Molecular Mechanisms of Gene Activation and Gene Expression mediated by CCAAT/Enhancer Binding Proteins

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Abstract

The transcription factor CCAAT/Enhancer-Binding Protein α (C/EBPα) coordinates proliferation arrest and differentiation of myeloid progenitors and adipocytes. C/EBPα acts as a transcriptional activator of lineage specific genes and blocks the cell cycle by repressing transcription of E2F-regulated genes. Data presented here suggest that also inversely E2F interferes with the transcriptional activity of C/EBPα, counteracting C/EBPα-mediated differentiation processes. Thus, E2F-C/EBPα are part of a switch mechanism between proliferation and differentiation. The mechanism by which E2F suppresses C/EBPα-mediated transactivation is novel in several aspects. This is the first time that E2F has been shown to act as a co-repressor of another transcription factor. E2F represses C/EBPα without binding to cis-regulatory elements, but by direct protein-protein interactions that abolish the binding of C/EBPα to DNA. This mechanism of transcriptional repression occurs independent of pocket proteins. Disturbed DNA binding of C/EBPα is often observed in AML patients suggesting a causative role in granulocytic disorders. Thus, it would be of main interest to analyze whether E2F mediates disruption of C/EBPα's DNA-binding in AML patients and whether therapies directed against E2F could restore granulocytic maturation.

Despite the extensive knowledge of mechanisms involved in the inhibitory function of C/EBPα, it has not been addressed whether C/EBPα may impinge on cell proliferation by affecting the ribosomal biogenesis of a cell. This work demonstrates an association of C/EBPα to the RNA Pol I transcription factor UBF1, both proteins retained in large chromosomal foci. Similarities to other focal structures associated to UBF1, suggest that C/EBPα may repress transcription of Pol I-transcribed rRNA genes, and thus affect ribosomal biogenesis. The enrichment of C/EBPα at sites of UBF1 is induced by the histone methyltransferase SUV39H1. Thus, C/EBPα may not only control lineage commitment and cell proliferation by regulating genes transcribed by RNA Pol II, but also may act as a repressor of RNA Pol I mediated rRNA synthesis.

Keywords:

C/EBPα, E2F, Differentiation, SUV39H1

Zusammenfassung

Der Transkriptionsfaktor CCAAT/Enhancer-Binding Protein α (C/EBPα) koordiniert Proliferationshemmung und Differenzierung von myeloiden Vorläuferzellen und Adipozyten. C/EBPα funktioniert als ein transkriptioneller Aktivator von abstammungspezifischen Genen und blockiert den Zellzyklus durch Repression von proliferationsfördernden E2F Zielgenen. Die in dieser Arbeit gezeigten Daten zeigen, dass auch umgekehrt E2F die transkriptionelle Aktivität von C/EBPα verhindert und somit C/EBPα abhängigen Differenzierungsprozessen entgegenwirkt. Somit besitzen E2F-C/EBPα eine zentrale Schalterfunktion zwischen Proliferation und Differenzierung. Der Repressionsmechanismus durch E2F ist in mehreren Aspekten neuartig: Zum erstenmal wurde gezeigt, dass E2F einen anderen Transkriptionsfaktor reprimieren kann. E2F reprimiert die transkriptionelle Aktivität von C/EBPα ohne Bindung an cis-regulatorischen Elemente, sondern durch direkte Protein-Protein Interaktionen, die die Bindung von C/EBPα an DNA verhindern. Diese Form der transkriptionellen Repression geschieht unabhängig von ”Pocket-Proteinen”. Patienten mit Akuter Myeloiden Leukämie (AML) weisen häufig eine gestörte DNA Bindung von C/EBPα auf, welche ursachlich für granulozitären Funktionsstörungen sein könnte. Daher wäre es wichtig zu analysieren ob E2F die DNA Bindung von C/EBPα in AML Patienten beeinträchtigt und ob auf E2F gerichtete Therapien granulozitäre Reifung wiederherstellen.

Trotz der vielen Mechanismen der Blockierung von Zellproliferation durch C/EBPα, ist es bislang unbekannt ob C/EBPα die ribosomale Biogenese einer Zelle beinflussen kann. In dieser Arbeit wurde gezeigt, dass C/EBPα mit UBF1, dem Co-Aktivator der RNA Polymerase I, an chromosomalen Foci positioniert wird. Eine Ähnlichkeit zu anderen fokalen Strukturen suggeriert, dass C/EBPα die Transkription von Polymerase I regulierten rRNA Gene reprimieren und somit ribosomale Biogenese beeinträchtigen könnte. Die Assoziation zwischen C/EBPα und UBF1 wird durch die Histon-Methyltransferase SUV39H1 stimuliert. Demnach könnte die antiproliferative Funktion von C/EBPα nicht nur auf der Regulierung von RNA Pol II-abhängiger Transkription, sondern auch auf der Repression von RNA Pol I regulierter rRNA Synthese basieren.

Schlagwörter:

C/EBPα, E2F, Differenzierung, SUV39H1

Im Grunde genommen ist Wissenschaft wie jede gewöhnliche kriminellen Aktivität:
Sie bedarf einer guten Planung, Ausdauer und eines Quäntchens Glück!

Dominik Nagl

Contents

Chapter 1

Introduction

1.1 Transcription factors of the CCAAT/Enhancer Binding Protein Family

CCAAT/enhancer binding proteins (C/EBP) are a family of bZIP (basic-region-leucine-zipper) transcription factors regulating multiple cellular proliferation and differentiation processes. Up to date six C/EBP members have been characterized (C/EBPα-C/EBPζ). The C/EBP genes are intronless, except C/EBPϵ and C/EBPζ which contain 2 and 4 exons, respectively (reviewed in (Ramji & Foka, 2002)). C/EBPs have a conserved carboxy-terminal bZIP domain, with over 90% sequence identity between the different members (Figure 1.1A). The bZIP is composed of a basic-amino-acid-rich DNA-binding region, and a leucine-zipper region mediating dimerization of two C/EBP proteins (Figure 1.1B). Dimerization is required for DNA binding since C/EBPs bind to conserved DNA sites as homo- or heterodimers. Besides the different C/EBP members, the main members C/EBPα and C/EBPβ encode for several proteins, increasing the diversity of this protein family. By alternative use of translation initiation codons in the same mRNA molecule due to a leaky ribosome scanning mechanism, from one mRNA several proteins can be translated (Descombes & Schibler, 1991);(Ossipow et al., 1993). In the case of C/EBPα, its mRNA encodes for a full-length isoform p42 and an amino-terminal truncated p30 protein. The C/EBPβ mRNA generates three isoforms: LAP* (full-length), LAP (a 21 amino-acid truncation of the amino-terminus) and LIP (large amino-terminal truncation). The amino-termini of full-length C/EBPα and C/EBPβ proteins contain a transactivation domain. Due to the fact that this domain is missing in C/EBPβ LIP and almost completely lacking in C/EBPα p30, these isoforms fail to induce transcription. Accordingly, LIP and p30 are suggested to act as dominant negative proteins by forming non-functional heterodimers with full-length isoforms (Descombes & Schibler, 1991);(Ossipow et

al., 1993).

The C/EBP members differ in their expression profile. High expression levels of C/EBPβ are found in liver, intestine, lung, adipose tissue, spleen, kidney and myelomonocytic cells (reviewed in (Ramji & Foka, 2002)). C/EBPα is expressed in adipose tissue, placenta, liver, intestine, adrenal gland, lung and peripheral-blood mononuclear cells (Ramji & Foka, 2002). While C/EBPγ and C/EBPζ are expressed ubiquitously, expression of C/EBPϵ is limited to myeloid and lymphoid cells. C/EBPδ, in turn, is expressed in adipose tissue, intestine and lung (Ramji & Foka, 2002).

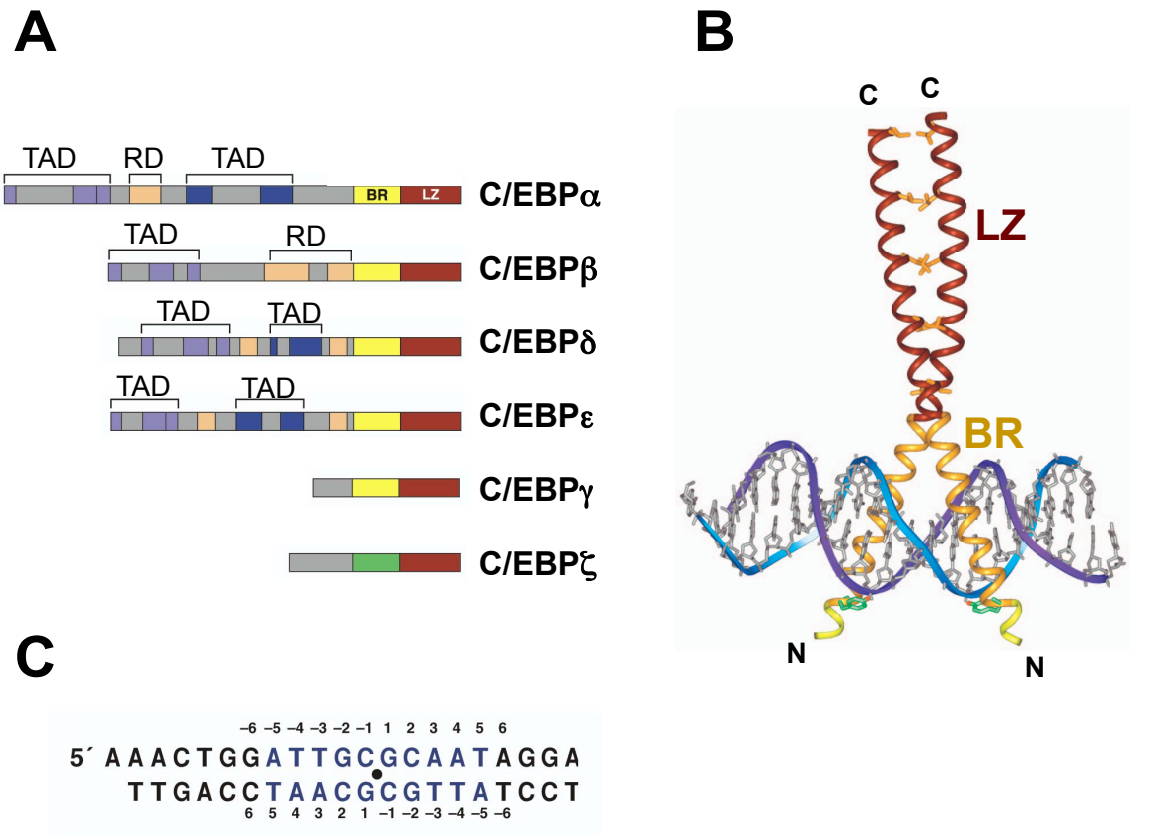

Figure 1.1: **The C/EBP family.** (A) Schematic representation of domains contained within the different C/EBP members. TAD = Transactivation domain; RD = regulatory domain; BR = basic region; LZ = leucine-zipper. Modified from (Johnson, 2005). (B) Crystal structure of the conserved bZIP region of a C/EBPα dimer bound to a consensus DNA site, modified from (Miller et al., 2003) (C) Sequence of the consensus C/EBP recognition element (blue), adapted from (Miller et al., 2003). A circle indicates the center of symmetry.

Specific physiological and pathophysiological situations change the expression levels of the different C/EBP members. One example is the sequential expression of C/EBPs during

differentiation processes. In vitro adipogenesis experiments have shown that C/EBPβ and C/EBPδ are induced during early differentiation phases, followed by expression of C/EBPα (Cao et al., 1991). Moreover, C/EBPα, C/EBPβ, C/EBPδ and C/EBPϵ are differentially expressed in myeloid cells. While C/EBPβ is up-regulated during macrophage differentiation (Natsuka et al., 1992), C/EBPϵ is more restricted to granulocytic differentiation (Yamanaka et al., 1997);(Morosetti et al., 1997). In turn, C/EBPα is up-regulated in early myeloid progenitors and its expression decreases as cells differentiate towards granulocytes (Scott et al., 1992). Finally, expression of C/EBPs is also affected upon an inflammatory response. Following inflammatory stimuli, the mRNA of C/EBPβ and C/EBPδ is induced in hepatocytes, macrophages, renal mesangial cells and astroglial cells, whereas C/EBPα expression is down-regulated in these cell types (reviewed in (Ramji & Foka, 2002)).

1.1.1 C/EBPα

C/EBPα is essential for glucose homeostasis as C/EBPα knock-out mice die perinatally from hypoglycemia. This hypoglycemia is due to defective induction of liver-specific enzymes required for glucose homeostasis, as for example glycogen synthase, phosphoenolpyruvate carboxy-kinase or glucose-6-phosphatase (N. D. Wang et al., 1995). The knock-out mice also showed that C/EBPα is essential for macrophages and granulocyte maturation, and for adipogenesis. Mature macrophages and macrophages progenitors are absent in fetal liver of C/EBPα -/- mice (Heath et al., 2004). In addition, C/EBPα deficient mice lack white adipose tissue (N. D. Wang et al., 1995), mature eosinophil and mature neutrophil granulocytes (Zhang et al., 1997). Accordingly, C/EBPα induces expression of both adipocytic and granulocytic genes (Table 1.1.1).

Gene name	**lineage**
peroxisome proliferator-activated receptorγ2 (*PPARγ2*)	adipocytic
apolipoprotein 2 (*AP2*)	adipocytic
neutrophil elastase	myeloid
myeloperoxidase	myeloid
granulocyte colony-stimulating receptor factor	myeloid

Table 1.1: C/EBPα controls adipogenesis and granulopoiesis by inducing lineage specific genes (Oelgeschlager et al., 1996);(Ford et al., 1996);(Smith et al., 1996).

1.1.2 C/EBPα and cell cycle arrest

The function of C/EBPα is not only limited to its transcriptional activity, but also possesses an antiproliferative capacity. To better understand the impact of C/EBPα on cell proliferation, a short introduction in cell cycle control will be given beforehand.

The eukaryotic cell cycle is divided into four distinct phases, G1, S, G2 and M. In the S- or synthesis-phase, the cell duplicates its DNA content, while during mitosis (M) the cell divides generating two daughter cells (Figure 1.2A). The duplication of protein mass and organelle DNA occurs during the intermediate phases, the S-phase preceding G1 (Gap 1) and the S-phase following G2 (Gap 2) phase (reviewed in (Sherr & Roberts, 1999)). S-phase entry and further cell cycle progression requires synthesis of proteins involved in DNA replication (e.g. dihydrofolate reductase) and of proteins regulating subsequent cell cycle events (e.g. cyclin A). These genes are induced by members of the E2F family of transcription factors in a cell cycle regulated manner. During G0 (quiescence) and early G1-phase, E2F is repressed by pRB, the retinoblastoma protein (Goodrich et al., 1991);(Chellappan et al., 1991);(Figure 1.2B). Upon mitotic growth signals, cyclin dependent kinase 4 (CDK4)/cyclinD and CDK2/cyclinE complexes are sequentially activated. These complexes phosphorylate pRB, which dissociates from E2F. Free E2F proteins, in turn, induce genes required for S-phase progression. Since the activation of CDKs conveys in cell cycle progression, it is not surprising that CDK function is tightly regulated by phosphorylation, by the synthesis of cyclins, by the degradation of both CDKs and cyclins, and by specific CDK inhibitors (Sherr & Roberts, 1999).

The antiproliferative activity of C/EBPα involves interaction with different cell cycle regulatory proteins (Figure 1.3A). Direct binding of C/EBPα to CDK inhibitor p21 has been proposed to stabilize p21 (Timchenko et al., 1996), causing an indirect repression of CDK2 and CDK4. In addition, C/EBPα can repress CDK2 and CDK4 by direct interaction, since C/EBPα disrupts the cyclin-CDK association, causing their inactivation (H. Wang et al., 2001). Furthermore, C/EBPα favors proteasomal degradation of CDK4 by enhancing the formation of CDK4-ubiquitin conjugates (H. Wang et al., 2002). The importance of these mechanisms, however, is questionable since C/EBPα blocks proliferation in p21-deficient mouse embryonic fibroblast (MEF) (C. Muller et al., 1999) and a knock-in strain of a C/EBPα mutant lacking the CDK-interacting region shows no apparent phenotype (Porse et al., 2006).

Another mode of cell cycle arrest by C/EBPα involves interaction with the SWI/SNF chromatin-remodeling complex. SWI/SNF complexes contain an ATPase/helicase activity inducing structural changes in chromatin by nucleosomal positioning either favoring or inhibiting the access of the basic transcriptional machinery to the DNA. In the case of C/EBPα,

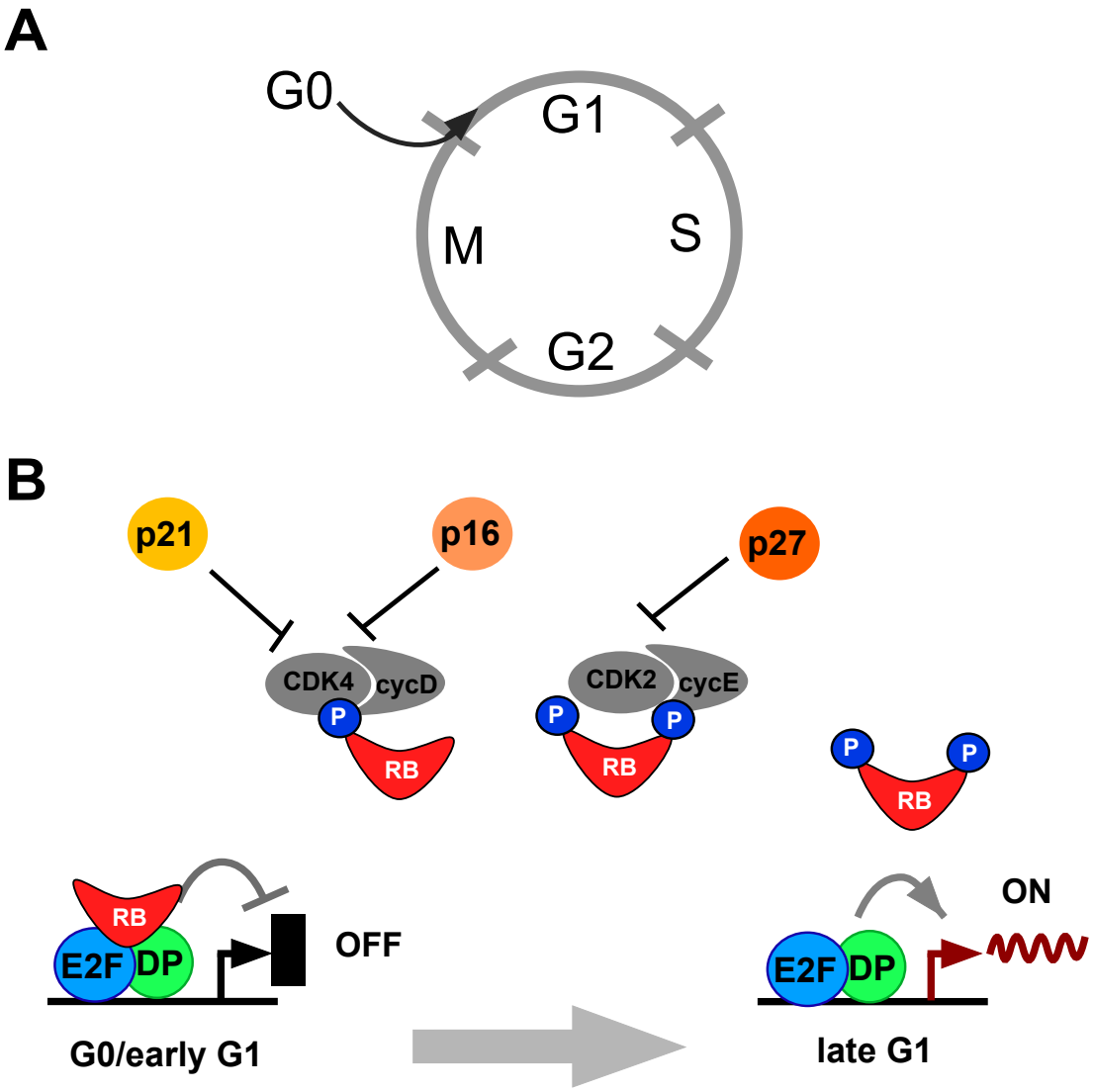

Figure 1.2: **Regulation of the cell cycle.** (A) The cell cycle is divided in G1, S, G2 and M-phase. Quiescent cells (G0) can reenter the cell cycle upon mitotic stimulation. (B) Control of cell cycle progression occurs during the G1/S-phase transition. In early G1, E2F binds to S-phase genes, but association with pRB maintains E2F repressed. The sequential activation of CDK4/cyclinD and CDK2/cyclinA complexes leads to sequential phosphorylation of pRB by these CDK-complexes. Phosphorylated pRB dissociates from E2F allowing the induction of E2F-regulated S-phase genes. Cyclin-dependent kinase inhibitors (CDKi) function as negative regulators of the cell cycle. Examples of CDKi are p21 and p16 which repress CDK4/cyclinD (Harper et al., 1993);(Serrano et al., 1993) and p27 which represses cyclin-E dependent kinases (Polyak et al., 1994).

SWI/SNF seems to favor proper induction of C/EBPα-target genes (Pedersen et al., 2001). The importance of this interaction is highlighted by the fact that abrogation of the C/EBPα-SWI/SNF binding disables C/EBPα to induce adipocytic genes. Furthermore, SWI/SNF is required for C/EBPα-induced cell cycle arrest, since in cells lacking a SWI/SNF subunit, C/EBPα fails to arrest proliferation (C. Muller et al., 2004).

Finally, C/EBPα-mediated proliferation arrest has been proposed to be a consequence of direct repression of E2F-regulated genes by C/EBPα. C/EBPα is recruited to E2F sites by direct interaction with E2F (Slomiany et al., 2000). Two mutants of the basic region of C/EBPα (Figure 1.3B), BRM2 and BRM5 have an impaired repression on E2F sites and fail to suppress cellular proliferation (Porse et al., 2001). In addition, BRM2 and BRM5 fail to promote granulocyte and adipocyte differentiation in vitro (D'Alo et al., 2003);(Porse et al., 2001). In agreement with the in vitro data, BRM2 and BRM5 knock-in mice strains lack white adipose tissue and are defective of mature neutrophils. Accordingly, repression of E2F through C/EBPα is thought to be essential for differentiation of both cell types. However, the transcriptional capacity and E2F binding of BRM2 and BRM5 is still debated (D'Alo et al., 2003);(Keeshan et al., 2003);(Miller et al., 2003).

Taken together, C/EBPα is a master regulator of differentiation that switches cells from an undifferentiated proliferative state to a cell cycle arrested and differentiated state, by blocking proliferation and inducing differentiation specific genes. These two C/EBPα-regulated functions can be uncoupled by the E7 oncoprotein of the "high-risk" human papilloma virus 16, which compromises C/EBPα-induced cell cycle arrest without affecting its transcriptional activity and its differentiation inducing activity (C. Muller et al., 1999).

1.1.3 Signaling to C/EBPα

A cell responds to its environment to control alternative cell fates such as cell division, quiescence or differentiation. Signaling pathways are essential for connecting the extracellular environment with the intracellular machinery. This is well-known for in vitro differentiation assays, were upon addition of specific growth factors or cytokines, intracellular cascades are activated which will lead to the induction of lineage specific genes and concomitant differentiation of the precursor cell. Cell proliferation also requires a communication to the exterior to ensure the availability of energy source to allow survival of daughter cells. A known signaling pathway stimulated by growth factors is the phosphatidylinositol 3-kinase (PI3K)-Akt pathway. Growth factors, which in cell culture are supplied by addition of serum, stimulate proliferation. Conversely, under serum deprivation, cells enter quiescence.

As most cell multiplication affecting mechanisms, the antiproliferative role of C/EBPα is

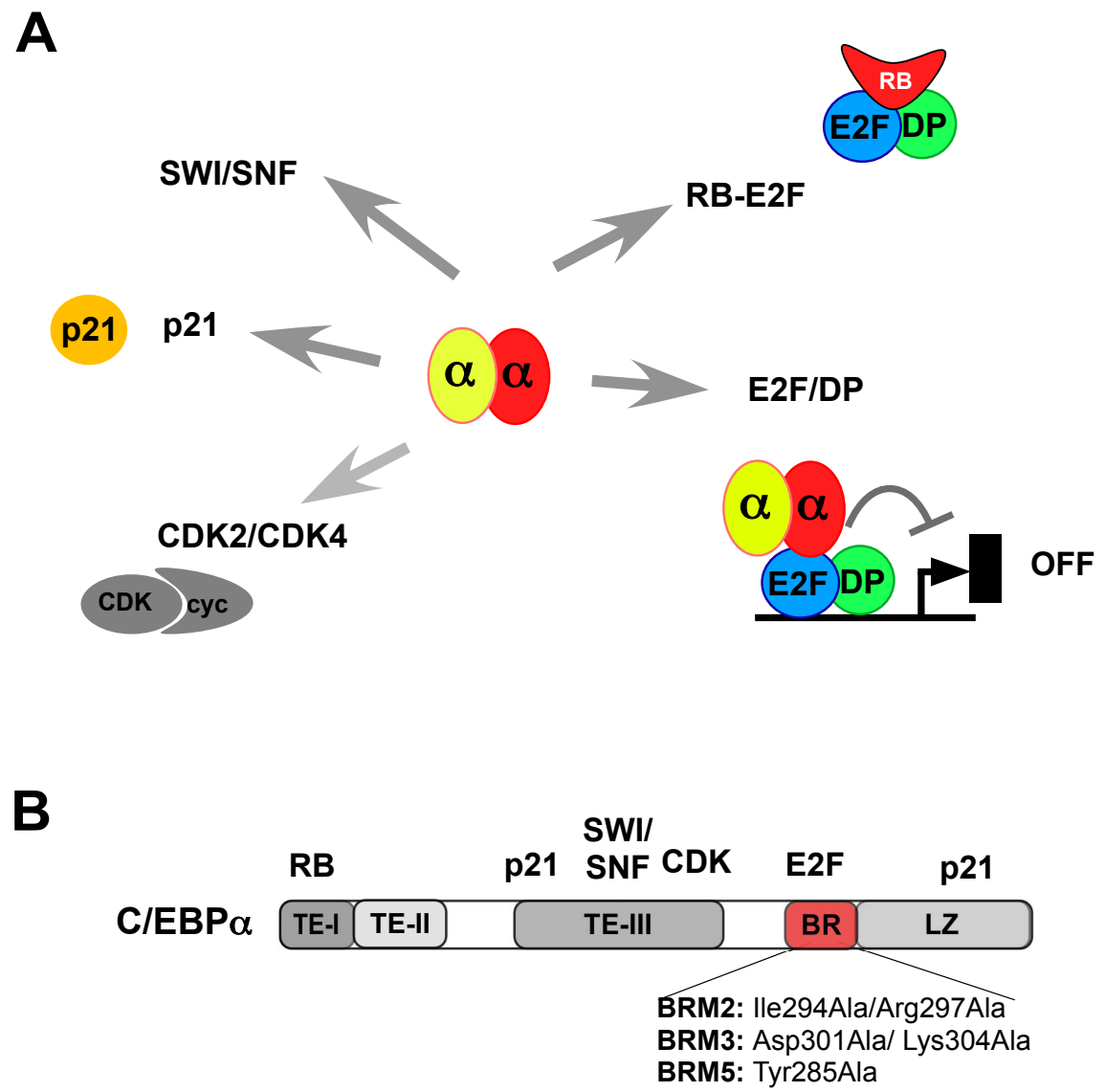

Figure 1.3: **C/EBPα and cell cycle arrest.** (A) C/EBPα interacts with the cell cycle regulatory machinery at different levels (see text for details). (B) Schematic representation of functional domains within C/EBPα and their interaction with cell cycle regulators. The transactivation elements (TE) interact with the transcription apparatus (TBP/TFIIB and CBP/p300). The leucine-zipper (LZ) is required for dimerization with other C/EBP molecules, while DNA-binding is mediated through the basic-region (BR). Point mutation within the BR can abolish the ability to repress E2F and to arrest cell cycle, as is the case for BRM2 and BRM5, but not for BRM3. The amino acids substitutions corresponding to these mutants are noted below.

regulated by signaling pathways. Activation of the PI3K-Akt pathway has been reported to convey in the dephosphorylation of C/EBPα at Ser193, which causes the inactivation of the antiproliferative activity of C/EBPα, without affecting its transcriptional activity (G. L. Wang et al., 2004). This regulatory pathway has been suggested to inactivate C/EBPα-mediated proliferation arrest in liver tumors and after partial hepatectomy, where hepatocytes proliferate despite high levels of C/EBPα.

Phosphorylation of C/EBPα also regulates its granulocytic function. Ras-signaling induced phosphorylation of Ser248 stimulates granulocytic differentiation (Behre et al., 2002). In contrast, phosphorylation of Ser21 on C/EBPα by extracellular signal regulated kinases 1 and 2 (ERK1/2), results in inhibition of its in vitro granulopoiesis potential (Ross et al., 2004).

1.2 Transcription factors of the E2F Family

The E2F family of transcription factors regulates cell cycle entry, progression and exit. Among the E2F target genes are found genes encoding enzymes involved in nucleotide biosynthesis (dihydrofolate reductase and thymidine kinase), genes encoding proteins of the DNA-replication machinery (such as Cdc6 and ORC1) and genes encoding cell cycle regulators such as cyclin A, Cdc25A or E2F1 (reviewed in (Bracken et al., 2004);(Attwooll et al., 2004)).

On the basis of sequence homology, the E2F family can be grouped into E2F and DP proteins. DP is the dimerization partner of E2F. Formation of E2F-DP heterodimers is essential for high affinity binding to cis regulatory sites in promoter regions and, thus, for efficient transcriptional activation.

To date three DP members and eight genes encoding for E2Fs (E2F1-E2F8) have been identified (Figure 1.4A). Additional isoforms arise from transcription of the E2F3 gene using alternative promoters, giving rise to two proteins, E2F3a and E2F3b. The well-characterized DP1 and DP2 proteins share high homology in their dimerization domain, mediating binding to E2Fs, and in their DNA-binding domain. The recently identified DP3 protein also possesses sequences homologous to the dimerization- and DNA-binding domain homologous sequences. However, while DP3 can dimerize with E2F, the DNA-binding domain is not functional. The resulting E2F-DP3 heterodimers fail to interact with E2F sites, causing inhibition of E2F-mediated transcription (Qiao et al., 2007).

E2F1-E2F6 harbor dimerization domains that mediate binding to DPs. This domain is absent in E2F7 and E2F8, which in turn posses two distinct DNA-binding domains mimicking E2F-DP heterodimers. The other E2F members only have one DNA-binding domain. In

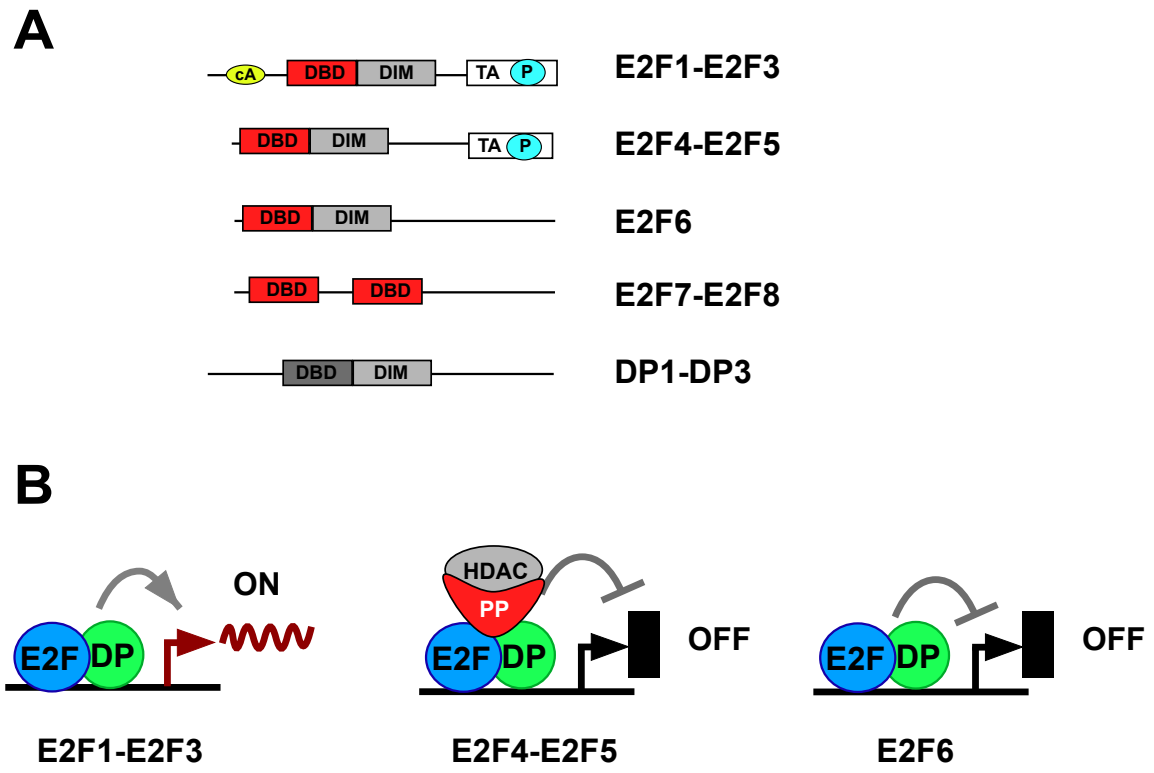

Figure 1.4: **The E2F family.** (A) Schematic representation of E2F members and their domains, modified from (Bracken et al., 2004). (cA = cyclinA binding domain, DBD = DNA binding domain, TA = transactivation domain, P = pocket protein interaction domain). (B) Functional differences between E2F members and their effects on targets genes. Left: E2F1-E2F3 activate E2F-targets genes in late G1-phase. Middle: E2F4-E2F5 bind to E2F sites in association with repressive complexes (PP = pocket proteins, HDAC = histone deacetylase). Right: An intact DNA-binding and dimerization domain allows E2F6 binding to E2F sites, however, lack of the transactivation domain leads to a repressive effect.

contrast to DP proteins, some E2F members (E2F1-E2F5) contain a transactivation domain required for transcriptional activity. Since this domain is absent in E2F6, E2F7 and E2F8, they are considered as transcriptional repressors (Figure 1.4B). Finally, a cyclin-A-binding domain (cA) has been described, which is only present in E2F1-E2F3 (Bracken et al., 2004).

E2Fs are the main controllers of the G1 to S-phase transition. The timely control of E2F-regulated genes involved in cell division occurs by changes in the association of E2F with its negative regulator pRB. Repression of E2F-controlled genes by pRB occurs through two mechanisms. pRB masks the E2F transactivation domain abrogating the recruitment of transcriptional machinery to the promoter. Moreover, pRB represses E2F-target genes by recruitment of repressive complexes which cause local structural changes in the chromatin. These repressive complexes are SWI/SNF chromatin remodelers (Strobeck et al., 2000) and histone modifiers, which will be explained in more detail in section 1.4.

pRB belongs to a family named pocket proteins, composed of pRB, p107 and p130. The name pocket proteins arises form their conserved carboxy-terminal domain, which mediates the binding to viral oncoproteins (e.g. adenovirus E1A) and to many cellular proteins (e.g. E2F1). While E2F1-E2F3 preferentially bind to pRB, E2F5 binds mainly to p130. E2F4 can bind to all three pocket proteins, although it is mainly regulated by p107 and p130. The domain required for interaction with pocket proteins is embedded inside the E2F transactivation domain. Since E2F6 and E2F7 lack a transactivation domain, accordingly, they do not interact with the pocket proteins.

