite for C/EBPα-mediated inhibition of proliferation.

Reciprocal regulation of C/EBPα and E2F-DP activity

Several studies proposed that the balance between C/EBPα and E2F-DP critically controls precursor cell proliferation and C/EBPα-mediated differentiation [31, 45, 46]. Our previous work demonstrated that E2F-DP complexes inhibit C/EBPα during adipocyte differentiation and that BRM2 was more sensitive to E2F-DP mediated repression, because DNA-binding of C/EBPα was decreased and binding to E2F-DP was enhanced [37]. We compared the activity of various individual C/EBPα mutations in reporter assays in order to dissect the individual contributions of the two BRM2 mutations in more detail. All mutants retained transactivation potential in C/EBP responsive reporter assays when expressed at high concentration (100 ng of plasmid transfected), however, impairment of transactivation of DNA-deficient mutants (BRM2, R297A and R297Q) was apparent at low concentration (1 ng) (data not shown). As shown in Figure 3A, E2F1-DP1 readily inhibited BRM2, R297A, and R297Q, as compared to WT C/EBPα

(expression controls are shown in Fig. 3B). The DNA binding enhanced mutants C/EBPα I294A, R297K both resisted E2F1-DP1 mediated repression. These results suggest that attenuation of the C/EBPα-DNA stability increases the susceptibility to E2F1-DP1 mediated C/EBPα repression.

C/EBPα represses E2F-regulated S-phase genes and induces proliferation arrest whereas mutations in the C/EBPα basic region (BRM2, BRM5) failed to inhibit E2F mediated transcription [31, 45]. The individual C/EBPα point mutations at amino acid positions 294/297 showed that DNA binding proficient mutants C/EBPα I294A and R297K readily repressed E2F-DP activity, whereas all DNA binding deficient mutants, BRM2, R297A, R297Q, were less able to do so (Fig. 3C; expression controls are shown in Fig. 3D). Repression of E2F without C/EBP binding sites in the promoter construct may be due to squelching effects or cryptic C/EBP sites somewhere in the plasmid. Removal of the 6xE2F sites from the reporter (leaving just the E1B-TATA element) revealed C/EBP responsiveness with DNA binding proficient WT, I294A, or R297K but less with DNA binding deficient BRM2 or R297A, favoring the notion of cryptic C/EBP binding sites in the reporter backbone (data not shown). Altogether, these data suggest that the association between C/EBPα and E2F-DP is not sufficient for repression of E2F-DP mediated transactivation, but additionally requires the DNA-binding function of C/EBPα.

C/EBPα-mediated adipogenesis

C/EBPα regulates adipogenesis together with the peroxisome proliferator activated receptor γ (*PPARγ*) [7, 8]. C/EBPα-/- MEF are defective for fat cell differentiation and were therefore employed to further examine the adipogenic capacity of the various C/EBPα mutants in tissue culture. As shown by chromatin immunoprecipitation in Figure 4A, DNA binding proficient WT C/EBPα, I294A, and R297K robustly associated with the *PPAR*γ and *ACSL1* gene promotors, whereas BRM2, R297A, and R297Q C/EBPα displayed strongly decreased association with both genes. In accordance, the adipogenic marker genes aP2, PPARγ, and adipsin are induced by DNA binding proficient WT and R297K C/EBPα, but not DNA binding deficient R297Q C/EBPα (Figure 4B). Importantly, the DNA binding proficient WT, I294A, R297K C/EBPα induced differentiation of C/EBPα-/- MEF into lipid laden fat cells, as revealed by Oil-red-O staining. In contrast, the DNA binding attenuated R297A, R297Q, or BRM2 C/EBPα mutants failed to induce fat cell differentiation (Figure 4C). In accordance with previous results [37], adipogenic differentiation of BRM2 and also of R297A C/EBPα mutants could be partially rescued by shRNA mediated knockdown of either DP1 or E2F3, whereas C/EBPα WT was

weakly affected and no effect of E2F-DP knockdown was detected with the I294A mutant (Figure 4D). Taken together, these data show that the positive charge at position 297 is important for the association of C/EBPα with target promoters and the DNA dependent interaction with DP1 and E2F to regulate cell proliferation and differentiation.

