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Ras^{V12}-Mediated Down-Regulation of CCAAT/Enhancer Binding Protein β in Immortalized Fibroblasts Requires Loss of p19^{Arf} and Facilitates Bypass of Oncogene-Induced Senescence

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Abstract

The transcription factor C/EBP β is involved in cellular responses to oncogenic and physiological Ras signals. C/EBP β is required for premature senescence of primary mouse fibroblasts induced by expression of H-Ras^{V12}, demonstrating its role in oncogene-induced senescence. Here we have investigated the mechanisms by which Ras inhibits proliferation of normal cells but transforms immortalized cells. We show that oncogenic Ras down-regulates C/EBP β expression in NIH 3T3 cells, which are immortalized by a deletion of the *CDKN2A* locus and therefore lack the p16^{Ink4a} and p19^{Arf} tumor suppressors. Ras^{V12}-induced silencing of C/EBP β occurred at the mRNA level and involved both the Raf/MEK/ERK and PI3K signaling pathways. Oncogenic Ras decreased C/EBP β expression in *Ink4a/Arf*^{-/-} MEFs but increased C/EBP β levels in wildtype MEFs. C/EBP β down-regulation in NIH 3T3 cells was reversed by expression of p19^{Arf} but not p53 or p16^{Ink4a}, highlighting a critical role for p19^{Arf} in sustaining C/EBP β levels. Ectopic expression of p34 C/EBP β (LAP) inhibited Ras^{V12}-mediated transformation of NIH 3T3 cells, suppressed their tumorigenicity in nude mice, and reactivated expression of the pro-apoptotic Fas receptor, which is also down-regulated by Ras. Our findings indicate that *CEBPB* gene silencing eliminates a growth-inhibitory transcription factor that would otherwise restrain oncogenesis. We propose that C/EBP β is part of a p53-independent, p19^{Arf}-mediated network that enforces Ras-induced cell cycle arrest and tumor suppression in primary fibroblasts.

Keywords

Ras; C/EBP β ; p19^{Arf}; oncogenesis; tumor suppression

Introduction

Ras genes frequently sustain oncogenic mutations in human and rodent cancers and many immortalized cell lines can be transformed by introduction of activated *Ras* alleles such as *H-Ras*^{V12} (1). In contrast, over-expression of oncogenic Ras in normal primary cells such as mouse embryo fibroblasts (MEFs) induces senescence, an irreversible state of cell cycle arrest that provides an intrinsic barrier to tumor development (2). Oncogene-induced senescence (OIS) requires activation of the p19^{Arf}-p53 and p16^{Ink4a}-Rb tumor suppressor pathways (3), and ablation of either pathway in fibroblasts leads to cellular immortalization and circumvents

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Ras-induced cell cycle arrest. Cellular transformation by *Ras* is associated with profound changes in gene expression, including increased levels of cell cycle stimulatory proteins such as cyclin D1 (1,4). In addition, activated Ras down-regulates an array of anti-oncogenic genes that promote apoptosis, cell cycle arrest, DNA repair, and differentiation. These include Fas (5,6), the p16^{Ink4a} tumor suppressor (7), lysyl oxidase (8), prostate apoptosis response 4 (Par4) (9), the cytoskeletal protein tropomyosin (10), PTEN (11), RhoB (12), Gadd153/CHOP (13), Egr-1 (14), and FOXO3a (15). Thus, Ras-induced transformation involves activation of oncogenic genes as well as silencing of many critical genes that suppress tumorigenesis.

CCAAT/enhancer binding protein β (C/EBP β) is a bZIP transcription factor that is post-translationally regulated by growth factor signals transmitted through Ras kinases and their downstream effectors, as well as by oncogenic H-Ras^{V12} (16–18). Studies using knockout mice have shown that C/EBP β is essential for development of skin tumors in the DMBA/TPA two-stage model of skin carcinogenesis (19), in which nearly all tumors carry *Ras* mutations. Moreover, using *Myc/Raf*-induced transformation of C/EBP $\beta^{-/-}$ bone marrow we found a critical role for C/EBP β in proliferation and survival of macrophage-like tumor cells (20). This requirement involves an anti-apoptotic activity of C/EBP β linked to expression of insulin-like growth factor I, which functions as an autocrine survival factor in these cells. In addition, dominant negative C/EBP β inhibits Ras^{V12}-induced transformation of NIH 3T3 fibroblast while low doses of C/EBP β facilitate Ras transformation (19). C/EBP β expression has also been linked to a variety of rodent and human cancers (21). Collectively, these studies show that C/EBP β regulates proliferation and survival of certain tumor cells.

In addition to its pro-oncogenic functions, C/EBP β exhibits tumor suppressor-like activity in some contexts. C/EBP β -deficient MEFs fail to undergo Ras^{V12}-induced senescence and continue to proliferate despite activation of Arf/p53, displaying a partially transformed phenotype (22). Over-expression of C/EBP β without Ras also induced proliferation arrest and a senescent-like cellular morphology in MEFs. C/EBP β decreased the proliferation of cells lacking p53 or p19^{Arf} and thus may act downstream or in a parallel pathway. The anti-proliferative activity of C/EBP β is dependent on RB:E2F, as cells lacking all three RB family members or expressing dominant negative E2F-1 undergo increased proliferation instead of growth arrest when C/EBP β is expressed. These studies show that C/EBP β , possibly in a hyper-activated form induced by Ras^{V12} signaling, cooperates with RB:E2F to enforce cell cycle arrest in Ras-expressing primary fibroblasts by repressing E2F target genes required for cell cycle progression (22). Recently, two groups reported that replicative and oncogene-induced senescence of human fibroblasts is mediated by secreted proinflammatory cytokines and chemokines and their receptors (23,24). These studies identified C/EBP β as an essential regulator of the oncogene-induced inflammatory secretome. Thus, several lines of evidence support the notion that C/EBP β contributes to OIS in fibroblastic cells.

