JunB suppresses cell proliferation by transcriptional activation of p16^{INK4a} expression

Emmanuelle Passegué and
Erwin F. Wagner¹

Research Institute of Molecular Pathology (IMP), Dr. Bohr-Gasse 7,
A-1030 Vienna, Austria

¹Corresponding author
e-mail: wagner@nt.imp.univie.ac.at

A role for the transcription factor JunB in proliferation control was investigated in genetically modified mouse fibroblasts. Increased JunB expression induced high levels of the cyclin-dependent kinase inhibitor p16^{INK4a}, leading to premature senescence in primary cells and reduced proliferation in 3T3 cells, whereas lack of JunB expression results in decreased p16 levels. Furthermore, JunB-mediated p16 induction in 3T3 cells completely abolished cyclin D-associated kinase activity, resulting in reduced pRb hyperphosphorylation and G₁-phase extension. Moreover, three AP1-like binding sites were identified in the p16 promoter through which JunB directly activates p16 transcription. Elevated JunB expression in 3T3 cells also inhibited Ras- and Src-mediated transformation and tumour growth *in vivo*. The suppressive effect of JunB on cell proliferation was shown to be dependent on p16 since it did not occur in *INK4a*^{-/-} fibroblasts that lack both p16 and p19^{ARF}. These results demonstrate that p16 is a direct transcriptional target gene of JunB and identify JunB as a negative regulator of cell proliferation.

Keywords: AP-1/JunB/p16^{INK4a}/proliferation/transformation

Introduction

The control of mammalian cell proliferation occurs mainly during the G₁ phase of the cell cycle (Harper and Elledge, 1996). During this period, the G₁-type cyclins bind and activate specific cyclin-dependent kinases (CDKs) 4–6 for D-type cyclins and CDK2 for cyclin E, thereby dictating when CDKs will phosphorylate appropriate substrates. The retinoblastoma protein pRb and related family members are critical targets for G₁-type cyclin–CDK complexes (Weinberg, 1995). In mid-G₁ phase, inactivating phosphorylation of pRb by cyclin D–CDK4–6 complexes and then later by cyclin E–CDK2 complexes enables transcriptional activation of genes required for S-phase entry and progression. Two families of cyclin-dependent kinase inhibitors (CKIs) block the activity of the G₁-type cyclin–CDK complexes (Sherr and Roberts, 1995). CKIs of the INK4 family (p16^{INK4a}, p15^{INK4b}, p18^{INK4c} and p19^{INK4d}) specifically block the cyclin D–CDK4–6 complexes. Interestingly, the *INK4a* locus consists of two overlapping genes, each regulated by its own

promoter, which through alternative reading frames encode for the two unrelated proteins p16^{INK4a} and p19^{ARF} (Serrano, 1997). In contrast to p16, p19^{ARF} is not a CKI but is involved in cell cycle control by regulating the MDM2/p53 pathway (Haber, 1997). CKIs of the CIP/KIP family (p21^{CIP1}, p27^{KIP1} and p57^{KIP2}) inhibit cyclin E–CDK2 complexes as well as other cyclin–CDK complexes operating throughout the cell cycle. Recently, p21 and p27 were found to activate CDK4 by promoting cyclin D–CDK4 assembly, stability and nuclear localization (LaBaer *et al.*, 1997; Cheng *et al.*, 1999). In quiescent cells, CKIs are present in excess to cyclin–CDK complexes therefore maintaining the cells in a non-proliferating state. Mitogen stimulation leads to the sequential synthesis of G₁-type cyclins and, as soon as the CKI inhibitory threshold is overcome, cyclin–CDK kinase activity appears and cells progress into S phase. CKIs are therefore critical mediators of antiproliferative signals that arrest the cell cycle and allow for processes such as DNA repair, terminal differentiation and senescence.

One of the immediate consequences of mitogenic signalling is the activation of the transcription factor AP-1 (activator protein-1), a collection of dimeric complexes composed of proteins from the Jun, Fos and ATF families, which convert extracellular signals into changes in gene expression (Angel and Karin, 1991). All Jun proteins (c-Jun, JunB and JunD) are similar in their primary structure and their DNA-binding specificity. However, JunB exhibits weaker DNA-binding and homodimerization affinity than c-Jun and was shown to act both as a transactivator and a transrepressor depending on the promoter context and on the heterodimerization partner (Deng and Karin, 1993; Hsu *et al.*, 1993; Li *et al.*, 1999). Interestingly, constitutive high JunB expression in transgenic mice does not lead to an obvious phenotype (Schorpp *et al.*, 1996), while inactivation of *junB* results in an early embryonic lethality (Schorpp-Kistner *et al.*, 1999).