DISCUSSION

The murine basic region mutant BRM2 of C/EBPα is frequently employed in studies of the antagonism between C/EBPα and E2F in proliferation, cell differentiation, and leukemia initiation. BRM2 fails to repress E2F-mediated transcription and to support differentiation, however, disparate views suggested impaired interaction with E2F-DP or DNA, respectively [31, 43]. Although structural analysis suggested R297 to interact with the phosphate group of G^1 in the DNA consensus site, alterations in DNA binding of R297 mutations have not yet been examined [42, 43]. In agreement with the structural data, our ITC results demonstrate diminished DNA binding for R297A (partially) and R297Q (strongly), whereas the substitution of R297 with lysine shows WT-like DNA binding (Figure 1C and Table 1). Thus, a positively charged side chain at residue 297 is critical for electrostatic interaction with the negatively charged DNA backbone to stabilize binding of C/EBPα to DNA. Additionally, we observe enhanced DNA binding of I294A, which might be due to improved space fitting to the DNA major groove, removal of the solvent exposed hydrophobic side chain of I294, or both.

All tested C/EBPα mutants with compromised DNA binding retained E2F1 and DP1 binding, or even showed enhanced interaction (BRM2, R297A), but failed to abrogate E2F-induced transcription. Concomitantly, E2F-DP readily inhibited transactivation by the same C/EBPα mutants. By contrast, the I294A mutant that displayed enhanced DNA binding also showed enhanced repression of E2F transcription, although interaction with E2F-DP was indistinguishable from WT C/EBPα. Moreover, mutation of R297 to lysine restored stable DNA binding, repression of E2F-DP induced transcription and protection from E2F-DP mediated repression. In accordance with this, WT C/EBPα, I294A, and R297K, but not R297Q, efficiently induced fat cell differentiation and bound to and activated endogenous C/EBPα responsive differentiation genes. Data presented here show that C/EBPα mutants with impaired DNA-binding dissociate more easily from E2F target genes and thus fail to restrict E2F-target gene expression, although they may still interact with E2F-DP. Accordingly, intact DNA-binding and interlinked bZip dimerization capacity of C/EBPα is a prerequisite for both, resistance to E2F-DP mediated inhibition (at low E2F-complex concentration from C/EBP-cis regulatory sites) and repression of transactivation by E2F-DP at elevated C/EBPα concentration such as during adipogenesis or granulopoiesis.

CONCLUSIONS

We conclude that repression of E2F target genes by C/EBPα requires efficient DNA binding. Intact E2F-DP-C/EBPα-DNA complex formation is indispensable for effective repression of E2F regulated S-phase genes and prevention of tumorigenesis. The DNA binding affinity of C/EBPα may represent a regulatory target to balance cell differentiation and proliferation arrest.

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ABBREVIATIONS

The abbreviation used are: C/EBPα, CCAAT/enhancer-binding protein-α; BRM2, basic region mutation 2; E2F, early gene 2 factor; DP, E2F-dimerization partner; DHFR, dihydrofolate reductase; ACSL1, acyl-CoA synthetase long-chain family member 1; GST, glutathione S-transferase; ITC, Isothermal titration calorimetric.

FIGURE LEGENDS

FIGURE 1. DNA binding of WT C/EBPα and variants.

A. General domain organization and amino-acid sequence alignment of the basic region of various C/EBP isoforms. Discussed mutations (BRMs and R297) are indicated. B. Overall fold of the crystal structure of the C/EBPα basic region leucine zipper complexed with DNA (left; PDB ID code 1NWQ) [43]. The enlarged view (right) of the region around R297 reveals its involvement in DNA binding by hydrogen bonding interactions (blue dashed lines) with the DNA backbone. Molecular drawings were created with PyMOL Molecular Graphics System (Version 1.3, Schrödinger, LLC). C. Differential DNA binding affinity of C/EBPα〈 wild-type and variant bZip proteins as revealed by ITC. Shown are the respective ITC isotherms (●, WT; ▪, BRM2; □, I294A; Δ, R297K; ✰, R297A, ○, R297Q). The data were fitted to a one-site binding model. Calculated parameters are summarized in Table 1. D. Off-rate EMSA showing the stability of the C/EBPα-DNA complex. WT or variant C/EBPα was bound to radiolabeled C/EBP DNA and subsequently incubated with a 100-fold excess of cold C/EBP probe for the time indicated.