E2Fs have different cellular localization depending on localization signals, protein interactions and post-translational modification. E2F1-E2F3 have a nuclear localization signal (NLS) embedded in the cyclinA-binding domain, which determines the constitutive nuclear localization of these E2F members. In contrast, E2F4-E2F5 have a nuclear export signal (NES) and are mostly cytoplasmic. Differences in localization are also observed between DP members. While DP2, harboring a NLS, is nuclear, both DP1 and DP3 are cytoplasmic. However, DP1 and DP3 localize to the nucleus by dimerization with those E2Fs that have a NLS (e.g. E2F1-DP1 heterodimers). Accordingly, E2F4-E2F5 have a nuclear distribution when complexed to DP2. Nuclear localization of E2F4-E2F5 can also be induced upon specific post-translational modifications or by complexing with pRB family members harboring a NLS (Magae et al., 1996);(Lindeman et al., 1997);(H. Muller et al., 1997);(Verona et al., 1997). E2F4-E2F5 binding to pocket proteins translocate into the nucleus, but then function as transcriptional repressors. In contrast, E2F1-E2F3, which localize in the nucleus independently of pocket protein association, are activators of transcription. The occupancy of S-phase genes by either E2F activators or repressors determines cell cycle progression.

In G0 and G1, E2F repressor complexes occupy genes required for S-phase progression. As cells enter late G1, E2F4-E2F5 are exported from the nucleus and activator E2F1-E2F3 occupy and activate the promoters of S-phase genes. The consequent induction of these genes leads to cell cycle progression (Figure 1.4). Accordingly, E2F1-E2F3 triple knock-out MEFs have an impaired proliferation (L. Wu et al., 2001), while E2F4/E2F5 are dispensable for proliferation (Gaubatz et al., 2000). Furthermore, E2F4/E2F5 double deficient MEFs fail to arrest G1 in response to the CDK inhibitor p16INK4a, indicative for a role of E2F4/E2F5 in pocket-protein-mediated G1 arrest (Gaubatz et al., 2000). The function of the different DP members during proliferation is still unclear. Loss of DP1 results in embryonic lethality, due to the failure of extraembryonic lineages to replicate DNA properly (Kohn et al., 2003). However, in a mouse model circumventing the placenta defect, DP1 is dispensable for cell proliferation (Kohn et al., 2004). Compound DP1-DP2 knock-out mice would solve the question whether DP2 compensates for loss of DP1.

Apart from regulating S-phase genes, E2F also regulates G2-specific genes, as for example cdc2 and cyclin B1 (W. Zhu et al., 2004), and genes involved in the G2/M checkpoint (Ren et al., 2002). Finally, E2F is in a negative feedback loop by inducing genes encoding for proteins that repress E2Fs transcriptional activity, as for example pRB (Shan et al., 1994), p107 (Ishida et al., 2001) or p21 (Hiyama et al., 1997).

1.2.1 Functions of E2Fs

Apart from its role in tuning the cell cycle, several other functions of E2F have been discovered. Among E2F targets, key DNA damage genes are found, both involved in DNA damage checkpoint (CHK1, TP53, ATM, BRCA1 and BRCA2) and in DNA damage repair (RAD51, RAD54, MLH1) (Ren et al., 2002);(Polager et al., 2002). This is indicative for a role of E2F in controlling the response to DNA damage. Another important function of E2F is the control of apoptosis. E2F1 induces apoptotic genes, including CASP3, CASP7 and TP73 (H. Muller et al., 2001);(Irwin et al., 2000). Accordingly, overexpression of E2F1 induces apoptosis (Ginsberg, 2002), while E2F1 -/- mice have a defective thymocyte apoptosis (Field et al., 1996).

Both E2F4 and E2F5 are involved in the control of differentiation. Loss of E2F4 results in abnormal development of various cellular lineages and growth retardation (Rempel et al., 2000). E2F4 plays an important role in erythroid maturation, since E2F4-/- mice have fetal macrocytic anemia (Humbert et al., 2000). Mice deficient for E2F5 develop hydrocephalus, indicating a role for E2F5 in brain development (Lindeman et al., 1998). Simultaneous inactivation of E2F4 and E2F5 results in neonatal lethality (Gaubatz et al., 2000), showing their

essential and overlapping role in late mouse development.

E2Fs have also been proposed to play a role in adipogenesis. E2F4 has a negative impact on adipogenesis, since E2F4 -/- MEFs have an increased propensity to undergo adipogenesis upon hormonal stimulation (Fajas et al., 2002). In contrast, E2F1 favors adipogenesis, since E2F1 deficient cells have a reduced ability to form adipocytes (Fajas et al., 2002). These studies suggested that E2F1 regulates the clonal expansion phase, in which cells undergo several rounds of division. After conclusion of clonal expansion, cells arrest and undergo terminal differentiation. Paradoxically, ectopic expression of E2F1 blocks adipogenesis (Porse et al., 2001).

Finally, E2F also regulates genes required for embryonic development. Mammalian development is controlled by the correct spatial expression of homeobox genes (Hox). These in turn are controlled by polycomb group (PcG) and trithorax group proteins (Lohuizen, 1998);(Deschamps et al., 1999);(Orlando, 2003). Both Hox and PcG genes are transcriptional targets of E2F1-E2F3 (H. Muller et al., 2001);(Stanelle et al., 2002);(A. P. Young et al., 2003). Accordingly, loss of E2F1 and E2F3 results in developmental defects such as growth retardation and testicular atrophy (Cloud et al., 2002). Another connection between E2Fs and PcG proteins exists at a protein-protein interaction level, since E2F6 forms complexes with them (Trimarchi et al., 2001), suggesting that the transcriptional repressive action of E2F6 involves recruitment of PcG proteins. Furthermore, E2F6 deficient mice have transformations of the axial skeleton, which are remarkably similar to those observed in mice lacking several different PcG proteins (Storre et al., 2002).

1.3 E2F and C/EBPα in diseases

The first tumor suppressor identified in humans was pRB, which was found mutated in retinoblastoma (Knudson, 1971). pRB is absent or mutated in one third of all human tumors (Weinberg, 1992). Significantly, almost all tumors that conserve a functional pRB, carry mutations in other regulators of the RB pathway as for example activating mutations in the cyclin D1 or CDK4 genes (Bartek et al., 1996) or inactivating mutations of the CDK4 inhibitor p16 (Sherr, 1996). Thus, the essential step during tumorigenesis involves mutations in components of the RB pathway, which will cause deregulation of E2F activity. Another deregulation of the RB/pathway involves binding and sequestering of pocket proteins by viral proteins, such as adenovirus E1A or human papilloma virus protein E7, resulting in derepression of E2F. Furthermore, deregulated E2F activity often correlates with poor prognosis (Gorgoulis et al., 2002);(Nevins, 2001);(Ebihara et al., 2004). Amplifications of E2F3 in urinary bladder

cancer are postulated to have a causative role (Oeggerli et al., 2004). However, so far no mutations in the E2F coding sequence have been described in human tumors.

The connection between E2Fs and cancer is better visualized when looking at the different mouse models of E2F genes. For instance, E2F1 deficient mice have a cancer predisposition (Yamasaki, 1999). Paradoxically, overexpression of E2F1 in transgenic mice can both promote or suppress tumorigenesis (Pierce, Fisher, et al., 1998);(Pierce, Gimenez-Conti, et al., 1998);(Conner et al., 2000). This hints towards a role for E2Fs as either oncogenes or tumor suppressors. In agreement with this idea, loss of E2F3 suppresses the formation of some pRB deficient tumors (pituitary tumors) while accelerating the progression of thyroid carcinomas (Ziebold et al., 2003). A possible explanation for these dual and opposite roles of E2F in tumorigenesis can be explained by the fact that E2F both accelerates proliferation and induces apoptosis.

C/EBPα is also known to be deregulated in several human tumor types. The strong antiproliferative effect of C/EBPα together with its role in controlling the differentiation of the granulocytic lineage are in accordance with dysfunction of C/EBPα in granulocytic leukemia. 9% of patients with acute myeloid leukemia (AML) carry mutations in the CEBPα gene (20% of the M2 subtype) (Nerlov, 2004). In addition, C/EBPα is down-regulated by oncogenic fusion proteins both at translational level (by BCR-ABL (Perrotti et al., 2002)) and transcriptional level (by AML-ETO (Pabst et al., 2001)). Many patients with AML carry mutations in both C/EBPα alleles. In one allele a frame-shift abrogates the expression of full-length p42 isoforms, thus, only the truncated p30 isoform is expressed. Mice expressing only the p30 isoform develop AML with complete penetrance (Kohn et al., 2004), demonstrating the AML-initiating role of mutations that abrogate expression of p42. The second allele of AML patients usually contains mutations in the basic region-leucine zipper. Mouse strains containing point mutations in the basic region of C/EBPα, known as BRM2 mutation (section 1.1.2. and Figure 1.3B), develop AML-like transformation of the granulocytic lineage. This mutation confers a predisposition for myeloproliferative granulocytic disorder and for transformation of the myeloid compartment of the bone marrow (Porse et al., 2005).

Decreased expression of C/EBPα has also been reported in several human epithelial tumors (reviewed in (Schuster & Porse, 2006)), as for example in lung cancer (Halmos et al., 2002) and in breast cancer (Gery et al., 2005). Based on a mouse model, a protective role of C/EBPα against skin tumorigenesis has been attributed (Loomis et al., 2007). Epidermal-specific C/EBPα knock-out mice were subjected to a DMBA/TPA two-stage carcinogenesis protocol, which results in the production of squamous papillomas. The majority of papillomas induced by this treatment contain an oncogenic mutation of Ras (Quintanilla et al., 1986).

In the absence of C/EBPα, chemical induced papillomas have an increased tumor growth rate and an increased rate of malignant progression (Loomis et al., 2007). Interestingly, the authors also showed that C/EBPα inhibits Ras-induced E2F transcriptional activity. Although only correlative, the data suggests that a failed repression of E2F could cause skin carcinogenesis. In agreement with this hypothesis, in a transgenic mouse model, overexpression of E2F1 or DP1 results in an enhanced Ras-induced skin carcinogenesis (D. Wang et al., 2001).

1.4 The Methyltransferase SUV39H1

1.4.1 Histone modifications and transcriptional control

The genetic material is organized by the structural units of chromatin, the nucleosomes (Figure 1.5A). A nucleosome is composed of 146 bp of DNA wrapped 1.5 times around an histone octamer, composed of two H2A/H2B dimers and an H3/H4 tetramer (Libertini et al., 1988). A linker histone, H1, connects nucleosomes, causing further compaction and formation of higher order chromatin. The highly basic amino-terminal histone tails protrude from the nucleosome core and are post-translational modified. The first modification described on histones was acetylation, which causes the repulsion of neighboring nucleosomes and, consequently, chromatin decondensation. A balance between acetylation and deacetylation, determined by histone acetyltransferases (HATs) and histone deacetylases (HDACs), regulates the level of chromatin condensation. Over the past years, additional histone modifications have been discovered such as phosphorylation, ubiquitylation, sumoylation and methylation. The dynamic, sequential and universal nature of these modifications has lead to the proposal of the 'histone code' hypothesis (Strahl & Allis, 2000). This hypothesis states that modifications are interdependent. The conjunction of modifications is read by other proteins as a code, to bring about distinct downstream events, mediating cellular responses such as transcriptional regulation, mitotic chromosomal condensation, and heterochromatin formation. Heterochromatin consists of genomic areas with highly dense packed chromatin fibers maintained throughout the interphase. This limits the access of transcriptional complexes of high molecular weight, therefore being transcriptionally inactive regions. Various functions have been attributed to heterochromatin including chromosome segregation, nuclear organization and gene silencing (Henikoff, 2000);(Jenuwein, 2001). Constitutive heterochromatin encompasses large gene poor regions found near the centromeres and telomeres of mammalian chromosomes that remain highly condensed throughout the cell cycle. In contrast, euchromatin, are less condensed areas of potentially transcriptional active genes located

along the gene rich chromosome arms. Structural changes from euchromatin to heterochromatin involve changes in the nucleosome compaction regulated by histone modifications (Figure 1.5A).

1.4.2 SET-domain methyltransferase SUV39H

Methylation has been found in a variety of histone lysine residues, including Lysine 4 (K4), K9, K27, K36 and K79 of histone H3, K20 in histone H4 and K26 of histone H1B. The methylation of these residues is catalyzed, with one exception, by SET-domain protein methyltransferases (Jenuwein, 2001). SET stands for Su(var)3-9, Enhancer-of-zeste, Trithorax and harbors the catalytic domain of those methyltransferases. The first SET-domain lysine methyltransferase to be characterized was SUV39H (Rea et al., 2000). SUV39H belongs to the Su(var) (suppressor of variegation) gene loci group, encoding for proteins such as histone deacetylase and heterochromatin associated proteins (Wustmann et al., 1989). The homologues of Drosophila Su(var)3-9 (Aagaard et al., 1999), are encoded by two loci in humans (SUV39H1 and SUV39H2) and in mice (Suv39h1 and Suv39h2) (O'Carroll et al., 2000). It has been shown that SUV39H1 specifically methylates K9 of histone 3 (H3K9). This methylation is regulated as postulated by the histone code hypothesis, since acetylation of histone H3 (H3K9ac) or phosphorylation of serine 10 (H3S10ph) via the Ipl1/aurora kinase blocks the methylation of K9 by SUV39H1 (Rea et al., 2000).

The main epigenetic function of SUV39H1 is the establishment of heterochromatinization. It has been proposed that trimethylation of H3K9 by Suv39H1 generates a binding site for heterochromatin protein-1 (HP1) (Bannister et al., 2001);(Lachner et al., 2001), leading to the propagation of stable heterchromatin and causing a local gene silencing (Figure 1.5B). Accordingly, Suv39h1/Suv39h2 double deficient mice have a reduced formation of pericentric heterochromatin. Furthermore, mouse embryonic fibroblasts (MEFs) isolated from these mice have an increased propensity to aberrant nuclear morphologies, indicating a role for Suv39h in chromosomal stability. The importance of Suv39h in the maintenance of stable heterochromatin is further highlighted by the enhanced tumor risk of these mice, associated with an increased genomic instability. Furthermore, male Suv39h deficient mice display a spermatogenic failure, probably a consequence of improper chromosomal interaction during male meiosis (Peters et al., 2001).

SUV39H-dependent H3K9 trimethylation accumulates at repetitive DNA elements, such as major satellite repeats (e.g. pericentric heterochromatin), LINEs and IAPLTRs (Martens et al., 2005). Genes which have been found to be transcriptional suppressed by SUV39H1-induced methylation are E2F-responsive S-phase genes, as for example cyclinA and PCNA

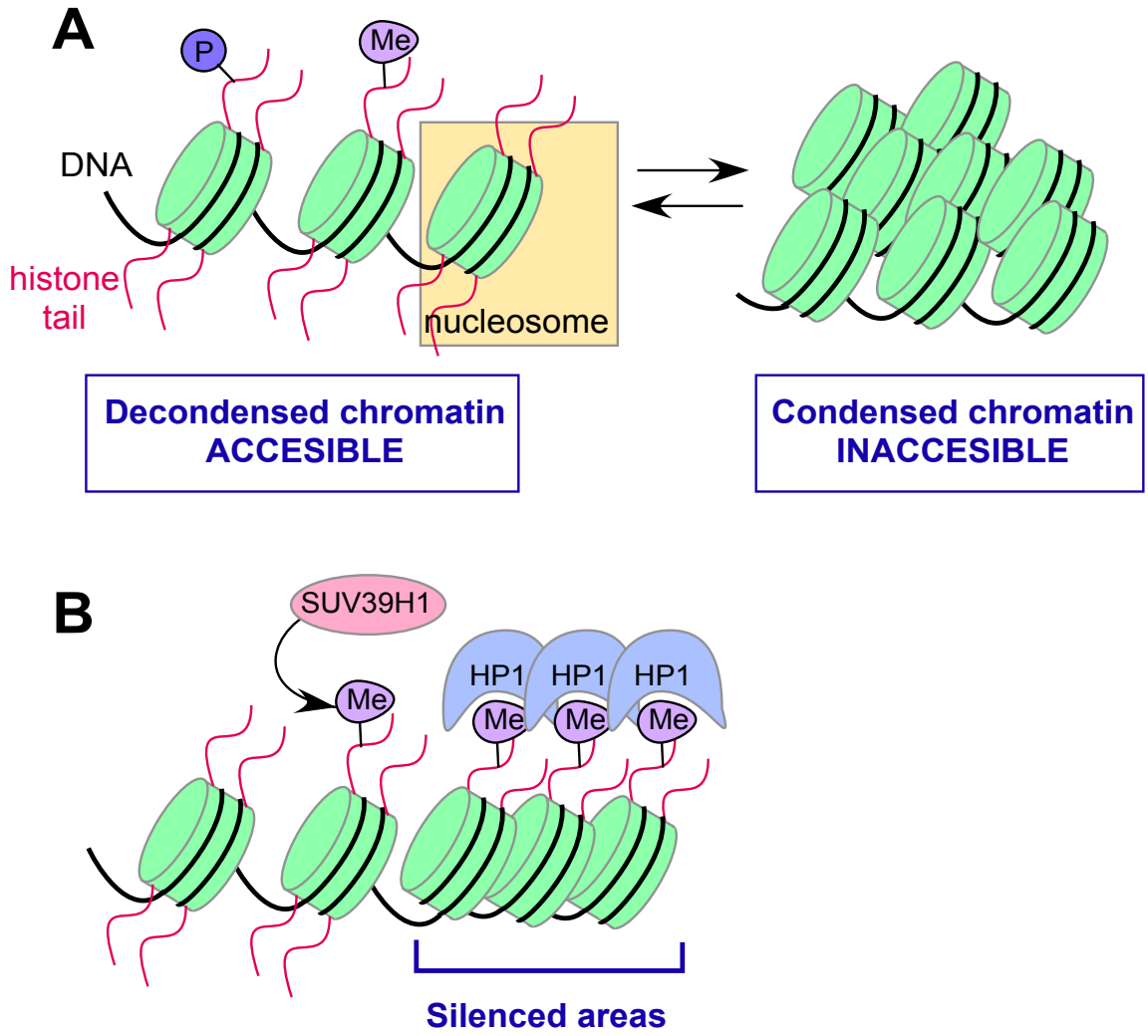

Figure 1.5: **Chromatin compaction, nucleosomes and histone modifications.** (A) Nucleosomes are composed of DNA wrapped around an histone octamer. Post-translational modifications of the protruding histone tails control chromatin condensation, regulating the access of the transcriptional machinery. (B) Methylation of H3K9 by SUV39H1 creates a binding platform for HP1, leading to local gene silencing. Adapted from (Nakayama et al., 2001) and (Sparmann & Lohuizen, 2006)

(Narita et al., 2003). Thus, E2F-regulated genes are a common target of SUV39H1 and C/EBPα (Johansen et al., 2001). SUV39H1 is one of the repressive histone modifiers known to be recruited by pRB to E2F target genes (Nielsen et al., 2001). The subsequent HP1 recruitment at and silencing of these E2F-target genes leads to a permanent cell cycle arrest, also known as senescence. The hallmarks of senescence will be described at continuation.

1.4.3 Senescence and lymphomagenesis

Primary fibroblasts have a limited proliferative capacity. Upon exhaustion of this capacity by long-term culturing, they undergo a stable form of cell cycle arrest, senescence (Hayflick, 1965), that in contrast to quiescence cannot be reversed. In addition to an arrest in G1, senescent cells display an upregulated senescence-associated β-galactosidase (SA-β-gal) activity (Campisi, 2001) and form senescence associated heterochromatic foci (SAHF) (Narita et al., 2003). Similar to apoptosis, senescence is a cellular response to stress that limits the proliferation of damaged cells (Campisi, 2001), thereby acting as protection against tumor progression. Accordingly, senescence can be induced by DNA damage, oxidative stress, or oncogenic signals, as for example oncogenic Ras (RasV12). Upon ectopic RasV12 expression, primary fibroblast irreversibly arrest in G1, by induction of the p16-INK4a-RB and the p19-ARF-p53 tumor suppressor pathways (Serrano et al., 1997). Furthermore, H3K9 trimethylation and an intense grainy HP1 staining becomes apparent, while SA-β-gal activity is induced (Braig et al., 2005).

Cells lacking Suv39h1 fail to show all these characteristic Ras-induced effects (Braig et al., 2005). Therefore, Suv39h1 is considered a key player in Ras-induced senescence. In agreement with the observation that cells escaping Ras-induced senescence show a transformed phenotype, loss of Suv39h1 promotes Ras-driven lymphomagenesis.

Remarkably, a role in Ras-induced senescence has also been proposed for C/EBPβ, since C/EBPβ-/- MEFs fail to senescence upon Ras overexpression (Sebastian et al., 2005). The common features of Suv39h1 and C/EBP suggest their integration in the same pathways. Accordingly, Ras-mediated induction of senescence by either SUV39H1 or C/EBP occurs through repression of E2F. Furthermore, C/EBPα protects from Ras-induced skin tumorigenesis, where the senescent failsafe mechanism is abrogated by chemical-induced mutations of the Ras-pathway (Loomis et al., 2007). Thus, both Suv39h1 and C/EBPα are key players counteracting the oncogenic effect of Ras.

Altogether these studies suggest that the ultimate target of Ras is E2F. Repression of E2F-regulated genes leads to senescence, while failed repression accounts for oncogenic transformation. Downstream of Ras are SUV39H1 and C/EBPα, which both repress E2F.

Given the findings that SUV39H1 represses E2F genes through heterochromatinization and that C/EBPα can associate at heterochromatic foci, it could be envisaged that repression of E2F may involve either sequentially or simultaneous action of SUV39H1, HP1 and C/EBPα at E2F target genes.

Chapter 2

Materials and methods

2.1 General equipment

- Avanti Centrifuge J-25 (Beckman)
- Centrifuge 5417R (Eppendorf)
- BioPhotometer (Eppendorf)
- Odyssey Scanner (Li-Cor)
- Electrophoresis Power Supply (Gibco)
- Agarose Electrophoresis Chamber (Roth)
- Mastercycler Gradient (Eppendorf)
- Thermomixer Compact (Eppendorf)
- GelDoc 2000 (Biorad)
- Protein Electrophoresis Chamber (Biorad)
- Transfer Chamber (Biorad)
- Nitrocellulose Transfer Membrane (Schleicher & Schuell)
- Nitrocellulose Filter (Schleicher & Schuell)
- Whatman Paper (Schleicher & Schuell)
- X-Omat AR Film (Kodak)

2.2 Working with DNA

2.2.1 Reagents and solutions

50 x TAE

4 M Tris-HCl

50 mM EDTA pH = 8.0

5.7% acetic acid

For 1 x TAE, dilute the 50 x stock solution with deionized water

Restriction endonucleases

All restriction endonucleases used were purchased from Roche.

DNA-Marker (Roth)

DNA loading dye (6 x)

15% (w/v) Ficoll 400

40 mM EDTA

0.1% (w/v) Bromophenolblue

0.1% (w/v) Xylene cyanol FF

Ampicillin-stock (1000 x)

1% (w/v) Ampicillin (Sigma-Aldrich)

Solve in deionized water.

Filter through 0.2μm nitrocellulose filter.

Store at -20°C.

Zeocin (InvivoGen)

Supplied as 1 mg/ml solution, store at -20°C.

Use as 4000 x stock for bacterial growth selection.

Add Zeocin prior use, protect from light.

X-Gal (Roth)

40 mg/ml solved in N,N-dimethylformamide

Store at -20°C.

IPTG (Roche)

Solve as 1 M solution in deionized water.

Store at -20°C.

LB-medium (Luria Bertani medium)

10 g/l bacto tryptone (DIFCO)

5 g/l bacto yeast extract (BP)

5 g/l NaCl

Dissolve in deionized water, autoclave and store at room temperature.

When containing antibiotics, store at 4°C

LB-agar plates

10 g/l bacto tryptone (DIFCO)

5 g/l bacto yeast extract

5 g/l NaCl

15 g/l bacto agar

Dissolve in deionized water, autoclave, cool to 50°C and add antibiotics.

For X-Gal plates, add Zeocin (25μg/ml), IPTG (5 mM) and X-Gal (80μg/ml).

Pour in 10 cm petri dishes and store at 4°C protected from light.

EMSA binding buffer (4 x)

50 mM HEPES (pH 7.9)

375 mM KCl

12.5 mM $MgCl_2$

0.5 mM EDTA

5 mM DTT

15% Ficoll

Dissolve in deionized water, store at -20°C.

EMSA loading buffer (6 x)

8% (w/v) Ficoll

500μl TE

10 mM EDTA

0.1% (w/v) Xylene Cyanol

0.1% (w/v) Bromophenol Blue

Dissolve in deionized water, store at -20°C.

2.2.2 Transformation of *E.coli* using the Nishimura Heat-Shock protocol

DNA was propagated using the *Escherichia coli* strain TOP10F' (Invitrogen), except for the psiRNA constructs, where the GT116 strain (InvivoGen) was used. Bacteria were transformed with plasmid DNA using the heat-shock protocol.

Competent bacteria were thawed and incubated with 1-100 ng plasmid DNA on ice for 30 min. Mixture was incubated for 1 min at 42°C and quickly chilled on ice for an additional minute. At continuation, 1 ml LB medium without antibiotics was added and the suspension was incubated for 1 hour at 37°C under gentle shaking (550 rpm). After the incubation, bacteria were pelleted and plated onto prewarmed LB agar plates containing the appropriated selection antibiotic. Plates were incubate overnight at 37°C. Colonies were picked and grown in LB medium with antibiotics under agitation (180 rpm, overnight, at 37°C).

2.2.3 DNA isolation

For preparative purposes, large amounts of highly purified plasmid DNA were obtained from 50 ml bacteria overnight culture using the QIAGEN Plasmid MAXI Kit (Qiagen), following manufacturer's specifications.

For sequencing of DNA (carried out by MWG, Ebersberg), small amount of highly purified DNA was isolated from a 5 ml bacteria overnight culture, using the Wizard®Plus SV Miniprep KIT (Promega). Alternatively, for purposed that did not require a high purification degree (e.g. analytical restriction endonuclease digestions), DNA was isolated with the alkaline lysis protocol using the P1, P2 and P3 buffers provided with the QIAGEN Plasmid MAXI Kit. 2 ml bacteria suspension was pelleted by centrifugation (5 min, 5000 rpm, RT) and resuspended in 90μl P1 buffer. After addition of 180μl P2 buffer, the tube was inverted three times. The mixture was incubated for 2 min, after which it was neutralized by addition of 135μl P3 buffer. The supernatant was transferred to a fresh tube and DNA was precipitated with 1 ml 96% ethanol. DNA was recollected by 10 min centrifugation at 14000 rpm, at RT. Pellet was washed once with 80% ethanol, vacuum dried and resuspended in 20μl sterile deionized water.

Plasmid DNA was stored at -20°C. DNA concentration was determined by measuring the absorbance of the diluted sample (usually 1:100) at 260 nm. Since an absorbance of 1 corresponds to 500μg/μl DNA, the concentration was calculated taking into account the dilution factor.

2.2.4 Restriction Endonuclease digestion

Restriction endonuclease digestions were performed by incubation of double-stranded DNA with an appropriate amount of restriction enzyme, in the buffer and at the temperature specified by the supplier. Typical digestions include a unit of enzyme per μg of starting DNA. For preparative purposes digestions were scaled up. Reactions were stopped by heat inactivation or by addition of loading dye. Fragments were analyzed or isolated by gel electrophoresis (see below). For cloning purposes, vectors were de-phosphorylated with Shrimp Alcaline Phosphatase (Roche) to avoid self-ligation. Vectors and fragments to be inserted were ligated with T4-DNA-ligase (Roche) overnight, at 14°C. TOP10F' were transformed with the ligation mixture using the Nishimura Heat-Shock protocol.

2.2.5 Polymerase-Chain-Reaction (PCR)

Specific restriction sites 5' and 3' of the coding sequence to be inserted in a given construct, were generated by PCR. Therefore, primers containing the required restriction site and annealing 5' and 3' of the coding sequence were used to amplify the insert. Primers were synthesized with HPLC purification by Biotez (Berlin). PCRs were carried out using the CombiZym System (Invitek, Berlin) following manufacturer's instructions; dNTPs (Desoxi-Nucleotid-Triphosphates) were purchased from Roche. PCR products were further digested and treated as described above.

2.2.6 Agarose Gel Electrophoresis and DNA Extraction from Agarose Gel

Depending on the size of the DNA fragments to be analyzed, gels with an agarose content of 1-2% (w/v) were boiled in 1xTAE. Once dissolved, solution was cooled down to approximately 50°C. Ethidium bromide (0.5μg/ml) was added and poured into casted gel chambers. Gels were run in 1xTAE, at 100V. DNA was loaded by addition of loading buffer and visualized under UV-light. For cloning purposes, DNA fragments of interest were excised with a scalpel and extracted using the QIAquick Gel Extraction Kit (Qiagen) following manufacturer's specifications.

2.2.7 Plasmids

pCMV-HA-hDP2, pCMV-HA-hDP1, the pE2Fx6-TATA-LUC reporter and the pRB binding defective point mutant E2F1 Y411C (Helin et al., 1993) were kindly provided by Dr. Claus Nerlov and Dr. Kristian Helin. The Myc-tagged SUV39H1 eukaroytic expression constructs (WT, catalytic inactive H324L and NChromo constructs) were supplied by Dr. Thomas

Jenuwein (Melcher et al., 2000), as well as the myc-tagged EZH2 construct. An HA-tagged SUV39H1 version was constructed by introducing a PCR product containing a BamHI and EcoRI site, respectively 5' and 3' of the coding region, into the equivalent pcDNA3 sites. This PCR product was also introduced into the pGEX4T2 construct, generating a GST-fusion protein for bacterial expression.

The pcDNA3-based amino-terminal HA-tagged E2F constructs were obtained from Dr. Stefan Gaubatz. The coding regions of E2F1 and E2F4 contained in the BamHI-EcoRI fragments of these constructs were introduced into the pGEX4T2 BamHI-EcoRI site, generating GST-fusion proteins. All DP1 and DP2 GST-fusion proteins were obtained by insertion of a PCR product containing a BamHI and NotI site, respectively 5' and 3' of the coding region.

The cyclin A binding deficient pcDNA1-E2F1Δ24 construct was provided by Dr. Liang Zhu (Krek et al., 1994). A BamHI fragment containing the E2F1 DNA binding deficient mutant E132 was cloned into pcDNA3 and into pGEX4T1.

The pBabePuro based retroviral C/EBPα basic region point mutants (BRM2: I294A,R297A; BRM3: D301A,K304A, BRM5: Y285A) were obtained from Dr. Claus Nerlov (Porse et al., 2001). For transient transfection, EcoRI-BamHI fragments of these mutants were fused to a carboxy-terminal triple FLAG, contained in a pcDNA3 construct (cloned by Dr. Elisabeth Kowenz-Leutz). The coding sequences of C/EBPα p30 and C/EBP$\alpha\Delta$126-200 were introduced in pcDNA3 construct with a carboxy-terminal single FLAG-tag (Pedersen et al., 2001). LAP*, LAP and LIP were expressed in pcDNA3 either without tag or with a carboxy-terminal FLAG-tag (cloned by Dr. Elisabeth Kowenz-Leutz). The C/EBP responsive -82 cMGF-luciferase reporter has been described previously (Sterneck et al., 1992).

For a complete listing of constructs, with information about DNA sequences, primers used for cloning, etc., see appendix.

2.2.8 Electrophoretic Mobility Shift Assay (EMSA)

The DNA binding activity of E2F or C/EBPα was investigated by using double-stranded oligonucleotides harboring either E2F or C/EBP binding sites. Double-stranded oligonucleotides were labeled with Klenow enzyme in a fill-in reaction with ^{32}P dCTP. Nuclear proteins were incubated with a ^{32}P-labeled oligonucleotide in EMSA binding buffer. Specific binding was inhibited using a 10 to 100-fold excess of unlabeled oligonucleotide, which was added 10 min prior to addition of labeled probe. For antibody supershift experiments, 10 min after addition of labeled probe, the binding reaction was incubated for additional 10 min with the corresponding antibody (1μl of a 1:5 dilution). All samples were subjected to electrophoresis on 4% polyacrylamide gels and visualized by autoradiography.

For in vitro band shift assays, binding of in vitro translated C/EBPα was examined (information about in vitro translation in section 2.4.6.). In vitro translation reaction was performed following manufacturer's specifications. 1μl of the C/EBPα in vitro translation reactions mixture was examined for binding. As negative control, 1μl of the pcDNA3 (empty vector) in vitro translation reaction mixture was added to binding reaction. Incubation steps were carried out as described for nuclear extracts.

Oligonucleotides used for EMSA:
E2F wt: 5' ATT TAA GTT TCG CGC CCT TTC TCA A 3'
E2F mut: 5' ATT TAA GTT TCG ATC CCT TTC TCA A 3'
C/EBP half-palindrome (HP): 5' ACA ATG AGG CAA T 3'
C/EBP palindrome (P): 5' ATG AGG CAA TCG GCA CTG TTG CCA CAT 3'

2.3 Working with proteins

2.3.1 Reagents and solutions

Tween-20 (Sigma)

Protease inhibitors Complete EDTA free (Roche)

Protein Ladder Page Ruler Prestained Protein Ladder (Fermentas)

Acrylamide (Biorad)
30% acrylamide/Bis Solution 29:1 (3.3% C)
Store at 4°C.

TEMED (Roth)

10% APS
Dissolve 1 g Ammonium persulphate in 10 ml deionized water.
Store at 4°C.

RIPA buffer
1% NP-40
0.1% SDS

50 mM Tris HCl pH 7.5

50 mM NaCl

Dilute in deionized water.

Add protease inhibitors (prior use).

Store at 4°C.

Triton buffer

50 mM Tris HCl pH 7.5

150 mM NaCl

1 mM EDTA

1% Triton X-100

Dilute in deionized water.

Add protease inhibitors (prior use).

Store at 4°C.

NP-40 buffer

0.4% NP-40

50 mM Tris HCl, pH 7.4

150 mM NaCl

1 mM EDTA

Dilute in deionized water.

Add protease inhibitors (prior use).

Store at 4°C.

Hypotonic buffer

0.2% NP-40

10% Glycerol

50 mM Tris HCl, pH 8

10 mM KCl

1 mM EDTA

Dilute in deionized water.

Add protease inhibitors (prior use).

Store at 4°C.

Hypertonic buffer

1% NP-40

10% Glycerol

50 mM Tris HCl, pH 8

10 mM KCl

400 mM NaCl

1 mM EDTA

Dilute in deionized water.

Add protease inhibitors (prior use).

Store at 4°C.

TBS

8 g NaCl

0.2 g KCl

3 g Tris

Dissolve in deionized water, adjust to pH 7.4 with HCl and fill up to 1000 ml.

Autoclave and store at 4°C.

Running buffer (10 x)

250 mM Tris

2 M Glycine

35 mM SDS

Dissolve in deionized water and store at RT.

SDS loading buffer (6 x)

600 mM DTT

350 mM Tris pH 6.8

10% SDS

10% glycerol

0.1 mg/ml bromophenol blue

Solve in deionized water.

Coomasie stock

Dissolve 2.5 g Coomasie Brillant Blue (FLUKA) in 500 ml methanol.

Stir overnight and add 500 ml deionized water.

Store at RT.

