3'UTR elements inhibit Ras-induced C/EBP β post-translational activation and senescence in tumour cells

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C/EBP β is an auto-repressed protein that becomes post-translationally activated by Ras-MEK-ERK signalling. C/EBP β is required for oncogene-induced senescence (OIS) of primary fibroblasts, but also displays pro-oncogenic functions in many tumour cells. Here, we show that C/EBP β activation by H-Ras^{V12} is suppressed in immortalized/transformed cells, but not in primary cells, by its 3' untranslated region (3'UTR). 3'UTR sequences inhibited Ras-induced cytostatic activity of C/EBP β , DNA binding, transactivation, phosphorylation, and homodimerization, without significantly affecting protein expression. The 3'UTR suppressed induction of senescence-associated C/EBP β target genes, while promoting expression of genes linked to cancers and TGF β signalling. An AU-rich element (ARE) and its cognate RNA-binding protein, HuR, were required for 3'UTR inhibition. These components also excluded the *Cebpb* mRNA from a perinuclear cytoplasmic region that contains activated ERK1/2, indicating that the site of C/EBP β translation controls de-repression by Ras signalling. Notably, 3'UTR inhibition and *Cebpb* mRNA compartmentalization were absent in primary fibroblasts, allowing Ras-induced C/EBP β activation and OIS to proceed. Our findings reveal a novel mechanism whereby non-coding mRNA sequences selectively regulate C/EBP β activity and suppress its anti-oncogenic functions.

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Introduction

Expression of activated Ras, Raf or other oncoproteins in primary cells often provokes cellular senescence, a permanent state of proliferative arrest that is activated by oncogenic stress (Campisi, 2005; Collado *et al*, 2007). This cytostatic response, termed oncogene-induced senescence (OIS), functions as an intrinsic tumour suppression mechanism in cells that have sustained critical levels of oncogenic flux (Sarkisian *et al*, 2007; Prieur and Peeper, 2008). OIS is implemented primarily through induction of the p19^{Arf}-p53 tumour suppressor pathway and CDK inhibitors such as p16^{Ink4a} and p21^{CIP1} that activate Rb-dependent checkpoints (Adams, 2009). Disruption of these and other anti-oncogenic effectors through mutation or epigenetic silencing leads to senescence bypass and renders cells susceptible to transformation by Ras and other oncogenes (Lowe *et al*, 2004).

Recent studies have also implicated the transcription factor C/EBP β and pro-inflammatory mediators such as IL-6, chemokines, and their receptors, comprising a 'senescence-associated secretory phenotype' (SASP), in senescence induction triggered by oncogenes or DNA damage (Sebastian *et al*, 2005; Acosta *et al*, 2008; Kuilman *et al*, 2008; Rodier *et al*, 2009). Genetic experiments demonstrate that C/EBP β is required for Ras- or BRAF-induced senescence of mouse embryonic fibroblasts (MEFs; Sebastian *et al*, 2005) and human diploid fibroblasts (Acosta *et al*, 2008; Kuilman *et al*, 2008), in part through its ability to activate SASP genes and p15^{Ink4b}. Although C/EBP β and SASP genes are important regulators of OIS, they differ from classical tumour suppressors in that they are rarely, if ever, inactivated in cancers and also exert pro-oncogenic effects in many transformed cells (Sebastian and Johnson, 2006; Mantovani *et al*, 2008). Currently, it is unclear how such factors can be critical for establishing senescence while promoting cancer in other contexts.

C/EBP β is maintained in a latent, low-activity state by several auto-inhibitory elements that suppress its DNA-binding and transactivation functions (Kowenz-Leutz *et al*, 1994; Williams *et al*, 1995; Lee *et al*, 2010a). In response to oncogenic Ras or other stimuli, C/EBP β becomes de-repressed by signalling through the RAF-MEK-ERK cascade (Nakajima *et al*, 1993; Kowenz-Leutz *et al*, 1994; Lee *et al*, 2010b), in part due to phosphorylation on Thr188 (mouse C/EBP β) by ERK1/2 that leads to altered binding of mediator complexes (Mo *et al*, 2004). Oncogenic Ras also stimulates C/EBP β 's anti-proliferative activity and increases the ratio of C/EBP β homodimers to C/EBP β :C/EBP γ heterodimers by a mechanism involving phosphorylation on leucine zipper residue Ser273 by p90^{Rsk} kinases (Lee *et al*, 2010b). These observations, together with the fact that C/EBP γ -deficient MEFs display severe proliferative defects, have led to the notion that the hyperactivated, homodimeric form of C/EBP β contributes to Ras-induced cell-cycle arrest and senescence in

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primary cells, whereas $\beta:\gamma$ heterodimers are permissive for, or actively promote, mitotic growth (Lee *et al*, 2010b). However, this model does not explain how transformed cells, particularly those harbouring *Ras* or *BRAF* oncogenes, evade the anti-proliferative effects of activated C/EBP β . In NIH 3T3 cells, endogenous C/EBP β expression is downregulated by Ras^{V12}, providing one possible mechanism (Sebastian and Johnson, 2009). Nevertheless, many transformed cells express relatively high levels of C/EBP β , suggesting that other means exist to constrain its anti-proliferative activity.

Here, we report the unexpected finding that Ras-induced post-translational activation of C/EBP β is inhibited by the 3' untranslated region (3'UTR) of its mRNA, suppressing the cytostatic and pro-senescence functions of C/EBP β selectively in immortalized and transformed cells. These observations, thus, identify a new function for 3'UTRs and suggest a further basis for senescence bypass in cancer cells.

Results

The *Cebpb* 3'UTR blocks the Ras-induced cytostatic functions of C/EBP β

Introduction of oncogenic H-Ras^{V12} in immortalized NIH 3T3 fibroblasts silences C/EBP β expression through a mechanism requiring absence of the tumour suppressor p19^{Arf} (Sebastian and Johnson, 2009). Ectopic C/EBP β expression in Ras^{V12}-transformed NIH 3T3 (3T3^{Ras}) cells suppresses proliferation and transformation and induces a flat, senescent-like morphology, suggesting that downregulation of endogenous C/EBP β is involved in neoplastic transformation of these cells. To explore a possible role for *Cebpb* 3'UTR sequences in C/EBP β downregulation by Ras^{V12} (e.g., via miRNA-mediated silencing), we used retroviral infection to introduce the C/EBP β coding region alone (C/EBP β ^{CR}) or the coding region plus 3'UTR (C/EBP β ^{UTR}) in NIH 3T3 or 3T3^{Ras} cells. The 3'UTR did not significantly affect C/EBP β protein levels (Figure 1A, bottom panel, lanes 4 and 6), indicating that 3'UTR elements do not confer C/EBP β silencing in this context. Interestingly, however, the proteins expressed from the two constructs exerted very different effects on cell proliferation. C/EBP β ^{CR} inhibited mitotic growth in a Ras-dependent manner, as observed previously (Figure 1A; Supplementary Figure S1A; Sebastian and Johnson, 2009). By contrast, C/EBP β ^{UTR} displayed greatly reduced anti-proliferative activity relative to C/EBP β ^{CR} despite similar nuclear protein levels and mRNA expression (Figure 1A; Supplementary Figure S1B). Cells expressing C/EBP β ^{UTR} also did not acquire the flattened morphology or expression of the senescence marker SA- β Gal that were elicited by C/EBP β ^{CR} (Figure 1B). Moreover, C/EBP β ^{CR} potentially inhibited the ability of 3T3^{Ras} cells to form transformed foci and grow as colonies in soft agar, whereas C/EBP β ^{UTR} was much less active in suppressing these transformed phenotypes (Supplementary Figure S1C). To ensure that the UTR effect was not an artefact of expressing C/EBP β from a retroviral transcript, we generated stable 3T3^{Ras} transfectants carrying inducible C/EBP β ^{CR} or C/EBP β ^{UTR} plasmid vectors. A comparison of two clones expressing equivalent levels of C/EBP β ^{CR} and C/EBP β ^{UTR} confirmed that the 3'UTR efficiently blocks the anti-proliferative activity of C/EBP β (Supplementary Figure S1D). Collectively, these observations