The involvement of AP-1 in the transmission of proliferative signals is supported by the observation that microinjection of neutralizing antibodies directed against the different AP-1 components inhibits cell cycle progression (Kovary and Bravo, 1991). However, AP-1 activity is also associated with the induction of growth arrest, terminal differentiation and apoptosis (Karin *et al.*, 1997). Analysis of fibroblasts either lacking or overexpressing one of the AP-1 components has recently shown that the different Jun family members may exert specific and distinct functions in controlling cell proliferation. A positive regulatory function of c-Jun on cell proliferation was established using *c-jun*^{-/-} fibroblasts that displayed reduced proliferation (Johnson *et al.*, 1993; Schreiber *et al.*, 1999). Furthermore, the impaired G₁–S phase progression in *c-jun*^{-/-} cells was shown to be due to

Fig. 1. JunB-expressing MEFs show premature entry into senescence. (A) Western blot analysis of JunB expression levels in wild-type (WT) and jB-Tg MEFs. The results from independent MEF lines of each genotype are shown. (B) Proliferation curves of WT and jB-Tg MEFs. The results from three independent MEF lines of each genotype are shown. (C) Senescence-associated β -galactosidase activity in passage 2, 4 and 6 WT and jB-Tg MEFs.

altered transcription of two cell cycle-regulating target genes, the tumour suppressor p53 and cyclin D1 (Schreiber *et al.*, 1999; Wisdom *et al.*, 1999). On the other hand, JunD was found to regulate fibroblast proliferation negatively by either establishing or maintaining the quiescent state of the cell cycle (Pfarr *et al.*, 1994). In this study, we have specifically investigated the function of JunB in proliferation control using genetically modified mouse fibroblasts.

Results

JunB-expressing mouse embryonic fibroblasts prematurely enter senescence

To determine the effects of high JunB protein levels on cell proliferation, primary mouse embryonic fibroblasts (MEFs) were derived from E12.5 transgenic embryos expressing the murine *junB* cDNA under the control of the human ubiquitin C promoter (Schorpp *et al.*, 1996). All *junB* transgenic (jB-Tg) MEFs expressed 5–10 times more JunB protein than the wild-type MEFs (Figure 1A). Until passage 4, no significant differences were found between the proliferation rates of wild-type and jB-Tg MEFs (Figure 1B). However, thereafter, the proliferation of jB-Tg MEFs was significantly reduced and by passage 6 all jB-Tg cells displayed characteristic features of senescence such as flattening, cytoplasmic enlargement, unresponsiveness to growth factors and expression of

acidic β -galactosidase in ~70–80% of the arrested cells (Figure 1B and C). These results indicate that jB-Tg MEFs undergo premature senescence in contrast to wild-type MEFs, which enter senescence by passage 8.

JunB-expressing 3T3 fibroblasts proliferate slowly due to an extended G₁ phase

Both wild-type and jB-Tg MEF lines were established according to the 3T3 protocol and were readily immortalized after a crisis period of 20–30 days (data not shown). However, all immortalized jB-Tg 3T3 lines showed a pronounced proliferation defect with a doubling time of 43 ± 2.5 h, compared with 19.4 ± 1.3 h in wild-type controls (Figure 2A). Although jB-Tg 3T3 cells displayed a dose-dependent proliferative response to increased amounts of serum, their saturation densities were on average 50% of the wild-type densities at all serum concentrations tested (Figure 2B). As comparably low percentages of dead and apoptotic cells were found in exponentially growing wild-type and jB-Tg cells (Figure 2C), increased cell death is most probably not the reason for the difference observed in the proliferation rates. In contrast, significantly lower amounts of BrdU positive cells were found in asynchronously growing jB-Tg cultures compared with wild-type cultures (Figure 2D). These decreased numbers of S-phase cells correlated with the reduction in total cell numbers

Fig. 2. JunB-expressing 3T3 fibroblasts proliferate slowly with an extended G₁ phase. (A) Proliferation curves of WT and jB-Tg 3T3 fibroblasts. The results from three independent 3T3 lines of each genotype are shown. (B) Saturation densities of WT and jB-Tg 3T3 fibroblasts under different fetal calf serum (FCS) concentrations. Values are the average $\pm$ SEM of two independent cell lines each measured in triplicate. (C) Numbers of dead cells (propidium iodide exclusion) and apoptotic cells (TUNEL assay) in exponentially growing WT and jB-Tg 3T3 fibroblasts. Values are expressed as a percentage of the total cell numbers and are the average $\pm$ SEM of two independent cell lines. (D) Comparative analysis of S-phase cell numbers and proliferation rates in WT and jB-Tg 3T3 fibroblasts. Cells were seeded at 0.4×10^6 cells per 10 cm dish and labelled for 1 h with 60 μ M BrdU before harvesting after 24, 48, 72 and 96 h of culture. The total cell numbers were determined (top panel; y-axis, $\times 10^6$) and the cells were analysed by two-parameter flow cytometry to determine the percentage of BrdU positive cells. (E) Analysis of cell cycle distribution in asynchronously growing WT and jB-Tg 3T3 fibroblasts. Cells were pulse-labelled for 1 h with 60 μ M BrdU, chased for the periods of time indicated and analysed by two-parameter flow cytometry to determine the percentage of BrdU positive cells in S, G₂/M and G₁ phase. The numbers of BrdU positive cells obtained after the 1 h pulse were normalized to 100% in the two cell populations and correspond to 62% of the total cell numbers in WT cultures and 24% in jB-Tg cultures.

observed after 24, 48, 72 and 96 h of culture, which suggests an altered cell cycle progression in jB-Tg 3T3 fibroblasts. To determine which phase of the cell cycle is impaired in JunB-expressing fibroblasts, exponentially growing wild-type and jB-Tg cells were analysed by BrdU pulse-chase labelling over a 14 h period (Figure 2E). Although both wild-type and jB-Tg cells exited from S phase, passed through G₂-M phase and entered the next G₁ phase with similar kinetics, their subsequent cell cycle progression was different. Wild-type fibroblasts left G₁ and entered the subsequent S phase after 8 h whereas jB-Tg fibroblasts remained in G₁ phase and did not enter the subsequent S phase during the 14 h of this experiment. These results, therefore, suggest that jB-Tg 3T3 fibroblasts have an extended G₁ phase.