FIGURE 2. Interaction between C/EBPα variants and E2F1-DP1 and colony formation capacity.

A. Interaction between C/EBPα, E2F1, and DP1. WT C/EBPα or mutants were expressed in HEK293T cells and cell lysates were incubated with GST-E2F1 or GST-DP1. Bound C/EBPα was examined by immunoblotting and quantified by Odyssey Infrared Imaging System. Error bars indicate the means ±SD of three individual experiments carried out in parallel. B. Colony assay of C/EBPα-/- MEFs transfected with various C/EBPα constructs, as indicated. Cells were seeded (1 ⃠ 10^5) and stained with crystal violet after six days. Neg: negative plasmid control lacking puromycin resistance gene. C. Cell proliferation assay. C/EBPα-/- MEFs were transfected with WT or mutant C/EBPα constructs, puromycin selected and seeded in tripilcates

(0,5 x 10^5). Cells were counted over a period of 6 days, as indicated (triplicats, means ±SD). D. Chromatin immunoprecipitation analysis (ChIP) of the DHFR promoter containing the E2F and C/EBP binding sites. C/EBPα-/-MEF cells were stably transfected with WT or C/EBPα mutations. ChIP analysis was performed after 96h treatment with adipogenic stimulation cocktail. Chromatin samples were immunopreciptated with antibody against C/EBPα or mouse IgG and analyzed by quantitative PCR, using primers flanking C/EBP-binding sites in the promoters of the DHFR gene. ChIP quantification data are expressed as anti-C/EBPα versus IgG and normalized to input. Error bars indicate the means ±SD of three individual experiments. P-values are ≤0.02 for all constructs.

FIGURE 3. Transcriptional activation and repression by C/EBPα variants and E2F-DP.

A. Transcriptional activity of C/EBPα variants in the presence of E2F-DP. HEK293T cells transfected with a C/EBP-responsive reporter and C/EBPα expression plasmids encoding WT, BRM2, I294A, R297A, R297Q, R297K (100 ng) in the absence or presence of indicated amounts of E2F, DP expression plasmids (50, 100ng each). Error bars indicate the means ±SD of three individual experiments. B. Protein expression in A) was analyzed by immunoblotting. C. Repression of E2F-DP mediated transactivation by C/EBPα variants. HEK293T cells were transfected with E2F-responsive gene reporter (100 ng) and E2F1, DP1 expression plasmids (50 ng each), in combination with increasing amounts (30, 60, 100 ng) of C/EBPα constructs, as indicated. Error bars indicate the means ±SD of three individual experiments carried out in parallel. ±D. Protein expression in C) was analyzed by immunoblotting.

FIGURE 4. Adipocyte differentiation induced by C/EBPα constructs.

A. C/EBPα association with target promoters. ChIP analysis of C/EBPα-/-MEFs stably transfected with WT C/EBPα and mutants or control vector. ChIP was performed with anti-C/EBPα or mouse IgG and primers flanking C/EBP-binding sites in the proximal promoters of the PPARγ2 and ACSL1 genes (left Y-axis). ChIP specificity was controlled by qPCR with negative control primers (right Y-axis). ChIP data are expressed as -fold enrichment of anti-C/EBPα versus IgG and normalized to input. B. Transcript levels of adipocyte genes as indicated, normalized to GAPDH levels. All ChIP and mRNA expression analysis were performed with cells harvested after 96 h treatment with adipogenic stimulation cocktail. Error bars indicate the means ±SD of three individual experiments. ChIP P-values for all constructs are ≤0.01 and ≤0.05 for RNA expression. C. Adipogenesis was induced in confluent C/EBPα-/-MEF cells stably expressing C/EBPα variants, as indicated. Lipid accumulation was detected by

Oil-Red-O staining after 10 days. Underneath: Protein expression control of C/EBPα constructs. D. C/EBPα-/-MEF cells stably expressing C/EBPα WT, I294A, R297A and BRM2 were transfected with scramble shRNA, shDP1 or shE2F3 in triplicates, as previously published [37]. One day after transfection, adipogenesis was induced by addition of the adipogenic stimulation cocktail. After 8 days, cells were stained with Oil-Red-O and 400 GFP-positive cells were quantified as adipocyte or non-adipocyte counts. Values were plotted as means ±SD.