suggested that the 3'UTR regulates C/EBP β function rather than its expression.

Electrophoretic mobility shift assay (EMSA) analysis of nuclear extracts from the NIH 3T3 cell lines demonstrated that C/EBP β ^{CR} DNA binding was enhanced by Ras^{V12} and homodimeric complexes were increased (Figure 1C and D; Lee *et al*, 2010b). However, C/EBP β ^{UTR} DNA binding was unaffected by Ras and homodimer levels remained low. Ras also stimulated C/EBP β ^{CR}-driven transactivation of a reporter gene (2xC/EBP-luc) in transiently transfected NIH 3T3 cells, and this response was blunted by the 3'UTR (see Figure 3E). The 3'UTR effect was not limited to NIH 3T3 cells, as Ras-induced DNA binding and transactivation by C/EBP β ^{UTR} was also reduced in transiently transfected human HEK293T (293T) cells (Figure 1E) and in HeLa cells (Supplementary Figure S2A). Thus, the 3'UTR blocks post-translational activation of C/EBP β in several immortalized/transformed cell lines.

C/EBP β DNA binding is auto-inhibited by three elements in the N-terminal region of the protein, and mutation of these sequences increases basal DNA-binding activity (Lee *et al*, 2010a). Appending the 3'UTR to this de-repressed triple mutant did not inhibit its constitutive DNA binding (Supplementary Figure S2B), showing that the 3'UTR specifically blocks signal-induced activation of C/EBP β and not its binding activity in general. Furthermore, co-expressing a transcript containing the *Cebpb* 3'UTR alone in *trans* did not decrease C/EBP β ^{CR} DNA binding (Supplementary Figure S2C). Hence, the 3'UTR acts in *cis* to suppress C/EBP β post-translational activation.

Since the related C/EBP α protein also has strong cytostatic activity (Johnson, 2005), we investigated whether the *Cebpa* 3'UTR exerts similar inhibitory effects. Like C/EBP β ^{CR}, C/EBP α ^{CR} suppressed NIH 3T3 cell proliferation in a Ras-dependent manner, and the *Cebpa* 3'UTR substantially blocked this anti-proliferative function without affecting C/EBP α expression (Supplementary Figure S3, upper panel). Moreover, fusing the *Cebpa* 3'UTR to the *Cebpb* coding region (C/EBP β ^{zUTR}) repressed C/EBP β 's ability to inhibit cell proliferation (Supplementary Figure S3, lower panel). Thus, the 3'UTRs of two *Cebp* genes contain transferable regulatory information that inhibits the Ras-induced cytostatic functions of both proteins.

An AU-rich element and its cognate binding protein, HuR, are required for 3'UTR inhibition

To identify 3'UTR sequences that inhibit C/EBP β activity, we tested a set of 3'UTR deletion mutants in transfected 293T cells. This analysis showed that a region near the 3'-end of the UTR was required to suppress DNA binding and transactivation (Figure 2A). The inhibitory region spans a sequence previously shown to contain A/U-rich elements (AREs; Gantt *et al*, 2005; Cherry *et al*, 2008), regulatory motifs found in many 3'UTRs that control mRNA decay and translation (Barreau *et al*, 2005). The *Cebpb* 3'UTR contains a class III ARE composed of several G(U)₄₋₆G repeats and lacks canonical AUUUA elements found in class I and II AREs (Xu *et al*, 1997). To test its role in C/EBP β inhibition, we deleted the 110-nt *Cebpb* ARE region. This mutation (Δ ARE) abolished the inhibitory effect of the 3'UTR (Figure 2B). Moreover, the ARE segment alone was nearly as effective as the complete 3'UTR in suppressing C/EBP β activity. Analysis of the mutant constructs in 3T3^{Ras} cells showed that Δ ARE was unable to

Figure 1 Suppression of Ras-induced C/EBPβ activation and its anti-proliferative functions by the 3'UTR. (A) Growth rates of control NIH 3T3 and 3T3^{Ras} cells infected with retroviruses expressing the C/EBPβ coding region alone (C/EBPβ^{CR}) or coding region + 3'UTR (C/EBPβ^{UTR}). Data are the mean values ± s.d. from three experiments, each point assayed in triplicate. Lower panel: C/EBPβ western blot of nuclear extracts from the infected cells. (B) Morphology and senescence features of 3T3^{Ras} cells transduced with C/EBPβ^{CR} or C/EBPβ^{UTR}. Cells expressing C/EBPβ^{CR} exhibit decreased proliferation and a flattened cellular morphology (left panels). SA-βGal staining (right) demonstrates senescent-like properties of many cells expressing C/EBPβ^{CR} but not C/EBPβ^{UTR}. The percentage of SA-βGal-positive cells is shown in the inset. (C) DNA-binding assays (EMSA) of nuclear extracts from cells described in (A). EMSA was performed using a canonical C/EBP site probe. The upper band represents a C/EBPβ homodimer; the intermediate band is composed of β:γ heterodimers and a second unidentified species. Bottom panel: C/EBPβ levels assessed by western blotting. (D) Antibody supershift assays were used to determine the composition of C/EBP EMSA complexes in C/EBPβ^{CR}-expressing NIH 3T3 cells. Lanes 1 and 2 show HEK293T cells transfected with C/EBPβ^{CR} ± Ras^{V12}; the upper complex corresponds to C/EBPβ homodimers. The faster-migrating complex present in C/EBPβ^{CR}-expressing 3T3^{Ras} cells (lanes 3–5) is a mixture of β:γ heterodimers and a second unidentified DNA-binding species. (E) DNA-binding and transactivation assays of C/EBPβ proteins transiently expressed in 293T cells. Cells were transfected with the indicated vectors and, after serum starvation, nuclear extracts were prepared, normalized for C/EBPβ levels and analysed by EMSA (upper). Transactivation (lower) was analysed using a C/EBP reporter (2xC/EBP-luc). Values represent fold activation over the reporter alone and are the mean values ± s.d. of four experiments. C/EBPβ protein levels are shown below the graph.

inhibit C/EBPβ-induced growth arrest, whereas the ARE alone efficiently repressed C/EBPβ (Figure 2C). EMSA confirmed that Ras-induced C/EBPβ DNA binding was also inhibited by the ARE in these cells (Figure 2D). Hence, the

ARE region is necessary and sufficient to suppress C/EBPβ activation by oncogenic Ras.