Coomasie solution

30% deionized water

30% ethanol 96%vol

20% acetic acid

30% Coomassie stock

Destain-solution

30% ethanol 96%vol

10% acetic acid

60% deionized water

Transfer buffer

25 mM Tris base

190 mM glycine

20% methanol

Dissolve in deionized water and add methanol.

Store at 4°C.

Ponceau S

1% Acetic acid

0.5% (w/v) Ponceau S

Dissolve in deionized water, store at RT.

10 x PBS

1.4 M NaCl

0.027 M KCl

0.018 M KH_2PO_4

0.1 M Na_2HPO_4

Dissolve in deionized water, autoclave and store at RT.

PBS-Tween

Make 1:10 of 10 x PBS stock, add 0.1% (v/v) Tween.

5% non-fat milk (blocking solution)

Solve 5% (w/v) non-fat milk powder (Merck) in 1 x PBS-Tween.

Store at 4°C.

Roti-Block (Roth)

Dilute 1:5 in deionized water

2.3.2 Cell protein extracts, SDS-PAGE and Immunoblotting

For detection of endogenous proteins, cells were lysed in RIPA buffer, while transiently transfected cells were lysed in Triton buffer. After 20 min incubation on ice, lysates were cleared by high-speed centrifugation at 4°C. Proteins were resolved by 10% or 12% SDS-polyacrylamide gels, prepared according to standard procedures (Maniatis Molecular Cloning Laboratory Manual). Gels were run at 120V until dye front was running out and blotted to nitrocellulose membranes (Schleicher&Schuell) using wet-blotting for 80 minutes at 80V.

After the transfer, membranes were rinsed shortly with deionized water and blocked with 5% non-fat milk for 1 hour at RT. Membranes were incubated with primary antibodies diluted in blocking milk overnight at 4°C. After extensive washes in PBS-Tween, membranes were incubated with secondary antibodies diluted in PBS-Tween for 1 hour at RT.

Antigen-antibody complexes were detected either by chemiluminescence (ECL system, Amersham) using secondary antibodies conjugated to horseradish peroxidase or by the Odyssey Infrared Imaging System using secondary antibodies conjugated to the fluorochrome IRDye™(Li-Cor). The latter allowed relative quantification of signals using the Odyssey Analysis software. When using the Odyssey detection system, the non-fat milk was substituted by Roti-block diluted 1:5 in deionized water.

2.3.3 Nuclear extracts

Transiently transfected cells were washed twice in ice cold PBS and collect by centrifugation. Cell pellet was resuspended in hypotonic buffer (600μl per 10 cm plate) and incubated for 5 min on ice. Nuclei were pelleted by centrifugation (6000 rpm, 3 min, at 4°C). Nuclear pellets were resuspended in hypertonic buffer (200μl per 10 cm plate). After 20 min incubation on ice, lysates were passed three times through a 27-gauge syringe and cleared by high-speed centrifugation at 4°C.

2.3.4 Relative protein concentration

The relative concentration of proteins present in lysates was determined with a Biorad reagent, based on the method of Bradford (1976). Prior use, the provided Bradford stock (Biorad) was diluted 1:5 with deionized water. An aliquot of each lysate (usually 2-5μl) was

mixed with 200μl diluted Bradford solution in the wells of a 96 well plate. After 10 min incubation at RT, the absorbance of the sample was measured at 595 nm. A plate reader spectroscope was used for this purpose (Molecular Devices Spectra Max 250). As a blank value, diluted Bradford reagent without lysate was used. To ensure a linear relation between absorbance and protein concentration, only absorbance values between 0.1 and 1 were considered for determination of relative protein concentration. When values were inferior or superior, the assay was repeated using respectively more or less amount of lysate.

2.3.5 Co-immunoprecipitation

For co-immunoprecipitation assays, $CaPO_4$-transfected cells were lysed in Triton buffer 48 hours post-transfection. The cleared lysates were incubated with anti-FLAG-M2-agarose (Sigma) for 50 min at 4°C. One fiftieth of the lysate was used as a control for protein expression (input). The agarose bound complexes were washed once with lysis buffer and three times with TBS (ice cold). To release bound complexes, the agarose pellets were resuspended in protein loading buffer and, together with the input sample, heated for 5 min to 95°C. Samples were resolved by SDS-PAGE and immunoblotting.

2.3.6 In vitro transcription/translation

The pcDNA3 constructs used in this work contained a T7 RNA polymerase promoter upstream of the coding sequence. For in vitro transcription and in vitro translation, the TNT®-Quick T7 coupled Reticulocyte Lysate System (Promega) was used following manufacturer's specifications. The reaction was carried out in the presence of ^{35}S-Methionine in Methionine-deficient reaction buffer, leading to the radioactive labeling of the resulting proteins.

2.3.7 Bacterial expression of GST-fusion proteins

BL21 (DE3) bacteria were transformed with the pGEX4T prokaryotic expression constructs encoding GST-fusion proteins. Bacteria were grown in 5 ml LB medium containing ampicillin (LB/Amp) overnight at 37°C. Next morning, the starter culture was transferred to 500 ml LB/Amp and grown at 37°C under agitation till an absorbance of 0.6 at 600 nm was reached. Protein expression was induced by addition of IPTG to a final concentration of 1 mM. After 4 hours shaking at 30°C, bacteria were pelleted by centrifugation (8000 rpm, 20 min, at 4°C). Pellet was resuspended in 20 ml PBS containing 1% Triton X-100 and protease inhibitors. After 20 min incubation on ice, the suspension was sonicated three times for 20 seconds,

using a Sonopuls HD70 sonicator. Lysates were cleared by centrifugation (10000 rpm, 20 min, at 4°C) and passed through a 0.45μm nitrocellulose filter (Schleicher&Schuell)

2.3.8 GST-pulldown

GST-fusion proteins were expressed and prepared like described in the previous section. For in vitro binding assays, 15μl ^{35}S-labeled in vitro translated proteins were incubated with equal amounts of affinity-purified GST-fusion proteins coupled to glutathione-sepharose for 1 hour at 4°C. Unfused GST protein served as a negative control. The beads were washed three times with NP-40 buffer and once with TBS (ice cold). Proteins were resolved by SDS-PAGE and visualized by autoradiography. GST fusion proteins were identified by Coomassie staining to verify that equal amounts were present in all reactions. Gels were incubated for 1 hour in Coomasie solution and washed in destain solution until the background staining was almost eliminated.

Alternatively, GST-proteins were incubated with an equal volume of lysates of 293T cells transfected with C/EBP expression plasmids. Bound proteins were detected by immunoblotting. GST fusion proteins were identified in immunoblots by Ponceau S staining. Membranes were stained for 3 min in Ponceau S and washed with deionized water. Staining was removed by washing 10 min in PBS-Tween.

2.4 Cell culture

2.4.1 Reagents, solutions and cell lines

DMEM+GlutaMAX™ (Invitrogen)

MEM-AlphaMedium+GlutaMAX™ (Invitrogen)

Fetal Bovine Serum (Gibco)

Penicillin / Streptomycin 100 x (PAA)

Trypsin-EDTA (PAA)

DMSO (Merck)

Puromycin (InvivoGen)

Zeocin (InvivoGen)

BSA (Boehringer Mannheim)

1 x PBS

137 mM NaCl

2.7 mM KCl

4.3 mM Na_2HPO_4

1.4 mM KH_2 PO_4

Dissolve in deionized water and autoclave, store at RT.

2 x HBS

50 mM Hepes (pH 7.0)

250 mM NaCl

1.5 mM Na_2HPO_4

Dilute in deionized water.

Filter sterilize (0.45μm filter) and store at -20°C

TE

10 mM Tris pH 8

0.1 mM EDTA

Filter sterilize (0.45μm filter) and store at 4°C

$CaCl_2$ (2.5 M)

Dissolve 36.76 g $CaCl_2$ in 100 ml deionized water.

Filter sterilize (0.45μm filter) and store at -20°C.

Polybrene® (Sigma)

Dissolve in deionized water to a final concentration of 4 mg/ml.

Filter sterilize (0.45μm filter) and store aliquots at -20°C.

Chloroquine (Sigma)

Dissolve chloroquine in deionized water (10 mM solution).

Filter sterilize (0.2μm filter) and store at -20°C.

IBMX (Sigma)

Dissolve 100 mg in 9 ml ethanol (50 mM stock).

Store at -20°C.

Insulin (Sigma)

Dissolve 100 mg in 10 ml acidified water (add 0.1 ml glacial acetic acid).

Store at -20°C.

Dexamethasone (Sigma)

Dissolve 40 mg in 10 ml ethanol (10 mM stock).

Store at -20°C.

1% BSA-blocking solution

Solve 1% BSA (w/v) in PBS.

Store at -20°C.

Prior use, centrifuge at 5000 rpm for 10 min, at 4°C.

Oil-Red-O stock solution

300 mg Oil-Red-O (Sigma)

Dissolve in 100 ml isopropanol and store at RT.

Oil-Red-O working solution (Prepare prior use)

Mix 3 part Oil-Red-O stock solution with 2 parts deionized water.

Let stand for 10 min and filter through a folded Whatman filter paper (Schleicher&Schuell).

Crystal violet

0.1% (w/v) crystal violet

10% ethanol

Solve in deionized water.

Store at RT.

ATP (Serva)

20 mM ATP

Dissolve in deionized water and adjust pH to 7.5 by NaOH addition.

Aliquot and store at -20°C.

Luciferin (Sigma)

Dissolve 10 mg Luciferin in 35.7 ml deionized water (low solubility, remains cloudy).

Aliquot and store at -20°C.

Light sensitive, keep dark.

Gly-Gly (Sigma)

25 mM Gly-Gly

Dissolve in deionized water and adjust pH to 7.8.

Store at -20°C.

Substrate-reaction buffer for Luciferase Assay

375μl Luciferin

250μl ATP (of 20 mM solution)

50μl $MgSO_4$ (of 1 M solution)

Dilute in 25 mM Gly-Gly.

Prepare prior use, protect from light.

Cell lines

HeLa: human, epithelial cells derived from cervix adenocarcinoma

Hek293T: human, kidney cells, with temperature sensitive gene of SV40 T-antigen

NIH3T3-L1: mouse, derived from embryonic fibroblasts, with insulin receptor

Phoenix-E: 293T-derived ecotropic retroviral packaging cells (Hofmann et al., 1996)

C/EBPα -/- MEFs: immortalized by Dr. Valerie Begay

pRB-/-/p107-/-/p130-/- MEFs: kind gift from Dr. Hein te Riele (Dannenberg et al., 2000)

2.4.2 Growth of Mammalian Cells

Cells were grown in DMEM+GlutaMAXTM, 10% fetal calf serum and antibiotics (Penicillin/ Streptomycin) in an incubator at 37°Cand 7.5% CO_2. When grown to confluency, cells were splitted. Therefore, medium was aspired, cells were washed once with 1 x PBS and incubate with trypsin for approximately 5 min at 37°C(1 ml trypsin per 10 cm dish). Once detached, cells were resuspended in an appropriate amount of medium, and the cell suspension was transferred in different dilutions to new tissue culture dishes.

2.4.3 Freezing and thawing of cells

Cells to be frozen were detached from a subconfluent 10 cm dish by trypsinization (see above). Detached cells were resuspended in 5 ml tissue culture medium and centrifuged in a 15 ml Falcon-tube for 5 minutes at 1100 rpm. Supernatant was aspired carefully, cell pellet was resuspended in 1 ml freezing medium (90% FCS, 10% DMSO) and transferred to a cryo-tube (NUNC). Cells were incubated for 15 min on ice and stored in liquid N_2.

To thaw cells, a 10 cm dish with 15 ml medium was prepared and incubated for 20 min at 37°C and 7.5 % CO_2. Cells were thawed in a 37°C water-bath and washed in 5 ml medium in order to eliminate DMSO. Cell pellet was resuspended and transferred to the medium containing plate. The next day, medium was changed or, if grown to confluency, cells were split.

2.4.4 Transfection with $CaPO_4$

$1*10^6$ 293T were plated on a 10 cm dish. The next day, a DNA solution was prepared containing 5μg of each construct, 20μl 2.5M $CaCl_2$ and TE to a final volume of 200μl. In a second Eppendorf tube, 200μl 2xHBS were pipetted. The DNA solution was added dropwise onto the HBS solution. After 30 min incubation, the solution was pipetted onto the cells.

For generation of viral supernatant, $2*10^6$ Phoenix-E per 10 cm dish were plated. 5 min prior transfection cloroquine was added into the medium to a final concentration of 25μM.

2.4.5 Liposomal transfection

Transfection with liposomal reagents offered two major advantages towards conventional $CaPO_4$-transfection, an higher efficiency and, since it is less prone to variations, an higher reproducibility. Two different reagents were used, Metafectene™(Biontex) or TransIT®-LT1 (Mirus), following the same protocol. Serum free-DMEM and the transfectant reagent were warmed to RT. A transfection stock containing transfection reagent and medium was prepared and incubated for 5 min at RT. Per 12-well to be transfected, either 0.5μl Metafectene™ or 1μl TransIT®LT1 were mixed with 37.5μl DMEM. Amounts were reduced to the half for 24-well transfection. The mixture was split into different tubes, were plasmid DNA was added (up to 0.5 μg per 12-well). After 25 min incubation, the mixture was added dropwise onto the cells. Cells were harvested 48 hours post-transfection.

2.4.6 Reporter assay

The transcriptional activity of proteins can be assayed by using responsive promoters fused to the luciferase gene. The increase of luciferase in the presence of a transcription factor is indicative for transcriptional activity. Luciferase catalyzes luciferin in the presence of ATP and Mg^{2+}, resulting in light emission. Measurement of the emitted light allows quantification of the luciferase amounts and, thus, quantification of the transcriptional activity.

To examine the transcriptional activity of C/EBPα, a luciferase reporter was used containing a fragment of the cMGF promoter (-82 to -41), with two palindromic C/EBP binding sites (Sterneck et al., 1992). For examination of the transcriptional activity of E2F, a reporter containing a TATA box and 6 conserved E2F binding sites was used (H. Muller et al., 1997). Transfection of either 293T or MEFs were carried out with respectively Metafectene™(Biontex) or TransIT®LT1 (Mirus) as described above. Cells were seeded 12 hours prior transfection ($3*10^5$ 293T per 24-well, $2*10^5$ MEFs per 12-well) and transfected with 50 ng reporter and 25-50 ng expression vector. Total DNA concentration was maintained equal by addition of empty pcDNA3. Transfections were carried out in duplicates. Luciferase assays were carried out 48 hours post-transfection. Cells were lysed in 100μl Triton buffer (see "working with proteins" section) and cleared by high-speed centrifugation (12000 rpm, 10 min, 4°C). 15μl of each lysate were transfered to an 96-well plate and measured with an Berthold Lumat LB9501 plate reader luminometer, which pipetted the substrate-reaction buffer automatically. Since the β-Galactosidase reporter showed responsiveness to C/EBPα, luciferase values were normalized to protein levels and protein expression was controlled by immunoblotting. The data are representative of three independent experiments, duplicates are graphed as the mean$\pm$SD.

2.4.7 Retroviral infection

For virus production, retroviral plasmids were transfected into the ecotropic virus packaging cell line Phoenix E using the $CaPO_4$ method. Culture supernatants were recollected 48 hours after transfection and passed through a 0.45μm PVDF filter (Millipore). Polybrene, a cationic polymer that increase the infection efficiency, was added to the viral supernatant (5μg/ml). Mixture was used to infect subconfluent layers of C/EBPα -/- MEFs. Infection was carried out overnight, replacing the viral supernatant with fresh medium the next morning. Cells were selected for 3 days in the presence of 2μg/ml puromycin (InvivoGen).

2.4.8 Small interference RNA (siRNA)

RNA interference is a technology that allows gene silencing. It bases on the finding that a 20-mer double-stranded RNA oligonucleotide, namely small interference RNA (siRNA), can target mRNA for degradation in a gene-specific manner (Elbashir et al., 2001). The siRNA can be expressed as synthetic duplex or alternatively be expressed by vectors as short hairpin RNAs (shRNAs). The latter technology was used in this work.

shRNAs were expressed by the RNA polymerase III promoter contained in the psiRNA construct (InvivoGen), harboring a Zeocin selection mar ker fused to an IRES GFP (Figure 2.1). The expressed hairpin RNA (approx.50-mer) contained two complementary sequences of 21 nucleotides, homologous to a segment of the gene of interest, separated by a short region of 5-9 nucleotides. shRNA oligos against DP1, E2F1, E2F3 or E2F4 were designed using the siRNA Wizard program from InvivoGen (http://www.sirnawizard.com/design.php) and subjected to BLAST search (http://www.ncbi.nih.gov/BLAST/) to exclude homology to any additional known sequence. As control, a non specific shRNA was used. Double-stranded DNA oligonucleotides were ligated into the BbsI site of the psiRNA construct (Figure 2.1). Since this site is flanking a bacterial lacZ α-peptide cassette, successful integration of the oligo duplex destroys the lacZ gene. Thus, growth on X-Gal plates allows discrimination between parental clones (blue) and insert-containing white clones (Figure 2.2). The oligo insertion was further confirmed by sequencing.

Sequences targeted by shRNAs:

Control 5' GTC CAT CGA ACT CAG TAG CT 3'

DP1 5' GCA GCA TCT CCA ATG ACA AAT 3'

E2F1 5' GCC AAG AAG TCC AAG AAT CAT 3'

E2F3 5' GCT CAC CAA GAA GTT CAT TCA 3'

E2F4 5' CGA GAG TGA AGG TGT CTG T 3'

2.4.9 Fluorescence activated cell sorter

The IRES GFP contained in the psiRNA allowed sorting of the transfected cells. Cells were detached from the plate by trypsinization and resuspended in PBS containing 2% serum and 2 mM EDTA. In order to get a single cell suspension, cells were passed through a cell strainer (BD Bioscience). The analog sorter FACSVantage SE was used for sorting the cells, which was carried out by Dr. Rahn (MDC, Berlin).

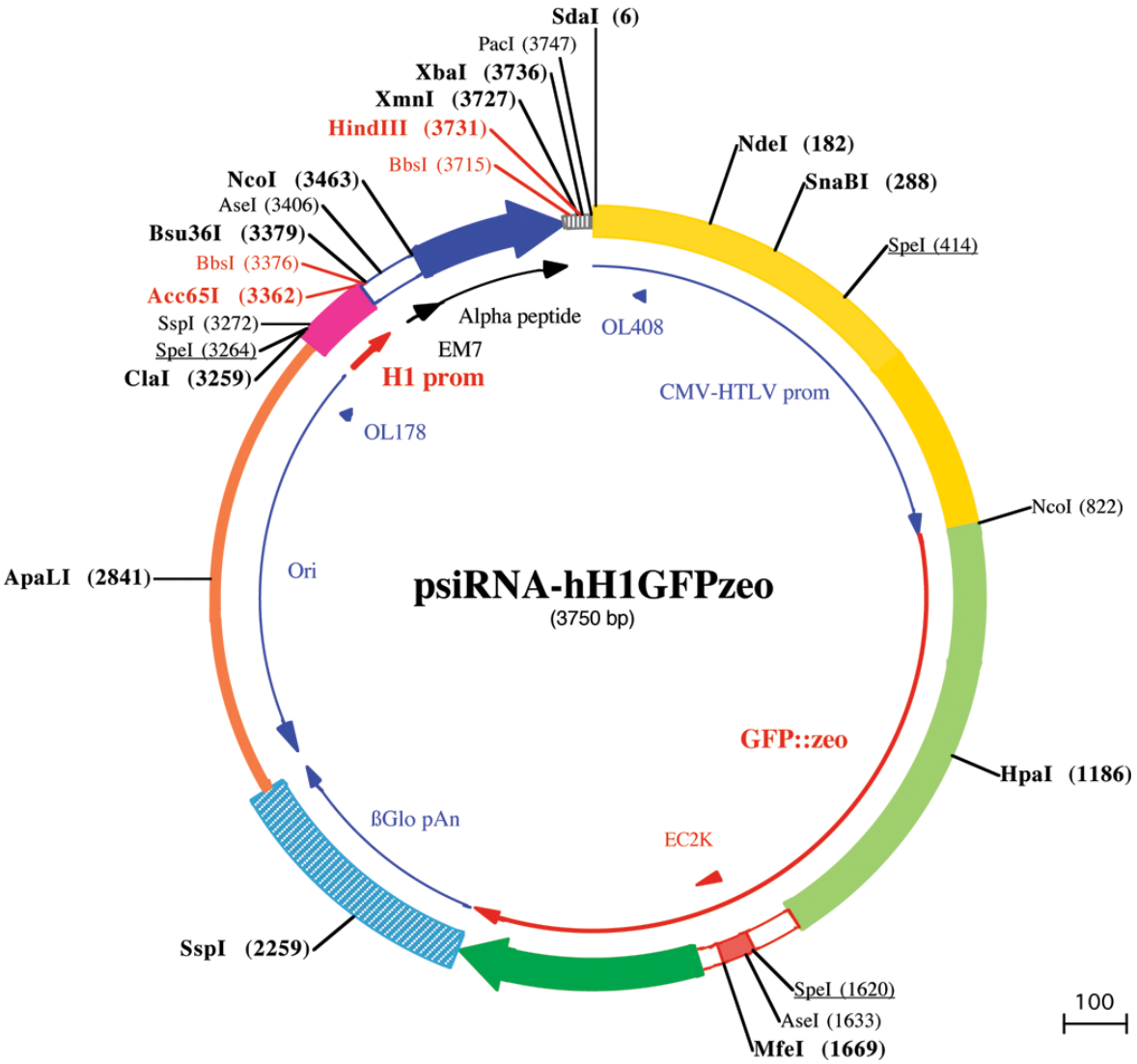

Figure 2.1: **psiRNA vector map (from www.invivogen.com)** The shRNA oligos were cloned downstream of the H1 RNA polymerase III promoter (H1 prom). Successful insertion of the oligo in the BbsI site destroys the bacterial lacZ α-peptide cassette (Alpha peptide). GFP::zeo is a fusion gene that encodes a red-shifted variant of the jellyfish GFP and resistance to Zeocin™ in mammalian cells. The GFP-zeo gene is under the control of the strong CMV-HTLV composite promoter (CMV-HTLV prom), allowing high expression in a constitutive manner in mammalian cells. The EC2K bacterial promoter expresses the Zeocin resistance gene in *E. coli*.

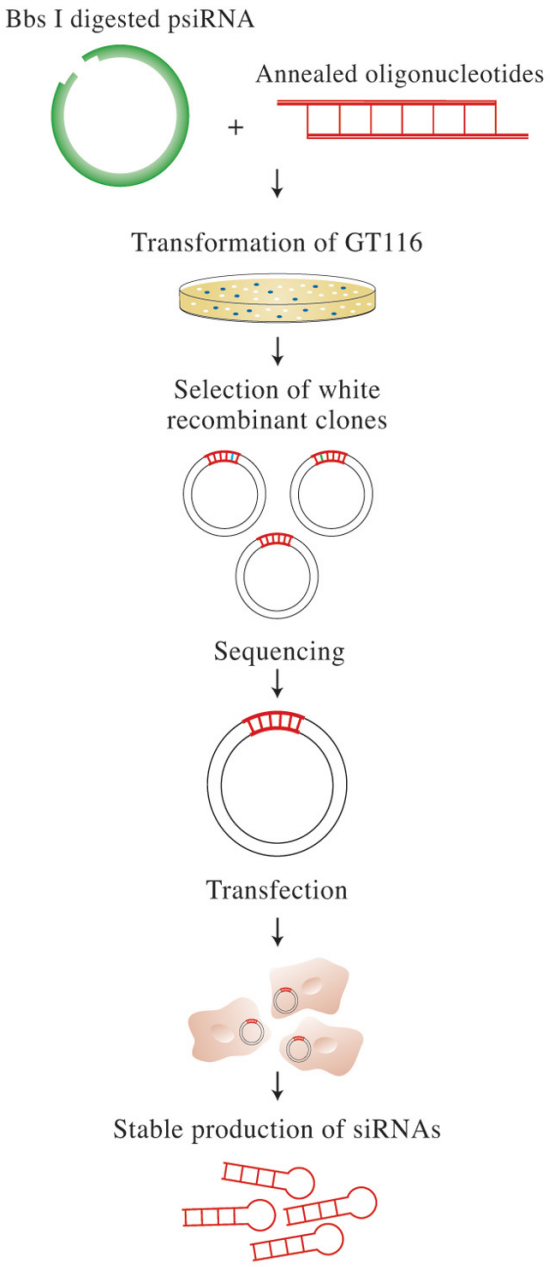

Figure 2.2: **Vector-mediated siRNA (from www.invivogen.com).** Schematic representation of the steps required for generation of vector-mediated siRNA. See text for details.

2.4.10 Adipogenesis

3T3-L1 or C/EBPα -/- MEFs were differentiated with MEM-AlphaMedium, 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 refreshed every second day. After 8 days drug treatment, cellular morphology was documented using bright-field microscopy and cell lysates were analyzed by immunoblotting.

2.4.11 Adipogenesis of shRNA-expressing cells

$2*10^5$ C/EBPα -/- MEFs were seeded in 24-well containing MEM-AlphaMedium, 10% serum and antibiotics. 12 hours later, cells were transfected with 50 ng psiRNA constructs. Drug treatment was started 24 hours post-transfection and continued as mentioned above. 8 days after treatment start, cells were washed twice in PBS and fixed 10 min with Roti®-Histofix 4% (Roth). The GFP contained in the psiRNA construct permitted the recognition of transfected cells, which were visualized under UV light with an AxioVert 100 (Zeiss) inverted microscope. Adipocytes were determined by cell morphology. GFP expressing cells were counted as either non-adipocytes or adipocytes (400 cells per double value). Finally, cells were stained with Oil-Red-O and analyzed by bright-field microscopy.

2.4.12 Oil-Red-O staining

Cells were washed once in PBS and fixed with Roti®-Histofix 4% (Roth) for 10 min. After two washes in PBS, cells were stained with Oil Red-O working solution for 15 min. Finally, cells were washed three times with deionized water.

2.4.13 Colony Forming Assay and Crystal violet staining

Transduced C/EBPα -/- MEFs were plated in duplicates at various densities (50, 200 or 1000 cells/6-well plate) and grown under puromycin selection. Colonies were grown for 2 weeks, after which they were stained with crystal violet. Therefore, plates were washed twice in PBS and stained for 10 min in crystal violet. After extensive washes with deionized water, plates were dried. Images were captured using a C5060 Olympus digital camera (Olympus).

2.4.14 Immunofluorescence

For immunofluorescence staining, cells were grown on glass coverslips of 12 mm diameter (Roth). In order to increase the attachment of 293T cells, coverslips were coated with Poly-L-Lysine (Sigma). Briefly, coverslips were immersed in ethanol, dried by evaporation and shortly rinsed with sterile water. Poly-L-Lysine solution was diluted 1:10 with deionized water and pipetted onto the coverslips. After 20 min incubation, coverslips were throughly washed in PBS, since any reminiscent of unbound Poly-L-Lysine causes cell death. Coverslips were inserted in 24-wells; medium and $3*10^5$ 293T cells were added. The next day, cells were transfected with Metafectene™, as described previously. 48 hours post-transfection, cells were washed twice in PBS for 5 min and fixed with Roti®-Histofix 4% (Roth) for 10 min. After two washes in PBS, cells were permeabilized with 0.1% Triton contained in 1%-BSA blocking solution for 15 min. Coverslips were washed twice in PBS for 5 min and blocked in BSA blocking solution for 45 min. Cells were incubated with primary antibody diluted in BSA blocking solution at RT for 1 hour (alternatively, incubate overnight at 4°C). After two washes in PBS, cells were incubated with fluorescence conjugated secondary antibodies diluted in BSA-blocking solution for 30 min. Cells were washed twice in PBS and stained with DAPI for 1 min. Coverslips were then washes twice in deionized water for 1 min, dried on Whatman filter paper and mounted on microscope slides (Menzel-Gläser) with Immu-mount (Thermo Shandon). Cells were analyzed using an AxioPlan 2 microscope (Zeiss) and images were captured with a Zeiss AxioCam Hr camera (Zeiss).

2.4.15 BrdU proliferation assay

C/EBPα -/- MEFs cells were grown on glass coverslips and transfected with the psiRNA constructs. 80 hours after transfection, cells were labeled for 16 hours with BrdU (10μM, Sigma). After BrdU incorporation, coverslips were fixed with Roti®-Histofix 4% (Roth). The BrdU-positive cells were identified by indirect immunofluorescence. The primary antibody (anti-BrdU mouse monoclonal antibody) was diluted in blocking buffer containing 3 mM $MgCl_2$ plus 100 U of DNase I (Roche) per ml. After 50 min incubation, cells were washed twice in PBS and incubated with an anti-mouse Alexa Fluor®555 conjugated antibody. Cells were counterstained with DAPI and analyzed using an AxioPlan 2 microscope (Zeiss). Percentage of BrdU incorporating cells was quantified by counting GFP expressing cells which stained positive for BrdU. At least 100 cells per duplicate were counted. Duplicates were graphed as the mean$\pm$SD. Images were captured with a Zeiss AxioCam Hr camera (Zeiss).

Chapter 3

Results

3.1 E2F and C/EBP crosstalk

3.1.1 Working hypothesis

C/EBPα-mediated proliferation arrest involves direct interaction between E2F and C/EBPα that leads to repression of E2F-regulated S-phase genes (Slomiany et al., 2000). Deregulation of this repressive action is believed to contribute to myeloproliferative disorders (Porse et al., 2005). In a yeast-two-hybrid screen, the dimerization partner of E2F (DP) was initially found as a C/EBPβ binding partner (unpublished result, Joschko S. and Leutz A.). This raised the possibility that not only E2F but also DP interacts with C/EBP. The aim of this work was to test this possibility and to analyze the molecular implication of the DP binding to C/EBP for the C/EBPα-E2F network. Moreover, it was analyzed whether this interaction has an impact on differentiation processes, where both E2F and C/EBPα play key roles.

3.1.2 C/EBPα binds to DP in vivo and in vitro

C/EBPα has been shown to repress E2F-mediated transcription through direct binding to E2F (Slomiany et al., 2000). Co-immunoprecipitation assays were carried out to investigate whether C/EBPα interacts with DP. As shown in Figure 3.1A, both highly homologous DP1 and DP2 members, formed complexes with C/EBPα.

To examine whether interaction of DP to C/EBPα was either direct or mediated through E2F, in vitro binding studies were carried out. In a GST-pulldown of GST-DP1, GST-DP2 and GST-E2F4, the binding of in vitro translated C/EBPα was assayed. As previously reported, C/EBPα interacted with GST-E2F4 (Figure 3.1B). Furthermore, both DP1 and DP2 complexed with C/EBPα. Thus, first hints suggest a direct binding of DP to C/EBPα.

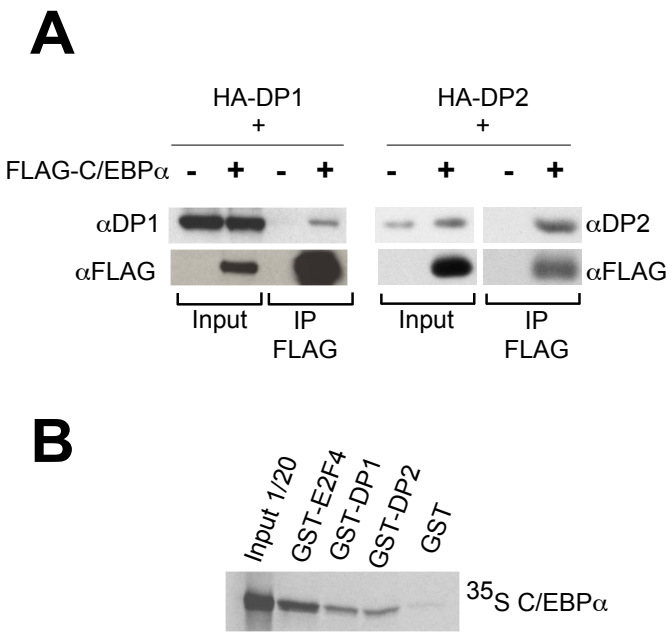

Figure 3.1: **C/EBPα interacts with the dimerization partner of E2F (DP).** (A) C/EBPα associates with DP1 and DP2 in vivo. Lysates of 293T cells transfected with FLAG-tagged C/EBPα and either HA-DP1 or HA-DP2 were immunoprecipitated with anti-FLAG and analyzed by immunoblot with anti-DP1, anti-DP2 and anti-FLAG antibodies. (B) C/EBPα interacts with DP1 and DP2 in vitro. GST-pulldown assay of either GST-E2F1, GST-DP1, GST-DP2 or GST alone, were incubated with in vitro-translated ^{35}S-labeled C/EBPα. Bound proteins were resolved by SDS-gel electrophoresis and analyzed by autoradiography.

3.1.3 C/EBPα and E2F bind different regions within DP

Amino- and carboxy-terminal DP2 deletion mutants were tested for interaction with C/EBPα. These DP2 mutants (Figure 3.2A) were expressed as GST proteins and assayed for their ability to bind to in vitro translated E2F4 or C/EBPα. E2F4 bound to DP2 mutants containing amino acids 83-236, while it failed to interact with the amino-terminus of DP2. In contrast, C/EBPα interacted with the DP2 mutants containing amino acids 1-72, 60-236 and to a lower extent 60-166. Moreover, C/EBPα failed to bind to DP2 mutants lacking the first 83 amino acids. Altogether, this indicates that C/EBPα interacts with the amino terminus of DP2, which is not bound by E2F4.

The region of DP2 required for C/EBPα binding (amino acids 60 to 82) is 87 % identical and 96 % homologous to DP1 (amino acids 105 to 127), as shown in the alignment in Figure 3.3B. In order to determine whether the binding of C/EBPα to DP2 and DP1 occurs through equivalent regions, two DP1 mutants, Δ105 and Δ127, which have amino terminal deletions of respectively 105 and 127 amino acids, were constructed. These mutants were expressed as GST proteins and their interaction with C/EBPα was assessed. As shown in Figure 3.2B, Δ105-DP1 bound C/EBPα comparable to full-length DP1, while Δ127-DP1 failed to do so. Figure 3.3A summarizes the mapping of the DP1 and DP2 interaction domains. An alignment of the DP1 and DP2 sequences suggests that C/EBPα binds to DP through a conserved basic region located in the amino-termini of DP1 and DP2 (Figure 3.3B). Together, these data indicated that interaction of C/EBPα with DP1 and DP2 occurs through a domain distinct from the E2F dimerization domain, excluding the possibility that E2F may act as a bridging factor between DP and C/EBPα, thus fully supporting the notion of a direct binding between DP and C/EBPα.

Alternative translation initiation from a single mRNA generates two C/EBPα isoforms, a full-length p42 isoform and the amino-terminal truncated p30 isoform (Figure 3.4). Both p42 and p30 interact with E2F (D'Alo et al., 2003), but p30 fails to repress E2F-mediated transcription (Porse et al., 2001). In order to determine whether DP interacts with both C/EBPα isoforms, p42 and p30 were expressed in 293T and their binding to GST-DP2 was analyzed. Bound C/EBPα was detected by immunoblotting using an anti-C/EBPα antibody. Alternatively, the binding of C/EBPα isoforms to GST-E2F4 was examined. As shown in Figure 3.4, DP2 associated with both isoforms. Thus, the amino-termini of C/EBPα is dispensable for interaction with E2F/DP.

