C/EBP β cooperates with RB:E2F to implement Ras^{V12}-induced cellular senescence

Thomas Sebastian¹, Radek Malik¹,
Sara Thomas¹, Julien Sage²
and Peter Frederick Johnson^{1,*}

¹Laboratory of Protein Dynamics and Signaling, NCI-Frederick, Frederick, MD, USA and ²Departments of Pediatrics and Genetics, Stanford University School of Medicine, Stanford, CA, USA

In primary cells, overexpression of oncogenes such as Ras^{V12} induces premature senescence rather than transformation. Senescence is an irreversible form of G₁ arrest that requires the p19^{ARF}/p53 and p16^{INK4a}/pRB pathways and may suppress tumorigenesis *in vivo*. Here we show that the transcription factor C/EBP β is required for Ras^{V12}-induced senescence. C/EBP β ^{-/-} mouse embryo fibroblasts (MEFs) expressing Ras^{V12} continued to proliferate despite unimpaired induction of p19^{ARF} and p53, and lacked morphological features of senescent fibroblasts. Enforced C/EBP β expression inhibited proliferation of wild-type MEFs and also slowed proliferation of p19^{ARF}^{-/-} and p53^{-/-} cells, indicating that C/EBP β acts downstream or independently of p19^{ARF}/p53 to suppress growth. C/EBP β was unable to inhibit proliferation of MEFs lacking all three RB family proteins or wild-type cells expressing dominant negative E2F-1 and, instead, stimulated their growth. C/EBP β decreased expression of several E2F target genes and was associated with their promoters in chromatin immunoprecipitation assays, suggesting that C/EBP β functions by repressing genes required for cell cycle progression. C/EBP β is therefore a novel component of the RB:E2F-dependent senescence program activated by oncogenic stress in primary cells.

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Introduction

Most immortalized rodent cell lines can be transformed by the Ha-ras oncogene (Ras^{V12}), leading to neoplastic growth in culture and tumorigenicity *in vivo* (Weinberg, 1989). In contrast, overexpression of Ras^{V12} or other activated oncogenes in many primary cells induces premature senescence, a permanent form of cell cycle arrest that is believed to play a role in tumor suppression (Serrano *et al*, 1997; Lin and Lowe,

2001). Cultured primary rodent fibroblasts also undergo 'spontaneous' senescence after 15–30 cell doublings, possibly resulting from prolonged exposure to mitogenic signals or accumulated DNA damage upon *ex vivo* culture ('culture shock'; Sherr and DePinho, 2000). The senescent state is characterized by expression of specific biochemical markers such as senescence-associated β -galactosidase and a distinctive cell morphology typified by a flattened, enlarged shape and increased focal adhesions (Goldstein, 1990; Campisi, 1996). The 'flat cell' phenotype is due in part to the assembly of actin stress fibers, which form a highly organized network of microfilaments and associations with other cytoskeletal proteins (Pawlak and Helfman, 2001).

Both Ras-induced and spontaneous senescence in mouse embryo fibroblasts (MEFs) require activation of the p19^{ARF}/p53 tumor suppressor pathway (Sherr and Weber, 2000). MEFs derived from p53^{-/-} or *INK4a* null mice (p19^{ARF} and p16^{INK4a} are *INK4a* gene products) are intrinsically immortalized, fail to undergo spontaneous or Ras-induced senescence, and are transformed by oncogenic Ras alone, underscoring the importance of the p19^{ARF}/p53 pathway in senescent cell cycle arrest. p19^{ARF} stabilizes p53 by sequestering the p53 E3 ligase, Mdm2. Induction of the p53 transcription factor subsequently provokes cell cycle arrest, although the mechanism by which p53 inhibits cell growth has not been fully elucidated. One transcriptional target of p53 that contributes to cell cycle arrest is the cyclin/Cdk2 inhibitor p21^{Cip1}, but other p53-regulated genes are likely to be involved (Groth *et al*, 2000).

The RB family of tumor suppressors (pRB, p107, and p130) also plays a critical role in cellular senescence by forming inhibitory complexes with E2F transcription factors that repress S-phase gene expression. pRB activity is controlled by the CDK inhibitor p16^{INK4a}, which is induced by Ras^{V12}. p16^{INK4a} impedes cell cycle progression by inhibiting the activity of Cdk4 and Cdk6, thus blocking phosphorylation of pRB and preventing its release from E2F. Senescence can be induced by overexpression of pRB (Xu *et al*, 1997; Alexander and Hinds, 2001), and MEFs doubly mutant for pRB and p107 or lacking all three RB family members are defective for Ras-induced cell cycle arrest (Dannenberg *et al*, 2000; Sage *et al*, 2000; Peeper *et al*, 2001). In contrast to p19^{ARF} or p53 null cells, *Rb*^{-/-}/p107^{-/-} MEFs are not transformed by Ras alone (Peeper *et al*, 2001). Hence, disruption of Ras^{V12}-induced senescence does not necessarily cause oncogenic transformation. pRB-imposed cell cycle arrest occurs even in cells lacking p53, indicating that pRB acts downstream of p53 or in another pathway (Alexander and Hinds, 2001). More recently, it was shown that E2F repressor complexes are downstream targets of p19^{ARF}/p53-induced proliferation arrest (Rowland *et al*, 2002), indicating a convergence of the p19^{ARF}/p53 and p16^{INK4a}/RB pathways at the level of E2F-RB.