AREs are recognized by several sequence-specific RNA-binding proteins, including members of the Elav1/Hu protein

Figure 2 An AU-rich element (ARE) region is necessary and sufficient for 3'UTR inhibition. **(A)** Analysis of *Cebpb* 3'UTR deletion mutants in 293T cells. The constructs depicted in the diagram were tested for C/EBP β DNA binding (left) and transactivation (right) in transiently transfected cells. Transactivation data are the mean values $\pm$ s.d. from three experiments. **(B)** Critical role of the ARE region for *Cebpb* 3'UTR repression. The constructs shown were analysed as described in **(A)**. Transactivation data are the mean values $\pm$ s.d. from three experiments. **(C)** Cytostatic activity of C/EBP β proteins expressed from WT and 3'UTR mutant constructs in 3T3^{Ras} cells. Data are the mean values $\pm$ s.d. from two experiments, each point assayed in triplicate. Western blot shows C/EBP β expression levels in the transduced cells. **(D)** DNA-binding assays of nuclear extracts from cells described in **(C)**. Extracts from non-transformed NIH 3T3 cells (i.e., without Ras^{V12}) infected with the same constructs are included for comparison.

family (Barreau *et al*, 2005). The ubiquitously expressed HuR protein binds to numerous mRNAs and controls their stability and translation (Abdelmohsen *et al*, 2008). Because HuR was shown to associate with the *Cebpb* ARE (Gantt *et al*, 2005; Cherry *et al*, 2008), we examined its role in 3'UTR inhibition. shRNA knockdown of HuR in 293T cells (HuR^{KD}) largely abolished 3'UTR inhibition of C/EBP β DNA binding and transactivation (Figure 3A and C), and inhibition was restored by re-expression of HuR (Figure 3B and C). HuR depletion did not affect activation of Ras effector kinases, as judged by induction of phosphorylated ERK, RSK, and Akt, nor was signalling disrupted by expression of the 3'UTR construct (Supplementary Figure S4). Thus, the inhibitory effects of the 3'UTR are not due to impaired Ras signalling.

We also depleted HuR in NIH 3T3 cells to assess its role in 3'UTR-mediated inhibition of C/EBP β cytotostatic activity. HuR silencing alone moderately decreased proliferation of control and Ras-transformed cells (Figure 3D). C/EBP β ^{CR} did not appreciably alter the growth of non-transformed 3T3-HuR^{KD} cells but blocked proliferation of 3T3^{Ras} cells, regardless of their HuR status. By contrast, C/EBP β ^{UTR} only weakly inhibited the expansion of 3T3^{Ras} cells but displayed potent cytotostatic activity in HuR^{KD}-3T3^{Ras} cells (Figure 3D;

Supplementary Figure S5A). Moreover, C/EBP β ^{UTR} elicited a flattened morphology in HuR^{KD}-3T3^{Ras} cells (Supplementary Figure S5B), but not in 3T3^{Ras} cells (Figure 1B). Hence, HuR is essential for the ability of the 3'UTR to suppress C/EBP β activation by Ras.

We further investigated whether HuR suppresses endogenous C/EBP β activity in human tumour cells, since this mechanism could be involved in establishing the transformed phenotype. HuR was ablated in A549 lung tumour cells and proliferation was assessed. HuR silencing decreased cell growth and colony formation (Supplementary Figure S6A), consistent with studies showing that HuR depletion inhibits proliferation of RKO colorectal carcinoma cells (Wang *et al*, 2000). To determine if this cytotostatic response involves C/EBP β , we depleted C/EBP β in conjunction with HuR. C/EBP β knockdown in A549 cells overcame the effect of HuR ablation and increased proliferation and colony formation to levels exceeding even those of control cells. HuR silencing also led to a modest increase in endogenous C/EBP β DNA binding (Supplementary Figure S6B). Similar results were obtained using MCF7 breast cancer cells and HeLa cells (Supplementary Figure S6C and D). Thus, HuR ablation impairs proliferation of several immortalized human tumour cell lines in a manner that is partially dependent on

Figure 3 HuR is required for 3'UTR inhibition. (A) 3'UTR inhibition is disrupted in HuR knockdown (HuR^{KD}) 293T cells. Nuclear extracts normalized for C/EBPβ levels were analysed by EMSA. Western blot shows HuR levels in control and HuR^{KD} cells. (B) Re-expression of HuR in HuR^{KD} cells restores 3'UTR inhibition. HuR was co-transfected with the indicated constructs in 293T-HuR^{KD} cells. (C) Transcriptional activity of C/EBPβ^{UTR} depends on HuR. Transactivation of the 2 × C/EBP-luc reporter was compared in control and HuR^{KD} cells. Re-expression of HuR restored inhibition by the 3'UTR without significantly affecting C/EBPβ^{CR} activity. Data are the mean values ± s.d. from two experiments. (D) HuR knockdown in NIH 3T3 cells abolishes 3'UTR inhibition of C/EBPβ cytostatic activity. Control NIH 3T3 cells (upper panel) or 3T3^{Ras} cells (lower panel) were infected with retroviruses expressing shHuR or the empty vector. The cells were subsequently infected with the indicated C/EBPβ constructs and proliferation rates were determined. Data are the mean values ± s.d. from two experiments, each point assayed in triplicate. Extracts were analysed for C/EBPβ and HuR expression by western blotting (right). (E) 3'UTR inhibition of C/EBPβ transactivation is impaired in HuR^{KD} NIH 3T3 cells. Transient transactivation assays using the 2 × C/EBP-luc reporter were performed in control and HuR^{KD} NIH 3T3 cells. Data are the mean values ± s.d. from two experiments. Ectopic C/EBPβ protein expression could not be assessed due to high levels of endogenous C/EBPβ in these cells.

C/EBPβ, supporting the notion that HuR suppresses C/EBPβ cytostatic activity in tumour cells.

Ras-induced post-translational modifications and homodimerization of C/EBPβ are suppressed by the 3'UTR

We next asked whether Ras-induced modifications on C/EBPβ are regulated by the 3'UTR. Ser64 and Thr188 are phosphorylated by Cdk and ERK1/2 kinases, respectively, in response to oncogenic Ras (Nakajima *et al*, 1993; Shuman *et al*, 2004). Analysis by western blotting using phospho-specific antibodies showed that both modifications were induced by Ras in C/EBPβ^{CR}, but were nearly undetectable in C/EBPβ^{UTR} (Figure 4A). By contrast, C/EBPβ^{UTR} underwent phosphorylation on Ser64 and Thr188 in HuR-depleted 293T cells (Figure 4B). Therefore, the 3'UTR blocks inducible phosphorylation on Ser64 and Thr188 in a HuR-dependent manner, consistent with C/EBPβ^{UTR} remaining in a low-activity state.