Specific induction of p16^{INK4a} in JunB-expressing fibroblasts

To identify the mechanism by which JunB could impair G₁ to S phase progression, the expression levels of the different CKI family members were analysed (Figure 3A).

Similar levels of the CIP/KIP proteins (p21, p27 and p57), the p21 transcriptional regulator p53, as well as the p15^{INK4b}, p18^{INK4c} and p19^{INK4d} proteins were detected in all cell lines irrespective of their genotype. However, p16^{INK4a} levels were dramatically elevated in all jB-Tg fibroblasts investigated whereas no changes were detected in the expression levels of p19^{ARF}, the second member of the *INK4a* locus (Figure 3A). Furthermore, jB-Tg cells showed a 3- to 5-fold increase in p16 mRNA levels, suggesting that JunB induces p16 expression at the transcriptional level (Figure 3B). Since the 3T3 protocol was reported to affect either p53 function or the *INK4a* locus, all 3T3 cell lines used in this study were investigated for the functionality of these two pathways. Beside the *junB*^{-/-} 3T3 clone that lost the entire *INK4a* locus during the immortalization process, none of the other wild-type and jB-Tg 3T3 cell lines (total of five) displayed detectable mutations, since all had wild-type functional p53 protein, expressed p16 and p19^{ARF} and remained diploid (data not shown).

Fig. 3. Increased p16 expression levels in JunB-expressing fibroblasts. (A) Western blot analysis of CKI expression levels in WT and jB-Tg 3T3 fibroblasts. The results from two independent 3T3 lines of each genotype are shown. (B) Northern blot analysis of p16 (exon 1 α probe) and GAPDH mRNA levels in WT and jB-Tg 3T3 fibroblasts. The results from two independent 3T3 lines of each genotype are shown. (C) Western blot analysis of JunB and p16 expression levels in an E12.5 wild-type embryo and wild-type MEFs at different passages. (D) Comparative analysis of p16 expression levels in WT and jB-Tg 3T3 cells and passage 3 MEFs. The results from two independent MEF lines of each genotype are shown. (E) Western blot analysis of JunB and p16 expression levels in *junB*^{-/-} ES cells and *junB*^{-/-} passage 3 MEFs. The monoclonal JunB antibody recognizes both fusion proteins obtained after single (JunB-neo) or double (JunB-hygro) targeting of the *junB* locus.

To establish a correlation between JunB and p16 levels, primary wild-type MEFs were investigated (Figure 3C). As previously reported, p16 levels progressively increased in culture with passage numbers to reach maximum levels when cells senesced at passage 8 (Palmero *et al.*, 1997; Zindy *et al.*, 1997). Interestingly, JunB displayed similar kinetics with barely detectable expression at early passages and with a progressive increase to reach maximal levels in senescing passage 8 MEFs. Since JunB-expressing primary cells displayed premature senescence, p16 expression levels were investigated in jB-Tg MEFs at passage 3 (Figure 3D). Although p16 protein already started to accumulate in passage 3 wild-type MEFs, jB-Tg MEFs displayed much higher p16 levels that appeared comparable to the ones observed in immortalized jB-Tg 3T3 fibroblasts. These results demonstrate that JunB expression parallels the increase in p16 levels in primary cells and suggest that the premature replicative senescence observed in jB-Tg MEFs could be due to elevated p16 expression.

Finally, two cell systems that lack JunB expression were analysed for p16 expression (Figure 3D). In ES cells, the loss of *junB* expression resulted in reduced p16 protein levels, an effect already detectable in *junB*^{+/-} ES cells. Similarly, reduced p16 levels were found in four inde-

Fig. 4. The suppressive effect of JunB on cell proliferation is dependent on p16. (A) Western blot analysis of JunB and p16 expression levels in WT and *INK4a*^{-/-} 3T3 fibroblasts infected with an empty retrovirus (pB-puro) or a JunB-expressing retrovirus (pB-junB). The results for two independent bulk cultures of each genotype are shown. JunB and p16 levels in non-infected WT, jB-Tg and *junB*^{-/-} 3T3 fibroblasts are shown for comparison. (B) Proliferation curves of pB-puro- and pB-junB-expressing WT and *INK4a*^{-/-} 3T3 fibroblasts. The results $\pm$ SD (bars included in the symbol when non-visible) from two independent bulk cultures of each genotype are shown. (C) Exponentially growing pB-puro- and pB-junB-expressing WT and *INK4a*^{-/-} 3T3 fibroblasts were labelled for 1 h with 60 μ M BrdU and analysed by two-parameter flow cytometry to determine the percentage of BrdU positive S-phase cells. The results $\pm$ SD from two independent bulk cultures of each genotype are shown.

pendently isolated *junB*^{-/-} MEFs. Although decreased p16 levels did not noticeably increase the proliferation rates of *junB*^{-/-} ES cells and MEFs, *junB*^{-/-} MEFs entered senescence on average three passages later than wild-type MEFs (data not shown).