Table 1

C/EBP〈 bZIP protein	**K_d (nM)**	**stoichiometry**
WT	699.3 ± 22.9	0.57 ± 0.0024
BRM2	1177.3 ± 57.2	1.21 ± 0.0077
I294A	207.5 ± 10.4	0.47 ± 0.0018
R297A	952.4 ± 28.4	0.55 ± 0.0025
R297Q	1377.4 ± 54.5	0.97 ± 0.0053
R297K	480.8 ± 22.9	0.42 ± 0.0026

Table 1. Summary of calculated parameters derived from ITC data using wild-type or variant C/EBPα bZip-domain proteins and C/EBP half-palindrome cMGF promoter site DNA.

Figure

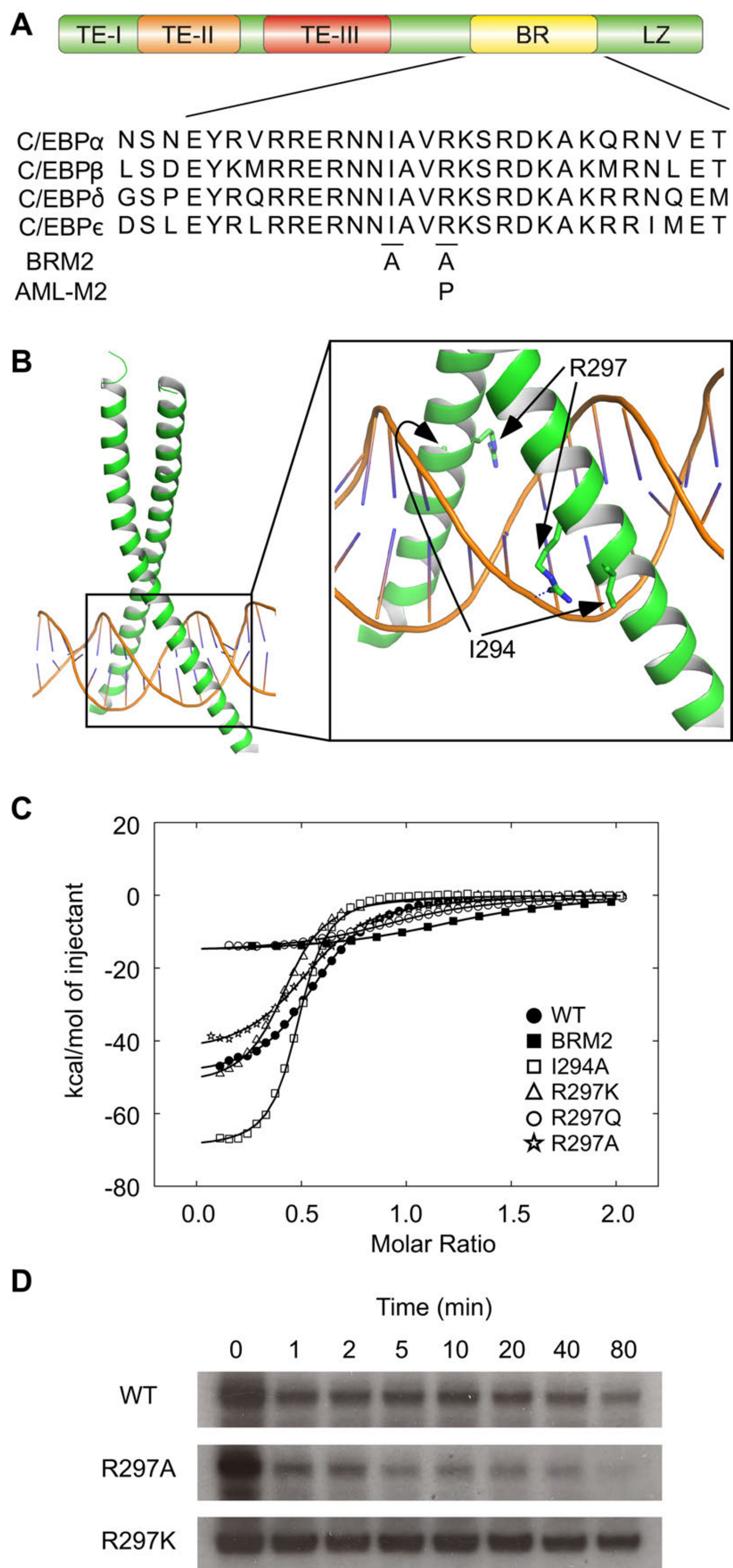

Figure 1. Kowenz-Leutz et al.

Figure

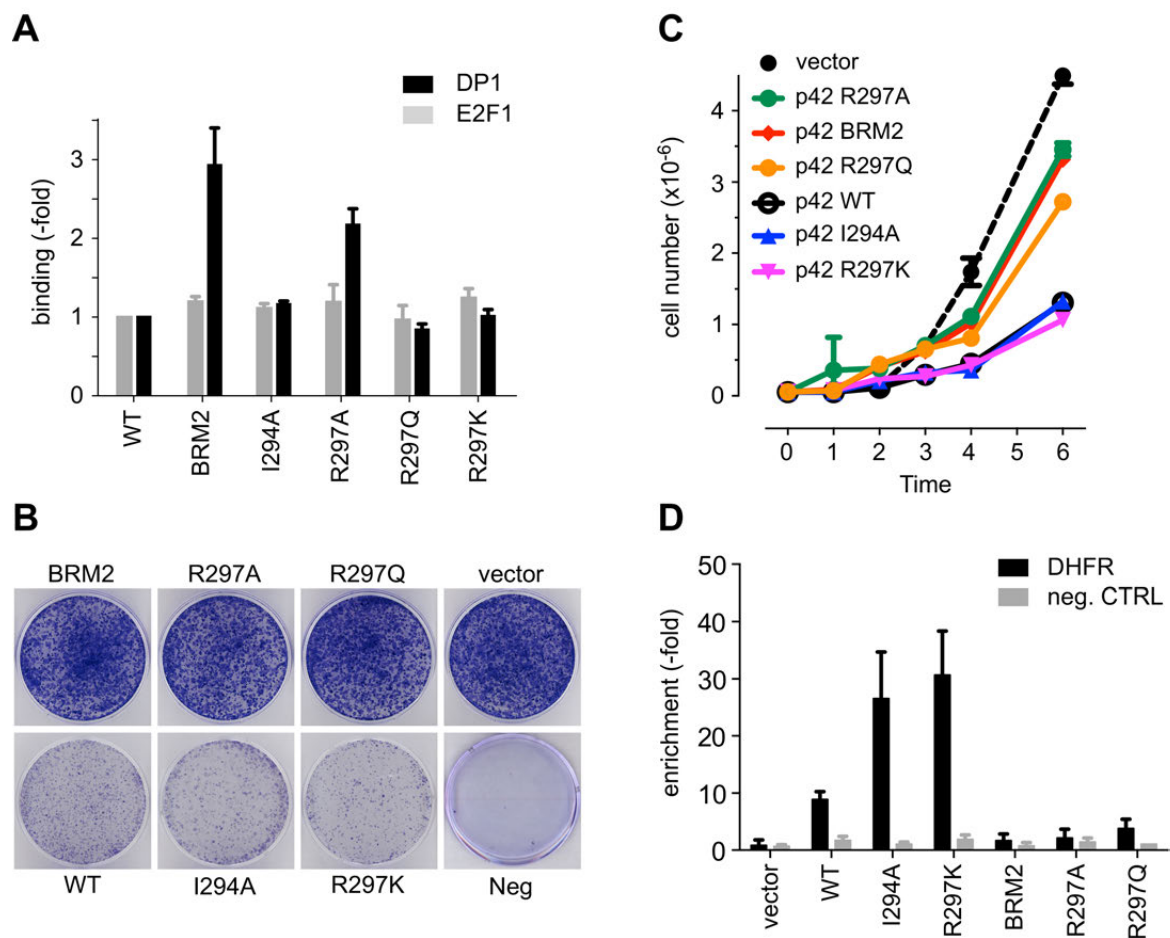

Figure 2. Kowenz-Leutz et al.