Ras-induced activation of C/EBPβ is associated with phosphorylation-mediated stabilization of homodimers that are resistant to displacement by C/EBPγ (Lee *et al*, 2010b). To determine whether the 3'UTR affects C/EBPβ dimerization

with C/EBPγ, we expressed C/EBPβ and Ras^{V12} in the presence of increasing amounts of C/EBPγ. EMSA showed that C/EBPβ^{CR} heterodimerization with C/EBPγ was relatively inefficient, whereas C/EBPβ^{UTR} readily formed heterodimers, especially at the highest dose of C/EBPγ (Figure 4C). Similar results were seen in reporter assays, where increasing amounts of C/EBPγ inhibited C/EBPβ^{UTR} transcriptional activity while that of C/EBPβ^{CR} was largely unaffected (Figure 4D). These findings indicate that the 3'UTR suppresses formation of transcriptionally active C/EBPβ homodimers, which promote cell-cycle arrest and OIS, and allows preferential heterodimerization with C/EBPγ.

The presence of the 3'UTR alters expression of C/EBPβ target genes

To confirm that C/EBPβ^{CR} and C/EBPβ^{UTR} possess distinct regulatory activities and to identify target genes that are differentially induced by these two proteins in the presence of oncogenic Ras, we used microarrays to compare the transcriptomes of 3T3^{Ras}, C/EBPβ^{CR}-3T3^{Ras}, and C/EBPβ^{UTR}-3T3^{Ras} cell lines. Using thresholds of ≥2-fold change and $P \leq 0.001$, 558 probes (409 genes) displayed significant upregulation by C/EBPβ^{CR} relative to control 3T3^{Ras} cells

Figure 4 The 3'UTR inhibits Ras-induced post-translational modifications and dimerization of C/EBPβ. (A) Ras-induced p-Ser64 and p-Thr188 modifications are suppressed by the 3'UTR. C/EBPβ^{CR} and C/EBPβ^{UTR} were expressed in 293T cells without or with Ras^{V12}. Nuclear extracts were analysed by western blotting using the indicated phospho-specific or conventional C/EBPβ antibodies. (B) 3'UTR inhibition of C/EBPβ phosphorylation is disrupted in HuR knockdown cells. The experiment described in (A) was performed using 293T-HuR^{KD} and control cells. (C) 3'UTR inhibition maintains efficient C/EBPβ heterodimerization with C/EBPγ in the presence of Ras signalling. C/EBPβ^{CR} and C/EBPβ^{UTR} were expressed with Ras^{V12} and increasing amounts of C/EBPγ in 293T cells, and nuclear extracts were analysed by EMSA. (D) C/EBPβ^{CR} and C/EBPβ^{UTR} transactivation of a luciferase reporter in the presence of Ras^{V12} and increasing amounts of C/EBPγ. Luciferase activity for the C/EBPβ constructs in the absence of C/EBPγ (control) was set to 100%, and the other values represent percent of control. Data are the mean values ± s.d. of three experiments.

(Figure 5A and B; Supplementary Table S1). By the same criteria, 208 probes (167 genes) were activated by C/EBPβ^{UTR}. In all, 124 probes (97 genes) constituted an intersecting set induced by both proteins. Thus, of the genes induced by C/EBPβ^{CR}, 76% (312/409) were not significantly activated by C/EBPβ^{UTR}. However, even this number underrepresents the inhibitory effect of the 3'UTR, since many of the transcripts upregulated by C/EBPβ^{UTR} were activated to a much greater extent by C/EBPβ^{CR} (Supplementary Figure S7; Supplementary Table S1). Interestingly, 84 probes (70 genes) were induced by C/EBPβ^{UTR} but not by C/EBPβ^{CR}, showing that the 'low-activity' C/EBPβ^{UTR} protein is able to stimulate gene transcription in a manner that is qualitatively distinct from the 'high activity' C/EBPβ^{CR} form.

We also examined expression of individual senescence-associated genes. Several inflammatory SASP genes (*IL-6*, *GROα/Cxcl-1*, *Cxcl-2*, *Cxcl-5*, and *Ccr-1*) ranked within the 400 transcripts showing highest fold induction by C/EBPβ^{CR}. Quantitative PCR demonstrated that these genes were induced to a much greater extent by C/EBPβ^{CR} than by C/EBPβ^{UTR} (Figure 5C). For example, *Cxcl-1* mRNA was induced 253-fold by C/EBPβ^{CR} (relative to 3T3^{Ras} cells) and only 4.3-fold by C/EBPβ^{UTR}. Similarly, *IL-1α*, *IL-1β*, and *S100a9* showed strong preferential activation by C/EBPβ^{CR}. In several cases, these transcripts were diminished in 3T3^{Ras} cells compared with control NIH 3T3 cells, possibly due to silencing of endogenous C/EBPβ expression by oncogenic Ras (Sebastian and Johnson, 2009). Other C/EBPβ^{CR}-activated genes identified in the arrays that are known to regulate cell-cycle arrest, cell adhesion/migration, or tumour suppression include *schlafen2*, *stefin A3*, *Btg2*, *p53 interacting protein 1*, *Vgll3*, and *thrombospondin 1 (Thbs1/TSP-1)* (Li *et al*, 2005; Tomasini *et al*, 2005; Kazerounian *et al*,

2008; Cody *et al*, 2009; Katsoulidis *et al*, 2009; Winkler, 2010). qPCR confirmed that each of these transcripts was significantly increased by C/EBPβ^{CR} but was induced much less efficiently by C/EBPβ^{UTR} (Supplementary Figure S7B). For example, *Stfa3* expression was increased 6600-fold by C/EBPβ^{CR} but only 290-fold by C/EBPβ^{UTR}, and *Vgll3* was induced 130-fold by C/EBPβ^{CR} versus 10-fold by C/EBPβ^{UTR}. Hence, the impaired activation of several SASP, anti-proliferative, and tumour suppressor genes is consistent with the diminished cytostatic potential of C/EBPβ^{UTR}.

We analysed the gene signatures induced by C/EBPβ^{CR} or C/EBPβ^{UTR} for associations with biological pathways or diseases using GeneGo MetaCore software. The 10 highest ranking associations for each category are shown in Figure 5D. C/EBPβ^{CR}-induced genes predominantly corresponded to pathways involved in inflammation and immune function (8/10 categories). By contrast, C/EBPβ^{UTR} targets were biased towards developmental pathways (4/10), cytoskeleton remodelling, and TGFβ signalling (4/10). Of note, C/EBPβ^{UTR}-activated genes were strongly correlated with neoplastic diseases (7/10 categories), while C/EBPβ^{CR} scored highly for cardiovascular diseases, inflammation, and wounds/injuries but not for cancers. The fact that the C/EBPβ^{UTR} signature is correlated with TGFβ signalling, epithelial-mesenchymal transition (EMT), and neoplastic diseases raises the intriguing possibility that this form of C/EBPβ has pro-oncogenic/metastatic functions.