JunB does not suppress cell proliferation in the absence of p16

To test directly whether increased p16 expression resulted from increased JunB expression, JunB was ectopically expressed in wild-type fibroblasts as well as in *INK4a*^{-/-} fibroblasts that lack both p16 and p19^{ARF} expression. *INK4a*^{-/-} fibroblasts were chosen because no singular p16 knockout cells are currently available and because neither the increase nor the lack of JunB expression affected p19^{ARF} levels (Figure 3A and data not shown). Wild-type

Fig. 5. Impaired cyclin D-associated kinase activity and reduced Rb phosphorylation in JunB-expressing 3T3 fibroblasts. Whole-cell extracts were prepared at the time points indicated post-serum stimulation from WT and jB-Tg 3T3 fibroblasts. (A) Western blot analysis of cyclin D1 and CDK4 expression levels. (B) Induction of cyclin D1-associated pRb kinase activity. The presence of CDK4 in the anti-cyclin D1 immunoprecipitate was confirmed by western blot analysis (ns, no substrate). (C) Induction of cyclin D2-associated pRb kinase activity. The presence of CDK4 in the anti-cyclin D2 immunoprecipitate was confirmed by western blot analysis. (D) Western blot analysis of cyclin E and CDK2 expression levels. (E) Induction of cyclin E-CDK2 kinase activity. The presence of CDK2 in the anti-cyclin E immunoprecipitate was confirmed by western blot analysis. (F) Western blot analysis of pRb expression and phosphorylation levels. A Molt-4 cell lysate was used as positive control (pc). (G) Western blot analysis of pRb expression levels in WT and jB-Tg 3T3 fibroblasts after serum starvation (s) or in an exponentially growing population (g). The results from two independent jB-Tg cell lines are shown.

cells infected with a JunB-expressing retrovirus (pB-*junB*; Matsuo *et al.*, 2000) displayed a specific increase in p16 levels compared with cells infected with an empty virus (pB-puro) (Figure 4A). Furthermore, pB-*junB*-infected wild-type fibroblasts displayed reduced proliferation rates compared with control cells, which result from decreased numbers of S-phase cells as shown by the 40% reduction of BrdU incorporation in asynchronously growing pB-*junB*-infected wild-type cultures (Figure 4B and C). Consistently, flow cytometric analysis revealed an increased percentage of G₁-phase cells in asynchronously growing pB-*junB*-infected wild-type populations ($90 \pm 0.2\%$) compared with control populations ($77 \pm 2.6\%$). In striking contrast, elevated JunB expression in *INK4a*^{-/-} fibroblasts did not reduce the proliferation rates (Figure 4B), the BrdU uptake (Figure 4C) and the percentage of cells in G₁ phase ($65 \pm 1.3\%$ versus $67 \pm 2.1\%$), strongly indicating that JunB cannot suppress proliferation in the absence of p16.

JunB-mediated p16 induction abolishes cyclin D-associated kinase activity

To investigate the consequences of increased p16 levels in jB-Tg cells, the activity of the different G₁-type cyclin-CDK complexes was analysed in serum-starved and serum-stimulated 3T3 fibroblasts. The basal level of

cyclin D1 was found to be 2–3 times lower in serum-starved jB-Tg cells compared with wild-type cells (Figure 5A). However, upon serum stimulation, cyclin D1 induction appeared comparable in both cell types except that the maximal levels were delayed by 6 h in jB-Tg cells, peaking after 18 h compared with 12 h in wild-type cells. Wild-type and jB-Tg cells showed similar levels of expression and kinetics of induction of the other D-type cyclins and comparable amounts of CDK4 and CDK6 (Figure 5A and data not shown). However, no cyclin D1-CDK4-6 kinase activity was detected in jB-Tg fibroblasts throughout the entire kinetics, while wild-type cells displayed a time-dependent increase in cyclin D1-CDK4-6 kinase activity correlating with normal progression through the cell cycle (Figure 5B). Furthermore, jB-Tg cells did not show any increase in cyclin D2-CDK4-6 kinase activity throughout the entire kinetics (Figure 5C). Finally, neither wild-type nor jB-Tg cells expressed any detectable cyclin D3-CDK4-6 kinase activity (data not shown).