While p53 and RB are essential mediators of oncogenic Ras-induced senescence, it is likely that additional pathways

*Corresponding author. Laboratory of Protein Dynamics and Signaling, NCI-Frederick, Building 539, Room 122, 7th Military Streets, Frederick, MD 21702-1201, USA. Tel.: +1 301 846 1627; Fax: +1 301 846 5991; E-mail: johnsopf@ncifcrf.gov

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or components of the p53 and RB pathways remain to be identified. In the present study, we have explored a possible role for the bZIP transcription factor C/EBP β (CCAAT/enhancer binding protein β). C/EBP β is broadly expressed and has diverse regulatory functions (Descombes *et al*, 1990; Poli *et al*, 1990; Cao *et al*, 1991; Williams *et al*, 1991). C/EBP β null mice display pleiotropic phenotypes, including defects in innate immunity (Tanaka *et al*, 1995) and female reproduction (Sterneck *et al*, 1997; Robinson *et al*, 1998; Seagroves *et al*, 1998) and impaired growth or differentiation of adipocytes (Tanaka *et al*, 1997; Tang *et al*, 2003), hepatocytes (Greenbaum *et al*, 1998), and keratinocytes (Zhu *et al*, 1999). C/EBP β activity is regulated by oncogenic Ras signaling (Nakajima *et al*, 1993; Kowenz-Leutz *et al*, 1994; Hanlon and Sealy, 1999; Zhu *et al*, 2002). Under basal conditions, C/EBP β is maintained in a latent, autoinhibited state by two negative regulatory domains (Kowenz-Leutz *et al*, 1994; Williams *et al*, 1995). However, in response to receptor tyrosine kinase activation or expression of Ras^{V12}, C/EBP β becomes derepressed and transcriptionally active (Nakajima *et al*, 1993; Kowenz-Leutz *et al*, 1994; Hanlon and Sealy, 1999). C/EBP β activation is mediated at least partly by ERK-dependent phosphorylation of a conserved threonine residue located in one of the regulatory domains (Nakajima *et al*, 1993).

Members of the C/EBP family, including C/EBP β , are implicated in regulating growth arrest of terminally differentiating cells and, when ectopically expressed, can inhibit cell proliferation (Umek *et al*, 1991; Hendricks-Taylor and Darlington, 1995). For example, forced expression of C/EBP β in HepG2 hepatoma cells induces cell cycle arrest at the G₁-S boundary (Buck *et al*, 1994), and C/EBP β inhibits colony formation of Balb/MK2 keratinocytes (Zhu *et al*, 1999). Conversely, C/EBP β knockout mice display mild epidermal hyperplasia, and C/EBP β -deficient primary keratinocytes show defective calcium-induced proliferation arrest *in vitro* (Zhu *et al*, 1999). These growth-inhibitory functions of C/EBP β , together with its role as a target of Ras^{V12} signaling, prompted us to investigate whether C/EBP β is involved in Ras-induced premature senescence.

Results

Impaired H-Ras^{V12}-induced senescence in C/EBP β ^{-/-} MEFs

To determine if C/EBP β has a role in oncogene-induced senescence, we examined the effect of overexpressing Ras^{V12} in C/EBP β ^{+/+} and C/EBP β ^{-/-} MEFs. Early-passage (P2/P3) MEFs were used throughout this study to avoid selecting immortalizing mutations in the p19^{ARF}-p53 pathway. Cells were infected with control or Ras^{V12}-expressing retroviruses and analyzed for proliferation over a 6-day time course (Figure 1A). As expected, expression of Ras^{V12} in wild-type MEFs provoked cell cycle arrest. However, Ras^{V12}-expressing C/EBP β ^{-/-} cells continued to proliferate. This response was observed for three independent C/EBP β ^{-/-} MEF populations, indicating that C/EBP β null cells are defective for Ras^{V12}-induced cell cycle arrest.

Analysis of nuclear extracts from wild-type MEFs showed only minor increases in C/EBP β expression and DNA-binding activity in Ras^{V12}-expressing cells (see Supplementary Figure 1). However, coexpression of Ras^{V12} stimulated C/EBP β -

mediated transcription of a C/EBP-driven reporter gene nearly 500-fold in transfected MEFs, whereas C/EBP β or Ras^{V12} alone caused only ~10-fold increases (Figure 1B). C/EBP β is thus strongly activated by Ras^{V12} signaling in MEFs, consistent with the idea that C/EBP β functions as a Ras effector in these cells.

We next asked whether Ras^{V12}-induced activation of the p19^{ARF}-p53 pathway is impaired in C/EBP β null MEFs, accounting for their failure to undergo cell cycle arrest. The levels of p19^{ARF}, p53, and other cell cycle regulators were examined on days 0 and 6 after drug selection (Figure 1C). p19^{ARF} and p53 expression was induced by Ras^{V12} to a similar extent in wild-type cells and C/EBP β ^{-/-} cells. p16^{INK4a} and p21^{CIP1} levels were also increased comparably in wild-type and C/EBP β ^{-/-} cells (Figure 1C and data not shown). Therefore, the inability of C/EBP β ^{-/-} MEFs to undergo Ras^{V12}-induced growth arrest is not due to defective engagement of the p19^{ARF}/p53 pathway or altered expression of CDK inhibitors. Cyclin A levels were diminished in Ras^{V12}-transduced wild-type MEFs relative to control cells, signifying G₁ arrest (Figure 1C, lanes 5 and 6), whereas cyclin A was unaffected or even increased by Ras^{V12} in C/EBP β ^{-/-} cells (lanes 7 and 8).

To determine whether proliferation of Ras^{V12}-expressing C/EBP β ^{-/-} MEFs is stably maintained or whether the cells eventually senesce, we propagated Ras-expressing and control C/EBP β ^{-/-} cells on a 3T3 protocol for four passages (Figure 2A). Cell numbers were comparable at each passage; moreover, the cells displayed similar growth rates before and after passaging (compare Figures 1A and 2B). The Ras-expressing cells also maintained elevated expression of p53 and p19^{ARF} (Figure 2C). Thus, Ras^{V12}-induced premature senescence is permanently disabled in C/EBP β ^{-/-} MEFs and proliferation remains unconstrained by induction of p19^{ARF}/p53 over many cell doublings.