The 3'UTR excludes *Cebpb* mRNA from a perinuclear cytoplasmic domain that contains activated ERK1/2 kinase

One possible mechanism for 3'UTR inhibition could involve localization of 3'UTR-containing transcripts to a cytoplasmic region where the newly translated protein cannot undergo

Figure 5 The *Cebpb* 3'UTR alters C/EBP β target gene specificity. (A) Heat maps depicting expression levels of genes selectively induced by C/EBP β^{CR} (left), C/EBP β^{UTR} (right), or both proteins (middle). Maps show microarray data from three independently derived cell lines each for 3T3 Ras , C/EBP β^{CR} -3T3 Ras , and C/EBP β^{UTR} -3T3 Ras cells. Darker shading indicates higher expression; dendrograms show hierarchical clustering patterns. (B) Venn diagram showing differential activation of target genes by C/EBP β^{CR} and C/EBP β^{UTR} in 3T3 Ras cells. Gene array data were filtered as described in the text to identify significantly upregulated genes. Genes induced significantly by both proteins are depicted in the intersection. (C) qPCR analysis of C/EBP β -induced SASP genes and other pro-inflammatory mediators. RNA from the indicated NIH 3T3 cell lines was analysed for expression of individual transcripts using quantitative PCR. Data are the mean values $\pm$ s.d. from two experiments, each assayed in triplicate. (D) Pathway and disease associations of gene signatures selectively activated by C/EBP β^{CR} or C/EBP β^{UTR} in 3T3 Ras cells. Genes corresponding to the non-overlapping groups shown in this figure and Supplementary Table S1 were analysed using GeneGo MetaCore software to identify associations with canonical biological pathways or disease states. Pattern matches were ranked by *P*-value (LOD score) and the top 10 categories are shown.

activation. To address this possibility, we analysed the cytoplasmic location of *Cebpb* mRNAs using an mRNA tagging method in which transcripts are tethered to a nuclear-targeted GFP reporter (GFP-MS2-nls) via the RNA-binding domain of MS2 phage coat protein (Rook *et al*, 2000). Multiple copies of

the MS2 binding site were appended to the *Cebpb*^{CR} and *Cebpb*^{UTR} transcripts and the constructs were analysed in transfected NIH 3T3 cells by confocal microscopy. The GFP reporter alone was exclusively nuclear due to the presence of the nuclear localization signal (Figure 6A). Co-expression of

Figure 6 The 3'UTR excludes *Cebpb* mRNA from a perinuclear region of the cytoplasm. (A) Localization of *Cebpb*^{CR} and *Cebpb*^{UTR} transcripts in NIH 3T3 cells using a GFP tethering assay. Cells were transiently transfected with the GFP-MS2-nls reporter alone or with tagged *Cebpb*^{CR} or *Cebpb*^{UTR} vectors. GFP-positive cells were imaged by confocal fluorescence microscopy. Nuclei were visualized by DAPI staining. The percentage of cells exhibiting the GFP fluorescence pattern depicted is indicated in parentheses. (B) The ARE is necessary and sufficient to direct *Cebpb* mRNA to the cytoplasmic periphery. Tagged *Cebpb*^{UTRΔARE} and *Cebpb*^{ARE} transcripts were expressed with GFP-MS2-nls in NIH 3T3 cells and imaged by confocal microscopy. (C) HuR is required for perinuclear exclusion of *Cebpb*^{UTR} mRNA. HuR^{KD} NIH 3T3 cells were transfected with tagged *Cebpb*^{CR} or *Cebpb*^{UTR} vectors and the GFP reporter. Control cells expressing the empty knockdown vector were analysed in parallel (Supplementary Figure S9).

tagged *Cebpb*^{CR} mRNA resulted in detectable cytoplasmic fluorescence, with the GFP signal distributed relatively uniformly throughout the cytoplasm. The *Cebpb*^{UTR} transcript also promoted cytoplasmic localization of the GFP reporter, but in this case GFP was excluded from a region surrounding the nucleus, with fluorescence concentrated in more peripheral areas. Although such perinuclear exclusion was not observed for every GFP-positive cell ($79.5 \pm 2.4\%$), the pattern was clearly distinct from that of *Cebpb*^{CR} mRNA, which was nearly always uniformly distributed ($93.3 \pm 1.8\%$). Similar results were obtained using HeLa cells (Supplementary Figure S8). Hence, the 3'UTR restricts *Cebpb* mRNA to a peripheral cytoplasmic domain.

Deleting the ARE led to uniform distribution of the *Cebpb*^{UTR} transcript (Figure 6B). Moreover, *Cebpb*^{ARE} mRNA was peripherally localized in a pattern similar to *Cebpb*^{UTR}. Compartmentalization of the *Cebpb*^{UTR} transcript required HuR, as it displayed more uniform distribution in HuR^{KD} NIH 3T3 cells compared with its peripheral restriction in control cells (Figure 6C; Supplementary Figure S9). Thus, the presence of *Cebpb* mRNA in the perinuclear region is strictly correlated with C/EBP β activation and induction of cell-cycle arrest, while exclusion from this domain is associated with diminished protein activity. To further test the mRNA localization model, we replaced the 3'UTR with three copies of the β -actin 3'UTR 'zipcode' element, a sequence that directs β -actin transcripts to the peripheral cytoplasm and leading lamellae of migrating cells (Kislauskis *et al*, 1994). The *Cebpb*^{3 $\times$ ZIP} transcript was predominantly localized to the periphery in NIH 3T3 cells, substantially mimicking the pattern observed for the *Cebpb* 3'UTR (Supplementary Figure S10). Significantly, the zipcode sequence also suppressed activation of C/EBP β DNA binding and transactivation in NIH 3T3 cells and 293T cells (Supplementary Figure S11), although its inhibitory effect was somewhat less than that of the 3'UTR. This quantitative difference may result from less efficient exclusion of the zipcode-containing transcript from the perinuclear region. Nevertheless, our results corroborate the notion that exclusion of *Cebpb* mRNA from the perinuclear compartment blunts C/EBP β post-translational activation by Ras signalling.

These data suggest that C/EBP β ^{UTR} protein synthesis in the cytoplasmic periphery could restrict its access to critical modifying enzyme(s). C/EBP β activation by Ras is highly dependent on Raf-MEK-ERK signalling, inducing direct phosphorylation on Thr188 by ERK1/2 and other modifications (Nakajima *et al*, 1993; Mo *et al*, 2004; Lee *et al*, 2010b). Since Thr188 phosphorylation was blocked by the 3'UTR (Figure 4A), we examined the subcellular location of activated ERK1/2 in NIH 3T3 cells expressing a 4-OHT-inducible H-Ras^{V12} protein, ER:Ras^{V12} (Dajee *et al*, 2002). Western blotting showed significantly increased levels of phospho-ERK 24 and 48 h after induction (Figure 7A). Immunofluorescent staining of the induced cells revealed a strong p-ERK signal that occurs in a punctate pattern confined mainly to the perinuclear region (Supplementary Figure S12). Nuclear p-ERK levels were low or undetectable in these cells, showing that activated ERK is predominantly perinuclear in cells undergoing sustained Ras signalling. Similar perinuclear p-ERK localization was observed in Ras^{V12}-expressing (senescent) normal human fibroblasts (Gaumont-Leclerc *et al*, 2004). Notably, simultaneous

imaging of p-ERK and *Cebpb* mRNA (GFP) revealed a striking overlap between p-ERK and the perinuclear region that is devoid of *Cebpb*^{UTR} mRNA (Figure 7B). These findings provide compelling evidence that C/EBP β protein synthesis in the perinuclear compartment facilitates coupling with p-ERK (and possibly other C/EBP β -activating kinases), while 3'UTR-mediated exclusion of the *Cebpb* transcript prevents functional interactions between nascent C/EBP β and Ras effector kinase(s).