Figure

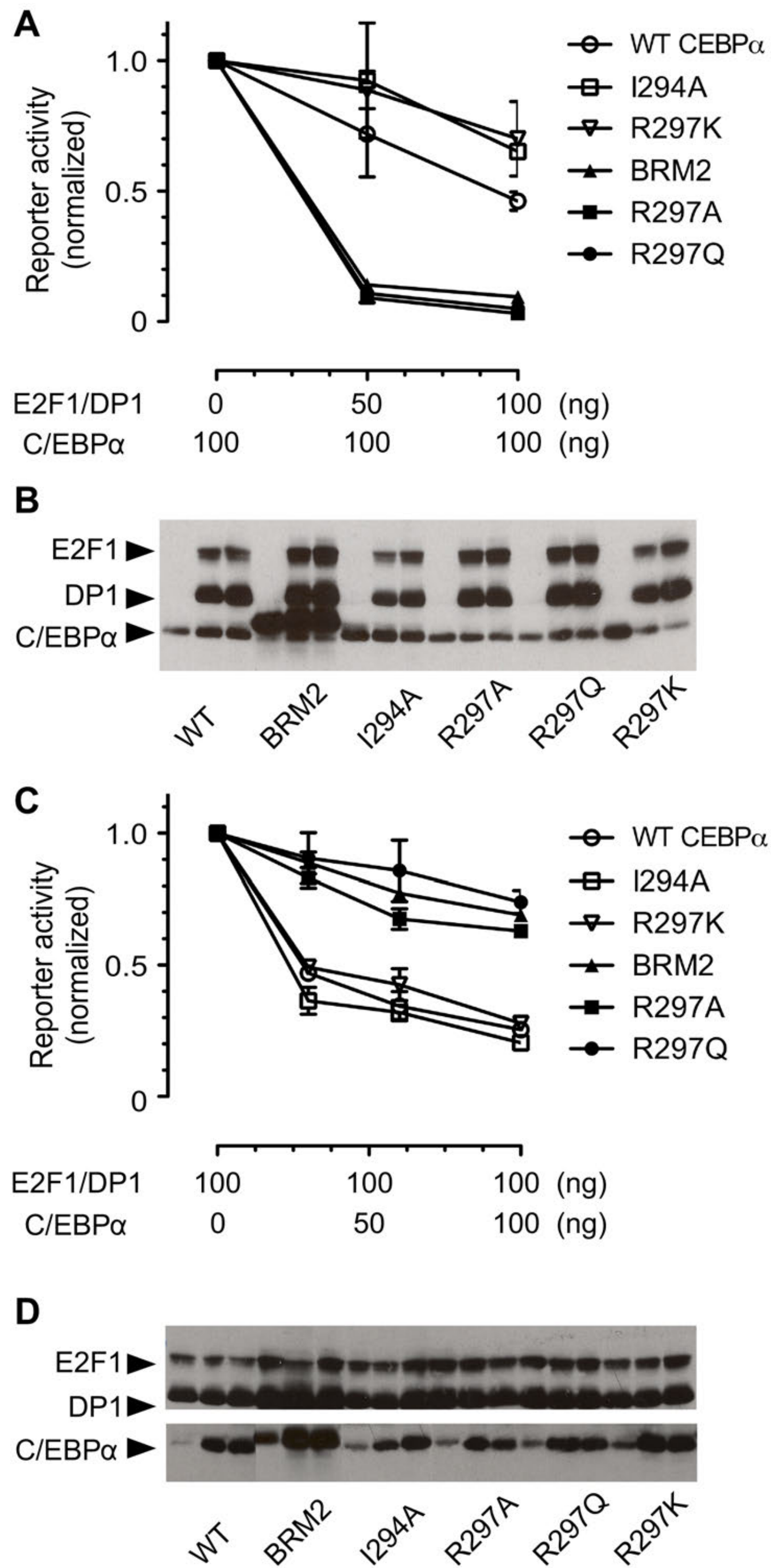

Figure 3. Kowenz-Leutz et al.