We also passaged wild-type and C/EBP β ^{-/-} primary MEFs on a 3T3 protocol to determine if the mutant cells exhibit defects in spontaneous senescence (see Supplementary Figure 2). C/EBP β ^{-/-} cells maintained a low rate of proliferation even after numerous passages, whereas the wild-type cells eventually senesced. Moreover, the mutant cells showed an increased tendency to become immortalized, coinciding with loss of p19^{ARF} and/or p53 expression (Supplementary Figure 2 and data not shown). MEFs lacking C/EBP β are therefore resistant to both spontaneous and Ras-induced senescence.

Partially transformed properties of Ras^{V12}-expressing C/EBP β ^{-/-} MEFs

In addition to inducing growth arrest, Ras^{V12} elicits characteristic changes in fibroblast cell morphology (Serrano *et al*, 1997). Expression of Ras^{V12} in C/EBP β ^{+/+} MEFs induced the flat, enlarged appearance typical of senescent cells (Figure 3A). By contrast, C/EBP β -deficient cells expressing Ras^{V12} were highly refractile, had spindle-like projections, and displayed reduced contact inhibition. Thus, Ras^{V12}-expressing C/EBP β ^{-/-} MEFs adopt morphological features usually associated with transformed cells. We also compared the colony-forming ability of Ras^{V12}-transduced wild-type and C/EBP β ^{-/-} MEFs plated at low density. Cells of both genotypes produced detectable colonies; however, cells displaying a transformed-like morphology were observed

Figure 1 *C/EBPβ*^{-/-} MEFs are defective for Ras^{V12}-induced cell cycle arrest. (A) Early-passage (P2) wild-type and *C/EBPβ*^{-/-} MEFs were infected with control or Ras^{V12}-expressing pBabe-puro retroviruses, drug selected, and used for growth assays. A total of 2.5×10^4 cells/well were plated and cell numbers were measured over a 6-day period. Each value was normalized to the cell number at day 0 (post drug selection). (B) Transactivation assays. *C/EBPβ*^{-/-} MEFs were transfected with a C/EBP reporter construct ($2 \times$ C/EBP-luc) either alone or with expression plasmids for C/EBP β and/or Ras^{V12}. The cells were harvested and luciferase activity was determined; reporter activity was normalized to protein levels and the value for the reporter alone (-) was set to 1. Data are plotted as fold activation and are the means $\pm$ s.e. of three experiments. (C) Induction of cell cycle regulatory proteins. Western blot analysis was performed on cell lysates prepared at days 0 and 6 from wild-type and *C/EBPβ*^{-/-} MEFs transduced with Ras^{V12} or empty vector. An 80–100 μ g portion of each protein extract was analyzed by Western blotting for the indicated proteins.

only with *C/EBPβ*^{-/-} MEFs (Figure 3B). Many of these colonies had a star-like shape and stained intensely with crystal violet, and under higher magnification the cells showed dense growth and loss of contact inhibition (Figure 3B, right panel). In contrast, *C/EBPβ*^{+/+} colonies stained much less intensely and the cells displayed a flattened, senescent appearance.

To assess the tumorigenicity of Ras^{V12}-expressing *C/EBPβ*^{-/-} MEFs, we examined their ability to grow anchorage-independently and to form solid tumors in nude mice. Wild-type cells infected with the Ras virus were unable to form colonies in soft agar (Figure 3C). *C/EBPβ*^{-/-} MEFs were also incapable of anchorage-independent growth despite being highly proliferative under adherent conditions. By comparison (and as previously reported; Peeper *et al*, 2001), Ras^{V12}-expressing p53^{-/-} MEFs formed numerous large colonies in soft agar. When wild-type or *C/EBPβ*^{-/-} MEFs expressing Ras^{V12} were injected into athymic nude mice (3.5×10^5 cells/flank, eight mice per group), neither population gave rise to tumors, even after 10 weeks.

However, Ras^{V12}-transformed NIH 3T3 cells transplanted in a parallel experiment were highly tumorigenic. Thus, by two criteria, Ras-expressing *C/EBPβ*^{-/-} MEFs lack a fully transformed phenotype even though they evade cell cycle arrest.

Ras^{V12} and C/EBP β cooperate to induce cell cycle arrest

We next investigated the ability of C/EBP β , when expressed alone or in combination with Ras^{V12}, to induce proliferative arrest. Ras^{V12} and/or C/EBP β were introduced into wild-type and *C/EBPβ*^{-/-} MEFs by retroviral infection and cell proliferation was monitored over a 6-day period (Figure 4A). Expression of Ras^{V12} or C/EBP β alone in *C/EBPβ*^{+/+} cells significantly reduced their proliferation rate. However, cell cycle arrest was incomplete, as cell numbers increased modestly at days 2 and 4. In contrast, Ras^{V12} and C/EBP β together provoked rapid and efficient arrest, with no measurable increase in cell number at any time point. *C/EBPβ*^{-/-} cells failed to arrest in response to Ras^{V12} but their growth was significantly inhibited by C/EBP β , and the combination of Ras^{V12} and C/EBP β completely blocked proliferation.

Figure 2 Enhanced proliferative capacity of Ras^{V12}-expressing C/EBP β ^{-/-} MEFs. (A) Two independent populations of C/EBP β ^{-/-} MEFs were infected and selected as described in Figure 1A and were propagated on a 3T3 protocol for four passages. (B) Growth curves of C/EBP β ^{-/-} MEFs infected with empty vector or Ras^{V12}-expressing retrovirus, after passage 4. (C) Western blot analysis of p53, p19^{ARF}, and Ras in lysates prepared from the indicated MEFs at P1 and P4.