NIH 3T3 fibroblasts are immortalized by loss of the *CDKN2A* locus, which encodes the tumor suppressors p19^{Arf} and p16^{Ink4a}; as a result, NIH 3T3 cells are transformed by oncogenic Ras alone. The fact that C/EBP β levels in normal NIH 3T3 cells are even higher than in MEFs raises the question of how these cells escape C/EBP β -mediated growth arrest after introduction of oncogenic Ras. Here we show that Ras^{V12} silences C/EBP β expression in immortalized fibroblasts. This decrease occurred at the mRNA level and was required for transformation, since forced expression of C/EBP β suppressed cell proliferation, focus formation, and tumor growth *in vivo*. In contrast, Ras^{V12} did not down-regulate C/EBP β in primary MEFs and, instead, caused a modest increase in its expression. Thus, C/EBP β levels are differentially regulated by Ras^{V12} in primary and immortalized fibroblasts. We show that C/EBP β down-regulation in immortalized cells requires loss of the tumor suppressor p19^{Arf}. C/EBP β also regulates transcription of the *Fas* gene, and down-regulation of C/EBP β is a key event in Ras-

induced silencing of Fas. Based on these findings, we propose that C/EBP β down-regulation is an important event during Ras transformation of fibroblastic cells.

Materials and Methods

Cell lines, culture conditions and reagents

NIH 3T3 cells were obtained from ATCC and maintained in DMEM medium with 10% calf serum. IMR-90 cells were also obtained from ATCC and maintained in DMEM with 10% Fetal Bovine Serum. WT and C/EBP $\beta^{-/-}$ MEFs were prepared and cultured in DMEM medium as described (22). The Phoenix ecotropic packaging cell line (provided by H. Young), *Ink4a/Arf* $^{-/-}$ MEFs (P11) (25), *p53* $^{-/-}$ MEFs (P3) (26) (provided by C. Stewart), MCF-7 cells (provided by E. Sterneck), and A549 cells (provided by L. Anderson) were grown in the same medium. U0126 and LY294002 were purchased from Calbiochem and Actinomycin D was obtained from Sigma-Aldrich.

Retroviral vectors and gene transfer

pBabe-C/EBP β vectors expressing the p34 (LAP) isoform of murine C/EBP β were generated by subcloning EcoR1/HindIII fragment from pcDNA3.1-C/EBP β . For knockdown of C/EBP β expression, pSuper retrovector-mediated RNAi technology was used. The mouse C/EBP β shRNA (GATGTTCTGCGGGGTTGT) was based on a sequence reported previously (27) and human C/EBP β shRNA (GAAGAAACGTCTATGTGTA) was based on published algorithms; both were cloned into pSuper-Retropuro. pBabe and pBabe-H-Ras^{V12} were kindly provided by S. Lowe; pWZL(hygro) and pWZL(hygro)-H-Ras^{V12} were from K. Vousden; pBabe(puro)-BRAF^{E600} and LZRS(zeocin)-p53-RFP constructs were from D. Peeper; pBabe(puro)-p16 was a gift from N. Sharpless. MSCV-p19^{Arf} (generated by C. Sherr) was obtained from D. Peeper; the Arf insert was excised and inserted into pBabe(puro). The murine p53 coding region was amplified by RT-PCR and ligated into pBabe(puro). Retroviruses were produced using Phoenix packaging cells and infections were performed as described (22). Infected cell populations were selected in puromycin (2.0 μ g/ml, 3 days) for pBabe-derived vectors, hygromycin (100–200 μ g/ml, 5 days) for pWZL(hygro)-based vectors, and zeocin (400–500 μ g/ml, 6–7 days) for LZRS vectors.

Growth curves

Cells were seeded at 2.5×10^4 cells per well in 6-well plates. At the indicated times, cells were washed with PBS, fixed in 10% formalin, and rinsed with distilled water. Cells were stained with 0.1% crystal violet (Sigma) for 30 min, rinsed extensively, and dried. The cell-associated dye was extracted with 10% acetic acid and absorbance was measured at 590nm. All values were normalized to day 0.

Colony assays

For standard colony formation assays, cells were plated at 1×10^4 or 2.5×10^4 cells per 10cm diameter plate. After two weeks, colonies were stained with crystal violet and counted. For soft agar colony assays, cells were resuspended in 0.35% agarose in DMEM supplemented with 10% calf serum at a density of 1×10^4 or 2.5×10^4 cells per 6cm plate and seeded onto solidified 0.5% agarose containing the culture medium. The cells were fed weekly and colonies were evaluated 2 weeks after plating.

Focus assays

NIH 3T3 cells were maintained in DMEM plus 10% calf serum and were transduced with retroviral vectors expressing C/EBP β and Ras^{V12}. The drug-selected cells were plated at a

density of 1×10^2 or 1×10^3 cells per 10 cm dish in a lawn of normal NIH 3T3 cells. Transformed foci were scored after 2 weeks.