Figure

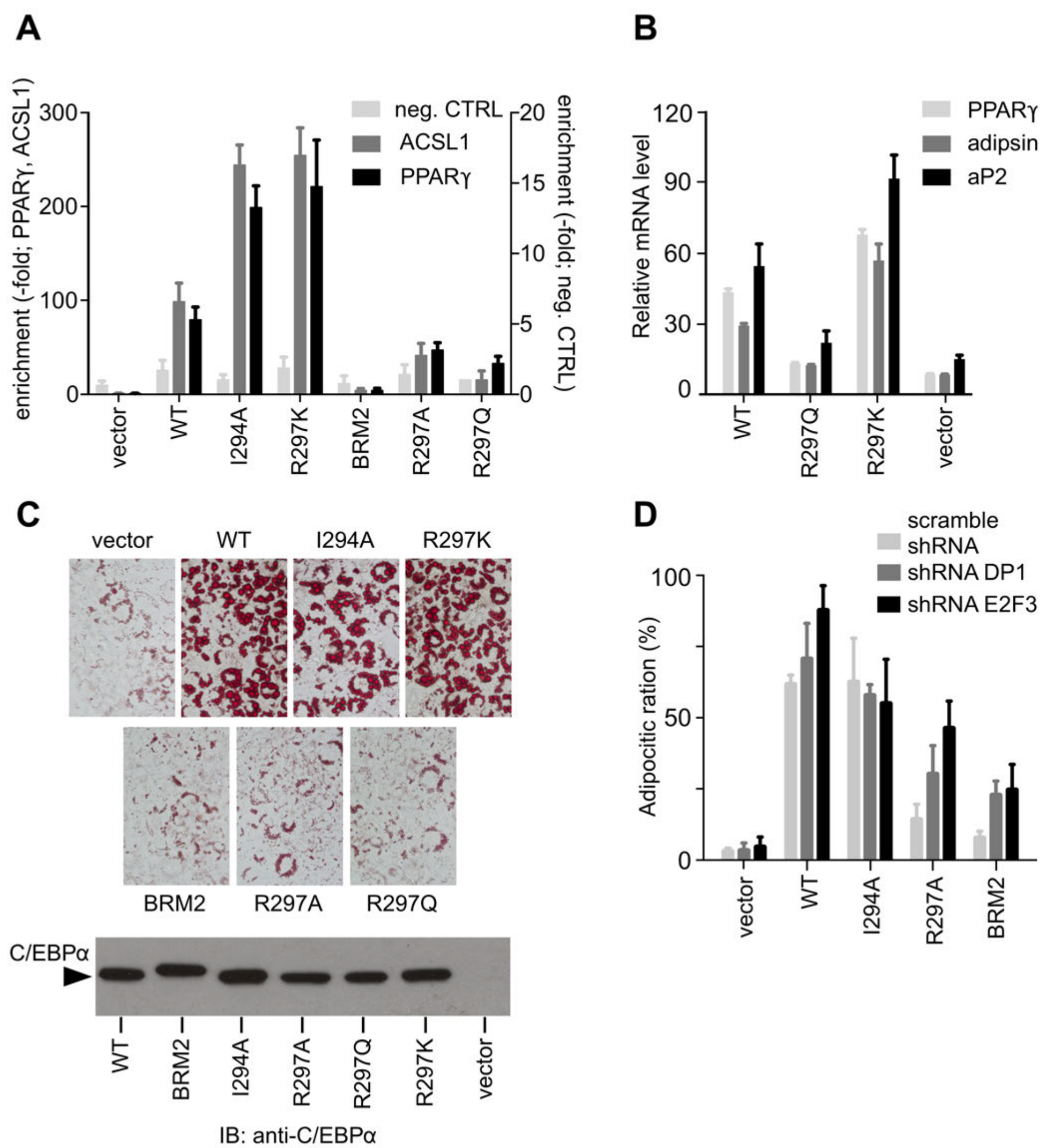

Figure 4. Kowenz-Leutz et al.