Therefore, Ras^{V12} and C/EBP β act synergistically to induce cell cycle arrest.

In addition to inhibiting proliferation, C/EBP β caused enlargement and flattening of C/EBP β ^{-/-} MEFs (Figure 4B). The cells were stained with phalloidin-FITC conjugate to examine actin-cytoskeleton rearrangements. In contrast to control cells, which were smaller, spindle-shaped, and lacked stress fibers, C/EBP β -transduced cells displayed a highly organized network of actin stress fibers (Figure 4B, right panels). Stress fiber formation induced by Ras^{V12} was also defective in C/EBP β ^{-/-} MEFs (data not shown). Together, these results demonstrate that C/EBP β regulates stress fiber formation and other morphological features associated with Ras^{V12}-induced senescence.

C/EBP β -mediated growth suppression is largely independent of p19^{ARF}/p53 but requires RB:E2F activity
MEFs deficient for p53, p16^{INK4a}/p19^{ARF}, or p19^{ARF} do not undergo replicative arrest and are transformed by Ras^{V12} alone (Serrano *et al*, 1996; Kamijo *et al*, 1997; Sharpless *et al*, 2001, 2004). To determine whether enforced C/EBP β

expression can arrest cells that lack *INK4a* gene products or p53, we expressed C/EBP β and/or Ras^{V12} in p53^{-/-}, p16^{INK4a}^{-/-}, and p19^{ARF}^{-/-} MEFs and analyzed cell proliferation. As expected, Ras^{V12} suppressed the growth of wild-type and p16^{INK4a}^{-/-} MEFs but not p19^{ARF}^{-/-} or p53^{-/-} cells (Figure 5A). C/EBP β decreased the proliferation of all genotypes, although to a lesser degree in p53 and p19^{ARF} null MEFs, and its inhibitory effects were significantly enhanced by Ras^{V12}. Cell proliferation was also assessed by colony formation in low-density plating assays (Figure 5B). Wild-type or p16^{INK4a}^{-/-} MEFs expressing Ras^{V12} did not produce detectable colonies, whereas p19^{ARF}^{-/-} and p53^{-/-} cells gave rise to large numbers of transformed colonies that stained intensely with crystal violet. The size and number of these colonies were reduced by C/EBP β coexpression. Collectively, the results of Figure 5 show that C/EBP β inhibits cell proliferation and Ras transformation in a manner that is largely independent of the p19^{ARF}/p53 pathway.

The properties of C/EBP β ^{-/-} MEFs are similar in several respects to those of cells lacking both pRB and p107 or all three RB family members (triple knockout, 'TKO')

Figure 3 Growth properties of Ras^{V12}-expressing MEFs. (A) Cell morphology of control or Ras^{V12}-expressing wild-type and *C/EBP β* ^{-/-} MEFs at day 6 postselection. (B) Colony formation assay of wild-type and *C/EBP β* ^{-/-} MEFs infected with control or Ras^{V12} retroviruses. A total of 2.5×10^4 cells were seeded into 10 cm plates, cultured for 2 weeks, and then stained with crystal violet. The right panels show higher magnification images of two selected colonies. Quantitation of intensely stained (i.e., transformed-like) colonies obtained from two MEF lines of each genotype is shown at the bottom. (C) Soft agar colony assays. Ras^{V12}- or control vector-infected MEFs of the indicated genotypes were seeded into soft agar (2.5×10^4 cells/6 cm dish). The plates were photographed after 2 weeks.

Figure 4 Effect of *C/EBP β* and Ras^{V12} on cell growth and cytoskeletal reorganization. (A) Growth curves of wild-type and *C/EBP β* ^{-/-} MEFs transduced with *C/EBP β* and/or Ras^{V12}. Each value was normalized to the cell number at day 0 (postselection). A representative example is shown from at least two independent experiments performed in duplicate. Bottom panel: Western blot analysis of *C/EBP β* expression. (B) Cell morphology of control and *C/EBP β* -transduced *C/EBP β* ^{-/-} MEFs. Cells were photographed at day 6 postselection. The cells were also stained for stress fibers using FITC-conjugated phalloidin (right panels). A representative example from two independent experiments is shown; photographs were taken at the same magnification.

(Dannenberg *et al*, 2000; Sage *et al*, 2000; Peeper *et al*, 2001). To determine whether *C/EBP β* arrests cells by an RB-dependent mechanism, we examined the effects of expressing *C/EBP β* in MEFs lacking *RB* genes (Figure 6). *C/EBP β* inhibited the growth of wild-type and *p107*^{-/-}/*p130*^{-/-} cells and also suppressed proliferation of *Rb*^{-/-}/*p130*^{-/-} and *Rb*^{-/-}/*p107*^{-/-} MEFs, albeit less efficiently. Remarkably, *C/EBP β* not only failed to inhibit the growth of TKO cells, but also significantly increased their proliferation rate. Western blot analysis showed that *C/EBP β* was over-expressed to similar levels in each cell line (lower panel). *C/EBP α* , which also potently inhibits proliferation of many cells (Nerlov, 2004), diminished the growth of wild-type and *p107*^{-/-}/*p130*^{-/-} cells and, to a lesser extent, *Rb*^{-/-}/*p130*^{-/-} and *Rb*^{-/-}/*p107*^{-/-} MEFs, yet it accelerated proliferation of TKO cells (Figure 6). Thus, both *C/EBP* isoforms require at least one RB family member to induce cell cycle arrest and can stimulate proliferation in the absence of all three pocket proteins.