3'UTR inhibition is absent in primary fibroblasts

C/EBP β is required for OIS in primary MEFs (Sebastian *et al*, 2005), and endogenous C/EBP β DNA-binding activity is stimulated by Ras^{V12} in these cells (Figure 8A; Lee *et al*, 2010b). These observations suggested that 3'UTR inhibition might be lacking in primary fibroblasts. Consistent with this prediction, both C/EBP β ^{CR} and C/EBP β ^{UTR} efficiently inhibited proliferation of Ras-expressing *Cebpb*^{-/-} MEFs and showed comparable transactivation of a reporter gene (Figure 8B and C). IMR90 human diploid fibroblasts also undergo C/EBP β -dependent senescence in response to oncogenic stress (Kuilman *et al*, 2008). Accordingly, C/EBP β ^{CR} and C/EBP β ^{UTR} displayed similar Ras-stimulated DNA-binding and transcriptional activity in these cells (Supplementary Figure S13A and B). Moreover, both transcripts showed uniform cytoplasmic distribution in MEFs and IMR90 cells (Figure 8D; Supplementary Figure S13C). Hence, 3'UTR inhibition is reduced or absent in primary fibroblasts and correlates with an absence of *Cebpb* mRNA compartmentalization, allowing C/EBP β to become activated and induce proliferative arrest in primary cells expressing oncogenic Ras.

We next asked whether cell-specific control of 3'UTR inhibition involves HuR. As HuR is predominantly nuclear but undergoes nucleocytoplasmic shuttling in a regulated manner (Doller *et al*, 2008), we investigated whether differential 3'UTR inhibition in MEFs and NIH 3T3 fibroblasts is associated with changes in HuR levels or subcellular partitioning. Cytoplasmic HuR levels were comparable in both control cell populations (Supplementary Figure S14, left panel, lanes 1 and 3). However, oncogenic Ras decreased the cytoplasmic HuR pool in MEFs, whereas it increased cytosolic HuR in NIH 3T3 cells (Supplementary Figure S14, left panel, lanes 2 and 4). Nuclear HuR levels were correspondingly increased or decreased, respectively (Supplementary Figure S14, right panel). Thus, elevated cytoplasmic HuR is observed in immortalized cells under conditions of oncogenic stress. This suggests that increased cytosolic HuR in transformed 3T3^{Ras} cells engages the C/EBP β 3'UTR repression mechanism. By contrast, primary cells undergoing OIS display reduced levels of cytoplasmic HuR and, concomitantly, lack C/EBP β 3'UTR inhibition.

Discussion

3'UTRs have well-established roles in controlling mRNA degradation and translation, often through the actions of miRNAs (Conne *et al*, 2000; Moore, 2005; Bartel, 2009). Here, we report a previously unrecognized function for 3'UTRs in regulating signal-induced activation of the protein product, a process that we term '3'UTR regulation of protein activity' or UPA (Figure 9A). UPA inhibits the cytostatic activities of both C/EBP β and C/EBP α in Ras^{V12}-expressing

Figure 7 Activated ERK1/2 localizes to the perinuclear region of Ras^{V12}-expressing NIH 3T3 cells. **(A)** Phospho-ERK1/2 is induced upon 4-OHT treatment of NIH 3T3 cells stably expressing ER:Ras^{V12}. Cells were starved for serum and lysates analysed for p-ERK and total ERK by immunoblotting following 4-OHT stimulation. p-ERK levels in non-transduced, serum-starved NIH 3T3 cells are shown for comparison. **(B)** Phospho-ERK1/2 is located in the perinuclear region that lacks *Cebp^{bUTR}* mRNA. 3T3^{ER:Ras^{V12}} cells were co-transfected with the GFP-MS2-nls reporter and tagged *Cebp^b* vectors, induced with 4-OHT for 48 h, immunostained for p-ERK (Texas Red), and both markers visualized by confocal fluorescence microscopy.

cells, showing that this mechanism regulates at least two members of the C/EBP family. At present, it is unclear how many other proteins are subject to UPA control. However, since an estimated 5–8% of human genes contain ARE-like sequences (Bakheet *et al*, 2003), similar mechanisms could theoretically regulate the activities of many proteins.

C/EBP β activity is constrained by auto-inhibition and heterodimerization with C/EBP γ , and these inhibitory effects are reversed by oncogenic Ras signalling via the MEK/ERK/RSK kinase cascade (Lee *et al*, 2010a,b). Our results show that UPA quells de-repression of C/EBP β by oncogenic Ras, providing another barrier to C/EBP β activation that prevents unscheduled cell-cycle arrest. UPA is restricted to immortalized and transformed cells, raising the possibility that genetic events rendering cells susceptible to transformation by Ras (i.e., loss of tumour suppressors) are involved in activating the C/EBP β UPA system. This mechanism could link inhibition of C/EBP β cytosolic activity with oncogenic transformation and may be essential for senescence bypass in tumour cells. Because C/EBP β also has pro-oncogenic functions and can promote cell proliferation and survival (Sebastian and Johnson, 2006), we suggest that UPA does not inhibit all

C/EBP β activities. Indeed, the C/EBP β ^{UTR}-induced gene signature was highly correlated with cancers whereas that of C/EBP β ^{CR} tended to be associated with inflammation and non-neoplastic diseases (Figure 5D). Thus, activation of genes associated with cancer, EMT, and TGF β signalling may underlie the known pro-tumorigenic effects of endogenous C/EBP β , which is expressed from a 3'UTR-containing transcript.