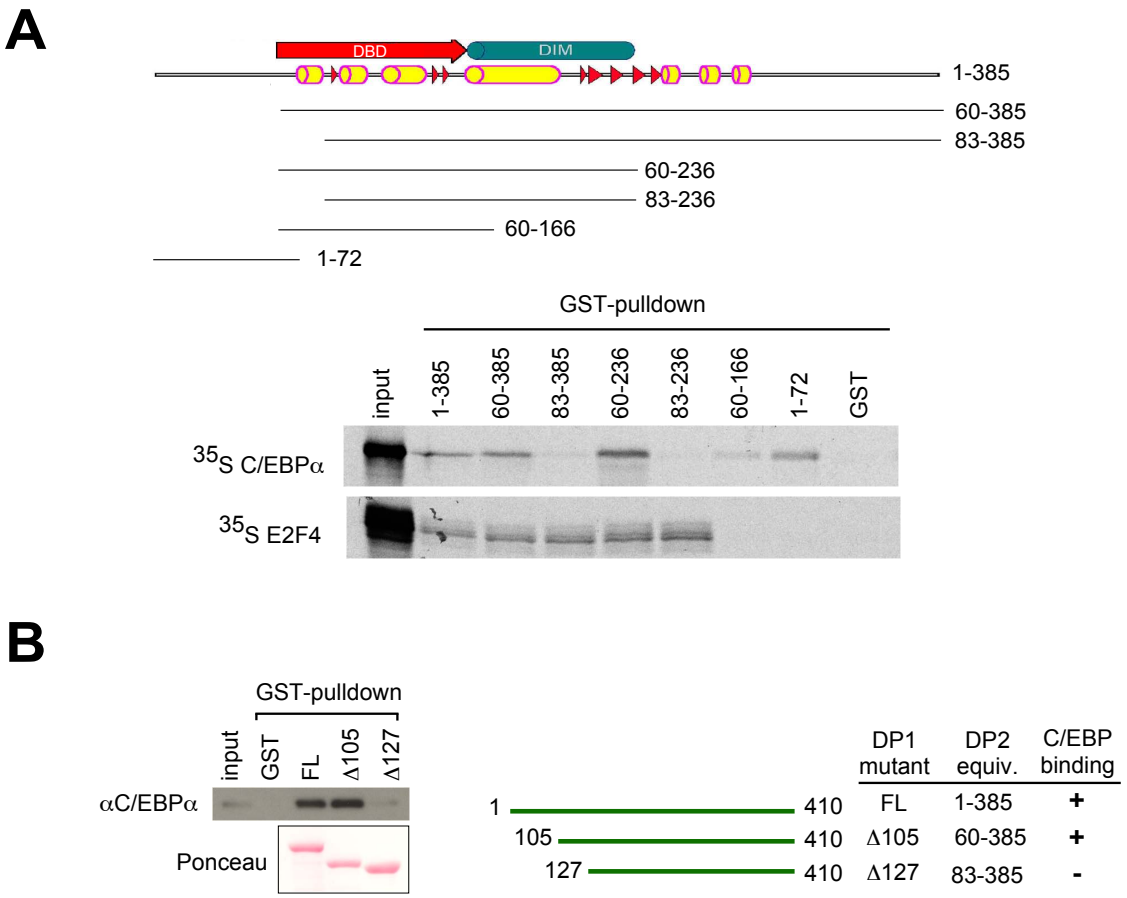

Figure 3.2: **E2F and C/EBPα interact with different DP domain.** (A) C/EBPα binds to the amino terminus of DP2. Schematic representation of the domains within DP2 and of the GST fusion deletion mutants (DBD: DNA-binding domain; DIM: Dimerization domain). The truncation sites were chosen taking into account the crystal structure of DP2 (cylinders: alpha-helix, triangle: beta-sheet (Zheng et al., 1999)) in order to avoid the disruption of secondary structure. GST-DP2 deletion mutants were incubated with in vitro-translated ^{35}S-labeled C/EBPα (top) or E2F4 (bottom). Bound protein were resolved by SDS-gel electrophoresis and analyzed by autoradiography. (B) Right: Schematic representation of DP1 deletion mutants and list of equivalent DP2 mutants. Left: Interaction of GST-DP1 mutants with C/EBPα expressed in 293T cells. Bound proteins were detected with anti-C/EBPα immunoblotting, GST-fusion proteins were visualized by Ponceau staining.

A

DP2 mutant	C/EBPα binding	E2F binding	DP1 mutant	C/EBPα binding
Full length	+	+	Full length	+
aa 60-385	+	+	Δ105	+
aa 83-385	-	+	Δ127	-
aa 60-265	+	+		
aa 1-72	+	-		

B

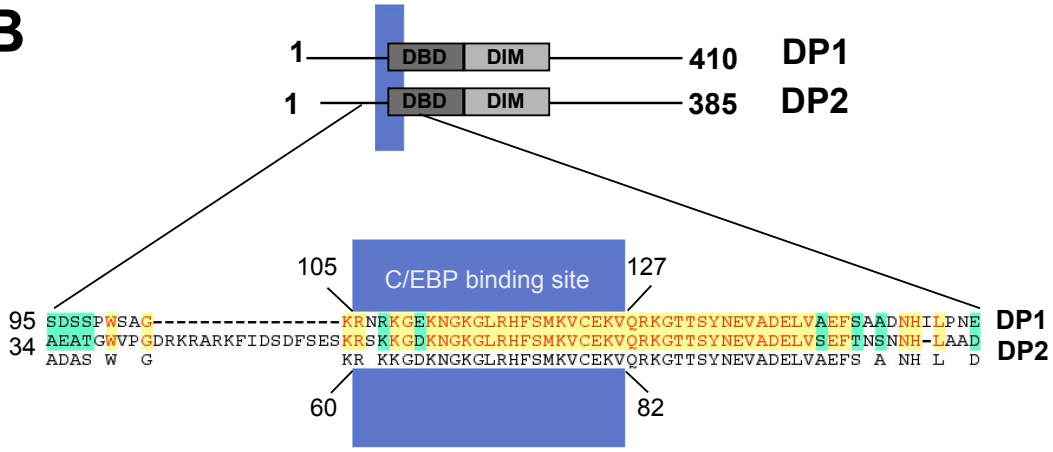

Figure 3.3: **DP interacts with C/EBPα via a different domain than with E2F (Summary).** GST fusion deletion mutants of DP2 and DP1 were tested in their ability to interact with C/EBPα. The results represented in Figure 3.2., are summarized in this table. (B) Schematic representation of the domains within DP1 and DP2 and alignment of the human sequences (DBD: DNA-binding domain; DIM: Dimerization domain). Yellow: Identity, Green: Similarity.

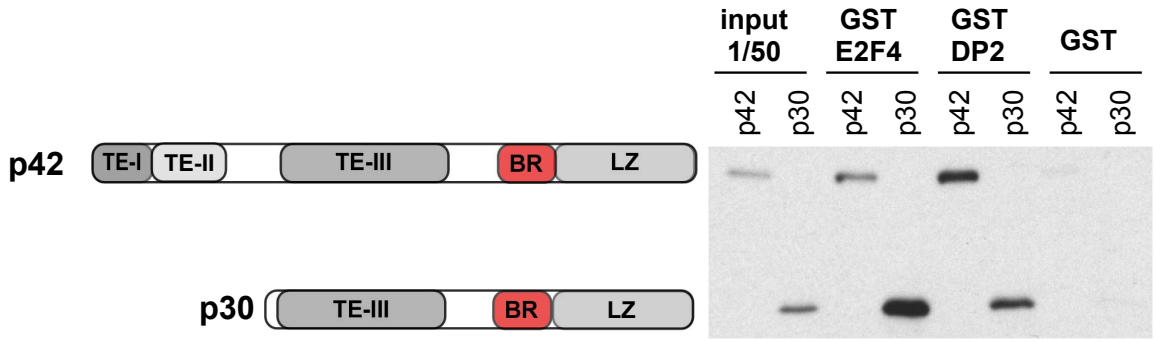

Figure 3.4: **The C/EBPα isoform p30 interacts with both E2F and DP.** (A) Schematic representation of C/EBPα isoforms generated from alternative translation initiation sites. The p42 isoform corresponds to full-length C/EBPα, while p30 has an amino-terminal truncation. Interaction of GST-E2F4 and DP2 to C/EBPα isoforms p42 and p30 expressed in 293T cells was analyzed. Bound proteins were detected with anti-C/EBPα immunoblotting.

3.1.4 E2F-DP repress C/EBPα-mediated transcription

Repression of E2F-mediated transcription by C/EBPα has been intensively studied, while it has not been addressed whether E2F affects the transcriptional activity of C/EBPα. Thus, it was assayed whether E2F affects induction of C/EBPα-mediated transcription of the C/EBP responsive cMGF luciferase reporter (Sterneck et al., 1992). Remarkably, the expression of both E2F1 and DP1 strongly repressed C/EBPα. However, on their own, E2F1 or DP1 repressed C/EBPα only partially, or not at all (Figure 3.5A). Consistent with the high homology of DP1/DP2, they have similar repressive effects on the activity of C/EBPα (Figure 3.5B).

On the basis of homology and function, E2F family members can be subgrouped into E2F1-E2F3 and E2F4-E2F5. E2F1-E2F3 share a cyclin-A-binding domain (cA) that is absent in E2F4 and E2F5. Furthermore, E2F1-E2F3 are thought to activate their target genes while E2F4-E2F5 are believed to suppress them (section 1.2). To test whether different E2F members repress C/EBPα, C/EBPα-mediated transcription was assessed in the presence of HA-DP1 and HA-tagged E2F1, E2F3, E2F4 or E2F5. Their expression was determined by immunoblotting using fluorescent dye-conjugated secondary antibodies, which allowed a relative quantification of signals (Figure 3.5C). As shown in Figure 3.5C, all E2F members repressed the transactivation by C/EBPα. E2F4 and E2F5 were less efficient in C/EBPα repression, however, both lack nuclear localization signals that might account for this deficit (Lindeman et al., 1997). Concluding, these data reveal that both activator and repressor E2Fs in combination with DPs, synergistically repress the transcriptional activity of C/EBPα.

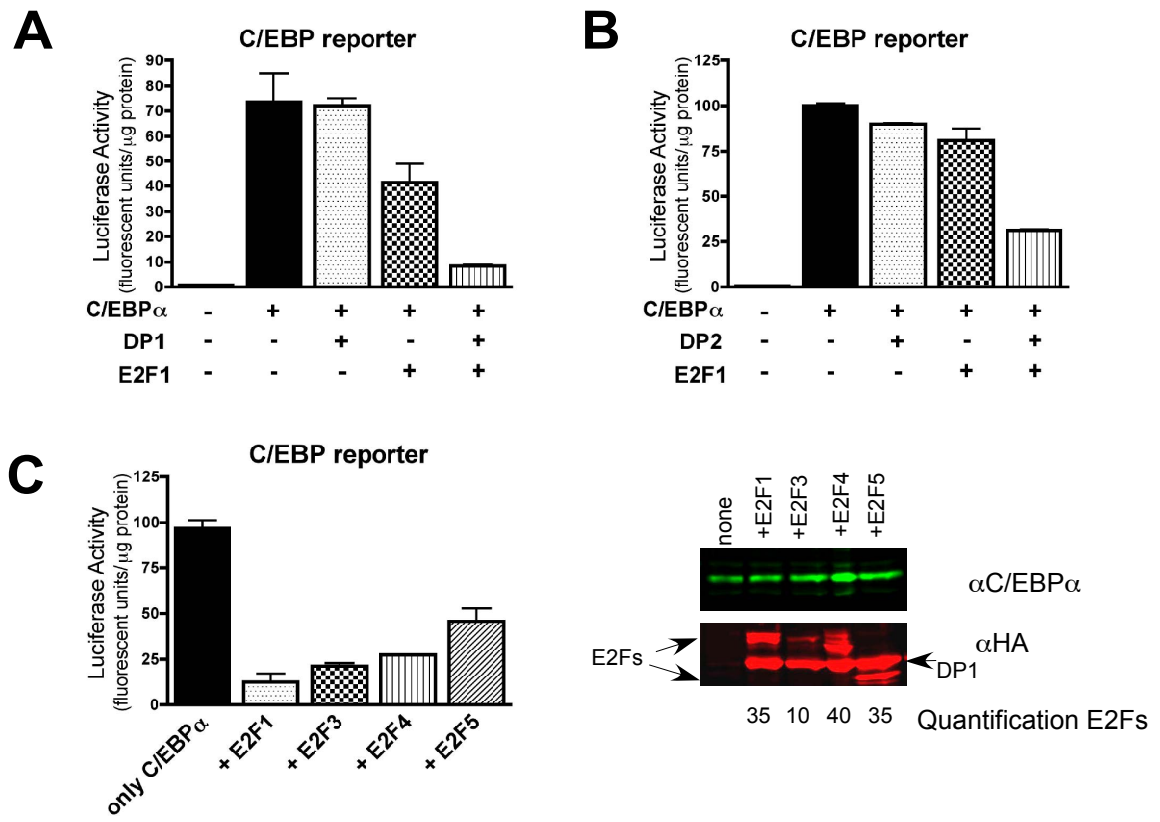

Figure 3.5: **E2F represses the transcriptional activity of C/EBPα.** (A) Reporter assay using the C/EBP responsive promoter cMGF, in 293T cells. Activation of the cMGF reporter (100ng) by wild type C/EBPα (100ng) was measured in the absence or presence of DP1 (100ng), E2F1 (100ng) or both. (B) Same as in (A), with DP2 substituting DP1. (C) Several E2F members can repress C/EBPα. 293T cells were transfected with C/EBP luciferase reporter, C/EBPα, HA-DP1 and HA-tagged E2F members expression constructs. Expression was tested by immunoblotting (Odyssey Infrared Imaging System) using anti-HA and anti-C/EBPα antibodies (right panel). E2F expression (anti-HA signal) was quantified. All luciferase reporter assays were done in duplicate, graphed as the mean$\pm$SD. Data are representative of at least three independent experiments.

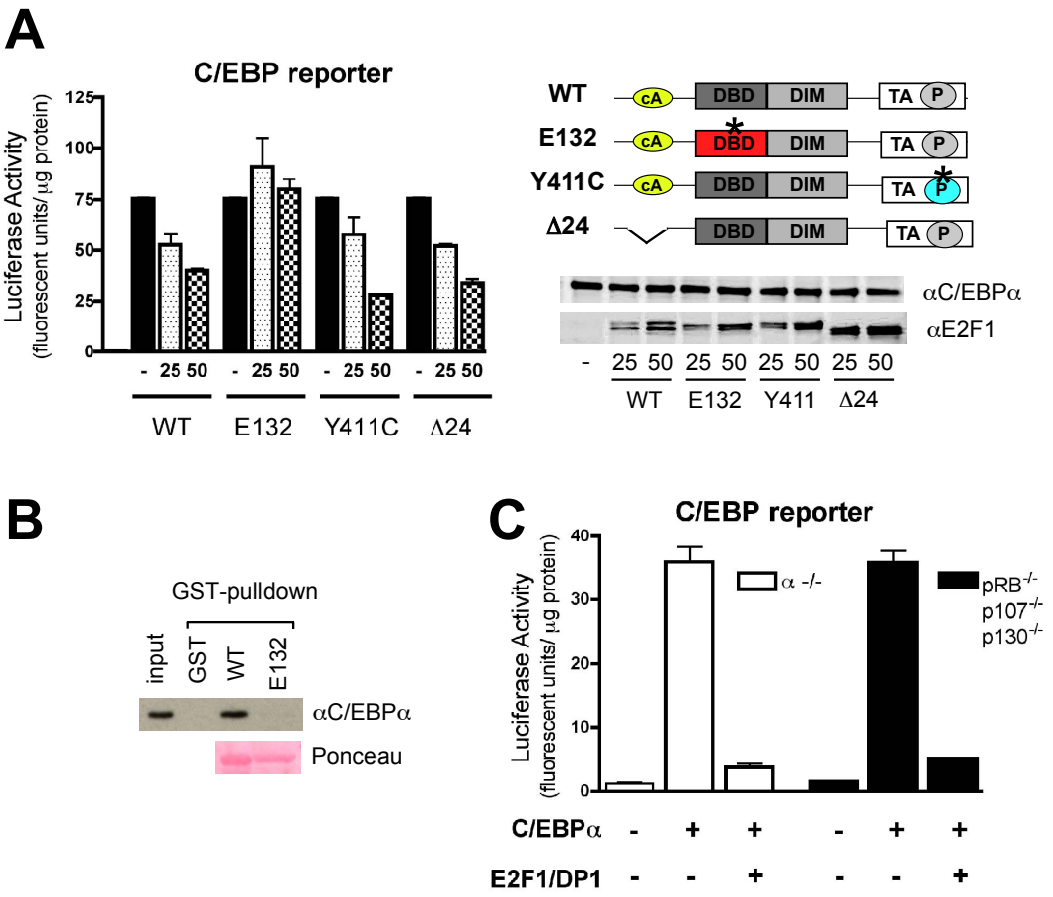

Figure 3.6: **E2F represses the transcriptional activity of C/EBPα independently of pocket proteins.** (A) Repression of C/EBPα occurs independently of cyclin A or pRB binding, but requires an intact DNA-binding domain. Different E2F1 mutants were tested in their ability to inhibit C/EBPα-mediated transcription: E132 (DNA-binding-deficient), Y411C (pRB-binding-deficient), Δ24 (cyclin A-binding-deficient). 293T cells were transfected with the C/EBP responsive cMGF promoter reporter, with C/EBPα (100 ng) and, where indicated, with E2F1 mutants (25 or 50 ng) expression constructs. Expression was tested by immunoblotting. (B) The E132 mutant fails to bind to C/EBPα. In a GST-pulldown assay, binding of C/EBPα expressed in 293T towards E2F1 WT or E2F1 E132 (GST-fusion proteins) was assessed. Bound proteins were detected by immunoblotting against anti-C/EBPα. (C) Inhibition of C/EBPα by E2F in the absence of pocket proteins. Luciferase assays done with either C/EBPα -/- MEFs or pRB-/-,p107-/-,p130-/- MEFs. Cells were transfected with C/EBP luciferase reporter and, where indicated, with C/EBPα (100ng), HA-DP1 (100ng) and HA-E2F1 (100ng) expression plasmids. All luciferase reporter assays were done in duplicate, graphed as the mean$\pm$SD. Data are representative of at least three independent experiments.

3.1.5 E2F represses the activity of C/EBPα in a pocket protein independent fashion

Next, the structural requirements of E2F on C/EBPα repression was examined. Employing E2F1 mutants that either fail to bind to DNA (E132), lack cyclin A-CDK2 binding (Δ24), or fail to bind to the retinoblastoma protein (Y411C) showed that repression of C/EBPα depends on the DNA binding domain (DBD) of E2F1, but not on the interaction between E2F and pRB or cyclinA/CDK (Figure 3.6A).

GST-pulldown interaction assays revealed that the E132 mutant fails to bind to C/EBPα (Figure 3.6B). This indicates that the repressive function of E2F on C/EBPα activity depends on protein-protein interactions, as the E2F mutant that fails to bind to C/EBPα also fails to repress gene activation.

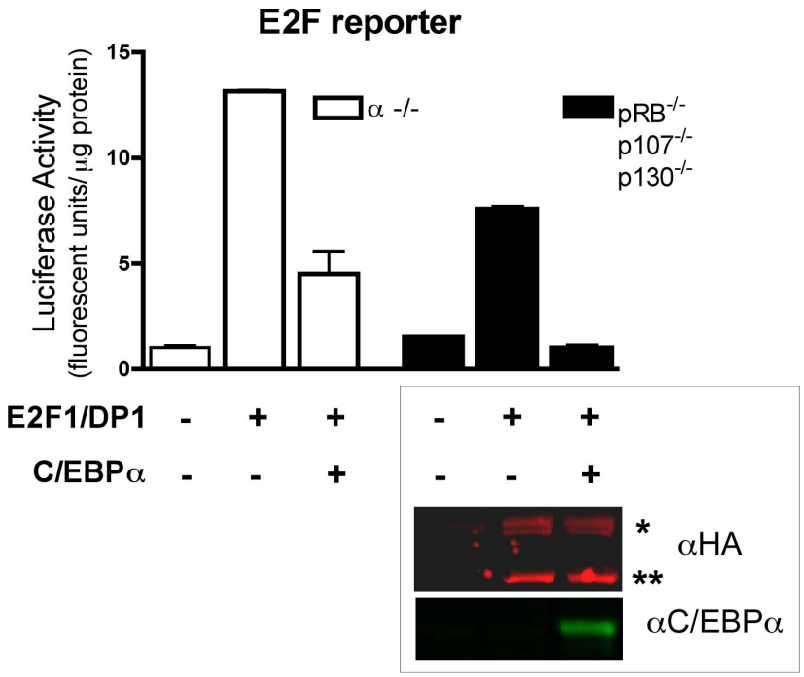

Figure 3.7: **C/EBPα-mediated repression of E2F's transcriptional activity does not require pocket proteins.** Reporter assays employing either C/EBPα -/- MEFs or pRB-/- ,p107-/-,p130-/- MEFs. Cells were transfected with an E2F-responsive luciferase reporter and where indicated with HA-E2F1 (50ng), HA-DP1 (50ng) and C/EBPα (100ng) expression plasmids. All luciferase reporter assays were done in duplicate, graphed as the mean$\pm$SD. Data are representative of at least three independent experiments. Expression was tested by immunoblotting. One asterisk marks band corresponding to E2F1, two asterisks mark band corresponding to DP1.

pRB belongs to the family of pocket proteins, including pRB, p107, and p130 that are involved in the regulation of the cell cycle and cell differentiation by sequestering "activator" E2Fs and repressing E2F target genes (Classon & Dyson, 2001). To further explore whether repression by E2F is independent of pocket protein family members, Mouse Embryonic Fibroblasts (MEFs) were employed lacking all three pocket proteins (pRB-/-,p107-/-,p130-/-) (Dannenberg et al., 2000).

In parallel, C/EBPα -/- immortalized MEFs were used. As shown in Figure 3.6C, E2F1-DP1 repressed C/EBPα to a similar extent in both C/EBPα -/- and pRB-/-,p107-/-,p130-/- MEFs. These results thus fully support the notion that E2F represses C/EBPα by a pocket protein independent mechanism. Whether the inverse repression, C/EBPα-mediated repression of E2F, requires pocket protein is under debate (Iakova et al., 2003);(Johansen et al., 2001);(Sebastian et al., 2005). Thus, pocket protein deficient MEFs were employed to determine whether repression of an E2F-responsive promoter by C/EBPα requires pocket proteins. As shown in Figure 3.7, C/EBPα repressed the transcriptional activity of E2F1-DP1 in both, C/EBPα-deficient and pocket protein deficient MEFs. Taken together, the reporter data indicate that E2F and C/EBPα repress each other's transcriptional activity independently of pocket proteins.

3.1.6 The Basic-region mutants bind DPs with different affinities

Two point mutants of the basic region of C/EBPα, BRM2 and BRM5, have been reported to display impaired repression on E2F sites and to fail to promote granulocyte and adipocyte differentiation (Porse et al., 2001). Accordingly, repression of E2F through C/EBPα is thought to be essential for differentiation of both cell types. The transcriptional activity and E2F binding capacity of these mutants is still debated (D'Alo et al., 2003);(Keeshan et al., 2003);(Miller et al., 2003)).

The transactivation by BRM2, BRM3 (a mutant largely indistinguishable from WT, (Porse et al., 2001)) and BRM5 was tested at high (100ng) and low (1ng) doses in reporter assays (Figure 3.8B). All C/EBPα mutants displayed similar transactivation potential at high plasmid concentration (Figure 3.8B, left graph). However, at low concentration, transactivation by both BRM2 and BRM5 was strongly diminished (Figure 3.8B, middle graph). This was not due to protein lability as both, BRM2 and BRM5, were expressed at similar or even slightly elevated amounts, compared to WT and BRM3 (Figure 3.8B, right graphs). Thus, transactivation by BRM2/BRM5 is more prone to inhibition by cellular factors, as transactivation by WT C/EBPα or BRM3.

To examine the possibility that repression by E2F-DP becomes rate limiting at high

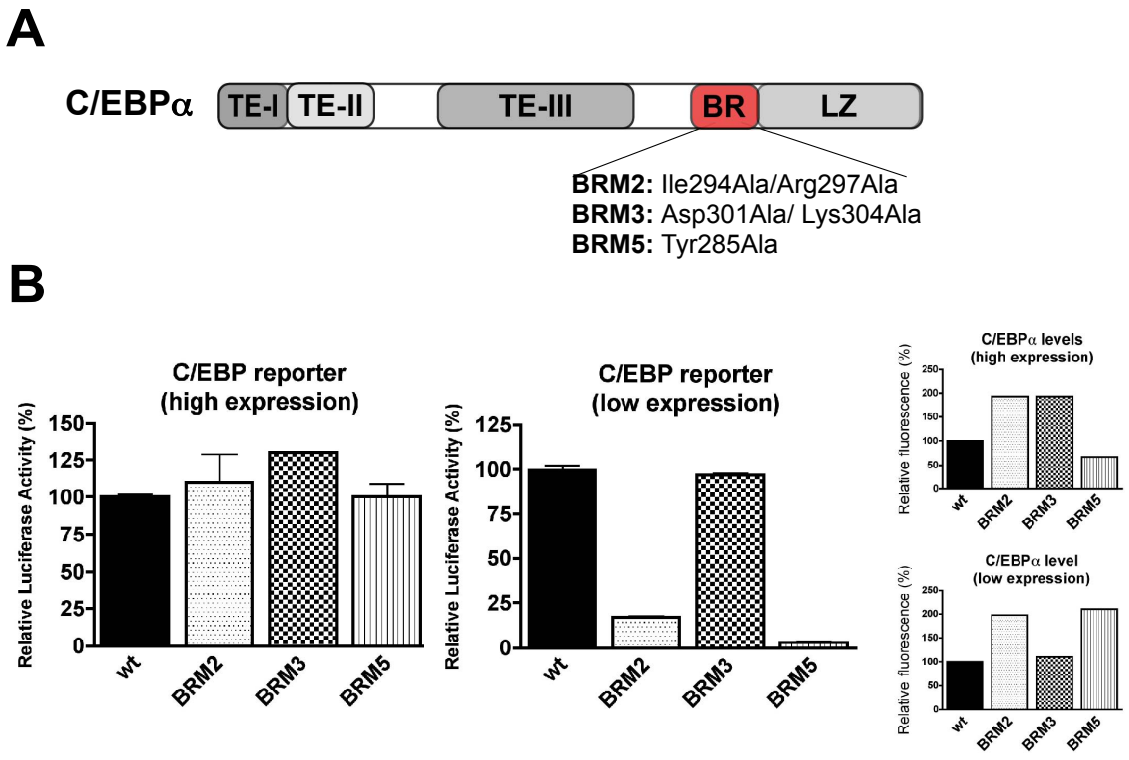

Figure 3.8: **Transcriptional activity of C/EBPα Basic-Region Mutants**. (A) Schematic representation of C/EBPα and the Basic-Region Mutants (BRMs). TE: transactivation element; BR: basic region; LZ: leucine zipper. (B) Transcriptional activation differences between BRMs and WT are concentration dependent. 293T were transiently transfected with a C/EBP-driven luciferase reporter (cMGF) and with either high amounts (100 ng) or low amounts (1 ng) of C/EBPα expression constructs. C/EBPα expression was quantified (right panels). Luciferase reporter assays were done in duplicate, graphed as the mean$\pm$SD. Data are representative of at least three independent experiments.

C/EBPα concentrations, WT or BR-mutants were transfected at high doses with or without E2F1-DP1 expression constructs (Figure 3.9A). Expression of the C/EBPα mutants alone yielded similar activities (black bars). However, BRM2 and BRM5 are much more susceptible to repression by E2F1-DP1 than WT C/EBPα or BRM3 (white bars).

Next, the affinity of BR-mutants to E2F/DP proteins was determined. GST-pulldown of bacterial expressed GST-DP1 or GST-DP2 were incubated with extracts of 293T cells expressing C/EBPα mutants (see input immunoblot, Figure 3.9A). Bound C/EBPα was quantified. Alternatively, the binding of C/EBPα mutants towards GST-E2F1 was examined. In agreement with Keeshan and colleagues (Keeshan et al., 2003), no significant difference was observed in the affinity of E2F1 to different BR-mutants relative to WT C/EBPα (Figure

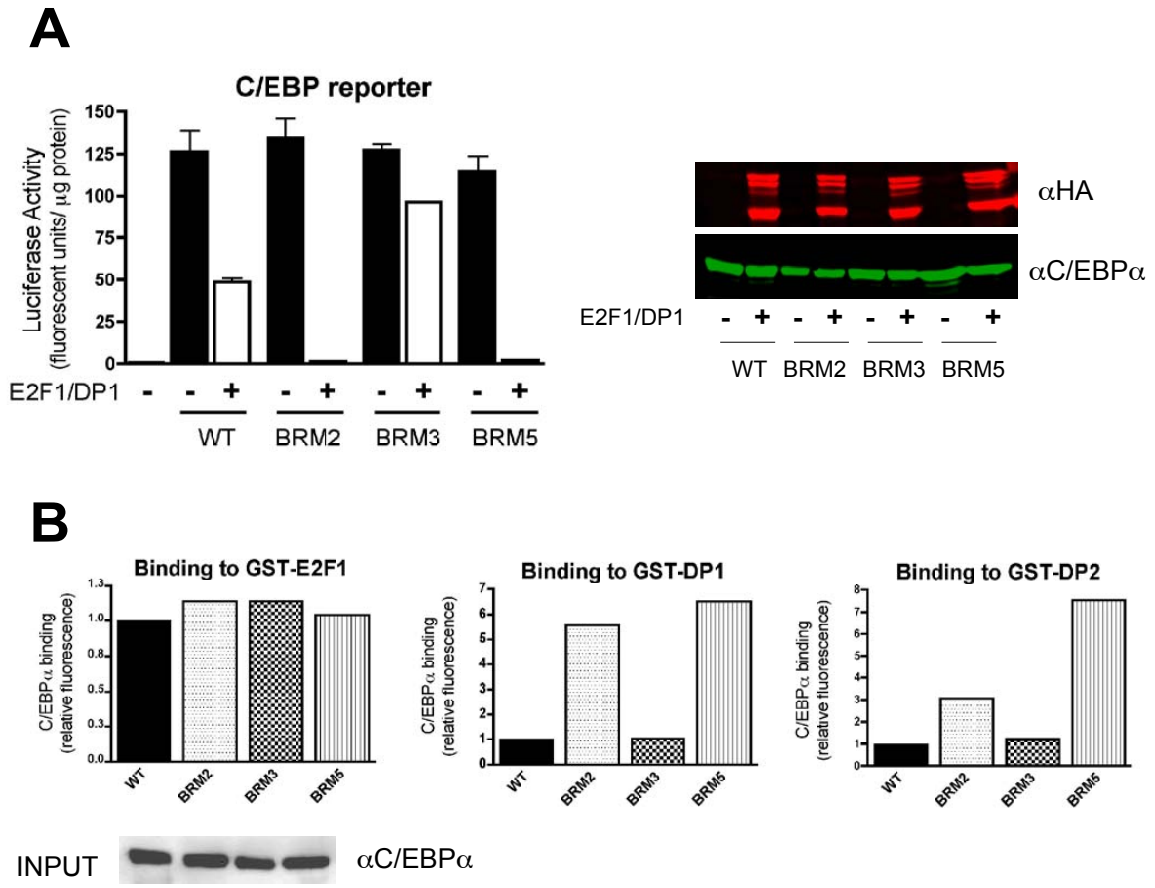

Figure 3.9: **C/EBPα Basic-Region Mutants: Enhanced binding to DP correlates with enhanced repression by E2F-DP.** (A) Repression of BRMs by E2F1-DP1 in 293T (C/EBP-luciferase reporter). Transcriptional activation by WT, BRM2, BRM3 or BRM5 (100ng) in the presence or absence of HA-E2F1/HA-DP1 (50ng each) was determined. Expression was analyzed by immunoblotting (right panel). All luciferase reporter assays were done in duplicate, graphed as the mean$\pm$SD. Data are representative of at least three independent experiments. (B) Increased binding of BRM2 and BRM5 to DPs. The binding of BRMs or WT C/EBPα expressed in 293T (input panel) towards E2F1, DP1 and DP2 (GST-fusion proteins) was examined in a GST-pulldown assay. Bound proteins were detected and quantified by immunoblotting. Results are representative of at least three independent experiments.

3.9A, left graph). In contrast, BRM2 and BRM5 interacted more avidly with DP proteins than WT or BRM3 (Figure 3.9A, middle and right graph).

Taken together, these data suggest that the transcriptional potential of the BRM2/BRM5 mutants was maintained. However, an augmented binding of these mutants to DP makes them prone to repression by E2F-DP, impairing their transcriptional activity.

C/EBPα represses E2F-mediated transcription, while BRM2/BRM5 fail to repress E2F-DP, as shown by luciferase assays in Q2bn fibroblasts (Porse et al., 2001). The reproducibility of these studies was examined employing 293T cells. Using the same E2F-responsive reporter (pE2Fx6-TATA-LUC), it could be shown in that BRM2 and BRM5 also failed to repress E2F/DP-mediated transcription in 293T cells (Figure 3.9).

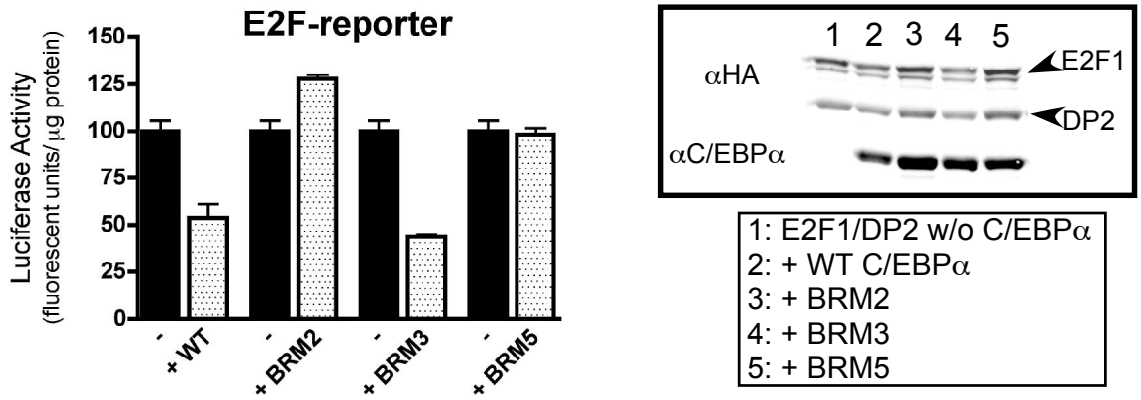

Figure 3.10: **Repression of E2F by the C/EBPα Basic-Region Mutants.** Luciferase assay using an E2F responsive promoter reporter (pE2Fx6-TATA-LUC). 293T cells were transfected with HA-E2F1 (50ng) and HA-DP2 (50ng). C/EBPα WT, BRM2, BRM3 or BRM5 were co-transfected (100ng) where indicated. Expression was analyzed by immunoblotting (right panel). Reporter assays were done in duplicate, graphed as the mean$\pm$SD. Data are representative of at least two independent experiments.

3.1.7 E2F-DP interferes with the binding of C/EBPα to its DNA recognition sites

The mutational analysis of E2F1 revealed some important facts about the molecular requirements of E2F-mediated repression of C/EBPα's transcriptional activity. However, the mechanism of this repression remained unsolved. E2F may repress C/EBPα by recruiting repressive complexes to C/EBPα-regulated promoters or by abrogating the binding of C/EBPα to DNA. To distinguish between these possibilities, gel shift assays were performed.