We next analyzed the effect of Ras^{V12} on proliferation of *C/EBP β* -expressing TKO MEFs (Figure 7A). As observed

Figure 5 Effect of C/EBP β and Ras^{V12} on proliferation of p53^{-/-}, p16^{INK4a}-/-, and p19^{Arf}-/- MEFs. **(A)** Primary MEFs of the indicated genotypes were infected sequentially with retroviruses expressing C/EBP β and Ras^{V12} and cell proliferation was analyzed over a time course. Each value was normalized to the cell number at day 0 (postselection). Each experiment was performed twice with similar results; data are the mean $\pm$ s.e. of triplicate time points from a representative experiment. **(B)** The same cells were plated at low density (2×10^4 cells/10 cm dish) and colonies were visualized 2 weeks later.

previously, C/EBP β and/or Ras^{V12} strongly inhibited proliferation of wild-type cells. However, Ras^{V12} failed to suppress the growth of TKO MEFs and also enhanced the

ability of C/EBP β to stimulate proliferation (most apparent at day 8 of the time course). C/EBP β also increased the colony-forming activity of TKO cells, as did Ras^{V12}

Figure 6 C/EBP β requires RB family members to induce growth arrest. Primary MEFs of the indicated genotypes were infected with C/EBP β or C/EBP α retroviruses and cell growth was analyzed over a time course. Each curve was performed at least twice, and time points were determined in triplicate. Bottom panel: Western blots of C/EBP β and C/EBP α expression.

(Figure 7B). Cells expressing both genes generated similar numbers of colonies but many of these stained more densely with crystal violet, indicating the presence of aggressively proliferating cells. Ablation of all three *Rb* genes therefore disrupts the antiproliferative effects of C/EBP β and/or Ras^{V12}.

RB proteins inhibit cell proliferation through their association with E2F transcription factors, which target RBs to specific growth-regulatory genes. Thus, we tested whether E2F function is required for the antiproliferative activity of C/EBP β by transducing wild-type and C/EBP β ^{-/-} MEFs with a dominant-negative form of E2F-1 ('E2F-DB') that lacks the transactivation and RB protein-binding domains (Krek *et al*, 1995; Rowland *et al*, 2002). The cells were subsequently infected with C/EBP β or control viruses and growth rates were analyzed (Figure 7C). E2F-DB alone had little effect on the proliferation rate, although there was a slight stimulation in wild-type MEFs. However, coexpression of C/EBP β and E2F-DB caused a significant increase in proliferation, in contrast to growth inhibition by C/EBP β alone. Therefore, disruption of the RB:E2F axis by deletion of all three *Rb* genes

or expression of dominant negative E2F reverses the cellular growth response to C/EBP β .

C/EBP β downregulates expression of E2F target genes and associates with their promoters

C/EBP β might induce G₁ arrest by inhibiting expression of E2F-regulated S-phase genes, as has been proposed for C/EBP α (Timchenko *et al*, 1999; Slomiany *et al*, 2000; Porse *et al*, 2001). We used RT-PCR to analyze mRNA levels of several E2F-regulated genes in C/EBP β ^{-/-} MEFs infected with C/EBP β virus (growth-arrested), Ras^{V12} (proliferating), or the empty vector (Figure 8A). C/EBP β decreased the expression of *E2F-1*, *c-Myc*, *DHFR*, *cyclin A2*, and *PCNA* to varying degrees, while *Cdc25A* levels were unaffected. The C/EBP β -induced decrease in gene expression, while modest, was greater than that observed in senescent wild-type MEFs overexpressing Ras^{V12} (data not shown). In contrast, Ras^{V12} generally increased expression of E2F-regulated genes in C/EBP β ^{-/-} MEFs (except for *cyclin A2*), consistent with the continued proliferation of these cells. We next used chromatin immunoprecipitation (ChIP) assays to examine C/EBP β

Figure 7 (A) Growth curves of TKO MEFs overexpressing C/EBP β and/or Ras^{V12}. Data are the means $\pm$ s.e. of triplicate assays. Right panel: Western blot for C/EBP β expression. (B) Colony assays of the TKO MEFs from panel A. (C) Dominant negative E2F disrupts C/EBP β -induced arrest. Wild-type or C/EBP β ^{-/-} MEFs were infected with vectors for C/EBP β and/or dominant negative E2F-1 (E2F-DB) and growth curves were performed. Right panel: Western blot analysis of E2F-DB and C/EBP β expression.

association with the proximal promoters of the same genes, using PCR primers that amplify sequences spanning the known E2F sites (Figure 8B). Using two different C/EBP β antibodies and an E2F4 antiserum, we detected specific binding of C/EBP β and E2F4 to the *E2F-1*, *c-Myc*, *DHFR*, *cyclin A2*, and *PCNA* promoters; E2F4 also bound to *Cdc25A*. Promoter binding was indicated by increased PCR signals with specific antibodies versus control IgG as well as signal reduction in the presence of blocking peptides. By these criteria, C/EBP β showed no binding to the negative controls (β -2 microglobulin and lamin B1) or to the *Cdc25A* promoter. Hence, C/EBP β is associated with several E2F target genes, and binding correlates with inhibition of expression. Analysis of the promoter sequences using the TESS search tool (www.cbil.upenn.edu/teess/) revealed several potential C/EBP binding sites that could mediate repression by C/EBP β (data not shown). In the case of *DHFR*, we identified a novel C/EBP site adjacent to the E2F element that binds C/EBP β and is required for transcriptional repression in reporter assays (Supplementary Figure 3). These data support a mechanism whereby C/EBP β elicits cell cycle arrest by repressing E2F target genes.