***In vivo* tumorigenesis assays**

1×10^4 cells were injected into the flanks of athymic nude mice (5 animals per group). Mice were sacrificed when tumors reached 2 cm in diameter or when the animals exhibited signs of morbidity.

SA- β gal assays

SA- β gal staining was performed according to a previously described method (28), with slight modifications. Cells were fixed with 3% formaldehyde solution in PBS for 10 minutes at room temperature, rinsed with PBS and incubated with a SA- β gal staining solution [PBS (pH 5.8), 150mM NaCl, 5mM potassium ferricyanide, 5mM potassium ferrocyanide, 2.5mM $MgCl_2$, and 1mg/ml 5-bromo-4-chloro-3-indoyl β -D galactoside] (X-gal). After staining the plates were washed, dried, and images taken. The percentage of cells expressing SA- β gal was quantified by inspecting 200 cells per well in two independent experiments.

Immunostaining

Cells were plated on coverslips and fixed in 3.7% formaldehyde/PBS for 10 min at room temperature. After washing with PBS, cells were permeabilized for 5 min with 0.2% Triton X-100 in PBS, washed with PBS, blocked with 1% BSA/PBS for 20–30 min, and incubated for 1 h with C/EBP β antibody (1:100) in PBS containing 1% BSA at room temperature in a humidified chamber. After washing in PBS, cells were stained with Rhodamine-conjugated secondary antibodies (1:200) for 1 h at room temperature, washed with PBS, and mounted on microscope slides using a mounting medium (Vector Laboratories) containing DAPI. Images were taken using an Olympus microscope.

Protein analysis

Whole cell extracts of retrovirally-transduced cells were prepared in NP-40 lysis buffer (50mM Tris-HCl (pH 8.0), 400mM NaCl, 1% NP-40, 1mM EDTA) containing a cocktail of protease inhibitors. After 30 min on ice, lysates were cleared by high-speed centrifugation. Nuclear extracts were prepared as described (29). Protein concentrations were determined using the Bio-Rad microassay. 15–25 μ g of nuclear extract or 50–60 μ g of whole cell lysate were separated on 12% SDS-PAGE and transferred to nitrocellulose membranes. The following primary antibodies were used for immunoblot analysis: C/EBP β (Santa Cruz, C-19, 1:1000); p53 (Novacastra, CM5, 1:2000); p19^{Arf} (Abcam, ab80, 1:500); p16^{Ink4a} (Santa Cruz, M-156, 1:1000); p21 (Santa Cruz, F-5, 1:1000); BRAF (Santa Cruz, F-7, 1:1000); Fas (Santa Cruz, SC-716, 1:500; a kind gift from N. Wajapeyee and M. Green); PTEN (Santa Cruz, A2B1, 1:1000); Actin (Santa Cruz, C-11, 1:1000). Secondary goat anti-rabbit or goat anti-mouse antibodies conjugated to horseradish peroxidase (Promega) were used to detect antigen-antibody by chemiluminescence (ECL detection system; Pierce).

Northern blot

Total RNA was extracted by using Trizol reagent. 10–15 μ g of total RNA were loaded in formaldehyde-agarose gels and transferred to Hybond N⁺ nylon membranes (Amersham Pharmacia Biotech). Blots were hybridized with ³²P-labeled probes specific for C/EBP β and a probe specific for β -Actin was used as a loading control.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared and incubated with ^{32}P -labeled oligonucleotide probe corresponding to a consensus C/EBP binding site for 20 min at room temperature, as described previously (29). The resulting DNA-protein complexes were fractionated on 6% nondenaturing polyacrylamide gels and visualized by autoradiography.

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were carried out as described previously (22). Briefly, cells were treated with the crosslinking agent, harvested and lysed in RIPA buffer. Chromatin was sonicated to obtain DNA fragments of 500–1000bp prior to centrifugation. The resulting supernatant was precleared with protein A/G agarose beads (Santa Cruz Biotech), incubated with 2.5 μg of C/EBP β antibody (C-19, Santa Cruz), and the antibody-bound complexes were precipitated with agarose beads. A blocking peptide specific to the C/EBP β antibody epitope was used in control reactions. Following extensive washing, bound DNA fragments were eluted and used for PCR amplification using primers near the transcription start site (TSS) of the *Fas* promoter, as described (30).

Results

C/EBP β inhibits proliferation and transformation of Ras^{V12}-expressing NIH 3T3 cells

To try to resolve how oncogenic Ras circumvents C/EBP β -mediated growth arrest in transformed fibroblasts, we investigated whether Ras^{V12} decreases C/EBP β activity or expression in NIH 3T3 cells. We first examined the anti-proliferative activity of C/EBP β by infecting H-Ras^{V12}-transformed or control cells with a C/EBP β -expressing retrovirus. After drug selection, cell growth rates were determined (Fig. 1A). While C/EBP β had only a minor effect on non-transformed NIH 3T3 cells, it strongly inhibited proliferation of Ras^{V12}-expressing cells even though similar levels of C/EBP β over-expression were detected in the two cell populations (see Fig. 2A). Thus, Ras^{V12} increases the cytostatic activity of C/EBP β in NIH 3T3 cells. C/EBP β over-expression also elicited a change in cellular morphology, causing the cells to adopt a flattened, less refractile appearance that is typical of senescent fibroblasts (Fig. 1B) (2). This morphological transition was much more pronounced in cells expressing C/EBP β and Ras^{V12} than C/EBP β alone, further indicating that C/EBP β senescence-inducing activity is enhanced by Ras signaling.