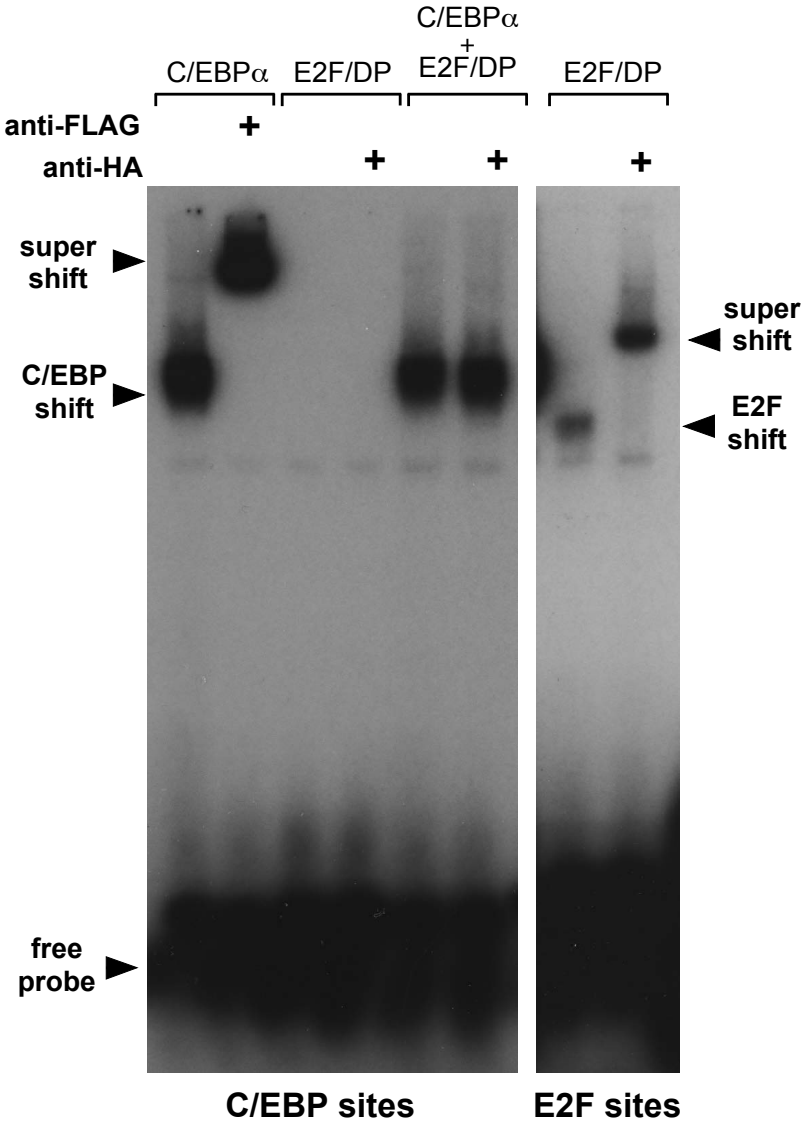

Figure 3.11: **E2F does not associate to consensus C/EBP DNA sites.** EMSAs using ^{32}P-labeled, double-stranded, oligonucleotides containing either a C/EBP binding site from the cMGF promoter (left panel) or an E2F consensus binding site (right panel). Nuclear extracts of 293T cells, transfected with FLAG-C/EBPα or with HA-tagged E2F1/DP1, were incubated with oligonucleotides and, where indicated, with either anti-FLAG or anti-HA antibodies.

The recruitment of repressive complexes would require binding of E2F to C/EBP sites, either directly or through C/EBPα. Thus, it was investigated whether E2F binds the palindromic C/EBP DNA recognition sequence, contained in the previously characterized cMGF promoter (Sterneck et al., 1992). Nuclear extracts of 293T cells transfected with HA-tagged E2F1/DP1 and/or FLAG-tagged C/EBPα expression constructs were subjected to gel retardation analysis. As shown in Figure 3.11, E2F1-DP1 did not bind to the cMGF promoter, not in the absence nor in the presence of C/EBPα (left panel). However, the C/EBP site was bound by C/EBPα as evidenced by antibody shift against the FLAG-tag of C/EBPα. As expected, E2F1-DP1 complexes associated to E2F sites and protein-DNA complexes were supershifted with an anti-HA antibody (Figure 3.11, right panel). Altogether this suggests that E2F-DP proteins do not associate to C/EBP sites.

Disruption of the DNA binding of C/EBPα could be a possible mechanism by which E2F-DP represses the transcriptional activity of C/EBPα. Mutations within the DNA-binding region of C/EBPα (BRM2 and BRM5) increase their association with DP and the efficiency in which E2F-DP abrogates their transcriptional activity (Figure 3.9A). Thus, it could be envisaged that association of E2F-DP to BRM2 interferes with the DNA binding activity of BRM2. This possibility was analyzed by assaying binding of in vitro translated WT and BRM2 to a consensus C/EBP site. As shown in Figure 3.12A, both WT and BRM2 complexed to C/EBP sites. This suggests that BRM2 has an intact DNA binding to C/EBP sites.

Next, binding activity of WT C/EBPα and BRM2 C/EBPα to C/EBP sites was assessed employing nuclear extracts of transfected 293T cells. The binding of C/EBPα to a ^{32}P-labeled C/EBP probe was competed with increasing amounts of unlabeled C/EBP (cold competitor). As shown in Figure 3.12C, the DNA-binding of BRM2 was slightly reduced compared to WT. Co-expression of E2F1-DP1 almost eliminated the DNA binding of BRM2 C/EBPα and slightly reduced the DNA binding of WT C/EBPα. Altogether, this suggests that E2F-DP proteins interfere with the DNA-binding activity of C/EBPα.

Finally, it was investigated whether addition of cold E2F-sites could restore the DNA-binding activity of C/EBPα. As shown in Figure 3.12D, the DNA binding activity of BRM2 was increased in the presence of cold E2F sites, achieving a binding activity similar to WT. In contrast, the binding of WT to DNA was slightly reduced by addition of cold E2F sites. Altogether this suggests that addition of E2F sites causes the dissociation of E2F-DP proteins from C/EBPα, rescuing the DNA-binding deficiency of BRM2.

Together these findings indicate that a correlation exists between E2F-mediated suppression of C/EBPα's transcriptional and DNA-binding activities. In the presence of E2F-DP, both the transcriptional activity and DNA-binding activity of BRM2 were strongly reduced.

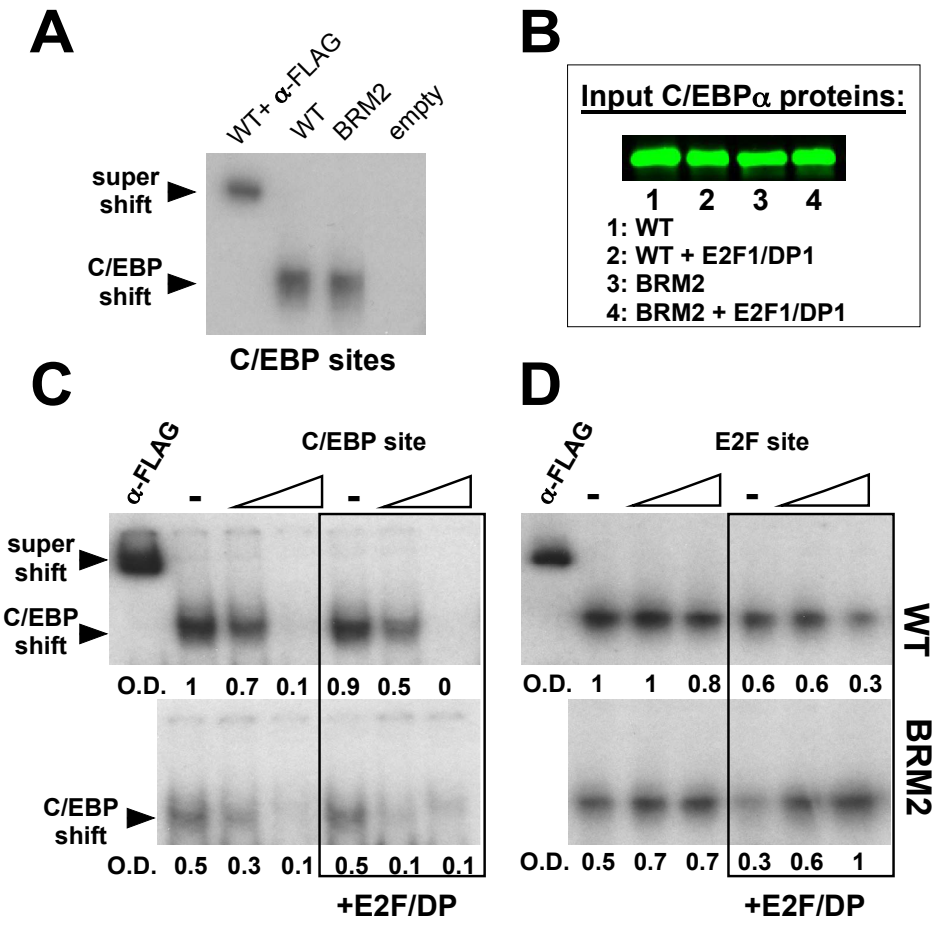

Figure 3.12: **E2F reduces the DNA-binding activity of C/EBPα.** EMSAs using ^{32}P-labeled, double-stranded, oligonucleotides containing the C/EBP binding site from the cMGF promoter. (A) In vitro translated FLAG-C/EBPα WT, FLAG-C/EBPα BRM2 or control vector pcDNA3 (empty), were incubated with oligonucleotides and, where indicated, with anti-FLAG antibody. (C+D) Nuclear extracts of 293T cells, expressing FLAG-C/EBPα (WT or BRM2) and, where indicated, HA-tagged E2F1/DP1 were incubated with oligonucleotides. "Cold C/EBP sites" (C) and "cold E2F sites" (D) refers to the addition of the unlabeled, double-stranded oligonucleotides harboring the respective binding sites. The unlabeled C/EBP oligonucleotides contained the complete palindromic C/EBP site, while the ^{32}P-labeled C/EBP oligonucleotides only contained the half-palindromic site. (B) Expression of WT and BRM2 proteins was analyzed by immunoblotting against C/EBPα.

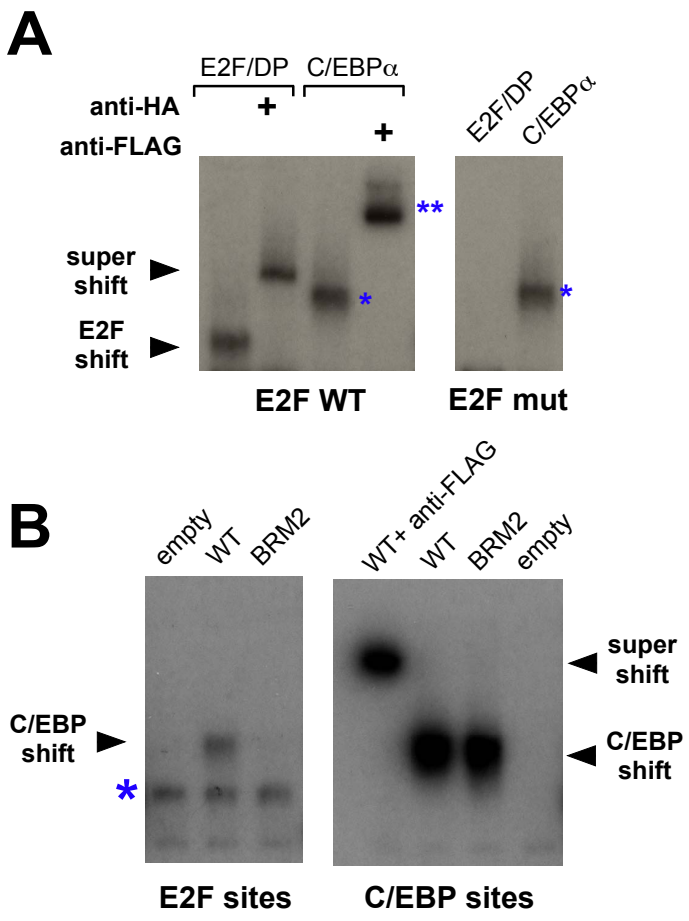

Figure 3.13: **Binding of C/EBPα to E2F sites.** (A) Nuclear extracts were incubated with ^{32}P-labeled, double-stranded, oligonucleotides harboring either an intact consensus E2F binding site (E2F WT) or a mutated version of this site (E2F mut). The asterisk marks a complex of C/EBPα protein at E2F site. Two asterisks mark the antibody-shifted complex. (B) EMSAs using ^{32}P-labeled, double-stranded, oligonucleotides containing either an E2F consensus binding site (left panel) or a palindromic C/EBP binding site from the cMGF promoter (middle panel). In vitro translated FLAG-C/EBPα WT, FLAG-C/EBPα BRM2 or control vector pcDNA3 (empty), were incubated with oligonucleotides and, where indicated, with anti-FLAG antibody. EMSAs were loaded on the same gel and detected by autoradiography (same exposure time). The asterisk marked bands are running at the same hight than over-expressed E2F1/DP1 proteins bound to E2F sites (not shown) and are thus, most probably, E2F/DP complexes contained in the reticulocyte lysate of the in vitro translation reaction.

Accordingly, outcompetition of E2F proteins restored the DNA-binding of BRM2. This suggests that E2F-DP may repress C/EBPα through disruption of its binding to target genes.

It has been suggested that E2F proteins recruit C/EBPα to E2F sites (Slomiany et al., 2000). However, recent studies have shown that E2F-regulated promoters may contain cryptic C/EBP binding sites which C/EBPα binds directly (Sebastian et al., 2005). Employing gel retardation analysis, the binding of C/EBPα to an intact E2F sites (E2F WT) or to a mutated version of this site (E2F mut) (Altiok et al., 1997) was compared. While E2F-DP only bound to wild-type E2F-site, C/EBPα bound both the mutated and the wild-type E2F site (Figure 3.13A). Thus, C/EBPα and E2F-DP proteins do not bind to the same sequence within E2F sites, suggesting that C/EBPα binding to E2F sites occurs directly and independently of E2F-DP proteins.

Next, binding of in vitro translated WT and BRM2 to E2F sites was analyzed. In agreement with previous reports (Porse et al., 2001), WT C/EBPα shifted E2F sites, while BRM2 failed to do so. The signal arising from WT C/EBPα bound to E2F sites was much weaker than WT C/EBPα bound to C/EBP sites, indicating a weaker binding affinity of C/EBPα to E2F sites than to C/EBP sites. This suggests that the binding of BRM2 to E2F sites may be more sensitive to disruption than the binding of BRM2 to C/EBP sites.

3.1.8 Rescue of BRM2 mediated adipogenesis by E2F/DP knockdown

BRM2 knockin mice lack white adipose tissue and BRM2, in contrast to WT C/EBPα, fails to induce adipogenic differentiation in NIH3T3 fibroblasts (Porse et al., 2001). The data presented in this work suggest that the failure of BRM2 to induce differentiation is due to super-repression by E2F-DP complexes.

Immortalized C/EBPα deficient fibroblasts were used to determine whether reduction of E2F/DP rescues the adipogenesis defect of BRM2. These cells are unable to differentiate to adipocytes when treated with standard inducers (Z. Wu et al., 1999). Activation of the adipogenic transcription factor *PPAR*γ and differentiation into fat cells were restored by expression of WT C/EBPα but not by BRM2 in C/EBPα deficient cells (Figure 3.14B and C). Expression of C/EBPα was monitored by immunoblotting. BRM2 expression levels were higher than WT C/EBPα, as reported for transduced NIH3T3 (Porse et al., 2001), while WT expression was slightly higher than the endogenous C/EBPα levels in NIH3T3-L1 differentiated to adipocytes. Furthermore, in contrast to WT C/EBPα, BRM2 failed to slow down proliferation as determined by colony forming assay (Figure 3.14A).

Reduction of E2F/DP levels was accomplished by small interference RNA (siRNA). For this purpose the psiRNA vector was employed which expresses hairpin siRNA (shRNA).

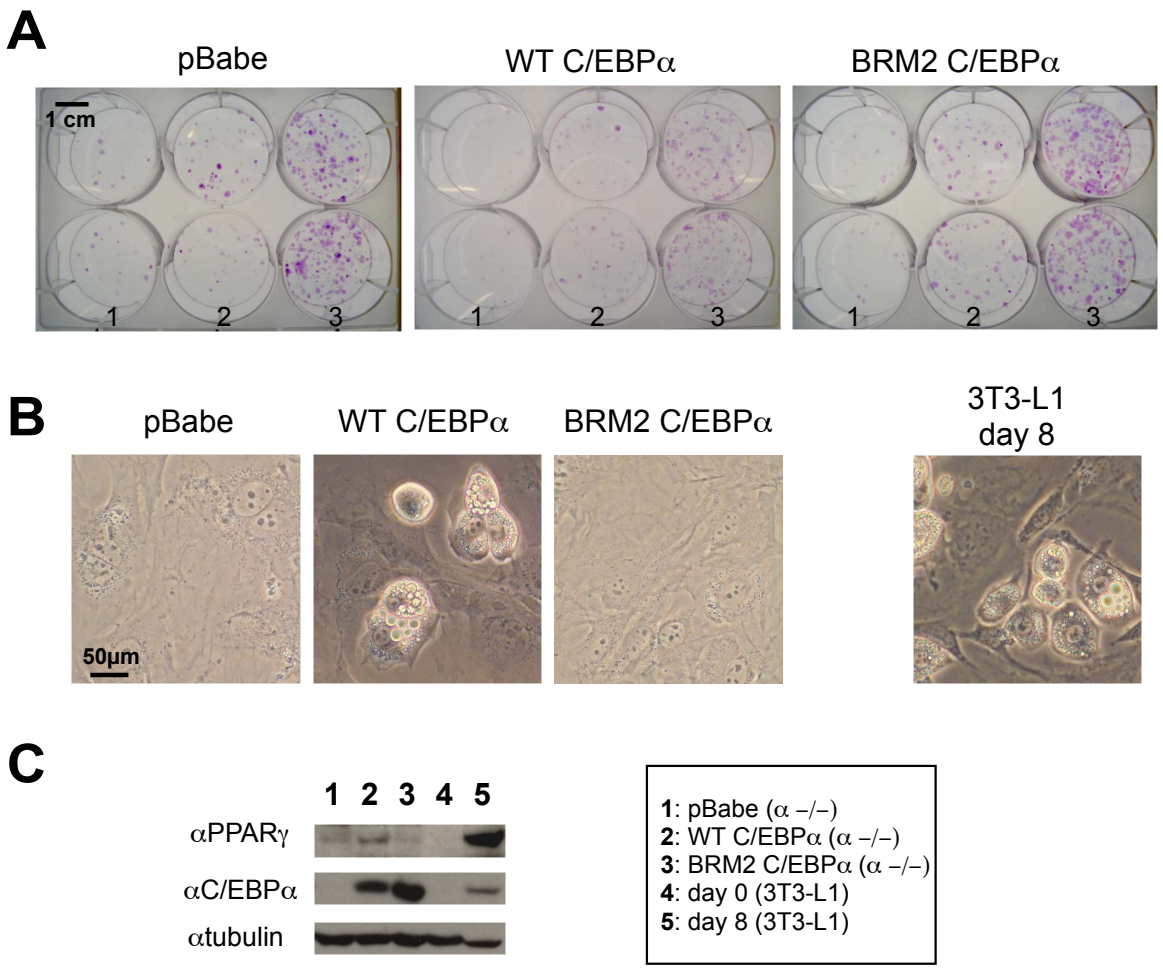

Figure 3.14: **The C/EBPα basic-region mutant BRM2 fails to arrest proliferation and adipogenesis in C/EBPα deficient MEFs.** (A) Cell proliferation of immortalized C/EBPα -/- MEFs transduced with either control vector (pBabe), wild-type (WT) or BRM2 C/EBPα, plated in duplicate: 50 cells (1), 200 cells (2) or 1000 cells (3). Cells were fixed and stained with crystal violet after 2 weeks under puromycin selection. (B+C) Adipogenesis of 3T3-L1 or C/EBPα -/- MEFs transduced with either control vector (pBabe), WT or BRM2 C/EBPα. After 8 days insulin/IBMX/DEX treatment, images were taken (B) and protein extracts were analyzed by immunoblotting (C). Expression of the adipocyte marker PPARγ and C/EBPα was analyzed. Untreated cells were used as negative control and αtubulin expression analyzed as a loading control.

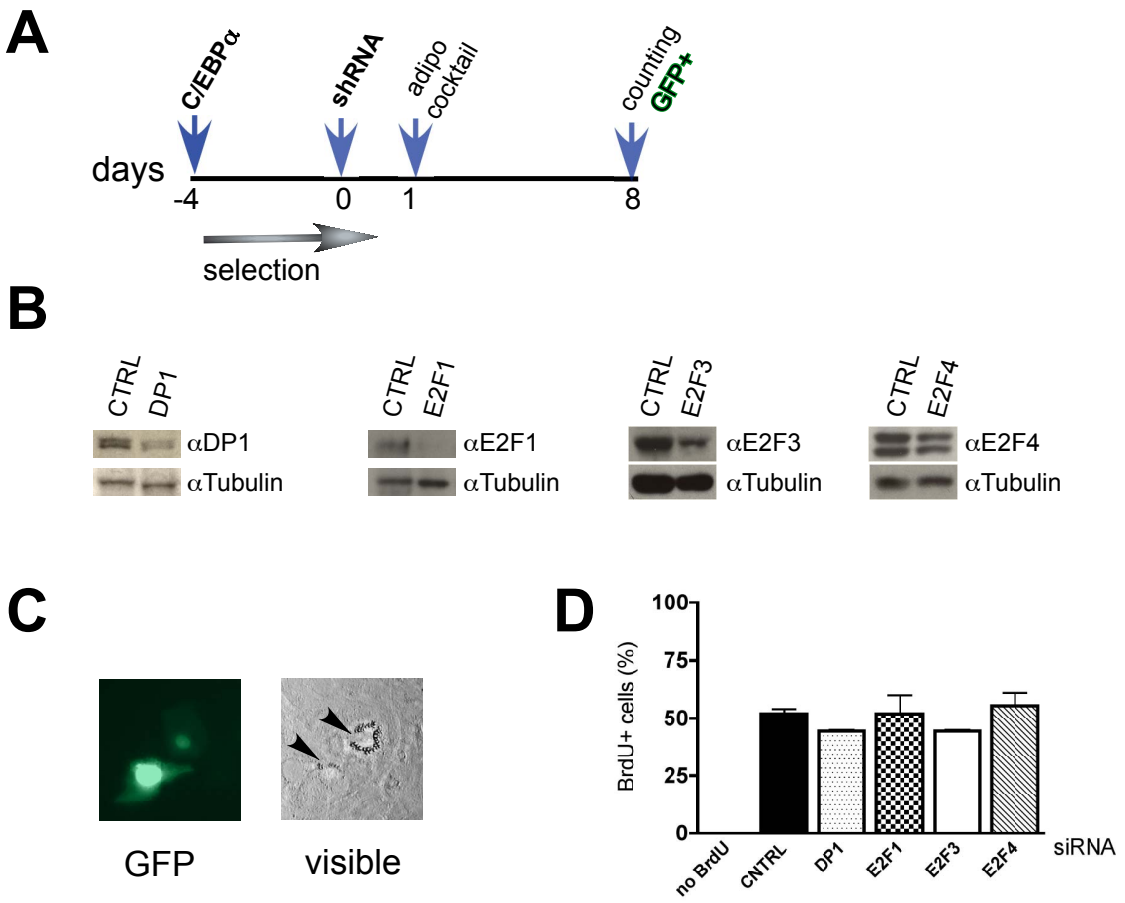

Figure 3.15: **Vector-mediated small interference RNA (siRNA) in C/EBPα -/- MEFs.** (A) Schematic representation of the experimental set-up. Immortalized C/EBPα-/- MEFs transduced with C/EBPα retroviral constructs (or control vector) were selected with puromycin. Once selection was completed, cells were transfected with psiRNA constructs (IRES GFP) against DP1, E2F1, E2F3, E2F4 or control siRNA (day 0). On day 1, the adipogenic stimulation cocktail (insulin/IBMX/DEX) was added. At day 8, adipocytes were quantified (see Figure 3.16). (B) Reduction of the targeted proteins was confirmed by immunoblotting analysis of GFP-positive sorted cells (72 hours post-transfection). (C) Representative picture of WT transduced C/EBPα-/- MEFs transfected with shRNA against E2F3. (D) Quantification of BrdU-incorporating GFP-positive cells. C/EBPα -/- MEFs transduced with WT C/EBPα were transfected with psiRNA constructs. 80 hours post-transfection, cells were grown in BrdU. BrdU incorporation of GFP-positive cells was determined by indirect immunofluorescence using an anti-BrdU antibody. Values represent mean of duplicates$\pm$SD.

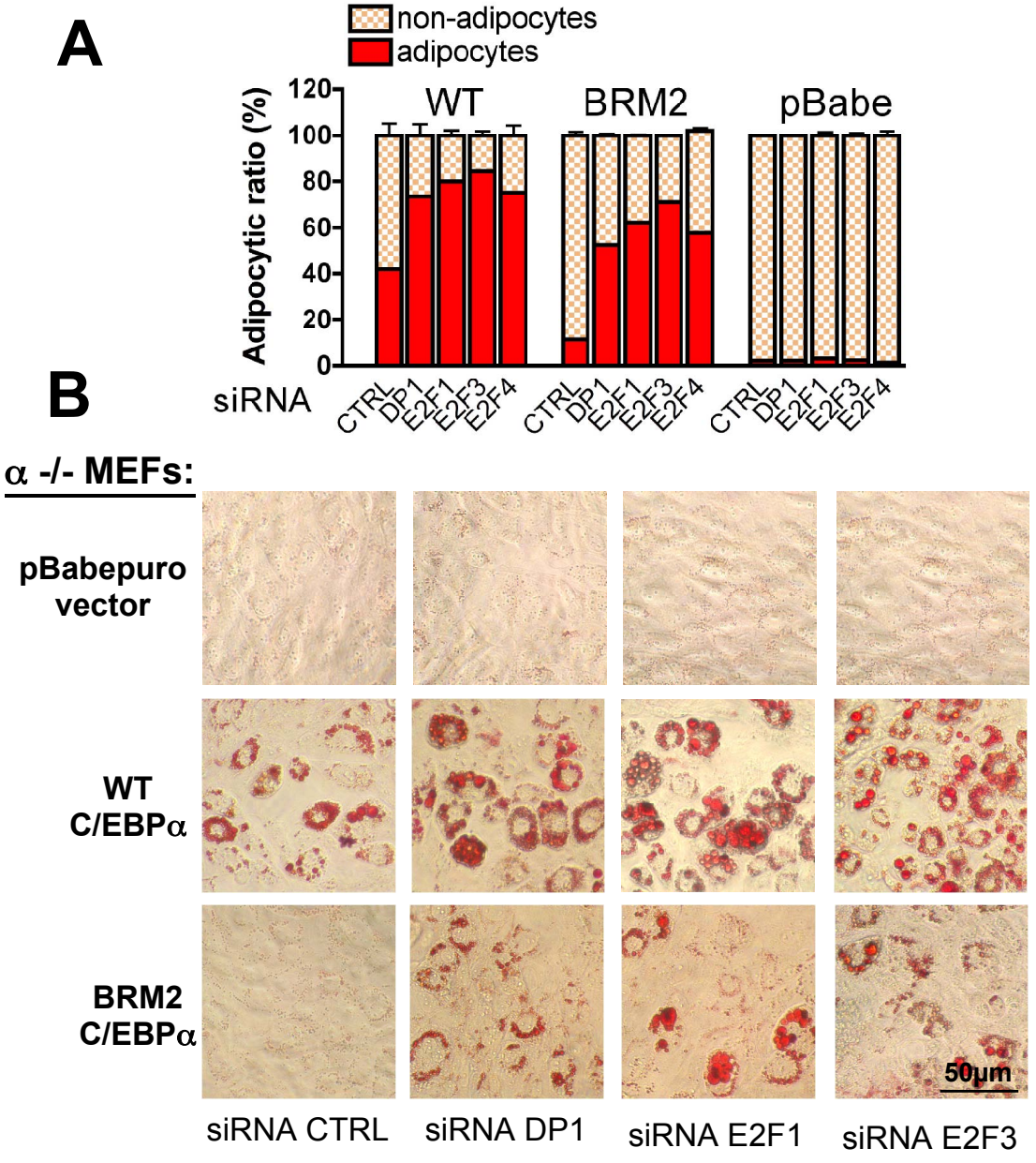

Figure 3.16: **Knock-down of DP/E2F enhances C/EBPα-mediated adipogenesis.** Adipogenesis of immortalized C/EBPα-/- MEFs, transduced with either control vector (pBabe), wild-type (WT) or BRM2 C/EBPα, and transfected with psiRNA constructs (IRES GFP) against DP1, E2F1, E2F3, E2F4 or control siRNA. After eight days insulin/IBMX/DEX treatment, GFP positive cells were quantified as adipocytes or non-adipocytes (A) and stained with Oil-Red-O (B). A minimum of 400 cells was counted in duplicate and graphed as the mean$\pm$SD. Data are representative of two independent experiments.

C/EBPα -/- MEFs were transfected with psiRNA against DP1, E2F1, E2F3 or E2F4. As a negative control, a non-specific siRNA was used. An IRES-GFP within the psiRNA constructs allowed identification of the transfected cells. Knock-down of targeted proteins was confirmed by sorting the transfected cells and analyzing the levels of DP or E2F proteins (Figure 3.15B).

Next, C/EBPα -/- MEFs transduced with WT C/EBPα, BRM2 C/EBPα, or control vector were transfected with psiRNA constructs encoding the different shRNA. 24 hours post-transfection cells were hormonally induced to adipocytes. After eight days, cells were examined for GFP expression. GFP positive cells were counted and classified as either adipocytes or non-adipocytes according to their morphology. Phase contrast microscopy allowed a clear recognition of accumulated fat within the adipocytes. Reduction of endogenous levels of DP1, E2F1, E2F3 or E2F4 enhanced WT C/EBPα induced adipogenesis (Figure 3.16A and B). Furthermore, cells expressing BRM2 could differentiate to adipocytes when E2F/DP were knocked-down, although generally with a lower fat content than WT transduced cells. However, no adipocytic differentiation was observed with shRNAs in C/EBPα deficient cells (Figure 3.16A and B). These results demonstrate the requirement of C/EBPα for adipogenic differentiation and exclude off-target effects of the shRNA constructs.

To determine whether siRNA of E2F/DP affects proliferation, long-term BrdU incorporation of shRNA-transfected cells was assessed. No significant differences in BrdU incorporation were observed in cells transfected with shRNA against control or E2F/DP (Figure 3.15D).

Taken together, these results show that the knock-down of E2F/DP favors formation of adipocytes in a C/EBPα-dependent fashion. Moreover, the data suggest that the BRM2 mutant maintains differentiation potential that is is unleashed by removal of E2F/DP. Finally, the siRNA against DP or E2F used in this study did not affect proliferation, allowing a cell cycle independent analysis of E2F function.

3.2 The methyltransferase SUV39H1 and the transcription factor C/EBPα

3.2.1 Working hypothesis

Over the last decade, the regulation of transcription by histone modification and chromatin remodeling has been extensively studied. While C/EBPα and C/EBPβ are associated with chromatin-remodeling activities, the H3K9-methylating activity of SUV39H1 establishes heterochromatic silenced areas by recruiting HP1. C/EBPα and C/EBPβ progressively accumulate to heterochromatic areas during adipocytic differentiation, suggesting that C/EBPs may accomplish an differentiation relevant function at these sites (Tang & Lane, 1999). The SUV39H1-induced heterochromatinization is believed to be important for cell differentiation (Ait-Si-Ali et al., 2004) and for senescence (Braig et al., 2005). Senescence, in turn, has been shown to require the presence of C/EBPβ (Sebastian et al., 2005) and of SUV39H1 (Braig et al., 2005). Taken together, these data suggest that an interplay between SUV39H1, C/EBPs and heterochromatin may exist during differentiation processes or senescence. This work investigated the possible involvement of SUV39H1 in the association of C/EBPs to heterochromatic areas.

3.2.2 C/EBPα and C/EBPβ interact with SUV39H1

Alternative translation initiation from a single mRNA generates three C/EBPβ isoforms (LIP, LAP and LAP*) and two C/EBPα isoforms (p42 and p30), as depicted in Figure 3.17. The ability of C/EBPs to interact with SUV39H1 was analyzed by GST-pulldown assay of GST-SUV39H1 incubated with C/EBPα and C/EBPβ isoforms expressed in 293T cells. As shown in Figure 3.17A, both C/EBPα isoforms interacted with SUV39H1. Moreover, C/EBPβ LAP* and LAP proteins, but not LIP, were found to associate with SUV39H1 (Figure 3.17B). In summary, SUV39H1 binds to the long isoforms of both C/EBPα and C/EBPβ. However, while SUV39H1 interacts with the amino-terminal truncated C/EBPα isoform p30, it fails to bind to the amino-terminal truncated C/EBPβ isoform LIP.