To understand how jB-Tg cells are still able to proliferate in the absence of any detectable D-type cyclin activities, the expression levels and the kinase activity of cyclin E, the second key G₁ cyclin that is not a p16 target, were investigated. The basal levels of cyclin E and cyclin E-CDK2 kinase activity were found to be lower in

Fig. 6. JunB mediates p16 transactivation through three AP-1-like binding sites. (A) Fibroblasts were co-transfected with 0.3 μ g of the p16 promoter reporter construct together with 0.2 μ g of empty plasmid (pB-puro) or plasmid expressing JunB (pB-junB) or c-Jun (pB-c-jun) and 0.2 μ g of a LacZ-expressing vector. The mean $\pm$ SD of one representative experiment performed in triplicate is shown. (B) Gel-shift assays performed with jB-Tg nuclear extracts and 32 P-labelled oligonucleotide encoding either a consensus AP-1 binding site or the three wild-type (A, B and C) or mutated (Amut., Bmut. and Cmut.) AP-1-like binding sites of the mouse p16 promoter. The arrowhead (SS) indicates the super-shift obtained with the JunB antibody. (C) Gel-shift assays performed with jB-Tg nuclear extracts, 32 P-labelled oligonucleotide encoding a consensus AP-1 binding site and 100 $\times$ cold competitors as indicated. (D) Fibroblasts were co-transfected with 0.3 μ g of the depicted luciferase reporter constructs together with 0.2 μ g of the LacZ-expressing vector. The results are normalized to the maximal stimulation obtained for the wild-type promoter in jB-Tg cells (100%) and are the mean $\pm$ SD of one representative experiment performed in triplicate. (E) Model for JunB-mediated p16 transcriptional regulation. In wild-type cells, JunB binds only site B, resulting in a basal level of promoter activity. In jB-Tg cells, JunB is bound to the three binding sites and the promoter activity is increased 3-fold. In *junB*^{-/-} cells, only the JunB-interacting proteins are bound and the promoter activity is decreased 2-fold.

serum-starved jB-Tg cells compared with wild-type cells, whereas no changes were detected in the levels of CDK2 (Figure 5D and E). However, upon serum stimulation, induction of both cyclin E expression and cyclin E-CDK2 kinase activity appeared comparable in both cell types except that the maximal levels of kinase activity were reached after 24 h of serum stimulation in jB-Tg cells, compared with 18 h in wild-type cells, a delay that is consistent with the slow proliferation rates of the cells. Finally, pRb phosphorylation upon serum stimulation was found to be strongly reduced in jB-Tg cells with only a minor increase in the hyperphosphorylated form of Rb detectable after 18 h (Figure 5F). In contrast, wild-type cells displayed a time-dependent increase in the pRb hyperphosphorylated form, starting after 6 h of serum stimulation, which correlates with normal progression through the cell cycle. Moreover, significantly lower amounts of pRb protein were found in exponentially

growing jB-Tg cells compared with wild-type cells, while similar levels were detected in serum-starved cells from both genotypes (Figure 5F and G).

JunB transactivates the p16 promoter through three AP1-like binding sites

To study JunB-mediated p16 transcriptional regulation, a 1.2 kb 5'-fragment of the mouse p16 promoter was cloned into a luciferase reporter construct (p16-Luc) and transient transfection experiments were performed with wild-type, jB-Tg and *junB*^{-/-} 3T3 fibroblasts (Figure 6A). Wild-type fibroblasts showed a moderate level of p16-Luc activity, consistent with the low levels of p16 mRNA and protein expressed in these cells. In contrast, jB-Tg cells exhibited three times higher p16 transcriptional activity whereas *junB*^{-/-} cells displayed only half of the wild-type activity. Expression vectors for JunB (pB-junB) and c-Jun (pB-c-jun) were then co-transfected with the p16-Luc

reporter construct (Figure 6A). Exogenous JunB expression in wild-type and *junB*^{-/-} 3T3 cells increased p16 promoter activity to the levels obtained in jB-Tg cells with the reporter alone. Co-transfection of JunB in jB-Tg 3T3 cells, however, did not further increase the p16 promoter activity, suggesting that it had already reached saturation level. In contrast, co-transfection of c-Jun resulted in a slight decrease in p16 promoter activity in wild-type cells and in a strong inhibition of JunB-mediated p16 transactivation in jB-Tg 3T3 cells. Increased p16 promoter activity was also observed in *c-jun*^{-/-} fibroblasts (data not shown), which further suggests that c-Jun represses p16 expression. Interestingly, while c-Jun levels in jB-Tg cells were similar to those in wild-type cells, *junB*^{-/-} cells showed increased levels of c-Jun protein and AP-1 binding activity (data not shown).

Sequence analysis of the mouse p16 promoter revealed five putative AP-1-like binding sites, and super-shift experiments performed with jB-Tg nuclear extracts demonstrated that three of them, termed A (TGACTGA, at -1189), B (TGACTTCA, at -783) and C (TGACACA, at -484), bind JunB (Figure 6B). Using wild-type nuclear extracts, JunB binding was detected only on site B and c-Jun binding on sites A and B (data not shown). Finally, these three sequences were able to compete out the AP-1 binding activity on a consensus AP-1 binding site (TGACTCA), thereby demonstrating that they are JunB/AP-1 binding sites (Figure 6C).