The *Cebp^b* ARE region is necessary and sufficient for UPA, which also requires the ARE-binding protein, HuR. HuR positively regulates cell proliferation, in part through binding to the 3'UTRs of cyclins A and B and stabilizing the mRNAs encoding these key cell-cycle regulators (Wang *et al*, 2000). C/EBP β depletion in several human tumour cell lines partially or completely abrogated the cytostatic response to HuR ablation (Supplementary Figure S6). Thus, C/EBP β is another important HuR target, and HuR may stimulate cell proliferation at least in part through the UPA mechanism. The connection between HuR and C/EBP β was also observed in NIH 3T3 cells, where HuR depletion converted C/EBP β ^{UTR} into a growth suppressor (Figure 3D). These experiments show that HuR inhibits the cytostatic activity of C/EBP β , an idea that is

Figure 8 C/EBPβ 3'UTR repression is lacking in primary fibroblasts. (A) DNA binding of endogenous C/EBPβ in MEFs is activated by oncogenic Ras. Nuclear extracts from MEFs infected with control or Ras^{V12} retroviruses were analysed for C/EBPβ DNA binding. Western blotting shows similar levels of C/EBPβ in the extracts. (B) Proliferation assays of low passage *Cebpb*^{-/-} MEFs infected with C/EBPβ^{CR} or C/EBPβ^{UTR} retroviruses. Data are the mean values ± s.d. from two experiments assayed in triplicate. (C) C/EBPβ^{CR} and C/EBPβ^{UTR} display equivalent Ras^{V12}-induced transcriptional activity in MEFs. *Cebpb*^{-/-} MEFs were transfected with 2xC/EBP-luc and C/EBPβ plasmids, with or without Ras^{V12}. Data are the mean values ± s.d. from two experiments. (D) The 3'UTR does not regulate *Cebpb* mRNA localization in MEFs. MEFs were transfected with the GFP-MS2-nls reporter, alone or with tagged *Cebpb*^{CR} or *Cebpb*^{UTR} vectors, and imaged by confocal fluorescence microscopy.

further supported by the fact that cytoplasmic HuR levels are decreased in senescent fibroblasts (Wang *et al*, 2001; Supplementary Figure S14), possibly due to increased energetic stress and elevated AMP-dependent signalling (Wang *et al*, 2003). The diminished cytoplasmic HuR pool in primary cells may alleviate negative regulation by the 3'UTR and permit C/EBPβ activation.

The UPA mechanism proposed here also relates to the reported association between HuR and cancer. Since elevated HuR levels and/or cytoplasmic abundance have been linked to increased incidence or malignancy of various cancers (Lopez de Silanes *et al*, 2003; Ido *et al*, 2008; Mazan-Mamczarz *et al*, 2008; Yoo *et al*, 2009), we propose that implementation of C/EBPβ UPA contributes to the progression of premalignant tumours (with senescent features) to fully malignant cancers. Although C/EBPβ^{UTR} is resistant to activation in immortalized/transformed cells, the protein is nonetheless expressed and transported to the nucleus. Thus, UPA provides a plausible mechanism for generating the low activity, mostly heterodimeric form of C/EBPβ that may have pro-oncogenic functions in advanced cancers.

Although the full details of UPA remain to be established, our studies strongly implicate the subcytoplasmic location of C/EBPβ synthesis as a critical factor in its post-translational activation by Ras signalling (Figure 9B). In tumour cells, the 3'UTR excludes *Cebpb* transcripts from a perinuclear domain through a HuR-dependent mechanism. HuR itself does not exhibit the same cytoplasmic distribution and tends to be localized throughout the cytosol in punctate structures (Zou *et al*, 2006), suggesting that HuR is only one component of a larger complex that assembles on the ARE region to regulate *Cebpb* mRNA localization. Importantly, the perinuclear region corresponds precisely to the site of active ERK1/2 in cells expressing oncogenic Ras. p-ERK and newly synthesized C/EBPβ are apparently capable of interacting in this compartment, allowing C/EBPβ to undergo activation before nuclear translocation. Since C/EBPβ de-repression involves additional modifications besides ERK1/2 phosphorylation on Thr188, it is likely that other kinases are also enriched in the perinuclear space. This possibility warrants further investigation. In further support of our model, *Cebpb* mRNA partitioning was not observed in

Figure 9 Model depicting 3'UTR regulatory functions and the proposed mechanism of C/EBP β 3'UTR inhibition. **(A)** The 3'UTR regulation of protein activity (UPA) mechanism identified in this study extends the previously known roles of 3'UTRs, which include control of mRNA decay and regulation of protein translation. **(B)** UPA is operative in immortalized/transformed cells but not in primary cells. The 3'UTR restricts *Cebpb* mRNA to the peripheral cytoplasm in tumour cells, in part due to increased levels of cytoplasmic HuR. Other factors are likely to contribute to this HuR effect, as depicted. In primary cells, *Cebpb* mRNA can enter the perinuclear region, where newly synthesized C/EBP β is available for activation by p-ERK and possibly other Ras effector kinases. Increased homodimerization of activated C/EBP β regulates inflammation, cell-cycle arrest, and senescence, whereas in tumour cells the predominantly heterodimeric form of C/EBP β may contribute to the transformed phenotype.

primary cells, consistent with the observation that oncogenic Ras can activate endogenous C/EBP β and induce senescence in these cells. Thus, UPA may be an important regulatory process that abrogates the senescence program in tumour cells but not in primary cells.

UPA has similarities to, but is distinct from, the 3'UTR zipcode mechanism that localizes β -actin mRNA to the leading edge of migrating fibroblasts (Kislauskis *et al*, 1994). Zipcode-directed mRNA trafficking restricts β -actin translation to a subcellular domain where high levels of actin are required. In contrast, UPA does not alter the ultimate destination of C/EBP β (the nucleus) but rather controls its post-translational activation. A study of mRNA localization in *Drosophila* embryos showed that 71% of the transcripts analysed were distributed in distinct spatial patterns (Lecuyer *et al*, 2007). Thus, differential mRNA localization may be a common means of controlling protein function and activity. The extent to which 3'UTRs specify this spatial patterning is unknown, but they are likely to have a significant role.

In light of similarities between cancer cells and stem/progenitor cells (Reya *et al*, 2001), an important physiologic function of UPA may involve constraining the anti-proliferative activity of C/EBP proteins in progenitor or transit amplifying cells during development. C/EBP α is expressed in proliferating haematopoietic progenitors but is also required for subsequent terminal differentiation of granulocytes and functions as a tumour suppressor in myeloid leukaemias (Nerlov, 2004). Therefore, UPA could regulate haematopoiesis and other developmental processes by restricting the anti-proliferative effects of C/EBP α and C/EBP β to the appropriate lineages and stage of differentiation. Future

studies should reveal whether this is an important biological role for C/EBP UPA. In addition, further elucidation of the proteins and pathways responsible for establishing UPA in tumour cells may reveal novel targets for cancer therapy, since disrupting UPA would be predicted to suppress tumour growth and induce senescence.

Materials and methods

Cell lines and reagents

NIH 3T3 cells were obtained from ATCC and cultured in DMEM medium with 10% calf serum. HEK293T cells, WT and *Cebpb*^{-/-}MEFs (Sebastian *et al*, 2005), A549 cells, MCF7 cells, HeLa cells, and IMR90 cells were cultured in DMEM with 10% fetal bovine serum. Phoenix ecotropic and amphotropic packaging cells were grown in the same medium. C/EBP β (C-19), β -actin (C11), HuR (3A2), and ERK1/2 (K23) antibodies were obtained from Santa Cruz Biotechnology. Phospho-C/EBP β (T188) antibody was obtained from Abcam (pT235) and phospho-S64 antibody has been described previously (Shuman *et al*, 2004). Phospho-p90^{RSK} (S380), Akt, phospho-ERK1/2 (T202/Y204), and pAkt (S473) antibodies were obtained from Cell Signaling Technology. RSK2 and RSK3 antisera were kindly provided by M Ernst and N Rice.