3.2.3 SUV39H1 causes nuclear relocalization of C/EBPα

SUV39H1 creates a methylation mark on H3K9 that induces the recruitment of HP1. Since C/EBPα has been shown to localize to heterochromatic areas enriched in trimethylated H3K9 (Berberich-Siebelt et al., 2006), the role of SUV39H1 on C/EBP localization was analyzed. 293T cells were transiently transfected with C/EBPα p42 and either HA-SUV39H1 or

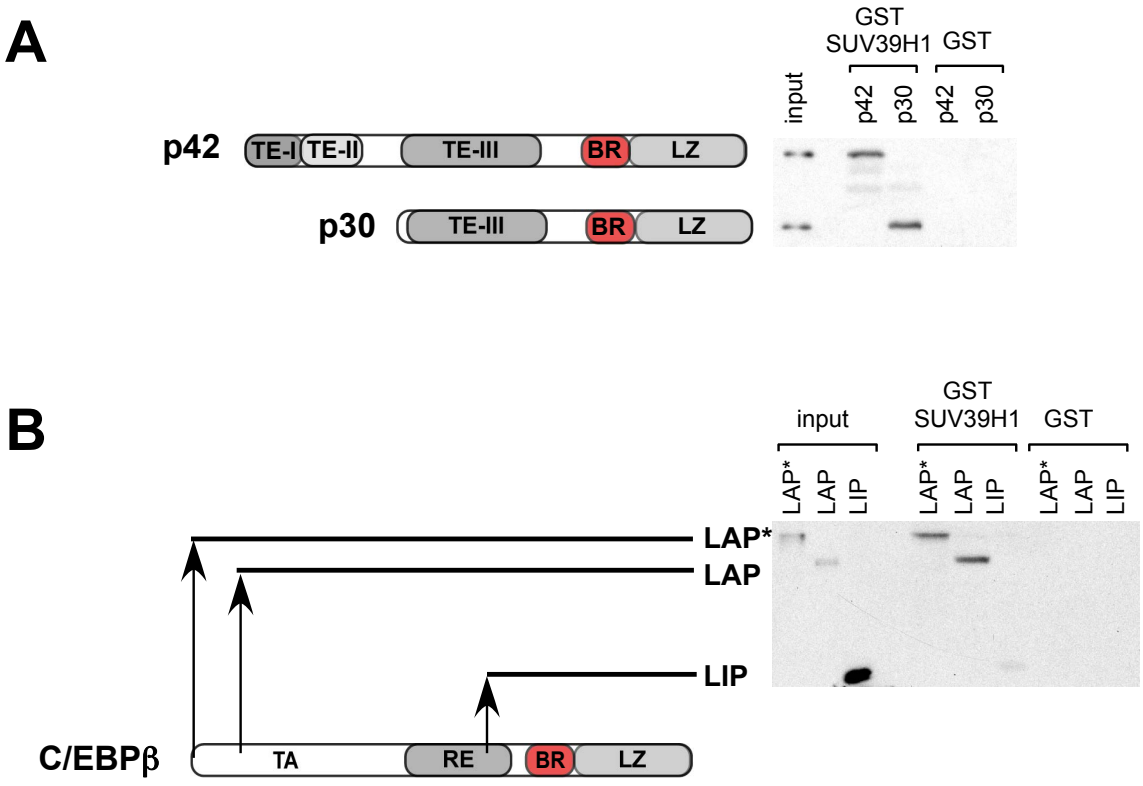

Figure 3.17: **SUV39H1 interacts with C/EBPα and C/EBPβ.** (A) Schematic representation of C/EBPα isoforms generated from alternative translation initiation sites. The p42 isoform corresponds to full-length C/EBPα, while p30 has an amino-terminal truncation. GST-pulldown of GST-SUV39H1 in the presence of extracts from 293T expressing either C/EBPα isoforms p42 or p30. Bound proteins were detected by anti-C/EBPα immunoblotting. (B) C/EBPβ isoforms generated from alternative translation initiation sites. Interaction of GST-SUV39H1 with C/EBPβ isoforms LAP*, LAP or LIP expressed in 293T cells was analyzed. Bound proteins were detected by anti-C/EBPβ immunoblotting.

control expression constructs. 48 hours post-transfection, ectopic protein localization was analyzed by indirect immunofluorescence. In interphase cells, overexpressed C/EBPα displayed a homogenous nuclear distribution, sparing the nucleoli (Figure 3.18A). However, upon co-expression of SUV39H1, C/EBPα was concentrated to defined nuclear foci (approx. 20-23 foci). In most cases C/EBPα foci localized around the nucleoli. Quantification data confirmed that in the absence of SUV39H1, the punctuated distribution of C/EBPα was a rare event, while in the presence of SUV39H1, C/EBPα foci appeared in most cells (Figure 3.18B). SUV39H1 displayed a broad nuclear distribution sparing the nucleoli. This localization was unaffected by the presence of C/EBPα.

Next, it was determined whether C/EBPβ also relocalized upon SUV39H1 co-expression. C/EBPβ LAP* was co-transfected with HA-SUV39H1 or control vector and visualized by indirect immunofluorescence. C/EBPβ displayed a nuclear homogenous distribution similar to C/EBPα. However, co-expression of SUV39H1 did not induce the formation of C/EBPβ foci (Figure 3.18B).

Taken together, ectopic expression of SUV39H1 induces the concentration of C/EBPα at discrete nuclear foci. Despite the fact that both C/EBPα and C/EBPβ interact with SUV39H1, the distribution of C/EBPβ remains unaltered by Suv39h1.

As the interaction studies in Figure 3.17A showed, both the long and the truncated p30 isoforms associated with SUV39H1. The capacity of SUV39H1 to form p30 C/EBPα foci was assessed by co-expression of p30 in the presence or absence of HA-SUV39H1. As shown in Figure 3.19B, SUV39H1 failed to induce the formation of p30-enriched foci. However, a C/EBPα mutant lacking a central region comprising amino acids 126 till 200 (Figure 3.19A)(Pedersen et al., 2001), formed foci upon SUV39H1 co-expression (Figure 3.19B). Together these data indicate that, while the amino-terminus of C/EBPα is dispensable for interaction with SUV39H1, it is required for the formation of SUV39H1-induced C/EBPα foci.

3.2.4 The HMTase activity of SUV39H1 is required to alter the nuclear localization of C/EBPα

The carboxy-terminal SET domain of SUV39H1 provides the catalytic domain responsible for the HMTase activity (Figure 3.20A). A point mutation within the SET domain, the substitution of Histidine 324 to Leucine (H324L), abolishes the HMTase activity of SUV39H1 (Lachner et al., 2001). Another evolutionary conserved domain within SUV39H1 is the amino-terminal chromo-domain, a protein-specific interaction surface that resembles an ancient histone-like fold (Ball et al., 1997). The SUV39H1 chromo-domain comprises the interaction surface with HP1 (Melcher et al., 2000). NChromo is a deletion construct lacking the carboxy-terminus

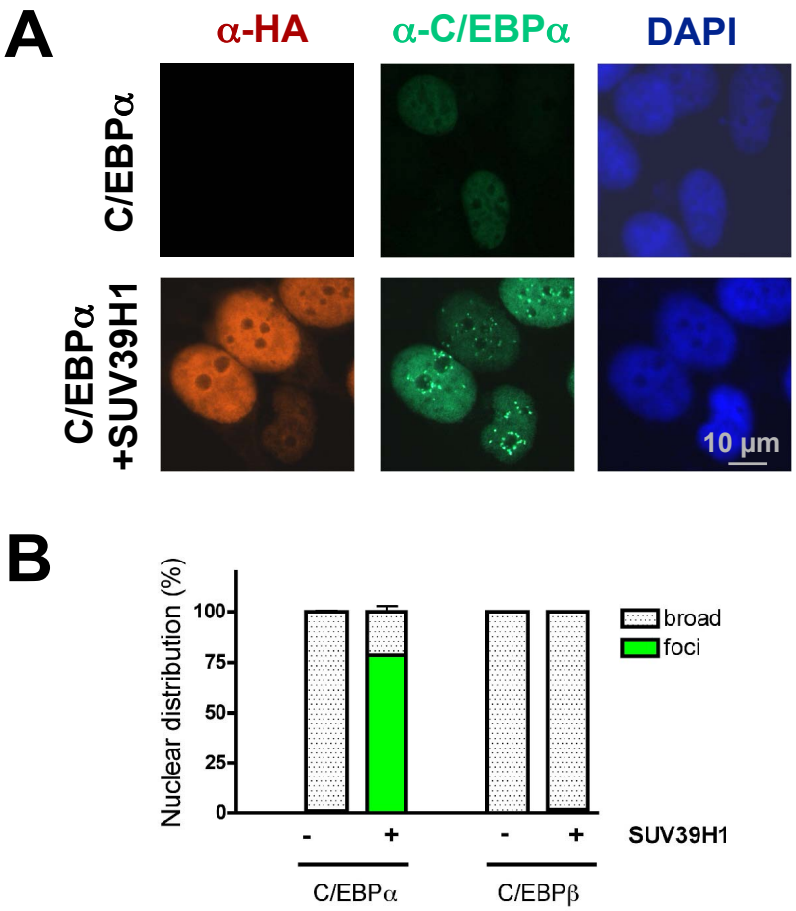

Figure 3.18: **SUV39H1 alters the nuclear distribution of C/EBPα, but not of C/EBPβ.** (A) Immunofluorescence staining of 293T cells transfected with C/EBPα alone or together with HA-tagged SUV39H1. Cells were co-stained with rabbit polyclonal antibodies directed against C/EBPα (secondary green) and mouse monoclonal anti-HA (secondary red). Nuclei were counterstained with DAPI. (B) Quantification of cells forming nuclear C/EBPα or C/EBPβ foci in the absence or presence of SUV39H1. Cells were transfected and stained as described above. Alternatively, cells were co-transfected with C/EBPβ and SUV39H1 and co-stained with rabbit anti-C/EBPβ (secondary green) and mouse anti-HA (secondary red). The double labeling allowed controlling for co-expression of SUV39H1. A minimum of 200 cells was counted in duplicate and graphed as the mean$\pm$SD. Data are representative of two independent experiments.

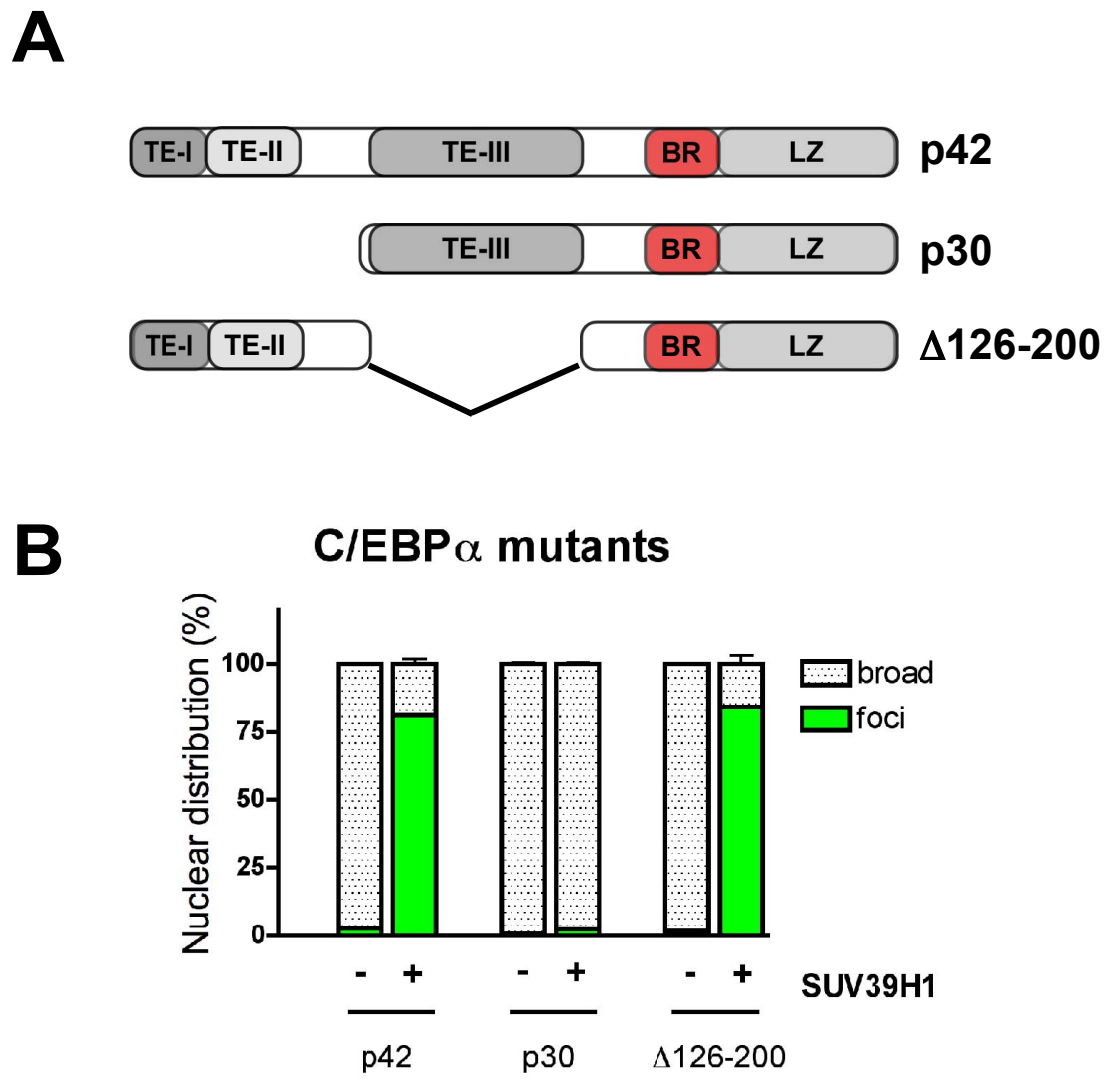

Figure 3.19: **The N-terminus of C/EBPα is required for SUV39H1-induced foci formation.** (A) Schematic representation of C/EBPα isoforms p42, p30 and the Δ126-200 deletion mutant. (B) Ability of different C/EBPα constructs to form nuclear foci in the absence or presence of SUV39H1. 293T cells transfected with C/EBPα constructs (p42, p30 or Δ126-200) in the absence or presence of HA-tagged SUV39H1 were co-stained with rabbit polyclonal antibodies directed against C/EBPα (secondary green) and mouse monoclonal anti-HA (secondary red). Nuclei were counterstained with DAPI. The double labeling allowed controlling for co-expression of SUV39H1. A minimum of 200 cells was counted in duplicate and graphed as the mean$\pm$SD. Data are representative of two independent experiments.

of SUV39H1 and consequently the HMTase activity. Myc-tagged version of the two HMTase deficient mutants NChromo and H324L were tested in their ability to induce C/EBPα foci formation (Figure 3.20A). As shown in Figure 3.20, WT SUV39H1 relocalizes C/EBPα, while both H324L and NChromo SUV39H1 failed to do so. In agreement with reported data (Melcher et al., 2000), the SUV39H1 mutants remained accumulated at discrete areas, while WT SUV39H1 displayed a broad distribution.

Enhancer of Zeste 2 (EZH2) belongs to the SET domain containing proteins with H3 methyltransferase activity. While SUV39H1 methylates H3K9, methylation of K27 has been attributed to EZH2 (Czermin et al., 2002);(Kuzmichev et al., 2002). It was examined whether redistribution of C/EBPα was specific for SUV39H1 or could be induced by EZH2. As shown in Figure 3.21, transient transfection of EZH2 had no effect on the distribution of C/EBPα, hence, EZH2 does not induce C/EBPα foci formation.

Methylation of H3K9 by SUV39H1 has been proposed to trigger the accumulation of HP1 at heterochromatic foci (Bannister et al., 2001);(Lachner et al., 2001). Remarkably, C/EBPα has been shown to be enriched at H3K9 trimethylated heterochromatic foci (Berberich-Siebelt et al., 2006). Thus, it could be possible that trimethylation of H3K9 catalyzed by SUV39H1 creates a binding platform for C/EBPα at heterochromatic foci. This possibility was analyzed by staining C/EBPα foci for trimethylated H3K9 or HP1. First, HA-SUV39H1 and FLAG-C/EBPα co-transfected cells were double labeled with anti-FLAG and anti-HP1β. In agreement with SUV39H1 overexpression studies, in SUV39H1-C/EBPα expressing cells, HP1β foci disassembled adopting a dispersed nuclear distribution (Figure 3.22A). Thus, no HP1β enrichment was observed in the C/EBPα foci. The homologous HP1γ member was also not accumulated at C/EBPα foci (data not shown). Staining of cells for tri- or dimethylated H3K9 (Figure 3.22B and data not shown) revealed that C/EBPα foci did not contain increased levels of these H3 methylation patterns. Finally, a panel of methylated histone residues were examined for localization with C/EBPα foci, including trimethylated H3K27, di- and trimethylated H3K4, and methylated H3K17. However, none of those methylated histones were enriched at C/EBPα foci (data not shown).

Together these data indicate that an intact and functionally active SET domain of SUV39H1 is required for the accumulation of C/EBPα into foci (Figure 3.20). The induction of these foci is specific for SUV39H1 and cannot be accomplished by other SET-domain histone methyltransferase. However, C/EBPα does not form classical heterochomatic foci, since it is not enriched in neither trimethylated H3K9 nor HP1.

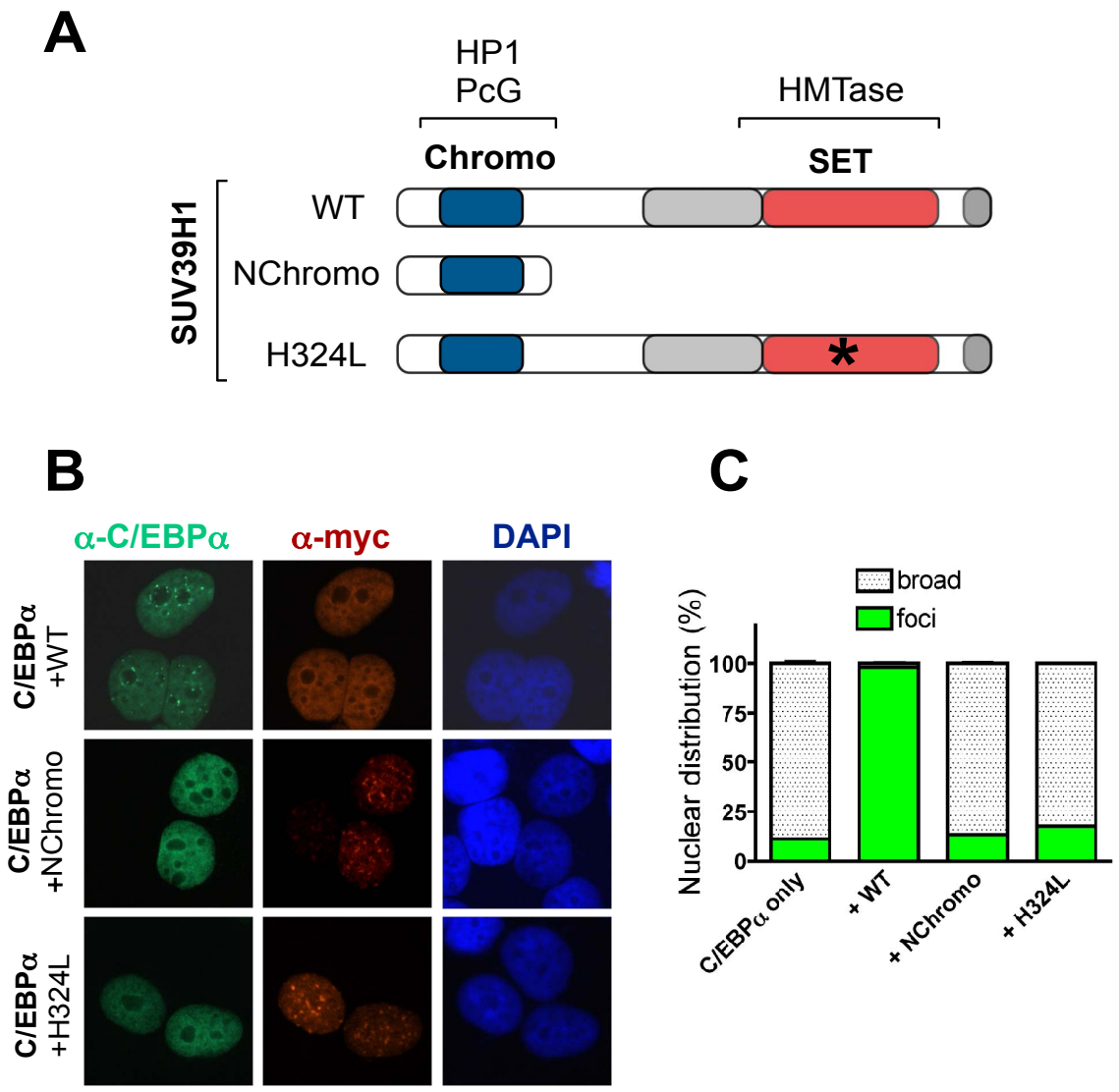

Figure 3.20: **The Methyltransferase Activity of SUV39H1 is required for induction of C/EBPα foci.** (A) Schematic representation of SUV39H1 wild-type (WT), NChromo and H324L mutants. (B) Immunofluorescence staining of 293T cells transfected with C/EBPα alone or together with triple-myc-tagged SUV39H1 constructs. Cells were co-stained with rabbit polyclonal antibodies directed against C/EBPα (secondary green) and mouse monoclonal anti-myc (secondary red). Only WT, but not NChromo or H324L SUV39H1, induces C/EBPα foci formation. (C) Quantification of cells stained as in (A) containing C/EBPα foci in the absence or presence of SUV39H1. The double labeling allowed controlling for co-expression of SUV39H1. A minimum of 200 cells was counted in duplicate and graphed as the mean$\pm$SD. Data are representative of two independent experiments.

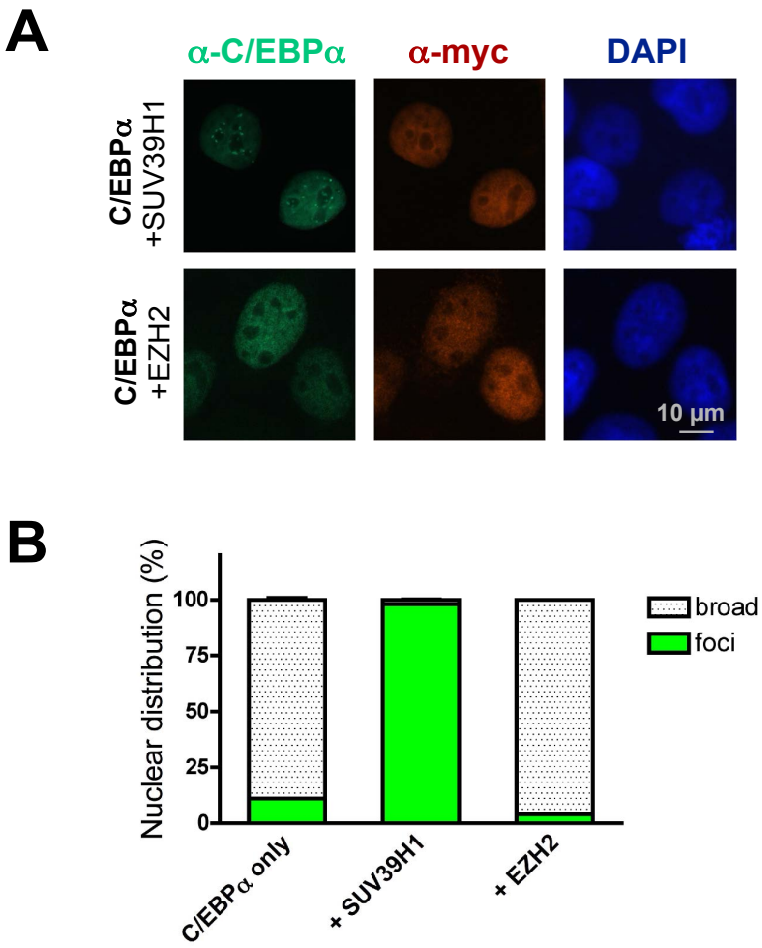

Figure 3.21: **The Histone-Methyltransferase EZH2 fails to induced C/EBPα foci.** Immunofluorescence staining of 293T cells transfected with C/EBPα and with either myc-tagged SUV39H1 or myc-EZH2. (A) Cells were co-stained with rabbit polyclonal antibodies directed against C/EBPα (secondary green) and mouse monoclonal anti-myc (secondary red). Nuclei were counterstained with DAPI. (B) Quantification of cells stained in (A), containing C/EBPα foci in the presence of either SUV39H1 or EZH2. The double labeling allowed to control for co-expression of SUV39H1 or EZH2. A minimum of 200 cells was counted in duplicate and graphed as the mean$\pm$SD. Data are representative of two independent experiments.

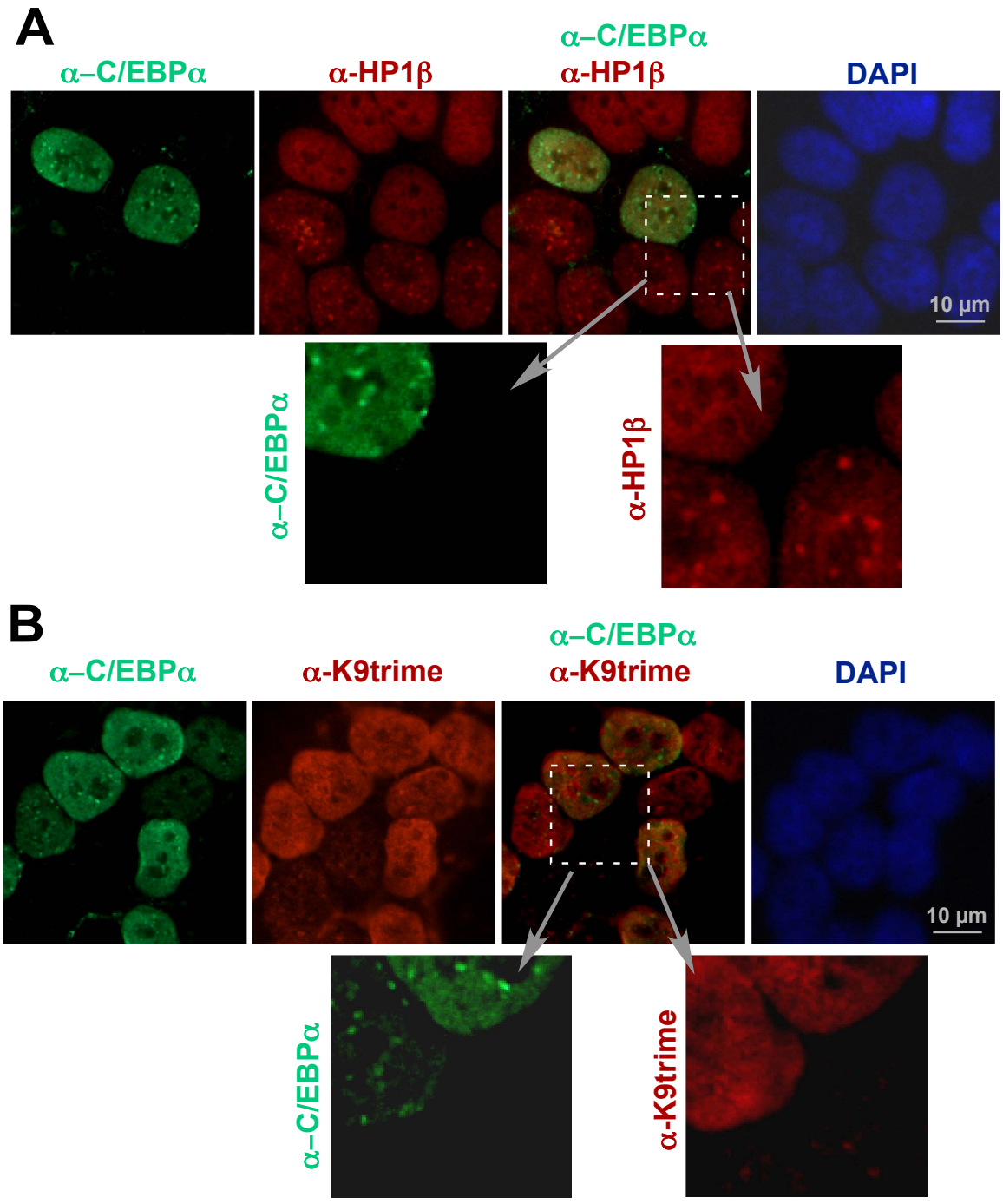

Figure 3.22: **SUV39H1-induced C/EBPα foci are not enriched in HP1β nor trimethylated H3K9.** Immunofluorescence staining of 293T cells co-transfected with FLAG-C/EBPα and HA-SUV39H1. (A) Cells were co-stained with rabbit polyclonal antibodies directed against HP1β (secondary red) and FLAG (secondary green). (B) Double labeling with anti-trimethylated H3K9 (secondary red) and anti-FLAG (secondary green). Nuclei were counterstained with DAPI.

3.2.5 Nuclear bodies

To determine the nature of the C/EBPα foci, other proteins that associate with distinct nuclear focal structures and that have a known connection with SUV39H1 were tested for colocalization. PML (promyelocytic leukemia) proteins form PML nuclear bodies and have been shown to interact with SUV39H1 (Chakraborty et al., 2003). Thus, it was assessed whether PML bodies colocalize with C/EBPα foci. Cells co-transfected with C/EBPα and HA-SUV39H1 were double stained with an antibody directed against PML and an anti-C/EBPα antibody. As shown in Figure 3.23A, the C/EBPα foci and PML bodies did not colocalize.

Overexpression of SUV39H1 in human cells causes the relocalization of components of the Polycomb repressive complexes 1 (PRC1) (Sewalt et al., 2002). The endogenous components of PRC1, as for example Bmi1 or RING1, display a fine granular nuclear distribution. However, upon stable ectopic expression of SUV39H1, Bmi1 and RING1 concentrate and colocalize at defined nuclear domains. These enriched domains are denominated polycomb bodies (Saurin et al., 1998). To assess whether C/EBPα foci accumulate at polycomb bodies, cells were co-stained against Bmi1 and C/EBPα. As shown in Figure 3.23B, the distribution of Bmi1 was concentrated in patches within the nucleus, varying in size, form and intensity. Most of these patches were larger than the C/EBPα foci and did not colocalize with them. Eventually, punctual Bmi1 patches were adjacent to C/EBPα foci. However, since most of the nucleus was covered by Bmi1 patches, these punctual colocalizations are probably irrelevant. Drawn together these data prove that C/EBPα foci do not colocalize with neither polycomb nor PML nuclear bodies. Moreover, C/EBPα foci are not locate at heterochromatic foci. Thus, the localization of C/EBPα foci is novel to the previously identified cellular structures targeted by SUV39H1.

3.2.6 Chromosomal association of C/EBPα foci during mitosis

C/EBPα foci were not exclusive of interphase cells, but associated to chromosomes at discrete areas along mitosis. Hence, it could be excluded that C/EBPα foci localize to the nuclear matrix.

As shown in Figure 3.24A, in metaphase cells, C/EBPα foci were arranged as duplets. Furthermore, C/EBPα foci also associated pairwise in prometaphase cells (Figure 3.24B).

To determine whether C/EBPα foci form at centromeres, cells were stained with an antibody directed against the centrosomal protein A (CENP-A). CENP-A is a H3 variant specialized in compacting centromeric DNA (Sullivan et al., 1994). As shown in Figure 3.25, CENP-A and C/EBPα foci failed to colocalize. CENP-A foci were more numerous than

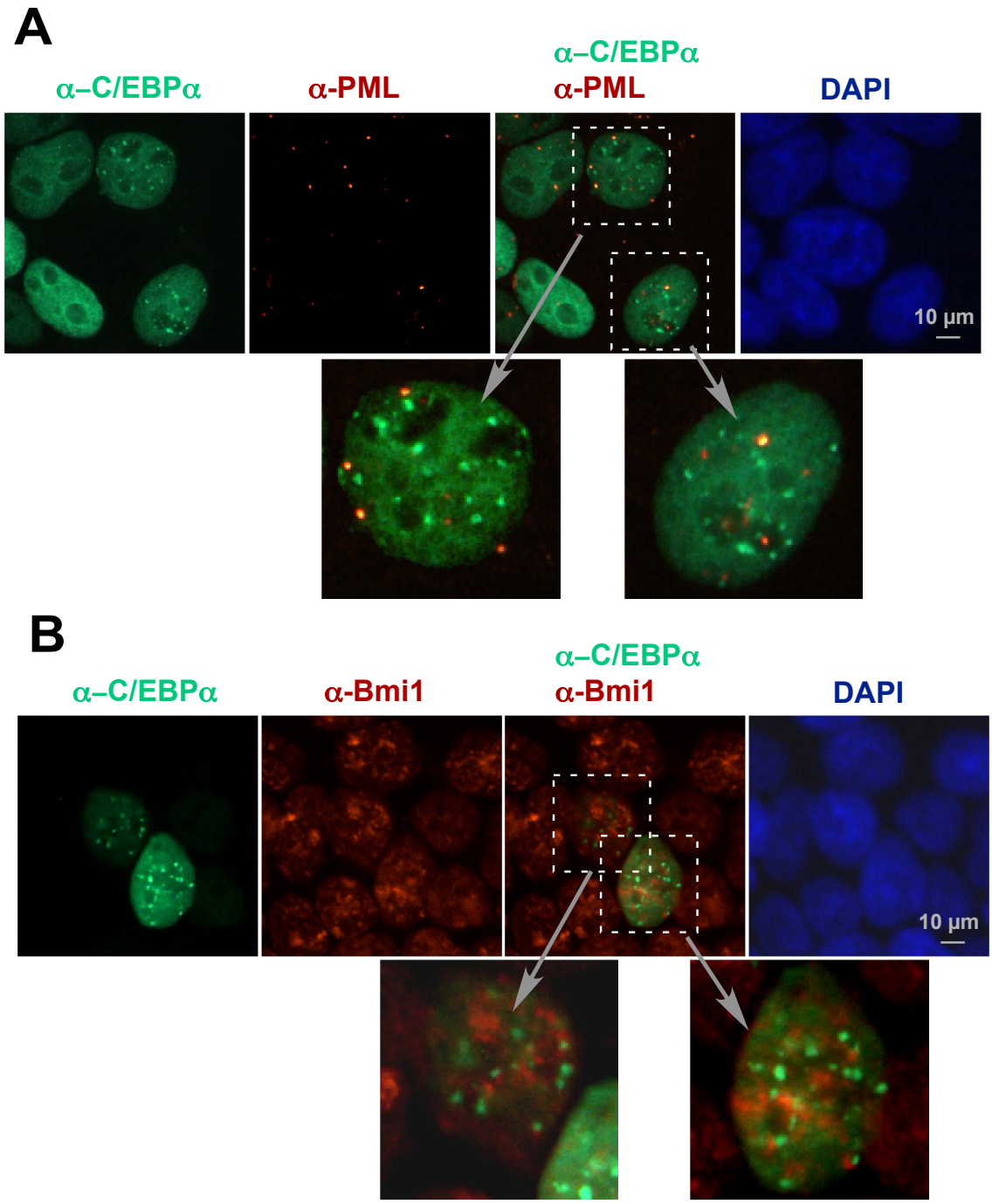

Figure 3.23: **SUV39H1-induced C/EBPα foci do not colocalize with PML, nor Bmi-1.** (A) Immunofluorescence staining of 293T cells transfected with C/EBPα and HA-tagged SUV39H1. Cells were co-stained with rabbit polyclonal antibodies directed against C/EBPα (secondary green) and mouse monoclonal anti-PML (secondary red). (B) Alternatively, cells were double labeled with anti-C/EBPα (secondary green) and mouse anti-Bmi1 (secondary red). In both cases nuclei were counterstained with DAPI.

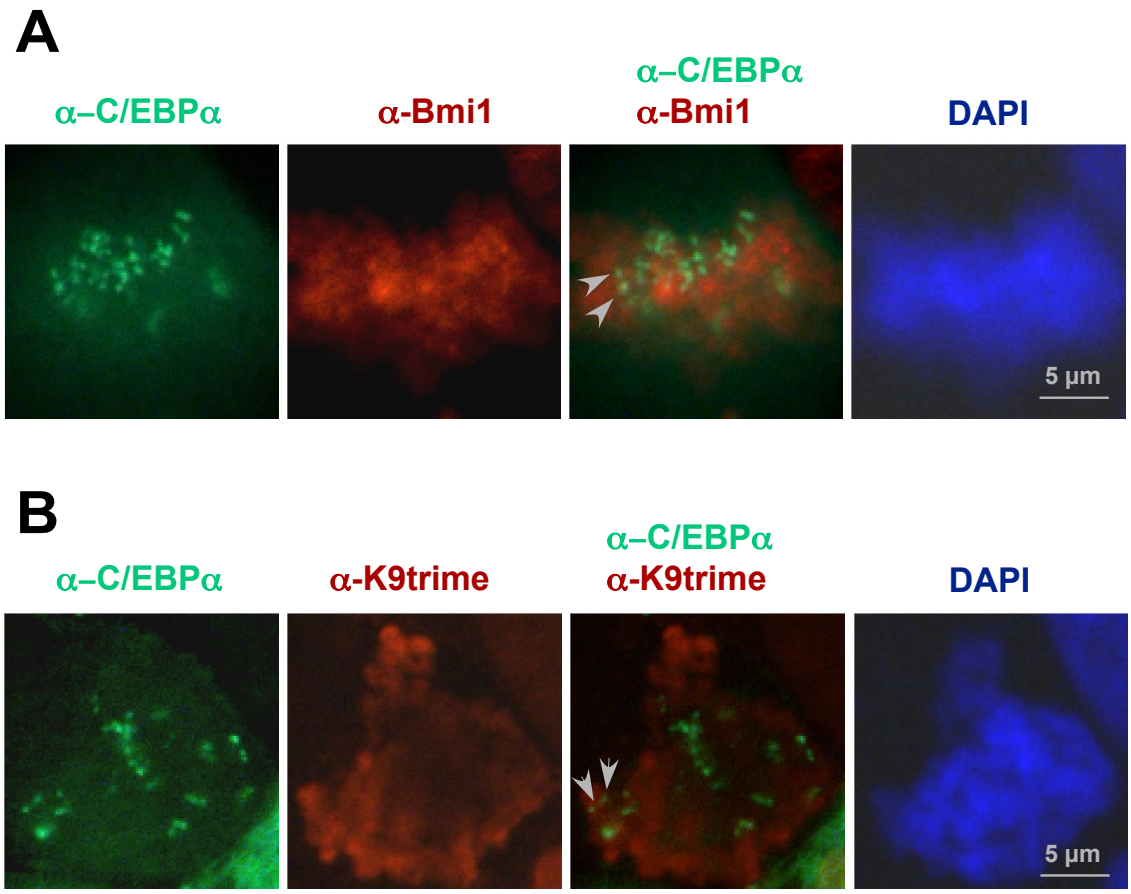

Figure 3.24: **SUV39H1-induced C/EBPα foci in mitosis.** Immunofluorescence staining of 293T cells transfected with FLAG-C/EBPα and HA-tagged SUV39H1. (A) Cells were co-stained with rabbit polyclonal antibodies directed against C/EBPα (secondary green) and mouse monoclonal anti-Bmi1 (secondary red). Nuclei were counterstained with DAPI. (B) Alternatively, cells were stained with mouse anti-FLAG (secondary green) and rabbit anti-trimethylated H3K9 (secondary red). The asterisk marks duplets.