Discussion

We have shown that C/EBP β null MEFs overexpressing Ras^{V12} fail to undergo senescence and instead display properties of partially transformed cells. C/EBP β mutant cells also show increased resistance to spontaneous senescence. Conversely, overexpression of C/EBP β in MEFs suppressed cell growth and, in conjunction with Ras^{V12}, induced complete cell cycle arrest. Cells transduced with C/EBP β also acquired a senescent-like morphology, displaying a flat, extended cell shape and prominent stress fibers. These findings establish C/EBP β as a novel regulator of the senescence program in primary fibroblasts.

C/EBP β -mediated cell cycle arrest requires RB-E2F

Induction of p19^{ARF} and p53 by Ras^{V12} was normal in C/EBP β -deficient cells, indicating that C/EBP β is not an upstream component of the p19^{ARF}/p53 pathway; moreover, C/EBP β was capable of suppressing the growth of p19^{ARF}^{-/-} or p53^{-/-} MEFs, particularly when coexpressed with Ras^{V12}. Thus, C/EBP β apparently functions independently or downstream of p19^{ARF}/p53 to implement cell cycle arrest and

Figure 8 C/EBP β downregulates and associates with E2F target genes. **(A)** RT-PCR analyses. Total RNA from C/EBP β ^{-/-} MEFs infected with control, C/EBP β , or Ras^{V12} viruses was analyzed by RT-PCR to detect expression of E2F-1, c-myc, DHFR, cyclin A2, Cdc25A, and PCNA. GAPDH was used as an internal standard. The PCR products were quantitated and normalized to the corresponding GAPDH levels, and are expressed relative to controls. **(B)** ChIP assays of NIH 3T3 cells overexpressing C/EBP β . Chromatin was immunoprecipitated with the indicated antibodies and the recovered DNA was analyzed by PCR using primers corresponding to the indicated genes. C/EBP β COOH-terminal (C-term), C/EBP β NH₂-terminal (N-term), and E2F4 antibodies were used, as well as a normal rabbit IgG control. Specific binding of the antibodies was determined by preincubating the antibodies with their respective blocking peptides (BP) overnight before using in the immunoprecipitation reaction. Input represents 2% of the total chromatin. β 2-Microglobulin (β 2M) and lamin B1 are negative controls and IL-6 is a positive control for C/EBP β binding.

senescence. Because C/EBP β did not fully arrest p19^{Arf}^{-/-} or p53^{-/-} MEFs, it is possible that the p19^{Arf}/p53 pathway also has C/EBP β -independent targets that contribute to growth arrest.

C/EBP β 's ability to suppress cell growth was reduced in Rb^{-/-}/p107^{-/-} MEFs and was completely abolished in TKO cells. C/EBP β diminished the proliferation of all combinations of double RB knockout MEFs but not TKO cells, demonstrating functional overlap among pRB, p107, and p130 in constraining cell growth (Dannenberget al, 2000; Sage et al, 2000). C/EBP β and pRB were previously found to associate *in vitro* and *in vivo*, and pRB can enhance the DNA-binding and transcriptional activities of C/EBP β (Chen et al, 1996b). These two proteins were also implicated in terminal differentiation of adipocytes (Chen et al, 1996a). Thus, C/EBP β may facilitate RB-mediated cell cycle exit in both senescent and terminally differentiating cells.

C/EBP β increased the proliferation of TKO MEFs; moreover, an RB binding-defective form of E2F-1 also disrupted C/EBP β -induced cell cycle arrest and rendered C/EBP β capable of stimulating proliferation. These findings strongly implicate RB:E2F complexes as targets of C/EBP β -dependent growth arrest. A previous study using E2F-DB demonstrated that E2F function is required for cellular senescence induced by p19^{Arf}, p53, and Ras^{V12} (Rowland et al, 2002). Apparently, both p53 and C/EBP β act on or in conjunction with RB:E2F complexes to implement senescence in cells overexpressing Ras^{V12}. Whether there is a direct connection between p53 and C/EBP β in this pathway remains to be established.

Ectopic expression of C/EBP β decreased transcripts from several E2F target genes and ChIP assays demonstrate binding to their promoters. Hence, C/EBP β may directly repress genes required for cell cycle progression. C/EBP β also represses transcription from a DHFR promoter-reporter in wild-type cells but not in TKO MEFs (Supplementary Figure 3), paralleling its effects on the growth of these cells. Transcriptional repression requires a C/EBP-like element adjacent to the E2F site in the DHFR promoter. The related C/EBP α protein can repress transcription from the DHFR promoter and other E2F-regulated genes such as Myc (Timchenko et al, 1999; Slomiany et al, 2000; Johansen et al, 2001; Porse et al, 2001). It was proposed that C/EBP α repression involves an indirect (i.e., non-DNA-binding) mechanism requiring the E2F sites in these promoters (Timchenko et al, 1999; Slomiany et al, 2000; Johansen et al, 2001; Porse et al, 2001). However, a more recent report suggests that C/EBP α can bind directly to the DHFR E2F site (Iakova et al, 2003). We detected weak binding of C/EBP β to the DHFR E2F motif but much stronger interaction with the adjacent C/EBP-like sequence (Supplementary Figure 3), further suggesting that the latter is the functional C/EBP β response element. Additional studies are required to determine whether other E2F target genes contain C/EBP sites that mediate transcriptional responses to C/EBPs.