The effect of C/EBP β was also assessed in several assays of oncogenic transformation. Over-expression of C/EBP β strongly suppressed focus formation of Ras^{V12}-transformed cells (Fig. 1C, left panel). The anti-oncogenic effect of C/EBP β was also evident in anchorage-independent growth assays and tumor formation in nude mice. Ras^{V12}-transformed NIH 3T3 cells formed numerous colonies in soft agar, and colony formation was potently inhibited when C/EBP β was co-expressed (Fig. 1C, right panel). Tumor development *in vivo* was assessed by injecting cells expressing Ras^{V12} with or without C/EBP β into the flanks of nude mice (Fig. 1D). Animals receiving Ras^{V12}-transformed cells succumbed to tumors approximately two weeks earlier than mice injected with cells expressing both Ras and C/EBP β . The majority of animals containing C/EBP β -expressing cells eventually succumbed to tumors. These tumors may have developed because of selection for subpopulations of cells that either initially expressed less C/EBP β or lost C/EBP β expression after transplantation. Nevertheless, the delayed onset of disease shows that C/EBP β over-expression inhibits the tumorigenicity of Ras-transformed NIH 3T3 cells.

Ras^{V12} down-regulates C/EBP β expression

Analysis of nuclear C/EBP β levels in Ras^{V12}-expressing and non-transformed cells showed that C/EBP β expression was strongly decreased in Ras^{V12} cells relative to controls (Fig. 2A, left panel, compare lanes 1 and 3). This decrease was also seen in cells expressing oncogenic K-ras (data not shown), which is more commonly mutated in human cancers. However, cells infected with the C/EBP β retrovirus displayed high levels of the protein even in the presence of Ras^{V12} (lane 4), indicating that C/EBP β down-regulation does not involve increased protein turnover. The decrease in C/EBP β levels was confirmed by EMSA, which showed a strong reduction of C/EBP β DNA-binding activity in Ras^{V12}-expressing cells but not in cells co-infected with the C/EBP β retrovirus (Fig. 2A, middle panel). Together with the data of Fig. 1, these results suggest that Ras^{V12}-induced down-regulation of C/EBP β is essential for oncogenic transformation of murine fibroblasts. When C/EBP β is artificially maintained at pre-transformed levels, proliferation and tumorigenesis is suppressed and the cells acquire a senescent-like morphology.

The *Cebpb* gene contains three in-frame initiation codons that produce proteins designated FL-LAP (p38), LAP (p34), and LIP (p20) (31), each of which has unique functions (32). LAP and LIP are generally the most abundant forms and were detected in NIH 3T3 cells (Fig. 2A). Both proteins were efficiently down-regulated in Ras-expressing cells (Fig. 2A, lane 3). Thus, increased LIP expression is not associated with oncogenic transformation of fibroblasts, as has been reported for mammary epithelial tumors (33). LIP was also observed in Ras transformed cells infected with the C/EBP β vector engineered to express the p34 (LAP) isoform of C/EBP β , and the ratio of the two isoforms was similar to endogenous levels (Fig. 2A, lane 4). Therefore, LIP is produced efficiently from this construct by translational or proteolytic mechanisms (29).

Northern blot analysis showed that the endogenous C/EBP β transcript was reduced by ~80% in Ras^{V12}-expressing NIH 3T3 cells, similar to the decrease in protein expression (Fig. 2A, right panel, lanes 1 and 3). The C/EBP β retroviral transcript was not down-regulated by Ras (lanes 2 and 4), consistent with the undiminished expression of C/EBP β in cells expressing ectopic C/EBP β . Ectopic C/EBP β also did not restore expression of endogenous C/EBP β mRNA, indicating that the *Cebpb* gene is not autoregulated. Thus, oncogenic Ras either decreases *Cebpb* gene transcription or increases the mRNA turnover rate. Analysis of C/EBP β mRNA levels after treatment with actinomycin D to block *de novo* transcription showed that the RNA decay rate was not increased in Ras-expressing cells compared to untransformed cells (Fig. 2B). This result demonstrates that enhanced mRNA turnover does not account for C/EBP β down-regulation in Ras-transformed cells and suggests the involvement of a transcriptional mechanism.

To identify the Ras effector pathway(s) that down-regulates C/EBP β , we first tested the effect of expressing activated BRAF. Raf kinases are activated by Ras, and oncogenic Raf mutants can induce senescence or transformation depending on the cellular context (34). Activated BRAF (BRAF^{E600}) decreased C/EBP β levels, indicating that the Raf-MEK-ERK cascade is involved in *Cebpb* gene silencing (Fig. 2C). Treatment of Ras-transformed cells with the MEK inhibitor U0126 also partially restored C/EBP β expression (Fig. 2D). The PI-3 kinase inhibitor LY294002 likewise incompletely restored C/EBP β levels, whereas both inhibitors together almost fully reversed C/EBP β down-regulation (Fig. 2D). These observations show that the Raf-MEK-ERK and PI3K effector pathways act in concert to silence *Cebpb* gene expression in Ras^{V12}-transformed cells.