C/EBPα ones but both foci type had a similar size. Moreover, CENP-A foci associated pairwise, with similar distance between each foci as neighboring C/EBPα duplets.

Together these data show that C/EBPα associates to chromosomes and not to the nuclear matrix. Furthermore, C/EBPα foci associate as duplets with similar size and distance between foci as centromeric-associated proteins. However, C/EBPα foci are not located at centromeres and do not associate to all chromosomes.

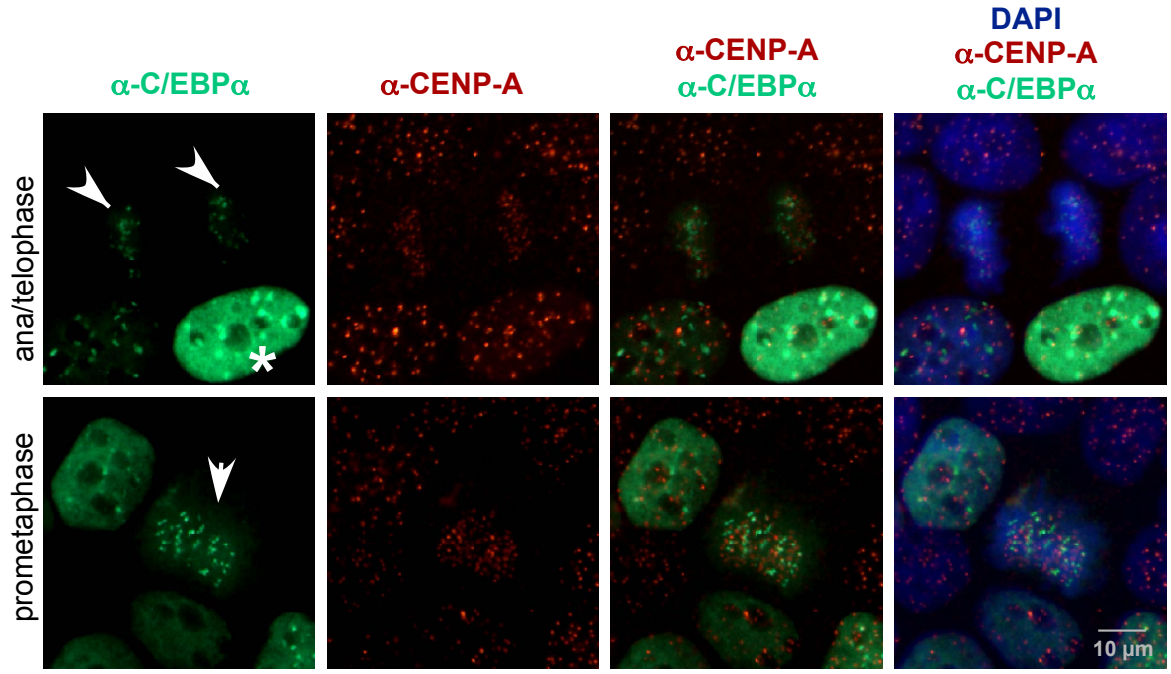

Figure 3.25: **SUV39H1-induced C/EBPα foci do not colocalize with centromeres.** Immunofluorescence staining of 293T cells co-transfected with C/EBPα and HA-tagged SUV39H1. Cells were co-stained with rabbit polyclonal antibodies directed against C/EBPα (secondary green) and mouse monoclonal anti-CENP-A (secondary red). Nuclei were counterstained with DAPI. Arrow = cell in the mitosis phase indicated at the left of the respective panel. Asterisk = cell in interphase.

3.2.7 C/EBPα foci associate with Upstream binding factor 1 (UBF1) foci.

An extensive bibliographic search for foci associating to chromosomes during mitosis and around the nucleolus during interphase, the hallmark of C/EBPα foci, was carried out. These requirements were fulfilled by the transcription factor RUNX2, which forms foci adjacent to Upstream binding factor 1 (UBF1), a transcriptional regulator of ribosomal RNA genes (rDNA) (D. W. Young et al., 2007). UBF1, which activates RNA Pol I, binds directly to rDNA and to mitotic nucleolar organizing regions (NORs) (Roussel et al., 1993). SUV39H1-

C/EBPα co-expressing cells were stained against UBF1 (Figure 3.26). Two differential UBF1 distributions were observed in interphase cells. UBF foci were either more numerous and spread along the whole nucleolus (data not shown) or numeric inferior and located more peripherally within the nucleolus. In this latter constellation, UBF1 foci were associated to C/EBPα foci (Figure 3.26A). Each UBF1 foci was paired to one C/EBPα foci, both foci having a similar size. The association of UBF1-C/EBPα foci became even more apparent in metaphase cells (Figure 3.26B). During mitosis, the UBF1 foci had an increased size and each foci was associated to one C/EBPα duplet. However, not all C/EBPα duplets were located at UBF foci, as the number of C/EBPα duplets (approx. 21-23) was superior to the UBF1 foci (approx.12). Taken together, a subset of C/EBPα foci associate to UBF ones, in a similar fashion as RUNX2 does to UBF (D. W. Young et al., 2007).

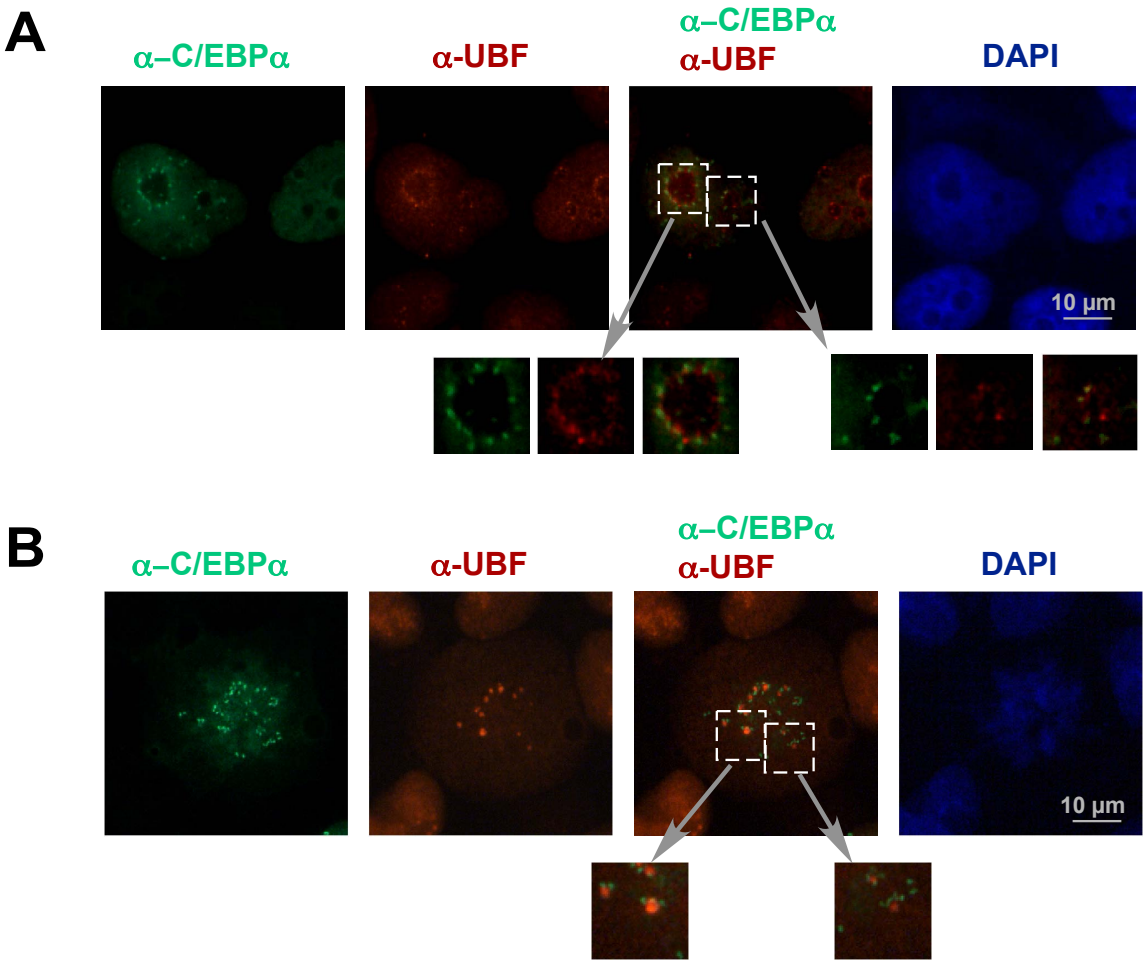

Figure 3.26: **C/EBPα foci associate to a rDNA-regulatory protein.** Immunofluorescence staining of 293T cells co-transfected with C/EBPα and HA-tagged SUV39H1. Cells were co-stained with rabbit polyclonal antibodies directed against C/EBPα (secondary green) and mouse monoclonal anti-UBF1 (secondary red). Nuclei were counterstained with DAPI. Images show cells in interphase (A) and a central positioned cell in metaphase.

Chapter 4

Discussion

4.1 The transcription factors C/EBPα and E2F/DP

The data described in this thesis unraveled a novel connection between the transcription factors C/EBPα and E2F. Both proteins repress each other's transcriptional activity. C/EBPα-mediated repression of E2F has been addressed in several studies. The present study expands the view of the E2F-C/EBPα interconnection, since E2F represses C/EBPα, stating a reciprocal control between both proteins. Given their opposing roles in proliferation and adipocytic differentiation, this mutual regulation may be the main determinant of whether a cell proliferates or differentiates.

4.1.1 E2F represses the transcription factor C/EBPα

The classical model for regulation of target genes by E2F implies activation of promoters by the members E2F1-E2F3 and repression by the members E2F4-E2F5 associated with pocket proteins. Pocket proteins recruit chromatin modifying enzymes which cause the transcriptional silencing of the E2F-target genes. However, gene expression profiling have also uncovered a large number of genes, that are repressed by E2F1-E2F3 (H. Muller et al., 2001). Some of these genes have been shown to be repressed by E2F1, independent of pocket proteins (Koziczak et al., 2000);(Croxton et al., 2002). However, up to date, it remains unclear how "activator" E2F1 represses transcription in a pocket protein independent way. Data presented here unravel that E2F1 represses transcription independent of pocket proteins by repressing the transcription factor C/EBPα.

In this work it was show that both members of classical activator as well as repressor E2Fs can repress the function of C/EBPα. Inhibition of C/EBPα by E2F occurs in a pocket protein independent fashion, as E2F1-DP1 inhibits C/EBPα in MEFs defective of all three

pocket-proteins (pRB-/-/p107-/-/p130-/-). Moreover, the pRB-binding defective E2F1 mutant Y411C, still suppresses C/EBPα.

The presented data showed that the DNA binding deficient E2F1 mutant E132 fails to associate and to suppress C/EBPα. Furthermore, E2F1 with an intact DNA-binding domain does not associate to C/EBP sites. Together this suggests that the failure of E132 to repress C/EBPα is a consequence of its failure to bind C/EBPα, rather than of its DNA-binding deficiency. Other genes reported to be repressed by E2F1 but not by its mutant E132 are *PAI-I*, *urokinase-type plasminogen activator* (Koziczak et al., 2000), *human telomerase reverse transcriptase* (Crowe et al., 2001), the Bcl-2 family member *Mcl-1* (Croxton et al., 2002) and the *androgen receptor* (Davis et al., 2006). The mechanism of repression by E2F1 of these target genes has not been addressed yet. Further studies will analyze whether C/EBPα is an activator of the before mentioned target genes and whether E2F1 represses their transcription through inhibition of C/EBPα.

The properties of E2F-mediated inhibition of C/EBPα regulated genes, are very similar to the ones described for repression of the PAI-I and the urokinase-type plasminogen activator promoter (Koziczak et al., 2000). This points towards the existence of a mechanism of transcriptional inhibition by E2F, that occurs independently of pocket proteins, without direct binding to promoter sites, but dependent on an intact DNA binding domain and involving other transcription factors. The function of E2F as a modulator of other transcription factors, allows an additional mode of regulation by E2F. Therefore, the presented data suggest a redefinition of E2F-regulated genes as ”direct”, through binding at E2F sites, or ”indirect”, through regulation of other transcription factors and without association of E2F to DNA.

4.1.2 A discrete role for the dimerization partner of E2F

The dimerization partner of E2F (DP) is required for correct binding and induction of E2F target genes. While many E2F interacting proteins have been identified, known DP binding partners are rare and usually not discriminated from E2F interaction. One exception is the transcription factor p53, that binds E2F and DP in an mutually exclusive way, causing the inhibition of E2F's activity by disruption of the E2F-DP dimer (Sorensen et al., 1996).

The presented data define a DP region required for interaction with C/EBPα that is not required for binding to E2F. Thus, DP binds directly to C/EBPα, independently of E2F. Binding assays to a series of C/EBPα basic-region mutants (BRM2, BRM3 and BRM5) further support that the DP-C/EBPα interaction can be discriminated from the E2F-C/EBPα interaction. While the binding of these C/EBPα mutants to E2F1 is indistinguishable, DP shows an enhanced binding to BRM2 and BRM5. In addition, reporter data have shown that the ac-

tivity of BRM2 and BRM5 is more efficiently repressed by E2F-DP than WT C/EBPα, which could be rationalized by their enhanced binding to DP. Taken together, these data suggest a central role for DP in determining the affinity of E2F complexes to C/EBPα, affecting the efficiency in which E2F-DP repress C/EBPα.

4.1.3 Constitutive repression of the C/EBPα mutant BRM2 by E2F-DP

The C/EBPα basic-region mutants BRM2 and BRM5 are defective in inducing adipogenesis and granulopoiesis (Porse et al., 2001). Several studies have analyzed the molecular determinants of this phenotype, offering apparent opposite explanations. These discrepancies were addressed taking into consideration the findings of this thesis.

BRM2 and BRM5 knock-in mice strains lack white adipose tissue and mature granulocytes (Porse et al., 2001). Although this shows a functional defect of the BRM2 and BRM5, the transcriptional capacity of these mutants is still not clear. While in the reporter studies from Keeshan and colleagues using 293T cells BRM2 achieved only 30% of the WT activation levels (Keeshan et al., 2003), in another study, both WT and BRM2 potently activated transcription (Q. F. Wang et al., 2003). Reporter data presented here, which were also done in 293T cells, offer an explanation to these apparent discrepancies as differences in activity between WT and basic-region mutants only became visible at very low C/EBPα expression levels. This indicates that experimental variation resulting in lower or higher C/EBPα expression levels could easily account for different outcomes of reporter studies.

The fact that differences in the transcriptional activity of WT and BRM2 only becomes discernible at low C/EBPα expression levels, suggests that endogenous proteins affect the transcriptional activity which are rate limiting at high C/EBPα concentrations. This could involve endogenous E2F-DP complexes which repress BRM2 more efficiently than WT C/EBPα, accounting for the reduced transcriptional activity of BRM2 when C/EBPα levels are low. Supporting this idea are data showing that differences in the transcriptional activity of overexpressed WT and BRM2 appear when co-expressing E2F/DP. This suggests that overexpression of all components (E2F and DP) involved in the inactive phenotype of BRM2, could mimic the endogenous scenario. Differences in the DNA binding affinity of WT and BRM2 become apparent when expressed in cells while in vitro translated WT and BRM2 bind to C/EBP consensus DNA sites with similar affinities, suggesting that endogenous E2F proteins may repress the DNA-binding activity of BRM2. Accordingly, co-expression of E2F1/DP1 reduces the binding of C/EBPα to C/EBP sites, almost abolishing the binding of BRM2, while addition of E2F sites restores the DNA binding of BRM2 to WT levels. This suggests that E2F/DP proteins binding to E2F sites detach from BRM2 and that free BRM2

proteins are no longer disrupted in their DNA binding. A competition of E2F and C/EBPα for C/EBP sites could be excluded, since E2F-DP do not bind these sites. More plausible therefore is that E2F-DP may compete with C/EBP sites for binding C/EBPα. In other words, C/EBPα may bind to either E2F protein or C/EBP sites, BRM2 binding preferentially to E2F-DP than to DNA. Drawn together these data suggest that strong binding of BRM2 to DP favors the disruption of this mutant from C/EBP sites, resulting in the repression of its transcriptional activity.

In contrast to WT C/EBPα, BRM2 fails to induce adipogenesis in C/EBPα deficient fibroblasts. Since the reduction of E2F/DP proteins by siRNA rescues the adipogenic defect of BRM2, this further supports the notion that endogenous E2F/DP complexes repress the activity of BRM2. Altogether, these results suggest that binding of E2F/DP proteins to C/EBPα disrupts C/EBPα binding to DNA sites. Accordingly, an increased binding affinity of BRM2 to E2F-DP causes an efficient disruption of DNA-binding, causing the inactivation of the transcriptional activity and differentiation capacity of BRM2.

4.1.4 Repression of E2F by C/EBPα

C/EBPα BRM2 fails to repress E2F (Porse et al., 2001). Given the discrepancies in the molecular determinants of this defect, this work analyzed several molecular characteristics of BRM2, such as protein-protein interactions and binding to E2F-sites.

C/EBPα associates to E2F DNA sites. While initial reports indicated that C/EBPα binding to E2F sites may be indirect and occur through E2F proteins acting as bridging factors (Slomiany et al., 2000), a recent report has postulated the existence of cryptic C/EBP sites in the proximity of consensus E2F binding sites (Sebastian et al., 2005). Band shift assays carried out in this thesis showed that C/EBPα associates to mutated E2F sites that do not bind E2F/DP proteins. It was further observed that C/EBPα binding to E2F sites was weaker than to consensus C/EBP sites. Altogether this indicates that C/EBPα binding to E2F sites is not mediated by E2F/DP proteins and suggests the presence of a cryptic C/EBP binding sequence within the E2F site.

In accordance with previous reports (Porse et al., 2001), gel shift assays confirmed that BRM2 shows impaired binding to E2F DNA sites. The inability of BRM2 to bind to and repress transcription from E2F sites has been suggested to be a consequence of its defective binding to E2F (D'Alo et al., 2003), as shown for E2F4 employing K562 cells. However, in 293T cells, E2F1 still associates with BRM2 (Keeshan et al., 2003). In the experimental settings used here the binding affinity of BRM2 to either E2F1 or E2F4 was comparable to WT. This result together with the finding that binding of C/EBPα to E2F sites is independent

of E2F-DP proteins, suggest that the failure of BRM2 to shift E2F sites is due to a disruption of its DNA binding capacity to these sites. Altogether, this suggests that repression of E2F involves binding of C/EBPα to E2F sites rather than association to E2F proteins. Failure to associate to E2F sites results in the inability to repress the transcriptional activity of E2F. Thus, the DNA binding activity of C/EBPα is crucial for two functions: induction of classical C/EBP target genes, and repression of E2F-regulated genes.

4.1.5 E2F-C/EBPα in adipogenesis

The switch of a proliferating cell into a differentiating adipocyte involves a dual role of C/EBPα: proliferation arrest through inhibition of E2F-regulated S-phase genes and induction of lineage specific genes. The data presented in this thesis provide evidence for a novel function of E2F during adipogenesis. It was shown that E2F actively represses C/EBPα-mediated transcription, offering an additional control level of differentiation processes (Figure 4.1).

Ectopic expression of E2F1 abolishes hormonally stimulated adipogenesis in 3T3-L1 cells whereas the E132 mutant fails to do so (Porse et al., 2001). The data suggested that overexpression of E2F1 circumvent C/EBPα-induced proliferation arrest required for adipogenesis. Accordingly, the DNA-binding deficient E132 mutant would fail to induce E2F-regulated S-phase genes and, hence, would fail to counteract adipogenesis. Given the present findings that E2F1 represses C/EBPα-mediated transcription, which the E132 mutation cannot, an additional interpretation of these data could be envisaged. Whereas E2F suppresses the transcriptional activity of C/EBPα on adipocytic genes, E132 fails to repress the activity of C/EBPα on these genes and fails to block adipogenesis. Taken together, this suggests that the proliferation/differentiation switch requires control of transcriptional activity of two different transcription factors, E2F and C/EBPα, which can repress each other's activity.

Studies of E2F1's function during adipogenesis have reported apparent discrepancies. While ectopic expression of E2F1 blocks adipogenesis (Porse et al., 2001), E2F1-/- MEFs form less adipocytes (Fajas et al., 2002). The latter study suggested that E2F1 is required for the clonal expansion phase that precedes adipogenesis. Furthermore, the study showed that after clonal expansion phase and as adipogenesis progresses, the E2F1 protein levels progressively decline. In light of the finding that ectopic expression of E2F1 blocks adipogenesis (Porse et al., 2001), down-regulation of E2F1 levels may be a cellular mechanism to allow adipocytic differentiation, once E2F1 has accomplished its function in the clonal expansion phase. This thesis analyzed the adipogenic function of E2F1 by siRNA-mediated

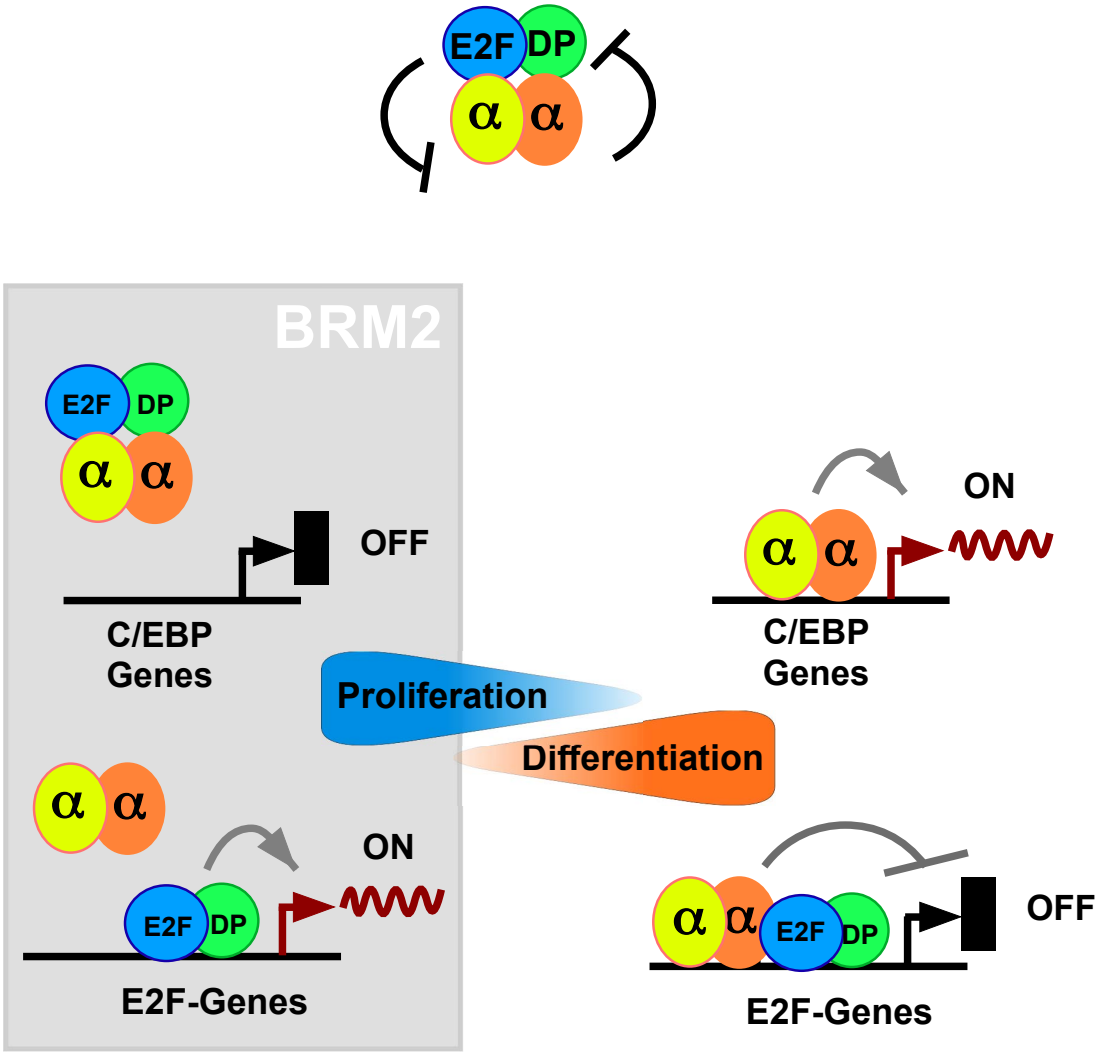

Figure 4.1: **The C/EBPα-E2F network.** Model of how E2F/DP and C/EBPα could control the proliferation/differentiation switch by repressing each others activity. Proliferation involves induction of E2F-genes and E2F/DP-mediated disruption of C/EBPα from E2F sites and C/EBP sites. During differentiation this repression is reversed, C/EBPα repressing the transcriptional activity on E2F-sites. In turn, C/EBP-target genes are activated. C/EBPα BRM2 fails to induce the switch towards differentiation, due to its failure to bind and repress E2F sites and to bind C/EBP sites, as a consequence of E2F/DP-mediated disruption of BRM2's binding to DNA.

knock-down of E2F1. The use of siRNA offered a way to bypass successively the clonal expansion phase, since a reduction of E2F1 using siRNA occurs 72 hours post-transfection, when adipogenesis is already initiated. Another advantage of the siRNA-approach towards knock-out studies is that expression of the targeted protein is reduced but not abolished. This would still allow E2F functions that do not require high expression levels. These functions could include control of S-phase genes, in agreement with the finding that siRNA against DP or E2Fs do not affect proliferation, as shown by BrdU incorporation assay. In conclusion, the use of siRNA offers main advantages to study the function of E2F during adipogenesis.

Data presented here show that reduction of E2F/DP by siRNA increases adipogenesis in C/EBPα-expressing cells, but not in cells depleted of C/EBPα. In addition, the adipogenic defect of BRM2 was partially rescued by siRNA against E2F/DP. These data suggest that E2F represses adipogenesis through inhibition of C/EBPα. The fact that the transcriptional and DNA-binding activity of BRM2 are constitutively repressed by E2F-DP, would rationalize why BRM2 fails to induce adipogenesis.

Already known was that C/EBPα favors adipogenesis by repressing E2F target genes and inducing adipogenic genes. Novel is the notion that E2F counteracts adipogenesis by repressing the activity of C/EBPα on adipocytic genes (Figure 4.1). Down-regulation of E2F1 expression, as occurs in differentiating cells (Fajas et al., 2002), eliminates this repressive action, allowing cell differentiation. According to this model, the BRM2 fails to induce adipogenesis as a consequence of its inability to bind to and inhibit E2F-sites, and of its inability to bind to and induce adipogenic genes (Figure 4.1). Taking together, the balance of E2F- and C/EBPα-mediated repression may determine the switch between alternative cell fates of proliferation and differentiation.

4.1.6 Pocket proteins in the E2F-C/EBPα regulatory axis

Pocket proteins mediated recruitment of repressive complexes has been considered as the main mechanism determining repression on E2F-target genes. Thus, a long discussed issue is whether C/EBPα-mediated suppression of E2F-regulated promoters requires pocket proteins. The family of pocket proteins is composed of pRB, p107 and p130. Experiments with the pRB-deficient Saos cell line suggest that C/EBPα blocks proliferation by formation of E2F-pRB-C/EBPα repressive complexes at the promoter of E2F-regulated genes (Iakova et al., 2003). In contrast, reporter assays show that C/EBPα still represses E2F-mediated transcription in Saos cells (Johansen et al., 2001). Since this cell line has other defects, as for example lack of p53, these results do not allow definite conclusions. So far, no reporter studies have addressed whether C/EBPα represses E2F in the absence of all three

pocket proteins. In this thesis reporter studies were carried out in pocket proteins deficient MEFs generated by Dannenberg and colleagues (Dannenberg et al., 2000). The presented data show that C/EBPα still represses E2F in these cells, stating that C/EBPα-mediated repression of E2F works independently of pocket proteins.

In contrast, Sebastian and colleagues have shown that the family member C/EBPβ fails to repress E2F in another pocket proteins deficient MEF cell line (Sebastian et al., 2005), which were generated by Sage and colleagues (Sage et al., 2000). A possible explanation could be that C/EBPβ represses E2F by a different mechanism, requiring pocket proteins, than C/EBPα does. Despite the homology between C/EBPα and C/EBPβ they can have antagonist function as in skin tumorigenesis, were lack of C/EBPα promotes chemical induced skin carcinogenesis (Loomis et al., 2007), while lack of C/EBPβ protect against skin tumorigenesis (S. Zhu et al., 2002). However, a second possibility could be that the different pocket protein deficient cell lines have acquired additional mutations or that other differences within the employed MEFs (targeting strategies, mice backgrounds) could account for different results. The latter option could also explain why both C/EBPα and C/EBPβ fail to arrest the cell cycle in MEFs from Sage et al. (Sebastian et al., 2005), while C/EBPα still blocks proliferation in MEFs from Dannenberg et al. (Mueller C. and Leutz A., unpublished results).

E2F4 has been shown to repress adipogenesis in pocket protein and cell cycle independent manner (Landsberg et al., 2003), an observation that is in accordance with our data showing that E2Fs do not require pocket proteins to repress C/EBPα. Both C/EBPα and pRB are required for adipogenesis (Chen et al., 1996). However, no correlation is observed between pRB-C/EBPα interaction and adipogenesis (Porse et al., 2001), while pocket protein binding deficient E2Fs are still repressed by C/EBPα (communicated by Porse and colleagues). This suggest that C/EBPα and pRB affect adipogenesis through parallel pathways.

4.1.7 Clinical implications and future plans

The presented data suggest that E2F disrupts the DNA binding and, consequently, the transcriptional activity of C/EBPα. Disturbed DNA binding of C/EBPα is often observed in AML patients. The disruption of DNA-binding is accomplished by either amino-terminal or carboxy-terminal deletion, or by direct mutations in the CEBPα DNA-binding basic region. In addition, the DNA-binding activity of non-mutated C/EBPα protein has been reported to be abolished by the t(15;17) translocation-derived PML/RARα fusion protein which alters the nuclear localization of C/EBPα (communicated by Lodie and colleagues). The results presented here suggest that the C/EBPα basic-region mutation BRM2, which confers a pre-

disposition to AML-like disease (Porse et al., 2005), is constitutively repressed in its DNA-binding and transcriptional activity by E2F-DP. The fact that different oncogenic mutations convey in the disruption of C/EBPα from DNA suggest that regulation of this disruption is a central cellular control mechanism. Thus, the E2F-DP mediated regulation of C/EBPα binding to DNA may determine malignancy. Therefore, it would be important to determine whether E2F-dependent C/EBPα binding to DNA repression is enhanced in malignant cells and whether elimination of this repression, for example by knock-down of E2F/DP, could reverse the malignant phenotype. A good model to study this issue is the myeloproliferative disorder of BRM2 mice, which serves as a model for C-terminal AML-C/EBPα mutations. As a consequence of impaired granulocytic maturation, these mice have an accumulation of myeloblasts and other promyeloblast. Future investigations will address whether blocking E2F-DP function in such cells would liberate the BRM2 protein and induce terminal differentiation of progenitors in accordance with the observation that the adipogenic defect of BRM2 could be overcome by siRNA-mediated E2F knock down.

Another cancer type in which C/EBPα could play a role is skin carcinogenesis, since loss of C/EBPα expression in the epidermis results in an increased susceptibility to Ras-induced skin tumorigenesis (Loomis et al., 2007). In contrast, in a transgenic mouse model, overexpression of E2F1 or DP1 results in an enhanced Ras-induced skin carcinogenesis (D. Wang et al., 2001). The opposite roles of E2F/DP and C/EBPα in skin carcinogenesis raises the question whether overexpression of E2F/DP increases tumor susceptibility by repression of C/EBPα. Thus, it should be determined whether the DNA-binding activity of C/EBPα is reduced in E2F1 or DP1-trangenic mice. Finding a DP1 mutant active on E2F-regulated genes, but unable to repress C/EBPα would be very informative, since Ras-induced carcinogenesis on mice carrying this mutation, could reveal whether repression of C/EBPα predisposes for skin transformation independent of E2F function on S-phase genes.

Future analysis will address which of the described C/EBPα target genes are repressed by E2F-DP and which of the genes shown to be repressed by E2F-DP are controlled by C/EBPα. Furthermore, based on the assumption that BRM2 is constitutively repressed by E2F-DP, microarray data comparing WT and BRM2 knock-in mice could lead to the identification of novel C/EBPα target genes.

Taken together, this work shows how deeply E2F-C/EBPα components are functionally interconnected. A novel function of E2F could be demonstrated: repression of other transcription factors by disruption of their DNA-binding. Furthermore, the presented data suggest that these E2F functions could be crucial for C/EBPα-mediated differentiation processes. Mutational analysis of AML-patients suggests that disruption of DNA-binding of

C/EBPα could be a main target in granulocytic disorders. Thus, it would be of main interest to analyze whether E2F mediates disruption of C/EBPα's DNA-binding in AML patients.

4.2 The methyltransferase SUV39H1 and the transcription factor C/EBPα

The transcription factor C/EBPα and the histone methyltransferase SUV39H1 are regulators of gene expression. Both C/EBPα and SUV39H1 proteins slow cellular proliferation by repression of common target genes. However, it has not been addressed whether they may coordinate each other's function as components of the same pathways. This work shows a functional dependence of C/EBPα on the histone methyltransferase SUV39H1. C/EBPα localizes at discrete foci adjacent to a RNA Polymerase I regulatory protein upon expression of a functional histone methyltransferase SUV39H1. This mechanism of foci induction is not a general function of C/EBP members and SET-domain methyltransferase, but is specific for C/EBPα and SUV39H1. SUV39H1 is known to catalyze di- and trimethylation of H3K9. The C/EBPα foci, however, are not enriched in these histone modifications, suggesting the existence of a novel SUV39H1 target.