C/EBP β functions as an effector of Ras signaling

Our studies examined C/EBP β function in primary fibroblasts overexpressing Ras^{V12}. These cells display constitutive high-level Ras signaling and sustained activation of the MEK/ERK cascade and are irreversibly growth-arrested (Serrano et al, 1997; Lin et al, 1998). In this context, C/EBP β is a key effector of Ras-induced senescence. Since C/EBP β activity is strongly potentiated by Ras^{V12} (Figure 1B) (Nakajima et al, 1993; Kowenz-Leutz et al, 1994; Zhu et al, 2002), its antiproliferative effects may also require post-translational activation

by downstream Ras effectors. Ras^{V12} expression induces phosphorylation on at least two C/EBP β residues, Thr188 (Nakajima *et al*, 1993) and Ser64 (Shuman *et al*, 2004), and C/EBP β mutants lacking these phosphoacceptor residues are functionally impaired in certain Ras-dependent activity assays (Nakajima *et al*, 1993; Kowenz-Leutz *et al*, 1994; Hanlon and Sealy, 1999; Zhu *et al*, 2002; Mo *et al*, 2004; Shuman *et al*, 2004). However, alanine substitutions at residues 188 and/or 64 do not disrupt C/EBP β 's ability to inhibit proliferation of C/EBP β ^{-/-} MEFs, even in the presence of Ras^{V12} (T Sebastian and PF Johnson, unpublished data). Hence, phosphorylation of these two sites is not essential for cell cycle arrest. We are currently investigating whether other Ras-induced modifications on C/EBP β may be involved.

Studies using conditional activation of an endogenous mutant *K-Ras* allele (*K-ras*^{G12D}) indicate that oncogenic Ras does not always cause growth arrest in normal cells (Tuveson *et al*, 2004). Activation of endogenous *K-ras*^{G12D} stimulates proliferation of primary MEFs, and in the lung and intestine it induces preneoplastic hyperplasias. Thus, it was suggested that senescence is elicited specifically by sustained, high-level Ras signaling, such as when oncogenic Ras is overexpressed (Tuveson *et al*, 2004). Conceivably, C/EBP β is part of the sensing mechanism for high-intensity Ras signaling, instructing cells to senesce under conditions of oncogenic stress instead of undergoing normal responses to Ras signals. In this regard, it will be important to examine the effect of C/EBP β deficiency on proliferation of cells expressing endogenous levels of oncogenic Ras.

Pro- and antiproliferative functions for C/EBP β

Our study demonstrates that C/EBP β is required for Ras^{V12}-induced growth arrest in primary fibroblasts and therefore might act as a tumor suppressor. Paradoxically, C/EBP β also promotes the growth and survival of some cells and tumors. For example, C/EBP β ^{-/-} mice are completely resistant to the development of papillomas in the two-stage model of skin carcinogenesis, which produces Ras-dependent tumors (Zhu *et al*, 2002). The protumorigenic effect of C/EBP β in skin cancer may involve its ability to suppress apoptosis. In addition, Myc/Raf-transformed murine macrophages are dependent on C/EBP β for survival; this antiapoptotic activity is mediated by autocrine production of IGF-I, whose gene is regulated by C/EBP β (Wessells *et al*, 2004). Forced expression of C/EBP β in transformed macrophages also greatly increases colony size, suggesting that C/EBP β promotes proliferation as well as survival of these cells. Similar promitogenic effects of C/EBP β were observed in mammary epithelial cells (Bundy and Sealy, 2003) and hepatic cells (Buck *et al*, 1999). Thus, depending on the cell type, C/EBP β can either augment or inhibit growth. Such opposite activities are reminiscent of E2F, which mediates the antiproliferative and tumor suppressor effects of pRB but can also stimulate growth by positively regulating transcription of S-phase genes when pRB is mutated or phosphorylated (Johnson, 2000). These parallels may indicate that C/EBP β , like E2F, interacts with RB proteins to promote cell cycle arrest in specific cells. Future studies should illuminate the molecular basis for cell-specific growth responses to C/EBP β .

Materials and methods

Plasmid constructs

The pcDNA3.1-C/EBP β vector was described previously (Shuman *et al*, 2004).

Cell culture and preparation of MEFs

Wild-type and C/EBP β ^{-/-} MEFs were prepared from day 13.5 embryos derived from mating C/EBP β ^{+/-} mice (Sterneck *et al*, 1997) on pure 129/Sv and C57BL/6 genetic strain backgrounds. Cells were cultured in DMEM (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Hyclone), 200 mM L-glutamine, and 100 U/ml penicillin-streptomycin (Gibco). Phoenix ecotropic packaging cells (provided by H Young), p53^{-/-} MEFs (P3) (Harvey *et al*, 1993), p16^{ink4a}^{-/-} (P5) and p19^{Arf}^{-/-} (P3) MEFs (Sharpless *et al*, 2001, 2004), and Rb^{-/-}/p130^{-/-}, Rb^{-/-}/p107^{-/-}, p107^{-/-}/p130^{-/-}, and Rb^{-/-}/p107^{-/-}/p130^{-/-} (TKO) MEFs (Danenberg *et al*, 2000; Sage *et al*, 2000) were propagated in the same medium. For 3T3 experiments, MEFs were maintained on a 3-day serial passaging protocol (Todaro and Green, 1963). Cells (3 × 10⁵) were plated in 6 cm dishes, and 3 days later the cell number was determined and 3 × 10⁵ cells were replated.