Plasmids and retroviral constructs

Details of the various expression vectors are presented in the Supplementary data.

Stable transfection and retroviral transduction

Retroviral infection was performed as described (Sebastian *et al*, 2005). After drug selection, cells were used for proliferation, colony formation, and focus formation assays. Knockdown of C/EBP β and HuR expression was achieved using pSuperRetro vector-mediated RNAi technology (Oligoengine). The siRNA sequences for C/EBP β (GATGTCCTGCGGGGTGT; Sebastian and Johnson, 2009) and HuR (GAGGCAATTACCACTTCA; Lal *et al*, 2004) were inserted

into pSuperRetro; retroviral supernatants were produced using Phoenix packaging cells. Permanent clones of HuR^{KD} HEK293T, A549, and MCF7 cells were selected, and individual colonies were expanded for further analysis. For inducible expression of C/EBP β , NIH 3T3 cells were transfected with pMTCB6-C/EBP β ($\pm$ 3'UTR) using CaPO₄ precipitation, transfectants were selected with antibiotics, and individual clones were isolated for proliferation assays. C/EBP β expression was induced with 50 μ M ZnSO₄.

Proliferation, transformation, and senescence assays

Cell proliferation and transformation were assayed using standard methods (see Supplementary data). For SA- β Gal staining, cells were plated in 12-well dishes (12×10^3 cells/well) and stained using a senescence detection kit (EMD Biosciences, USA) according to the manufacturer's instructions. Positive cells were quantified by scoring 100 cells each from two different wells.

Transient transfection and luciferase assays

HEK293T and IMR90 cells were transfected with 200 ng C/EBP β vector and 100 ng of pcDNA3-H-Ras^{V12} in 60 mm dishes. In all, 700 ng HuR vector was used for HuR re-expression experiments. After transfection, cells were cultured in complete media for 24 h and then serum starved overnight before harvesting. For transactivation assays, 293T cells, NIH 3T3 cells, HeLa cells, IMR90 cells, or WT and *Cebpb*^{-/-} MEFs were transfected with 100 ng 2xC/EBP-luc reporter and 5 ng C/EBP β vector with 10 ng Ras^{V12} plasmid. Cells were lysed in $1 \times$ passive lysis buffer (Promega) and luciferase activity was measured using a Monolight 2010 luminometer. Luciferase values were normalized to total protein concentration of the lysate. Data are presented as fold activation of the reporter alone and represent mean values $\pm$ s.d. of at least three independent experiments.

Protein analysis and EMSA

Nuclear and cytoplasmic extracts were prepared from the retrovirally infected or transiently transfected cells, as described (Sebastian and Johnson, 2009). EMSA was performed by incubating nuclear extracts (normalized for C/EBP β levels) with ³²P-labelled oligonucleotide probe corresponding to a consensus C/EBP binding site, as described (Lee *et al*, 2010b). In these experiments, β -actin controls were not included because the extracts were equalized for C/EBP β levels. For supershift assays, extracts were pre-incubated with 200 ng antibodies against C/EBP β or C/EBP γ (Parkin *et al*, 2002) for 30 min before the binding reaction. DNA-protein complexes were resolved on 6% polyacrylamide/1 $\times$ TBE gels.

mRNA localization

The GFP-MS2-nls reporter system has been described previously (Rook *et al*, 2000). The pcDNA-Cebpb-MS2b vector was constructed by cloning the multiple MS2 binding sites from the RSV-lacZ-MS2b vector (Rook *et al*, 2000) downstream of the *Cebpb* coding region, without or with the 3'UTR, using *Bam*HI/*Bgl*II sites. The *Cebpb*^{CR}-MS2b vector was modified to *Cebpb*^{3 $\times$ ZIP}-MS2b by cloning three copies of the 54-nt β -actin zipcode element (5'-ACCGGACTGT-TACCAACACCCACACCCTGTGATGAAACAAAACCCATAAATGC-3') (Kislauskis *et al*, 1994) downstream of the MS2 binding sites using *Bam*HI/*Hind*III restriction sites. NIH 3T3 cells, HeLa cells, IMR90 cells, and WT MEFs were plated at 5×10^4 cells/35 mm dish and transfection was carried out using Polyfect (Qiagen) (for NIH 3T3), Fugene 6 (Roche) (for HeLa), and Transpass D2 and Transpass V (New England Biolabs) (for IMR90 and MEFs, respectively). After 36 h, the cells were fixed with 3.6% formaldehyde for 10 min and stained with 1 μ g/ml DAPI (Sigma). Fluorescence images were taken with a Zeiss confocal microscope.

Immunofluorescence

Immunostaining was performed as described (Sebastian *et al*, 2005) with minor modifications. Cells (5×10^4 /35 mm dish) were fixed with 4% paraformaldehyde for 10 min and incubated with phospho-ERK1/2 primary antibody followed by goat anti-rabbit IgG secondary antibody conjugated with Texas red (Santa Cruz

Biotechnology sc-2780). Fluorescence images were taken with a Zeiss confocal microscope.

Microarray gene expression profiling

Genome-wide expression analysis was carried out using RNA isolated from the indicated NIH 3T3 cell populations (RNeasy, Qiagen). Each cell population was generated by retroviral transduction, in triplicate, and RNAs from each replicate were used for independent microarray hybridization analysis (LMT/Affymetrix Group, SAIC-Frederick). Labelled cDNAs were hybridized to GeneChip Mouse Genome 430 2.0 arrays (Affymetrix). The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar *et al*, 2002) and are accessible through GEO Series accession number GSE30834 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE30834>) > 30834 <<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE30834>>). Detailed statistical methods are described in Supplementary data.

qPCR

RNA isolated from NIH 3T3 cell populations was used to determine expression levels of individual genes. cDNA was generated (Reverse Transcription Kit, Qiagen) from total RNA and qPCR was performed using SYBR Green with QuantiTect Primers (Qiagen) and normalized against β -actin or β 2-microglobulin. Analysis was performed using an Applied Biosystems 7500 Real Time PCR System and SDS Software (version 1.4). For each gene, a random representative of the triplicate RNA samples used for microarray analysis was selected for qPCR. Relative gene expression values were determined twice (in triplicate) and the data were averaged. Error bars represent standard error of mean expression levels. For primer sequences, see Supplementary data.

Gene function and pathway analysis

The association of gene expression signatures with biological pathways and diseases was performed using MetaCore software (MetaCoreTM, GeneGo, St Joseph, MI). Gene symbols of transcripts specifically upregulated by C/EBP β ^{CR} (312 genes) or C/EBP β ^{UTR} (70 genes) (i.e., the non-overlapping sets in Supplementary Table S1) were uploaded to GeneGo and the data were compared with the gene signatures for canonical biological pathways or specific diseases. Associations were ranked by *P*-value (LOD score; logarithm of the odds) and the top 10 matches were selected.

Supplementary data

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

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Conflict of interest

The authors declare that they have no conflict of interest.

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