4.2.1 C/EBPα associates to sites of ribosomal RNA gene regulation

Over the last decades the antiproliferative function of C/EBPα has been extensively studied (section 1.1.2.). Interaction of C/EBPα with components of the cell cycle machinery regulating DNA replication, such as E2F, conveys in cell cycle arrest. Unattended has been the other major checkpoint which limits proliferation: the regulation of cell growth. Prior to cell division, proliferating cells must increase their mass, which requires an elevation of protein synthesis. One mechanism to increase the translational capacity of a cell is to augment ribosomal biogenesis. A major rate-limiting step in the biogenesis of the ribosomes is the transcription of ribosomal RNA genes (rRNA genes or rDNA). rRNA genes are arranged in tandems of hundreds within the nucleoli (Gonzalez & Sylvester, 2001). RNA Polymerase I (Pol I) transcribes the genes that encode the 45S ribosomal RNA precursor of the 18S, 5.8S and 28S rRNA. Upstream binding factor 1 (UBF1) is a regulator of RNA Pol I which binds directly to rDNA and to mitotic nucleolar organizing regions (NORs) that are the precursors to interphase nucleoli (Gebrane-Younes et al., 1997). A subset of proteins, with known regulatory functions on RNA Pol II, have recently been shown to regulate RNA Pol I-mediated transcription. This includes the tumor suppressors p53 and pRB, which both have been shown to repress transcription of rDNA by disrupting the interaction of UBF with a central com-

ponent of the Pol I transcriptional machinery (Zhai & Comai, 2000);(Hannan et al., 2000). Moreover, the transcription factor Runx2, which has a known role in lineage commitment and cell proliferation through regulation of the protein-coding genes transcribing RNA Pol II, has also been found to repress RNA Pol I (D. W. Young et al., 2007). Immunofluoresence analysis revealed that in interphase cells Runx2 exhibits a punctated distribution throughout the nucleus, of which a subset associates to UBF1 foci. These almost superimposed UBF1-Runx2 foci reside at nucleolar sites of rRNA synthesis, Runx2 localizing at the periphery of the nucleolus, while UBF1 is situated versus the nucleolus center. Association of Runx2 to these sites represses rDNA promoter activity. This is an example where a transcription factor colocalizes with UBF1 and the RNA Pol I machinery. The transcription factor C/EBPα normally is distributed homogenously throughout the nucleus sparing the nucleoli. However, upon expression of SUV39H1, C/EBPα is retained in discrete foci. Data presented here revealed that C/EBPα is enriched at discrete foci, which mainly are located around the nucleolus. Furthermore, these foci associate in close proximity to UBF1 foci, both foci locating like the Runx2-UBF1 foci. C/EBPα foci are retained on condensed chromosomes. This mitotic association is also observed for Runx2 foci (D. W. Young et al., 2007). Most interphase cells (human diploid 293T cells) have 20-23 C/EBPα foci, while a subset of interphase cells have the double amount of foci, arranged as duplets. Up to 23 C/EBPα duplets are also observed on mitotic chromosomes. As revealed by co-staining against centromeric-associated proteins, the distance between paired foci is similar to paired centromeres, indicating that C/EBPα foci are formed on two sister chromatids. This suggests that the formation of either single foci or duplets correlates with the duplication of DNA. The number and size of UBF1 foci is very dynamic, with reduced number (approx. 12) and increased size during mitosis. Each of these rather irregular mitotic foci are most probably composed of two foci in close proximity, since it has been shown that UBF1 forms one focus on each sister chromatid (D. W. Young et al., 2007). In summary, C/EBPα foci are numeric superior to UBF1 foci and, accordingly, a subset of C/EBPα foci is not associated with UBF1, a characteristic also shared by Runx2 foci. Altogether, the phenotype of C/EBPα and Runx2 foci are remarkably similar, suggesting that C/EBPα may have a similar effect on rDNA as Runx2. Notably, C/EBPα and Runx proteins are known for their synergistic actions. For instance, Runx1 protein and C/EBPα, which both are crucial for normal granulopoiesis (Q. Wang et al., 1996);(Zhang et al., 1997), have been shown to activate myeloid specific promoters in a synergistic manner (Lausen et al., 2006);(Petrovick et al., 1998). Furthermore, Runx proteins and C/EBPα are both antiproliferative. Thus, it could be envisaged that C/EBPα and Runx proteins also cooperate to repress rDNA. Future experiments will be required to deter-

mine whether C/EBPα represses rDNA synthesis upon SUV39H1 expression and whether this may be a determinant for C/EBPα-mediated proliferation arrest.

4.2.2 SUV39H1 as a possible upstream effector of ribosome biogenesis

SUV39H1 plays a major role in gene silencing (section 1.4.2.). In contrast to promoter-specific gene repression, gene silencing acts in a regional manner generating large domains of DNA that are inaccessible to DNA binding proteins. Silencing involves the assembly of specialized proteins, which are recruited to the chromatin by interactions with histones. Heterochromatin protein 1 (HP1) and PcG proteins, which are the major silencing proteins, associate to chromatin upon H3 trimethylation catalyzed by SUV39H1 (Bannister et al., 2001);(Lachner et al., 2001);(Sewalt et al., 2002). Gene silencing is often associated with repetitive DNA sequences. In agreement with its silencing function, SUV39H1 has been found to act on repetitive DNA elements, such as major satellite repeats (e.g. pericentric heterochromatin), LINEs and IAPLTRs (Martens et al., 2005). Data presented here suggest that SUV39H1 induces the association of C/EBPα at rDNA clusters. Thus, a role for SUV39H1 in regulating the rDNA repeats, would be in agreement with its known functions on repetitive elements. In fact, the budding yeast homolog of SUV39H1, Set1, has been shown to mediate rDNA silencing (Briggs et al., 2001). The size of the C/EBPα foci suggests that C/EBPα binds to the entire rDNA region, thus may act as a silencing protein in an analogous fashion to HP1 and PcG. Common to HP1 and PcG proteins is also that the methylating activity of SUV39H1 catalyzes the recruitment of C/EBPα to specific sites, which is visualized as foci. Taking together data presented here and previously reported, it could be envisaged that mammalians have a SUV39H1-mediated mechanism to silence rDNA, as has been described for the budding yeast. The methylating activity of SUV39H1 localizes C/EBPα across rDNA repeats areas, eventually leading to silencing of ribosomal genes. In agreement with the antiproliferative function of SUV39H1 and C/EBPα, this work suggests that the coordinated action of these proteins repress rDNA which is known to convey in cell cycle arrest.

4.2.3 Mechanism of SUV39H1-mediated foci formation

Data presented here show that expression of SUV39H1 causes the formation of C/EBPα foci on chromosomes. The concentration of C/EBPα protein to discrete sites, caused by SUV39H1, appears not to be a mere recruitment effect. One could expect that if SUV39H1 would recruit C/EBPα to discrete sites, it should be enriched at these sites. However, this is not the case. Although full-length C/EBPβ and truncated C/EBPα (p30) also associate with

SUV39H1, they are not relocalized by SUV39H1 as full-length C/EBPα. In addition, the HMTase activity of SUV39H1 is crucial for the relocalization, as catalytical inactive SUV39H1 mutants fail to induce C/EBPα foci formation. However, EZH2, which also is a SET-domain HMTase, does not affect C/EBPα distribution. Altogether this clearly shows that the methylating activity of SUV39H1 specifically causes assembly of C/EBPα foci.

It remains unresolved what the SUV39H1 substrate is that triggers the formation of C/EBPα foci. The only known substrate of mammalian SUV39H1 is H3K9. Immunofluorescence analysis did not reveal an enrichment of either di- or trimethylated H3K9 at C/EBPα foci, suggesting that methylation of H3K9 is not involved in the induction of C/EBPα foci. However, additional analysis are required to exclude enrichment of H3K9 at C/EBPα foci, such as protein interaction studies. The presented data suggest that a novel SUV39H1-methylated protein could be involved, causing C/EBPα relocalization. Three scenarios can be imagined. First, SUV39H1 could methylate a chromatin-associated protein creating a docking site for C/EBPα analogous to how SUV39H1 does for HP1 (Figure 4.2, model A). This protein could be either a histone residue different from H3K9 or some other chromosome-associated protein. Several methylated histone residues, such as trimethylated H3K27, di- and trimethylated H3K4, and methylated H3K17, were examined for enrichment at C/EBPα foci. Given the finding that none of them are enriched at C/EBPα foci, they could be excluded as SUV39H1-substrates attracting C/EBPα. Other chromosome associated proteins which could be methylated by SUV39H1 are components of the transcriptional machinery. A previous report demonstrated that classical HMTase can also methylate proteins different from histones. SET9 methylates a subunit of the basic transcriptional machinery, favoring its association to RNA Pol II and, thus, stimulating transcription (Kouskouti et al., 2004). Analogous, SUV39H1-mediated methylation of components of the RNA Pol I transcriptional machinery could create a binding site for C/EBPα, recruiting C/EBPα to rDNA clusters, where C/EBPα could regulate transcription.

The second possibility is that C/EBPα itself is methylated by SUV39H1. Upon methylation, C/EBPα could gain high affinity to either specific DNA elements or to chromatin-associated proteins, causing its local enrichment on chromosomes (Figure 4.2, model B). Candidate residues for this possible methylation are two lysines contained in the amino terminus of C/EBPα, since this region is required for C/EBPα's ability to form foci. Future analysis will address whether mutations of these lysines in C/EBPα abolish foci formation and whether these lysines are methylated using in vivo and in vitro methylation assays.

Another option is that SUV39H1 may not act directly on C/EBPα or C/EBPα-bound proteins, but may activate a signaling cascade, which will convey on relocalization of C/EBPα.

Which of these three options cause C/EBPα relocalization will be analyzed in future studies.

4.2.4 Functional relevance of C/EBPα foci formation

In multicellular organisms, the adaptation of a cell to extracellular signals is fundamental for developmental processes, tissue maintenance or immunological responses, resulting in the correct coordination of the whole organism. Main cell fate determinants are growth factors that trigger cell proliferation and lineage stimulating factors inducing lineage commitment. The cellular response to these extracellular factors is mediated by signaling pathways. Extracellular factors bind to specific transmembrane receptors which will transmit a signal to the cell interior, leading to the activation of a signaling cascade. One of the main growth effector pathways is the phosphatidylinositol 3-kinase (PI3K)-Akt pathway. Stimulation of this pathway by growth factors results in multiple molecular effects, which will ultimately convey in cell proliferation. For example, the PI3K-Akt pathway activates rDNA transcription (James & Zomerdijk, 2004), which is required for cell growth. Remarkably, signaling through the PI3K-Akt pathway abolishes C/EBPα's antiproliferative capacity (G. L. Wang et al., 2004). Data presented here, in conjunction with the findings mentioned above, suggest that accumulation of C/EBPα at rDNA clusters may decrease the synthesis of rRNA. Therefore, it would be interesting to determine whether the PI3K-Akt pathway stimulates rDNA transcription by counteracting the formation of C/EBPα foci. Given the findings that SUV39H1 induces the formation of C/EBPα foci, SUV39H1 and C/EBPα may act sequentially as components of a signaling pathway, SUV39H1 being an upstream effector of C/EBPα. Thus, activation of the PI3K-Akt pathway might inhibit the formation of C/EBPα foci by repressing the methylating activity of SUV39H1.

Signaling pathways also regulate the lineage commitment of a cell. Some differentiating processes, such as granulocytic differentiation, are associated with a reduction of cell mass. This reduction might be accomplished by reducing the expression of molecules implicated in certain aspects of ribosome biogenesis (Iritani et al., 2002). During granulocyte differentiation, MAD1, a member of the MAD family of transcription repressors, represses transcription of rDNA, while the MAD1 antagonizer c-myc activates rDNA transcription (Poortinga et al., 2004). Remarkably, c-myc and C/EBPα also have antagonizing functions during granulopoiesis. Myeloid precursor cells (myeloblasts) from C/EBPα-null mice exhibit an early block in differentiation (Zhang et al., 1997), while ectopic expression of c-myc forces myeloblasts to remain undifferentiated (Johansen et al., 2001). Thus, C/EBPα favors, whereas c-myc inhibits granulocytic maturation. Both proteins have been shown to suppress each others expression. C/EBPα-mediated down-regulation of c-myc is required for granu-

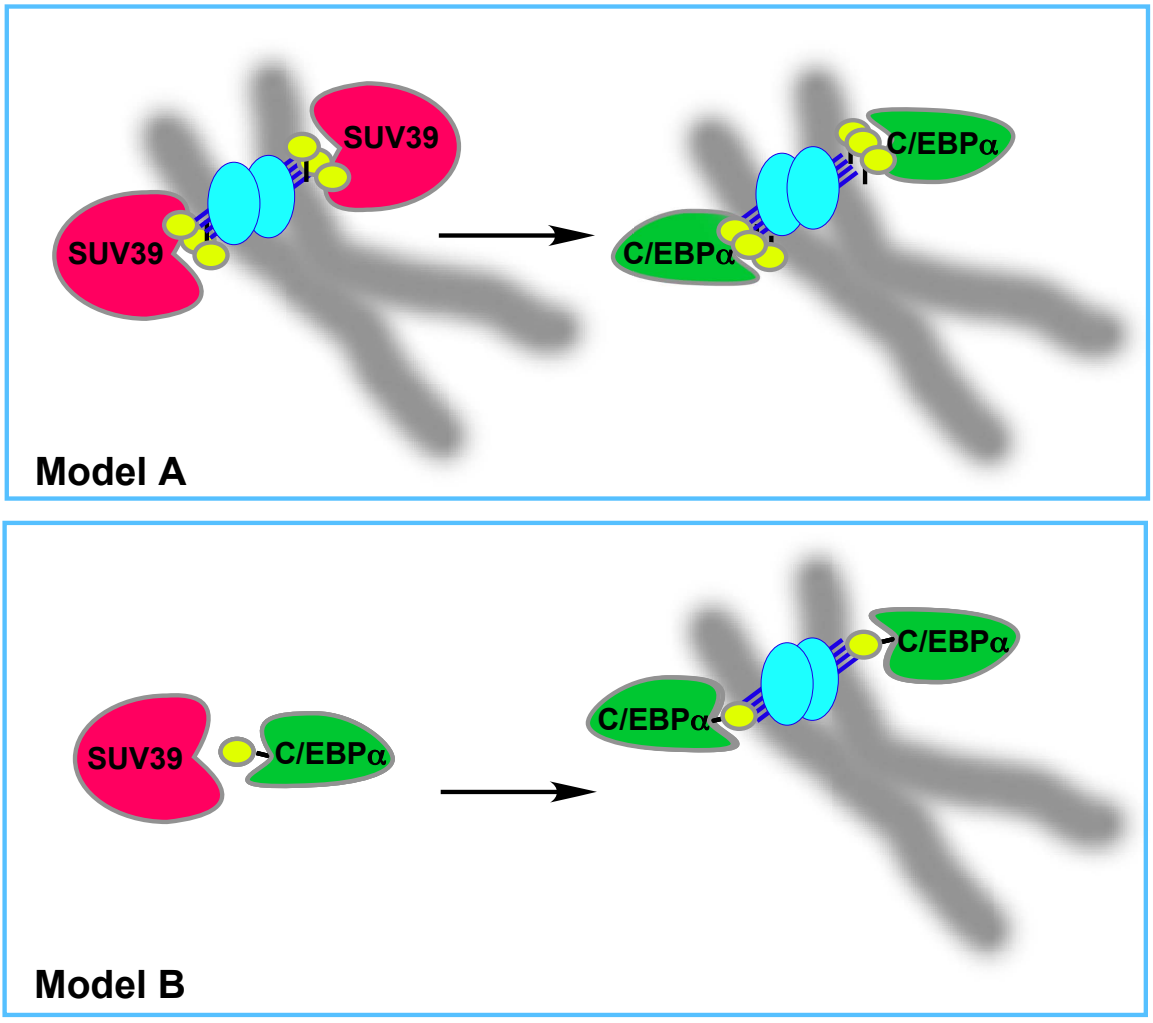

Figure 4.2: **Formation of C/EBPα foci on chromosomes.** Proposal of two model of how methylation by SUV39H1 could account for the accumulation of C/EBPα in discrete foci at chromosomes (see text for details). The blue bars represent rDNA clusters. The yellow circles are methylation marks, which are either set on chromosome-associated proteins (model A) or directly on C/EBPα (model B). The blue circle represent UBF foci which by immunofluoresence are visible as one rather deform foci located between two C/EBPα foci. The depictions are schematic, no attempt has been made to accurately display these structures.

lopoiesis (Johansen et al., 2001), while c-myc inhibits the expression of the C/EBPα gene in preadipocytes, preventing C/EBPα-induced differentiation (Freytag & Geddes, 1992);(Li et al., 1994). Furthermore, c-myc has been shown to suppress C/EBPα-mediated transactivation of genes (Mink et al., 1996). The effect of these proteins on cell cycle progression is also antagonistic, since C/EBPα blocks proliferation (section 1.1.2.) and c-myc counteracts cell cycle arrest (Spencer & Groudine, 1991). Given the antagonistic functions of C/EBPα and c-myc, it could be envisaged that these proteins also play opposite roles in rDNA regulation. As c-myc activates rDNA transcription (Poortinga et al., 2004), C/EBPα may repress rDNA transcription. The maintenance of c-Myc expression can induce myeloid leukemia (Felsher & Bishop, 1999), while C/EBPα deactivation are frequently found in AML patients. The reciprocal regulation and antagonizing functions of these proteins may determine the development of acute myeloid leukemia (AML). Cancers with deregulated expression of c-myc and loss of function of C/EBPα proteins might have enhanced ribosome biogenesis accompanying rDNA transactivation and thus may display sensitivity to cancer therapies directed at protein synthesis.

4.2.5 Implications for C/EBPβ

Data presented here show that the C/EBP family members C/EBPα and C/EBPβ associate with SUV39H1. It was further shown that SUV39H1 favors the formation of C/EBPα foci but not of C/EBPβ. The specificity of SUV39H1 for C/EBPα indicates that a certain amino acid sequence is required for foci formation which is not conserved between C/EBPα and C/EBPβ. Although SUV39H1 does not cause relocalization of C/EBPβ, their interaction could have other functional consequences. Both proteins are involved in Ras-induced senescence, as MEFs lacking either SUV39H1 or C/EBPβ fail to senesce upon Ras treatment (Braig et al., 2005);(Sebastian et al., 2005). Their common phenotypes suggests they may act in the same pathway. Furthermore, SUV39H1 and C/EBPβ have been shown to repress E2F-regulated genes required for S-phase genes (Narita et al., 2003);(Sebastian et al., 2005), which is believed to be a crucial point in the induction of senescence associated cell cycle arrest.

Alternative translation initiation from a single mRNA generates three C/EBPβ isoforms. An antiproliferative function has been attributed to the longer isoforms, LAP* and LAP, while the shorter LIP isoform has been speculated to enhance proliferation. Data not shown here also indicate that, despite the fact that LIP interacts with E2F, it fails to repress E2F's activity. The present work shows that SUV39H1 interacts with LAP and LAP*, but fails to interact with LIP. Thus, C/EBPβ may require binding to SUV39H1 to repress E2F. Repression of E2F

by C/EBPβ could involve the recruitment of SUV39H1 to E2F-regulated genes, analogous to the function of the E2F-repressor pRB in recruiting SUV39H1, HDACs and other repressive histone modifiers. Whether a C/EBPβ isoform interacts with SUV39H1 or not, could determine whether it represses E2F-regulated S-phase genes, and thus, its antiproliferative potential.

4.2.6 Concluding remarks

Data presented here demonstrate a role for SUV39H1 in regulating the cellular localization of C/EBPα to sites of ribosomal RNA genes. However, many questions remain open, the probably most intriguing being whether C/EBPα regulates the transcription of rDNA. The known function of C/EBPα and the observed similarities between C/EBPα foci and Runx2 foci, suggest that C/EBPα may repress rDNA transcription and thus abolish ribosomal biogenesis. Since suppression of rDNA often conveys with cell cycle arrest, SUV39H1-C/EBPα-mediated inhibition of rDNA may be the main determinant of the antiproliferative function of both SUV39H1 and C/EBPα. The antagonizing functions of C/EBPα and c-myc have been shown to determine granulocytic differentiation. Repression of rDNA by C/EBPα would also antagonize c-myc function, and thus, may play a role during granulopoiesis. Furthermore, the regulation of C/EBPα's localization may allow a spatial control of its function on known target genes. Finally, it remains to be determined whether C/EBPs and SUV39H1 might repress E2F-regulated genes in a synergistically manner. These questions will be the focus of future investigations.

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Appendix A

A.1 Abbreviations

μg microgram

μl microliter

μM micromolar

aa amino acids

APS Ammoniumpersulfate

AML Acute Myeloid Leukemia

BRM Basic Region Mutant

BSA Bovine serum albumin

bp base pair(s)

bZIP basic leucine zipper

C/EBP CCAATT/Enhancer Binding Protein

CDK Cyclin-dependent kinase

DAPI 4',6-diamidino-2-phenylindole

DMEM Dulbecco's modified Eagle medium

DMSO Dimethylsulfoxid

DNA Desoxyribonucleic acid

DP Dimerization Partner of E2F

DTT Dithiotreitol

E2F Early gene 2 factor

ECL Enhanced Chemiluminescence

E. coli Escherichia coli

EDTA Ethylenediaminetetraacetate

ER Estrogen receptor

EZH2 Enhancer of Zeste Homolog 2

FACS Fluorescence activated cell sorter

FCS Fetal calf serum

FITC Fluoresceinisothiocyanat

GFP Green-fluorescent protein

GST Glutathione S-transferase

H3K9met3 Tri-methylated Lysine 9 of Histone H3

HBS HEPES buffered saline

HDAC Histone-Deacetylase

HEPES N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)

HMTase Histone Methyltransferase

HRP Horseradish peroxidase

HP1 Heterochromatin protein 1

IBMX 3-isobutyl-1-methylxanthine

IF Immunofluorescence

IPTG Iso-propylthio-β-D-galactopyranoside

IRES internal ribosome entry site

MEF Mouse Embryonic Fibroblast

mg milligram

ml milliliter

mM millimolar

min minutes

mRNA Messenger-Ribonucleic acid

ng nanogram

nm nanometer

NP-40 Nonidet P-40

Oil Red O 1-8-[4-(Dimethylphenylazo)dimethylphenylazo]-2-naphthalenol

PAGE Polyacrylamide Gel Electrophoresis

PAI-I Plasminogen-Activator Inhibitor I

PBS Phosphate Buffered Saline

PcG Polycomb Group

PCR Polymerase-chain-reaction

PML Promyelocytic leukemia

Polybrene hexadimethrine bromide

PPARγ peroxisome proliferator-activated receptor γ

pRB Retinoblastoma protein

PRC Polycomb complex

PVDF Poly vinylidene difluoride

rpm Rotations per minute

rDNA ribosomal RNA genes

RNA Ribonucleic acid

rRNA ribosomal RNA

SET Su(var)3-9, Enhancer-of-zeste, Trithorax

SA-β-Gal Senescence-associated β-galactosidase

SDS Natriumdodecylsulfat

siRNA small interference RNA

Su(var)3-9 Suppressor of variegation 3-9

TAE Tris/acetate/EDTA buffer

TBS Tris-buffered Saline

TEMED (N,N,N',N'-Tetramethylethylenediamine)

TKO triple-knockout

Tris Tris(hydroxymethyl)aminomethane

Triton X-100 Octylphenoldecaethylenglycolether

Tween-20 Polyoxyethylensorbitanmonolaurat

UBF Upstream binding factor

UV Ultraviolet

X-Gal 5-bromo-4-chloro-3-indolyl β-D-galactoside

WT wild type

A.2 Primers

Cloning of...	Primers used for cloning
DP2 aa1 Forw	5'-CGG GAT CCA TGG TGC CCC AAA TGA TTA TAA GCA CAC CAC AG-3'
DP2 aa385 Rev	5'-CGG GAT CCG CGG CCG CTT ATT CTG GGG AGG AGG AAT CCT CCT-3'
DP2 aa72 Rev	5'-CGG GAT CCG CGG CCG CTT ATC TCA AGC CTT TCC CAT TTT TAT CTC C-3'
DP2 aa60 Forw	5'-CGG GAT CCA TGG TGC GAA GCA AAA AAG GAG ATA AAA ATG GG-3'
DP2 aa83 Forw	5'-CGG GAT CCA TGG TGC AAC GAA AAG GTA CAA CAT CG-3'
DP2 aa236 Rev	5'-CGG GAT CCG CGG CCG CTT AAA ACT TGT CAC TGG AGA TGC-3'
DP2 aa166 Rev	5'-CGG GAT CCG CGG CCG CTT ACC TCT GCT TCT CTA TCT CCA GAT-3'
DP1 aa105 Forw	5'-CG GGA TCC ATG GTG CGC AAC AGG AAA GGA GAG AAG AAT-3'
DP1 aa127 Forw	5'-CG GGA TCC ATG GTG CAG AGG AAA GGG ACC ACT TCC-3'
DP1 aa410 Rev	5'-CGG GAT CCG CGG CCG CTC AGT CGT CCT CGT CAT TC-3'
E2F1 aa1 Forw	5'-CGG GAT CCC ATA TGG CTT TGG CCG GGG CC-3'
E2F1 aa437 Rev	5'-CCG GAT CCG CGG CCG CTC AGA AAT CCA GGG GGG T-3'
mSuv39H1 BamHI Forw	5'-CAG TGG ATC CGC CAC CAT GGC GGA AAA TTT AAA AGG-3'
mSuv39H1 EcoRI Rev	5'-GCA CGA ATT CCT AGA AGA GGT ATT TTC GGC AAG CCG-3'

Table A.1: Primers used for cloning expression constructs. A forward and a reverse primer annealing with the source plasmid were used to generate a PCR product to be inserted in the target construct. aa = aminoacids, For = forward, Rev = reverse

Cloning of...	**Primers used for cloning**
scramble sense	5'-ACC TCG TCC ATC GAA CTC AGT AGC TTC AAG AGA GCT ACT GAG TTC GAT GGA CTT-3'
scramble antisense	5'-CAA AAA GTC CAT CGA ACT CAG TAG CTC TCT TGA AGC TAC TGA GTT CGA TGG ACG-3'
DP1 sense	5'-ACC TCG CAG CAT CTC CAA TGA CAA ATT CAA GAG ATT TGT CAT TGG AGA TGC TGC TT-3'
DP1 antisense	5'-CAA AAA GCA GCA TCT CCA ATG ACA AAT CTC TTG AAT TTG TCA TTG GAG ATG CTG CG-3'
E2F1 sense	5'-ACC TCG CCA AGA AGT CCA AGA ATC ATT CAA GAG ATG ATT CTT GGA CTT CTT GGC TT-3'
E2F1 antisense	5'-CAA AAA GCC AAG AAG TCC AAG AAT CAT CTC TTG AAT GAT TCT TGG ACT TCT TGG CG-3'
E2F3 sense	5'-ACC TCG CTC ACC AAG AAG TTC ATT CAT CAA GAG TGA ATG AAC TTC TTG GTG AGC TT-3'
E2F3 antisense	5'-CAA AAA GCT CAC CAA GAA GTT CAT TCA CTC TTG ATG AAT GAA CTT CTT GGT GAG CG-3'
E2F4 sense	5'-ACC TCC GAG AGT GAA GGT GTC TGT TTC AAG AGA ACA GAC ACC TTC ACT CTC GTT-3'
E2F4 antisense	5'-CAA AAA CGA GAG TGA AGG TGT CTG TTC TCT TGA AAC AGA CAC CTT CAC TCT CGG-3'

Table A.2: Primers used for cloning siRNA expression constructs. Sense and antisense primer were annealed and inserted in the BbsI site of the psiRNA construct.

A.3 Constructs

Construct name	Source details
pGEX4T2	GST-tag (for Nt fusion)
pGEX4T2 DP2 FL	human FL (aa1-385)
pGEX4T2 DP2 aa1-72	human mut coding aa1-72
pGEX4T2 DP2 aa60-385	human mut coding aa60-385
pGEX4T2 DP2 aa83-385	human mut coding aa83-385
pGEX4T2 DP2 aa60-236	human mut coding aa60-236
pGEX4T2 DP2 aa83-236	human mut coding aa83-236
pGEX4T2 DP2 aa60-166	human mut coding aa60-166
pGEX4T2 DP1 FL	human FL (aa1-410)
pGEX4T2 DP1Δ105	PCR fragment 5'-BamHI and 3'-NotHI
pGEX4T2 DP1Δ127	human
pGEX4T1 E2F1 WT	FL human WT
pGEX4T1 E2F1 E132	FL human E132: L132E, N133F substitution
pGEX4T2 E2F4	FL human
pGEX4T2 Suv39H1	mouse FL

Table A.3: Procaryotic expression constructs. FL = full-length, WT = wild-type, aa = aminoacids, del = deletion, mut = mutant, Ct = carboxi-terminal, Nt = amino-terminal

Construct name	Source details
pBabepuro	empty vector, puro selection
pBabepuroC/EBPα WT	FL rat WT, puro selection
pBabepuroC/EBPα BRM2	FL rat BRM2, puro selection

Table A.4: Retroviral expression constructs. FL = full-length, WT = wild-type, puro = puromycin

Construct name	**Source details**
pcDNA3	empty vector
pcDNA3-HA-DP1	human FL with Nt HA-tag
pcDNA3-HA-DP2	human FL with Nt HA-tag
pcDNA3-HA-E2F1	human FL with Nt HA-tag
pcDNA3-HA-E2F3	human FLwith Nt HA-tag
pcDNA3-HA-E2F4	human FLwith Nt HA-tag
pcDNA3-HA-E2F5	human FLwith Nt HA-tag
pcDNA3-E2F1 WT	human FL
pcDNA3-E2F1 E132	human L132E,N133F
pcDNA3-E2F1Y411C	human Y411C substitution
pcDNA1-E2F1Δ24	human del. aa79-103
pcDNA3-HA-Suv39h1	mouse FLwith Nt HA-tag
pcDNA3-(3xmyc)SUV39H1 WT	human FL with Nt triple-myc-tag
pcDNA3-(3xmyc)SUV39H1 H324L	human with Nt triple-myc-tag
pcDNA3-(3xmyc)SUV39H1 N-Chromo	human del. mut. with Nt triple-myc-tag
pcDNA3-(3xmyc)EHZ2	human FL sequence with Nt triple-myc-tag
pcDNA3-(3xFLAG) C/EBPα WT	FL rat WT with Ct triple-FLAG-tag
pcDNA3-(3xFLAG) C/EBPα BRM2	FL rat I294A,R297A, Ct triple-FLAG-tag
pcDNA3-(3xFLAG) C/EBPα BRM3	FL rat D301A,K304A, Ct triple-FLAG-tag
pcDNA3-(3xFLAG) C/EBPα BRM5	FL rat Y285A, Ct triple-FLAG-tag
pcDNA3-(3xFLAG) C/EBPα p30	p30 rat isoform with Ct triple-FLAG-tag
pcDNA3-FLAG-C/EBPα WT	FL rat WT with Ct FLAG-tag
pcDNA3-FLAG-C/EBP$\alpha\Delta$126-200	rat del. aa126-200 with Ct FLAG-tag
pcDNA3-FLAG-C/EBPβ LAP*	LAP* rat isoform with Ct FLAG-tag
pcDNA3-FLAG-C/EBPβ LAP	LAP rat isoform with Ct FLAG-tag
pcDNA3-FLAG-C/EBPβ LIP	LIP rat isoform with Ct FLAG-tag

Table A.5: Eucaryotic expression constructs. FL = full-length, WT = wild-type, aa = aminoacids, del = deletion, mut = mutant, Ct = carboxi-terminal, Nt = amino-terminal

A.4 Antibodies

Antibody	Host	Application	Dilution	Company
anti-C/EBPα (14AA)	rabbit	WB, IF	1:500	Santa Cruz
anti-C/EBPβ (C-19)	rabbit	WB, IF	1:500	Santa Cruz
anti-Bmi1 (F6)	mouse	IF	1:100	Upstate
anti-BrdU (BU-33)	mouse	IF	1:200	Sigma
anti-CENP-A (3-19)	mouse	IF	1:200	Abcam
anti-DP1 (K-20)	rabbit	WB	1:200	Santa Cruz
anti-DP1 (Ab-6)	mouse	WB	1:500	NeoMarkers
anti-DP2 (C-20)	rabbit	WB	1:500	Santa Cruz
anti-E2F1 (KH95)	mouse	WB	1:200	Santa Cruz
anti-E2F1 (C-20)	rabbit	WB	1:200	Santa Cruz
anti-E2F3 (C-18)	rabbit	WB	1:200	Santa Cruz
anti-E2F4 (C-20)	rabbit	WB	1:500	Santa Cruz
anti-H3 dimethyl K9	rabbit	IF	1:1000	Abcam
anti-H3 trimethyl K9	rabbit	IF	1:1000	Abcam
anti-HP1β	rat	IF	1:100	Abcam
anti-αtubulin (B-7)	mouse	WB	1:250	Santa Cruz
anti-PPARγ (H-100)	rabbit	WB	1:250	Santa Cruz
anti-PML (PG-M3)	mouse	IF	1:100	Santa Cruz
anti-UBF1 (F-9)	mouse	IF	1:100	Santa Cruz
anti-HA (12CA5)	mouse	WB,IF	1:1000	Roche
anti-GST	goat	WB	1:2000	Amersham
anti-FLAG M2	mouse	WB, IF	1:1000	Sigma
anti-FLAG M2 agarose	mouse	IP	-	Sigma
anti-myc (9E10)	mouse	IF	1:500	Santa Cruz

Table A.6: Primary antibodies. WB = Western Blot, IF = Immuno-Fluorescence, IP = Immunoprecipitation

Antibody	**Host**	**Detection**	**Dilution**	**Company**
anti-mouse IgG HRP	sheep	ECL	1:10000	GE Healthcare
anti-rabbit IgG HRP	donkey	ECL	1:10000	GE Healthcare
anti-goat IgG HRP	donkey	ECL	1:10000	Santa Cruz
anti-mouse IgG Alexa-Fluor®680	goat	Odyssey	1:3000	Invitrogen
anti-rabbit IgG Alexa-Fluor®680	goat	Odyssey	1:3000	Invitrogen
anti-mouse IgG IRDye 800	goat	Odyssey	1:3000	Invitrogen
anti-rabbit IRDye 800	goat	Odyssey	1:3000	Invitrogen
anti-mouse Alexa-Fluor®594	goat	IF	1:1000	Invitrogen
anti-rabbit Alexa-Fluor®555	goat	IF	1:1000	Invitrogen
anti-rat Alexa-Fluor®594	goat	IF	1:1000	Invitrogen
anti-mouse Alexa-Fluor®488	goat	IF	1:400	Invitrogen
anti-rabbit Alexa-Fluor®488	goat	IF	1:400	Invitrogen

Table A.7: Secondary antibodies. HRP = horseradish peroxidase, IF = Immuno-Fluorescence, ECL= Enhanced chemiluminescence

Appendix B

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List of Figures

List of Tables

Selbständigkeitserklärung

Ich versichere hiermit, dass die von mir vorgelegte Dissertation selbständig angefertigt wurde und ich die Stellen der Arbeit, die anderen Werken in Wortlaut oder Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe. Diese Dissertation wurde noch keiner anderen Fakultät zur Prüfung vorgelegt.

Berlin, 25. Juni 2008

Katrin Zaragoza Dörr