Retroviral vectors and viral infection

MEFs were infected with a pBabe-puro vector expressing human H-Ras^{V12} cDNA (pBabe-H-Ras^{V12}) provided by S Lowe; expression of H-Ras^{V12} using pLXSP3 or pWZL-hygro gave similar results. pWZL-hygro and pBabe-puro were used to generate retroviruses expressing the p35 form of murine C/EBP β (Descombes and Schibler, 1991) or the p42 form of rat C/EBP α . pWZL-hygro and pWZL-H-Ras^{V12}-hygro were obtained from K Vousden. pBabe-puro expressing truncated E2F-1 (residues 1–368; 'E2F-DB') was kindly provided by B Rowland (Krek *et al*, 1995; Rowland *et al*, 2002). Retroviral plasmids were transfected into the Phoenix packaging line using a standard CaPO₄ method. At 24–72 h after transfection, viral supernatants were collected every 5–6 h, pooled, filtered (0.45 μ m), supplemented with 5 μ g/ml polybrene, and used to infect P2-P3 MEFs. Three infections were performed and the cells were selected for 3 days in 2 μ g/ml puromycin or for 5 days in 100 μ g/ml hygromycin. Multiple genes were introduced by sequential infection and drug selection.

Growth curves

Retrovirally infected MEFs were seeded at 2.5 × 10⁴ cells/well in six-well plates. At the indicated times, cells were washed with phosphate-buffered saline (PBS), fixed in 10% formalin, rinsed with water, stained with 0.1% crystal violet (Sigma) for 30 min, rinsed extensively, and dried. The dye was extracted with 10% acetic acid and absorbance measured at 590 nm. All values were normalized to day 0.

Colony assays

MEFs were plated at 2 × 10⁴ cells/10 cm dish in DMEM containing 10% FBS, and after 2 weeks colonies were visualized by crystal violet staining. For soft agar colony assays, cells were placed into 0.35% agar in DMEM containing 10% FBS at 2.5 × 10⁴ cells/6 cm plate and seeded onto solidified 0.7% agar containing culture medium. The cells were fed weekly and colonies were evaluated after 2 weeks.

Reporter assays

C/EBP β ^{-/-} MEFs were plated 12 h prior to transfection (7 × 10⁴ cells/well in six-well plates). A 1 μ g portion of 2 × C/EBP Luc reporter plasmid was cotransfected with 100 ng of murine C/EBP β expression vector (pcDNA3.1-C/EBP β) and/or H-Ras^{V12} (pcDNA3.1-Ras) using Fugene6 (Roche Molecular Biochemicals). At 16 h prior to harvesting, the cells were placed into medium containing 0.5% serum. At 48 h after transfection, the cells were lysed and analyzed using the Luciferase assay system (Promega). Luciferase values were normalized to protein levels; the data represent the average of three independent determinations and are graphed as the mean ± s.e.

Immunoblotting

Lysates from retrovirally transduced MEFs were prepared in NP-40 lysis buffer (50 mM Tris-HCl (pH 8.0), 400 mM NaCl, 1% NP-40,

1 mM EDTA) containing protease inhibitors and cleared by high-speed centrifugation. Nuclear extracts were prepared as described (Baer *et al*, 1998). A 25 μ g portion of nuclear extract or 80–100 μ g of whole cell lysate was resolved by 12% SDS-PAGE and blotted to nitrocellulose membranes. Primary antibodies used were as follows: C/EBP β (Santa Cruz, C-19, 1:1000), C/EBP α (Santa Cruz, 14AA, 1:1000), p53 (Novacastra, CM5, 1:1000), p19^{ARF} (Abcam, ab80, 1:500), p16^{INK4a} (Santa Cruz, M-156, 1:1000), cyclin A (Santa Cruz, C-19, 1:1000), E2F-1 (Santa Cruz, KH-95, 1:1000), and actin (Santa Cruz, C-11, 1:1000). Secondary antibodies conjugated to horseradish peroxidase were used to detect antigen-antibody by chemiluminescence (ECL detection system; Pierce).

Immunofluorescence staining of actin stress fibers

MEFs grown on 18 mm glass coverslips were fixed with 3.7% formaldehyde in PBS for 10 min, permeabilized with 0.2% Triton X-100 in PBS for 5 min, and blocked with 1% bovine serum albumin in PBS for 30 min. To visualize actin stress fibers, samples were incubated with FITC-conjugated phalloidin (Molecular Probes) for 20 min at room temperature. The cells were washed three times with PBS and mounted on glass slides using Vectashield (Vector Laboratories). Fluorescence images were obtained using a Zeiss microscope.

RT-PCR analysis

Total cellular RNA was prepared using Trizol reagent (Invitrogen). A 1 μ g portion of total RNA was reverse transcribed using the first strand cDNA synthesis kit (Super Array). After reverse transcription, cDNAs were amplified using primers for GAPDH (control), E2F-1, c-myc, DHFR, cyclin A2, Cdc25A, and PCNA. PCR products were separated on agarose gels and visualized by ethidium bromide staining. The gene-specific primers and Hot-start PCR reagents were purchased from SuperArray Bioscience.

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Chromatin immunoprecipitation

ChIP assay was performed according to Spencer *et al* (2003) with minor modifications. In brief, subconfluent cell cultures (15 cm dish per one ChIP reaction) were crosslinked by addition of 1% formaldehyde and incubated for 10 min at room temperature. Cells were washed with PBS and resuspended in lysis buffer (0.1% SDS, 0.5% Triton X-100, 150 mM NaCl, 20 mM Tris-HCl, pH 8.1) and sonicated to obtain DNA fragments of 500–1000 bp. Immunoprecipitation was performed using 5 μ g of the following antibodies: C/EBP β C-terminal (C-19, Santa-Cruz), C/EBP β N-terminal (Williams *et al*, 1991), and E2F-4 (C-20, Santa-Cruz). In control reactions, antibodies were preincubated overnight with their respective blocking peptides. Samples were incubated with antibodies overnight at 4°C and then StaphA cells (Calbiochem) were added and incubated for 30 min at 4°C. Precipitates were washed and processed for DNA purification. DNA was amplified by PCR using sequence-specific primers (30–35 cycles). Primers sequences are available upon request.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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