To determine whether the effect of JunB on p16 expression is mediated through these three AP-1-like sites, we mutated each of them by site-directed mutagenesis. These mutated sites lost JunB binding activity (Figure 6B) and failed to compete out AP-1 binding activity (Figure 6C). Luciferase reporter constructs containing all the combinations of wild-type and mutated sites were then generated and tested in transient transfections (Figure 6D). In jB-Tg fibroblasts, mutation of one or two of these binding sites decreased the expression of the p16-Luc reporter by ~30% while mutation of all three abolished 70% of the promoter activity. Similar residual promoter activity was obtained with the triple mutant reporter construct in wild-type and *junB*^{-/-} cells. In *junB*^{-/-} cells, both the triple mutant and the wild-type reporter showed similar activity levels, therefore suggesting that these three binding sites mediate the entire JunB-induced p16 transactivation. Finally, in wild-type fibroblasts, a single mutation on site B gave similar residual promoter activity to the triple mutant, which, together with the data of the super-shift experiments, argues for a major role of site B in JunB-induced p16 transactivation. These results are summarized in Figure 6E, describing a model of how p16 promoter activity could be regulated by the amount of JunB protein expressed in the cell.

JunB suppresses *v-ras*- and *v-src*-induced cellular transformation and tumour growth

Finally, we investigated whether oncogenic transformation is affected by high JunB expression since this cellular process critically depends on cell proliferation and is often associated with increased AP-1 activity. Wild-type and jB-Tg 3T3 fibroblasts were infected with an empty retrovirus or retroviruses expressing activated Ras and Src oncogenes. As shown in Figure 7A for *v-ras*-infected

fibroblasts, both wild-type and jB-Tg cells exhibited the typical morphology of transformed cells such as increased light refraction and an elongated shape. Loss of contact inhibition is a characteristic of oncogenic transformation that can be monitored *in vitro* by a focus formation assay (Figure 7B). Wild-type and jB-Tg fibroblasts infected with an empty virus remained contact-inhibited and did not show any signs of oncogenic transformation. Expression of activated Ras and Src in wild-type cultures efficiently induced loss of contact inhibition as shown by the high number of foci. In contrast, jB-Tg cultures exhibited greatly reduced numbers of foci after *v-ras* and *v-src* infection. Out of five jB-Tg cell lines tested, two exhibited no signs of focus formation whereas the three others showed a 60–70% decrease. To test whether JunB can still suppress cell transformation in the absence of p16, pB-puro- and pB-*junB*-infected *INK4a*^{-/-} fibroblasts were used in the same assay (Figure 7B). The lack of both p16 and p19^{ARF} expression has already been reported to cause spontaneous transformation and to increase susceptibility to oncogenic transformation by Ras (Serrano *et al.*, 1996). Consistently, control *INK4a*^{-/-} cells infected with an empty virus readily formed numerous foci, which increased in numbers following *v-ras* and *v-src* infection. Most importantly, both control *INK4a*^{-/-} and JunB-expressing *INK4a*^{-/-} fibroblasts displayed similar numbers of foci after infection with *v-ras*, *v-src* and empty virus, indicating that JunB cannot suppress cell transformation in the absence of p16.

The ability of the infected cells to form tumours when injected into immunosuppressed mice was analysed next (Figure 7C). Neither wild-type nor jB-Tg cells infected with an empty virus formed tumours, whereas small tumours were obtained with *INK4a*^{-/-} cells at 5 weeks post-injection. However, within 2 weeks, mice injected with *v-ras*-infected wild-type fibroblasts developed large tumours (120 ± 40 mg) while *v-ras*-infected jB-Tg fibroblasts from two independent cultures gave rise to only very small tumours (15 ± 7 mg). In addition, these small tumours did not increase in size when evaluated at 5 weeks post-injection. The lack of p16 and p19^{ARF} expression resulted in increased tumour mass since tumours derived from *v-ras*-infected *INK4a*^{-/-} fibroblasts were found to be significantly larger (214 ± 106 mg) than those derived from *v-ras*-infected wild-type fibroblasts. Similar results were obtained for tumours derived from *v-src*-infected fibroblasts, i.e. dramatically reduced jB-Tg-derived tumours and enlarged *INK4a*^{-/-}-derived tumours (data not shown). These results demonstrate that elevated JunB expression suppresses both neoplastic transformation and tumour growth induced by *v-ras* and *v-src*. Finally, no changes in tumour size were observed between tumours derived from *v-ras*-infected control *INK4a*^{-/-} and JunB-expressing *INK4a*^{-/-} fibroblasts, indicating that JunB cannot suppress tumour growth in the absence of p16 (Figure 7C).

Discussion

In this study, we have shown for the first time that JunB suppresses cell proliferation by direct transcriptional activation of the CKI p16^{INK4a}, thereby providing a novel molecular link between AP-1 complexes and the cell

Fig. 7. JunB suppresses *v-ras*- and *v-src*-induced tumour growth. (A) Phase-contrast photographs of WT and jB-Tg 3T3 fibroblasts infected with empty-neo or *v-ras*-expressing retroviruses. (B) Focus formation assay 2 weeks after infection with empty-neo or *v-ras* and *v-src*-expressing retroviruses. (C) *v-ras*-infected cells were injected subcutaneously into nude mice and the individual tumours were dissected and weighed after 2 weeks. One representative tumour of each genotype is shown in the upper part and the weight of each tumour is depicted in the lower part.

cycle machinery. Although the levels of p16 protein do not fluctuate in response to mitogenic stimulation, their control is critical in determining the length of the G₁ phase of the cell cycle and consequently the proliferation rates of the cells (Harper and Elledge, 1996).