C/EBP β down-regulation by oncogenic Ras requires loss of *Ink4a/Arf*

We previously observed a modest increase in C/EBP β levels, rather than down-regulation, in Ras^{V12}-expressing primary MEFs that are undergoing premature senescence (22). To confirm this observation, we analyzed proliferation and C/EBP β expression in wt MEFs infected with Ras^{V12} or C/EBP β retroviruses. C/EBP β and Ras^{V12} each decreased cell proliferation, and both together induced nearly complete cell cycle arrest (Fig. 3A, left panel). Introduction of Ras^{V12} increased endogenous C/EBP β levels by ~2-fold, in contrast to the down-regulation observed in NIH 3T3 cells. Thus, C/EBP β expression is oppositely regulated by Ras in primary MEFs and immortalized NIH 3T3 fibroblasts. C/EBP β expression was also increased in human diploid IMR90 fibroblasts undergoing Ras^{V12}-induced senescence (data not shown).

Since loss of *Ink4a/Arf* is the immortalizing lesion in NIH 3T3 cells (35), we asked whether this mutation accounts for the differential regulation of C/EBP β by Ras^{V12} in primary and immortalized cells. Introduction of Ras^{V12} in *Ink4a/Arf*^{-/-} MEFs caused a marked reduction in C/EBP β expression (Fig. 3A, right panel), similar to the decrease observed in NIH 3T3 cells. Ectopic expression of C/EBP β decreased proliferation of *Ink4a/Arf*^{-/-} cells and this effect was augmented by Ras although, unlike wt MEFs, these cells maintained some mitotic activity. Thus, loss of *Ink4a/Arf* together with expression of oncogenic Ras silences the *Cebpb* gene. Because ectopic C/EBP β inhibits proliferation of Ras-expressing cells, its down-regulation may be an important event in Ras transformation.

p19^{Arf}, but not p53, restores C/EBP β expression in Ras^{V12}-transformed cells

The above results suggest that one or both products of the *Ink4a/Arf* locus positively regulate *Cebpb* gene expression. To identify which protein is involved, we used retroviral vectors to express p16^{Ink4a} or p19^{Arf} in control and Ras^{V12}-transformed NIH 3T3 cells. p19^{Arf} substantially reversed the Ras-induced decrease in C/EBP β expression, restoring it to approximately one-half the level seen in non-transformed cells (Fig. 3B, lane 6). In contrast, p16 did not detectably influence C/EBP β expression (Fig. 3B, lane 4). To determine if p16 might act cooperatively with p19^{Arf} we introduced both genes into control and Ras^{V12}-expressing cells. However, p16 did not further augment C/EBP β levels (data not shown), indicating that p19^{Arf} alone is sufficient to up-regulate *Cebpb* gene expression. p19^{Arf} also partially restored C/EBP β mRNA expression (Fig. 3C). In addition, p19^{Arf} increased C/EBP β levels in Ras^{V12}-expressing *Ink4a/Arf*^{-/-} MEFs (Fig. 3D, lane 4) and modestly augmented C/EBP β expression in non-transformed cells (lane 3). These results show that p19^{Arf} prevented Ras^{V12}-induced down-regulation of C/EBP β in murine fibroblasts, whereas p16^{Ink4a} had no discernable effect.

The p53 transcription factor is the primary target of p19^{Arf} signaling in many cells and hence could potentially mediate the effects of Arf on *Cebpb* gene expression. Therefore, we asked whether p53 could restore C/EBP β expression in Ras-transformed cells. Murine p53 expressed from a retroviral vector did not increase C/EBP β levels (Fig. 4A, left panel). The presence of functional p53 was demonstrated by Western blotting as well as expression of a p53 target gene, p21 (Fig. 4A), and suppression of cell proliferation (Fig. 4B). Note that endogenous p53 was weakly induced by Ras^{V12} (Fig. 4A, left panel, lane 2) and over-expressed p53 was strongly augmented, even though NIH 3T3 cells lack Arf. Arf-independent stabilization of p53 by oncogenic Ras has been observed previously (28). A human p53 protein (p53-RFP fusion) also failed to restore C/EBP β levels (Fig. 4A, right panel). In addition, Ras^{V12} had only a slight effect on C/EBP β expression in p53^{-/-} MEFs (Fig. 4C). Thus, p53 is dispensable for Arf-mediated regulation of C/EBP β in fibroblasts.

Growth-inhibitory effects of endogenous C/EBP β in human fibroblasts and tumor cells

To assess the influence of C/EBP β on proliferation of human cells, we expressed C/EBP β or Ras^{V12} in IMR-90 human diploid fibroblasts, which undergo senescence in response to various oncogenes. Over-expression of either protein suppressed proliferation and increased the nuclear levels of C/EBP β , as determined by Western blot and immunofluorescence (Fig. 5A); hence, Ras^{V12} stimulates C/EBP β expression in these cells. Both proteins also induced a senescent cellular morphology and activated a marker of senescence, SA β -galactosidase, in ~80% of the cells (Fig. 5B). These results show that ectopic C/EBP β can activate a senescent phenotype in the absence of an oncogenic signal such as Ras^{V12}, extending our observations in mouse fibroblasts (22).

We next used siRNA to ablate C/EBP β expression in IMR-90 cells. shRNA knockdown of C/EBP β increased the proliferation rate (Fig. 5C), demonstrating that endogenous C/EBP β partially inhibits growth of human fibroblasts. However, in contrast to results from murine fibroblasts (22), loss of C/EBP β did not prevent Ras^{V12}-induced cell cycle arrest (data not shown), indicating that other tumor suppressor pathways such as p16^{Ink4a}-Rb may be capable of activating senescence in human cells in the absence of C/EBP β . Alternatively, the residual level of C/EBP β in shC/EBP β -expressing cells could be sufficient to support Ras-induced senescence. We also analyzed the effect of ablating C/EBP β on growth of two human tumor cell lines. Knockdown of C/EBP β in A549 lung tumor cells (which carry an activated K-Ras oncogene) or MCF-7 breast cancer cells increased their proliferation *in vitro* (Fig. 5C). Collectively, the data of Fig. 5 demonstrate that C/EBP β impedes proliferation of normal and transformed human cells, suggesting that it has partial tumor suppressor activity. Since most human cells resist transformation by a single oncogene, it is difficult to determine whether C/EBP β is down-regulated during transformation of human cells by Ras or Raf and whether this is required to circumvent senescence, as was observed for NIH 3T3 cells.

C/EBP β down-regulation mediates Ras-induced silencing of *Fas*

Several pro-apoptotic and growth-inhibitory genes, including *Fas* (5,6) and PTEN (11), are down-regulated in Ras-transformed NIH 3T3 cells. Since previous reports indicated that the *Fas* promoter is regulated in part by C/EBP β (36,37), we asked whether Ras-induced silencing of *Fas* involves down-regulation of C/EBP β . As expected, *Fas* levels were significantly decreased in H-Ras^{V12} transformed cells; however, this decrease was prevented by over-expression of C/EBP β (Fig. 6A). shRNA knock-down of C/EBP β in control cells also reduced *Fas* levels to those seen in Ras-transformed cells (Fig. 6B). Thus, *Fas* expression is dependent on the presence of C/EBP β . ChIP analysis showed that C/EBP β associates with the 5' flanking region of the *Fas* gene (Fig 6C), consistent with the location of known C/EBP sites in the proximal promoter (36,37). The ChIP signal was diminished in Ras-expressing cells, as expected from the reduction in C/EBP β levels. The effect of C/EBP β on *Fas* expression was specific because levels of PTEN, another Ras-silenced gene (11), were neither restored by C/EBP β over-expression in transformed cells nor decreased by C/EBP β knockdown in normal cells (Fig. 6A and B). Thus, C/EBP β positively regulates transcription of the *Fas* gene and loss of C/EBP β is a critical event in Ras-induced silencing of *Fas* but not PTEN.

Discussion

We have shown that C/EBP β is down-regulated in H-Ras^{V12}-expressing NIH 3T3 fibroblasts and the reduction in C/EBP β levels facilitates oncogenic transformation. Maintenance of high C/EBP β levels in Ras-expressing cells suppressed proliferation and transformation and induced a senescent-like cellular morphology. These results support and extend our previous findings showing that C/EBP β promotes cell cycle arrest in Ras^{V12}-expressing MEFs and that Ras-induced senescence is bypassed in C/EBP β -deficient cells (22). Taken together, our

observations indicate that C/EBP β down-regulation in transformed NIH 3T3 cells removes an anti-proliferative signal that would otherwise suppress oncogenesis.

Oncogenic BRAF also diminished C/EBP β levels, suggesting that the silencing pathway downstream of Ras involves the Raf-MEK-ERK cascade. This conclusion is supported by the fact that MEK1/2 inhibition partially restored C/EBP β expression in Ras^{V12}-transformed cells. Inhibiting PI3K activity also led to partial re-expression of C/EBP β , implicating this Ras effector pathway as well in C/EBP β silencing. Since treatment with both inhibitors was necessary to restore C/EBP β levels, we conclude that the two pathways converge to repress *Cebpb* gene expression, as depicted in Fig. 6D. Presently, the distal kinases in these pathways and their presumptive targets in the transcriptional machinery that mediate *Cebpb* repression are unknown but are under investigation in our laboratory.

We have tested reporter constructs containing up to 3 kb of the C/EBP β promoter to investigate Ras-induced down-regulation of transcription. However, such constructs were not silenced by oncogenic Ras, hampering studies of *cis*-regulatory elements that mediate down-regulation of the *Cebpb* gene. The absence of repression in reporter assays suggests that silencing of the *Cebpb* promoter requires an appropriate chromatin environment, which is generally not recapitulated on transiently transfected DNA templates. An epigenetic silencing mechanism was suggested by the reversal of Ras-induced C/EBP β down-regulation by TSA treatment (data not shown), which indicates that increased histone acetylation activates transcription of the *Cebpb* gene. Further studies are required to identify the epigenetic mechanisms involved in down-regulation and the possible role of differential histone modifications.