A correlation between JunB and p16 levels is first observed in fibroblastic cell lines derived from transgenic mice that constitutively express high *junB* proteins. In both jB-Tg primary MEFs and immortalized 3T3 cells, a specific increase in p16 expression is found, which correlates with impaired proliferation. Primary jB-Tg MEFs display decreased proliferation rates after 4–5 passages and undergo premature senescence compared with wild-type MEFs. The CKI p16 has been directly implicated in inducing cellular senescence based on the observations that p16 levels are rapidly increasing when primary cells approach senescence (Palmero *et al.*, 1997; Zindy *et al.*, 1997) and that *INK4a*^{-/-} MEFs do not senesce (Serrano *et al.*, 1996). Similarly, JunB levels rapidly increase in wild-type MEFs when cells approach senescence. These results suggest that JunB contributes to establish p16 levels during senescence and further support the conclusion that elevated p16 levels are causal to the premature onset of replicative senescence in jB-Tg MEFs. In immortalized jB-Tg 3T3 fibroblasts, the proliferation defect results from a complete inhibition of cyclin D-associated kinase activity, which, in turn, leads to reduced pRb hyperphosphorylation and to an extension of the

G₁ phase of the cell cycle. Since p16 specifically inhibits the cyclin D–CDK4–6 complexes, its presence at high levels is likely to be the cause of the alteration in the cyclin D–pRb cascade that affects S-phase transition in jB-Tg 3T3 fibroblasts. Furthermore, ectopic JunB expression in wild-type fibroblasts results in increased p16 levels and reduces proliferation. Most importantly, proliferation rates are unchanged following ectopic JunB expression in p16-deficient *INK4a*^{-/-} fibroblasts, thereby directly demonstrating that the suppressive effect of JunB on cell proliferation cannot occur in the absence of p16. *INK4a*^{-/-} fibroblasts also lack p19^{ARF} but since the expression of this cell cycle regulator is not affected by JunB, the lack of p16 appears likely to be the cause for the absence of a JunB-mediated effect in *INK4a*^{-/-} cells. However, it would be important to confirm these data in a pure p16 knockout background when such a cell system becomes available. The correlation between JunB and p16 levels is also confirmed in cells lacking JunB since decreased p16 protein levels are found in both *junB*^{-/-} ES cells and *junB*^{-/-} MEFs. However, none of these cell types exhibits increased proliferation rates. Proliferation of *junB*^{-/-} ES cells might not be affected because undifferentiated ES cells do not express D-type cyclins and are therefore insensitive to p16 levels (Savatier *et al.*, 1996). The unaffected proliferation rates of *junB*^{-/-} MEFs may simply reflect the fact that p16 levels are reduced and not abolished as in *INK4a*^{-/-} MEFs. However, in agreement

with decreased p16 levels, *junB*^{-/-} MEFs do undergo a delayed senescence.

Ectopic expression of p16 has been shown to induce G₁ arrest in cell lines containing functional pRb (Serrano, 1997). In contrast, the elevated p16 levels induced by exogenous *junB* expression do not fully arrest jB-Tg 3T3 cells and, more surprisingly, do not affect cyclin E-CDK2 kinase activity. This result is striking since active forms of cyclin D-CDK4-6 complexes contain the CKIs p21 and p27, which are released upon p16 inhibition of the cyclin D-CDK4-6 complexes. As a consequence, the free p21 and p27 block the activity of cyclin E-CDK2 complexes, thereby arresting the cells in G₁ (Sherr and Roberts, 1995; LaBaer *et al.*, 1997; Cheng *et al.*, 1999). In jB-Tg cells, release of p21/p27 only delays the induction of cyclin E-CDK2 kinase activity, thereby contributing to the G₁-phase extension and reduced proliferation. Another intriguing observation is the reduced pRb protein levels found in exponentially growing jB-Tg 3T3 fibroblasts. However, since this effect is not observed in serum-arrested jB-Tg cells, it is most probably a secondary consequence of the slow proliferation rates of the cells rather than a direct effect of JunB on pRb expression. Moreover, mutations in the p16/Rb pathway, mainly deletions of the entire *INK4a* locus, and mutations of the tumour suppressor gene p53 are the two major known events leading to cellular immortalization and cancer development (Harper and Elledge, 1996). We therefore expected that in jB-Tg cells p16 and/or p53 would have been inactivated during immortalization. However, we found that jB-Tg MEFs became immortalized with similar kinetics to wild-type MEFs and that spontaneously immortalized jB-Tg 3T3 cells maintained a functional p53 and *INK4a* locus.