We determined that C/EBP β is a positive regulator of *Fas* and that C/EBP β down-regulation is critical for *Fas* silencing. Using an RNAi screen, Gazin et al. recently identified 28 genes that are necessary for Ras-induced epigenetic silencing of *Fas* in NIH 3T3 cells (30). *Fas* expression is repressed at least in part by DNA methylation, and many of the genes uncovered in this screen are involved in DNA methylation, chromatin remodeling and transcriptional regulation. An additional class of genes is associated with cellular signaling, and the encoded proteins presumably affect pathways linking oncogenic Ras to *Fas* silencing. We note that C/EBP β would not have been identified in this RNAi screen, which was designed to uncover genes whose ablation allows reexpression of *Fas*. Several other transcription factors are down-regulated by oncogenic Ras in NIH 3T3 cells, including the C/EBP-related protein gadd153/CHOP (13), AP-1 family members (JunB, c-Fos, FosB, and Fra-2 (38)), Egr-1 (14), and FOXO3a (15). It is likely that many of these proteins regulate tumor suppressor genes that become silenced in Ras-transformed cells, leading to a global gene expression pattern that is permissive for oncogenesis.

In addition to the oncogenic Ras signal, C/EBP β down-regulation requires loss of the tumor suppressor p19^{Arf}. Forced expression of Arf in NIH 3T3 cells or *Ink4a/Arf*^{-/-} MEFs largely abolished the Ras-induced decrease in C/EBP β levels, whereas p16^{Ink4a} had no effect. The positive effect on C/EBP β expression is not simply due to the growth-inhibitory activity of Arf, since p53, p16^{Ink4a}, and C/EBP β all strongly suppressed cell proliferation but did not increase endogenous C/EBP β levels. Although p53 is a critical effector of the Arf tumor suppressor pathway, several observations including the fact that mice lacking both p53 and Arf develop tumors more rapidly and of a wider spectrum than animals carrying either mutation alone (39) demonstrate that Arf also has p53-independent functions (40). We propose that C/EBP β is one such target, acting as part of an Arf-dependent network in primary fibroblasts and possibly other cells to regulate Ras-induced senescence (Fig. 6D). The connection between Arf and C/EBP β was also supported by a gene expression array study showing that C/EBP β is one of many genes whose expression is increased by Arf in non-transformed NIH 3T3 cells (41).

Arf maintains C/EBP β expression in the face of oncogenic signals such as activated Ras or Raf. The mechanism by which Arf controls *Cebpb* gene transcription is presently unclear. Arf was reported to interact with transcription factors such as c-Myc (42–44) and the E2F heterodimerizing protein DP-1 (45), altering the transcriptional activities of Myc and E2F and raising the possibility that Arf directly regulates certain genes by association with DNA-binding proteins. Using ChIP we found strong associations of Arf with the *Cebpb* gene, but this also occurred with negative control genes and other sequences tested (data not shown). These apparent ChIP signals are most likely non-specific and may reflect the fact that Arf is a “sticky” protein that can bind to many proteins and surfaces (40). Thus, whether Arf acts directly to regulate target genes such as *Cebpb* remains an open question, and further studies are necessary to elucidate the pathway linking Arf with C/EBP β and other downstream targets.

C/EBP β expression was diminished but not completely eliminated in Ras-transformed cells. The residual endogenous C/EBP β may in fact promote oncogenesis, since low levels of ectopic C/EBP β were found to stimulate Ras-induced focus formation in NIH 3T3 cells while dominant negative C/EBP β inhibited transformation (19). The pro-oncogenic function of C/EBP β could involve its anti-apoptotic activity, which has been observed in several kinds of tumor cells (46). It is likely that C/EBP β has differential effects on cell growth and transformation depending on its concentration and the cellular context (21). We suggest that there is a critical level of C/EBP β in NIH 3T3 cells, below which it facilitates Ras^{V12}-mediated transformation and above which it exerts anti-proliferative and tumor suppressive effects. This threshold might be established by heterodimeric partners such as C/EBP γ (47,48) or ATF proteins (49). The growth inhibition seen at higher concentrations of C/EBP β could be due to increased formation of C/EBP β homodimers, which may preferentially induce cell cycle arrest (S. Lee, T.S., P.F.J. et al., unpublished data) and would accumulate when the pool of heterodimeric partners is exceeded. The most likely targets of such dimers are E2F-regulated genes involved in cell cycle progression such as Myc that are known to be repressed by C/EBP β (22,50). Conversely, the block to Ras-induced transformation elicited by expression of dominant negative C/EBP β may result from disruption of pro-oncogenic C/EBP β heterodimers.

Ras and other oncogenes can activate senescence or apoptosis, which provide intrinsic barriers to tumor development *in vivo* (3). Disruption of senescence and cell death checkpoints by mutation or silencing of tumor suppressor genes is a critical event in oncogenesis and accounts for the observed cooperativity between oncogenes and tumor suppressor mutations in many cancers. Our studies show that Arf loss plays a key role in down-regulation of C/EBP β and subsequent silencing of *Fas* in Ras-transformed, immortalized mouse fibroblasts. It will be of considerable interest to elucidate the role of Arf loss on dis-regulation of other genes during Ras transformation. The current findings advance our understanding of the regulatory pathways that suppress tumorigenesis and provide further insights into the aberrant regulation of such networks in cancer cells. Since endogenous C/EBP β also suppresses proliferation of human fibroblasts and tumor cells (Fig. 5), future studies will address the mechanisms regulating C/EBP β expression and activity in human cancers.

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Figure 1.

C/EBP β and Ras^{V12} cooperate to induce growth arrest and a senescent cellular morphology in NIH 3T3 fibroblasts. **A**, growth curves of cells infected with retroviruses expressing p34 C/EBP β (LAP) and/or Ras^{V12}. Data are the average of triplicate assays ($\pm$ SE). **B**, phase contrast microscopy of cells expressing C/EBP β and/or Ras^{V12}. **C**, cells were seeded into monolayers of non-transformed NIH 3T3 cells to assess focus formation (left panel) or plated in semisolid medium to examine anchorage-independent colony formation (right). **D**, 1×10^4 cells expressing Ras^{V12} and/or C/EBP β were injected into the flanks of athymic nude mice. Tumors were monitored and animals were sacrificed when tumor diameters reached 2 cm.

stability is not decreased in Ras^{V12}-transformed cells. Cells were treated with 5μg/ml actinomycin D and harvested at the indicated times. Northern blots show the relative decay of C/EBPβ mRNA in control and Ras^{V12}-expressing cells. C/EBPβ mRNA levels were quantitated by phospho-imaging, normalized to actin, and plotted relative to time 0 (right panel). *C*, C/EBPβ expression in NIH 3T3 cells transformed with oncogenic BRAF. *D*, effects of MEK1/2 and PI3K inhibitors on C/EBPβ down-regulation. Ras-transformed NIH 3T3 and control cells were treated in the absence or presence of 5μM UO126 (MEK1/2) or 20μM LY294002 (PI3K) for 24 hr and nuclear extracts were analyzed for C/EBPβ expression.

Figure 3.

Ras-mediated down-regulation of C/EBP β expression in mouse fibroblastic cells involves loss of p19^{Arf}. **A**, Ras^{V12} and/or C/EBP β were expressed in WT MEFs (left panel) or *Ink4a/Arf^{-/-}* cells (right) and C/EBP β levels were determined by Western blotting. Growth curves for each cell population are shown in the lower panels. Data are the average of triplicate assays. **B**, Ras^{V12}-expressing NIH 3T3 cells were infected with retroviruses encoding p19^{Arf} or p16^{Ink4a} and levels of endogenous C/EBP β and the over-expressed proteins were determined by Western blotting. Cell proliferation was evaluated by colony assays (lower panel). **C**, C/EBP β mRNA levels in control and Ras^{V12}-expressing *Ink4a/Arf^{-/-}* cells. RNA was prepared from the cells described in panel **B** and analyzed by Northern blotting using a radiolabeled C/

EBP β probe. The blot was reprobbed for β -actin as a loading control. *D*, C/EBP β Western blots in *Ink4a/Arf*^{-/-} MEFs transduced with p19^{Arf}. Growth curves are shown in the lower panel.

Figure 4.

Ectopic p53 does not restore C/EBP β expression. **A**, p53 and/or C/EBP β were expressed in Ras^{V12}-transformed NIH 3T3 cells and C/EBP β levels were determined by Western blotting. **B**, growth curves of p53-expressing cells. Data are the average of triplicate assays. **C**, C/EBP β Western blot in p53^{-/-} MEFs transduced with Ras^{V12}.

Figure 5.

Endogenous C/EBP β inhibits proliferation of human fibroblasts and tumor cells. **A**, IMR-90 human diploid fibroblasts were infected with retroviral vectors for hC/EBP β or Ras^{V12} and cell proliferation was analyzed. Western blotting (inset) shows the levels of nuclear C/EBP β LAP and LIP isoforms. The cells were also immunostained for C/EBP β (right panel). **B**, the cell populations described in panel **A** were stained for SA- β galactosidase (left panel) and the percentage of SA- β gal positive cells was scored (right panel). **C**, effect of C/EBP β knockdown on proliferation of IMR-90, MCF-7 (breast tumor), and A549 (lung tumor) cells. Cells were infected with shC/EBP β and control retroviruses and growth rates were determined. Nuclear C/EBP β levels were analyzed by Western blotting (insets).

Figure 6.

The pro-apoptotic Fas receptor is regulated by C/EBPβ. *A*, nuclear extracts were prepared from NIH 3T3 cells expressing C/EBPβ and/or Ras^{V12} and analyzed for Fas and PTEN expression. β-Actin was used as a loading control. *B*, effect of C/EBPβ knockdown on expression of Fas and PTEN. NIH 3T3 cells infected with C/EBPβ shRNA or Ras^{V12} vectors were analyzed for Fas and PTEN levels by Western blotting. *C*, C/EBPβ binds to the *Fas* promoter *in vivo*. Chromatin was prepared from normal and Ras^{V12}-expressing cells and immunoprecipitated with C/EBPβ antibody. DNA was analyzed by PCR using primers corresponding to promoters of the indicated genes. Specificity of the C/EBPβ antibody was verified by including a C/EBPβ blocking peptide (BP) in the immunoprecipitation reaction. Input represents 2% of the total chromatin. β2-Microglobulin (β2M) and IL-6 were used as negative and positive controls, respectively, for C/EBPβ binding (22). *D*, a model for Ras-induced cell cycle arrest and tumor suppression in murine fibroblasts. Sustained Ras signaling activates the p16^{Ink4a}-RB and p19^{Arf}-p53 pathways which, together with C/EBPβ, repress cell cycle genes required for G1-

S progression. In the presence of Arf, C/EBP β expression is maintained. When Arf is absent, *CEBPB* gene transcription is down-regulated by a mechanism requiring MEK/ERK and PI3K signaling, thus establishing conditions permissive for cell proliferation and transformation. Loss of C/EBP β expression also promotes silencing of other possible tumor suppressor genes such as the pro-apoptotic Fas receptor. Dashed lines represent pathways identified in the present study.