Accumulation of p16 mRNA and protein has been reported in response to various stimuli or conditions such as cellular senescence and overexpression of oncogenic Ras in rodent cells or inactivation of pRb in human cells (Serrano, 1997). More recently, Jacobs *et al.* (1999) have shown that the polycomb-group gene *bmi-1* is a repressor of the entire *INK4a* locus that regulates the expression of both p16 and p19^{ARF} proteins. In this study, we have identified JunB as one of the first transcription factors that directly regulates the p16 promoter. We also propose a mechanism explaining how p16 transcription levels might be determined by the amount of JunB protein expressed in the cell (Figure 6E). In wild-type cells, where JunB is expressed at a low level, only one site (B) is occupied. Interaction of JunB with other(s), as yet unknown, transcription factor(s)/co-factor(s) results in a basal level of p16 transactivation. Since p16 mRNA and protein are remarkably stable, with a half-life of >24 h for the mRNA and ranging from 8 to 18 h for the protein, this low level of transactivation is sufficient to ensure a constant low level of p16 protein throughout the cell cycle. When JunB levels rise, the two other AP-1-like sites (A and C) are occupied, resulting in a 3-fold increase in p16 promoter activity. Owing to the high stability of the mRNA and protein, this minor increase in transcription produces a robust and constant accumulation of p16 protein. Conversely, in the absence of JunB protein none of these sites are occupied and the low residual transactivation is ensured by the associated partner(s). Co-transfection experiments have

shown that c-Jun contributes to p16 regulation since c-Jun represses the basal p16 transcriptional activity and inhibits JunB-mediated p16 transactivation. Interestingly, elevated c-Jun protein levels are found in *junB*^{-/-} cells that display reduced p16 transactivation. However, the multiple DNA-protein complexes obtained on each of these AP-1-like sites suggest that the other(s) JunB heterodimerization partner(s) may differ from the usual Fos and ATF family members. Finally, in light of the recently published paper by Li *et al.* (1999), it will be interesting to investigate the importance of JunB phosphorylation in p16 regulation.

JunB has been postulated to be a growth arrest mediator since increased *junB* expression was found in different cell types such as myeloid cells or T helper cells undergoing terminal differentiation (Lord *et al.*, 1990; Li *et al.*, 1999). Moreover, treating fibroblasts as well as human mammary carcinoma cells and neuronal cells with *junB*-specific antisense oligonucleotides led to increased cell proliferation (Schlingensiepen *et al.*, 1993). However, in none of these cell types has a clear molecular mechanism been described, and the inhibitory effect of JunB on cell proliferation has rather been linked to its ability to decrease AP-1 transactivation. Besides this indirect effect, which may contribute to inhibition of cell proliferation by reducing the transactivation potential of positive regulators such as c-Jun, we show that JunB can also directly control the expression level of at least one very important negative regulator of the cell cycle, p16. In a recent paper, Bakiri *et al.* (2000) have identified a second target gene for JunB in the same regulatory pathway, since they could demonstrate that JunB negatively regulates the cyclin D1 promoter. In agreement with their observations, we detected reduced steady-state levels and delayed serum induction of cyclin D1 in jB-Tg cells, which may also contribute to their proliferation defect. Since Bakiri *et al.* (2000) were using two different immortalized cell lines that happen to lack the entire *INK4a* locus, the effect of JunB was only observed on cyclin D1 regulation with consequently a less dramatic reduction in the proliferation rates. Nevertheless, these two convergent findings support the notion that JunB, in contrast to c-Jun, is a negative regulator of cell proliferation and show that JunB can directly transactivate the expression of some critical cell cycle regulators.

JunB is widely expressed throughout embryonic development and its expression is maintained at a low level in adult tissues (Wilkinson *et al.*, 1989; Schorpp *et al.*, 1996; Schorpp-Kistner *et al.*, 1999). In contrast, p16 is not expressed during embryonic development and only low levels of mRNA have been detected by RT-PCR in the lung, spleen and testis of old mice (Zindy *et al.*, 1997). Furthermore, *INK4a*^{-/-} mice develop normally and do not show obvious abnormalities in adulthood besides a higher incidence of tumour development (Serrano *et al.*, 1996). Interestingly, although *junB*^{-/-} embryos are severely retarded in growth and development, leading to an early embryonic lethality at ~E9.5, cellular proliferation is apparently not impaired (Schorpp-Kistner *et al.*, 1999). It is therefore likely that other JunB target genes, different from p16, are involved in mediating JunB functions during embryogenesis. In adult mice, JunB and p16 are co-expressed in all organs where p16 is present, which suggests that in the organs where *junB* is expressed but

p16 is not, other regulatory mechanisms, as yet unknown, are able to down-regulate p16 expression. To corroborate the findings in fibroblasts with other cell types *in vivo*, p16 expression levels were analysed by RT-PCR in different organs of *junB* transgenic mice. Interestingly, the spleen of *junB* transgenic mice showed significantly increased p16 mRNA levels, and preliminary analysis of *junB* transgenic B and T cells *in vitro* indicates reduced proliferation upon mitogenic stimulation (data not shown). These observations therefore suggest that the JunB/p16 pathway may well act *in vivo* to regulate lymphocyte proliferation and we are presently analysing whether *junB* transgenic mice are immune-deficient.

The oncogenic potential of the Jun family members has been extensively studied and efficient neoplastic transformation of rodent cells has been found to require cooperation with activated oncogenes such as Ras and Src (Angel and Karin, 1991). In cooperation with oncogenic Ras, the different Jun proteins display distinct transforming capacities: c-Jun, a positive regulator of cell proliferation, transforms very efficiently (Schütte *et al.*, 1989a), while JunD, a negative regulator of cell proliferation, antagonizes *ras*-induced transformation (Pfarr *et al.*, 1994). JunB, similarly to JunD, displays a poor transforming activity and was found to inhibit c-Jun/*ras*-induced transformation (Schütte *et al.*, 1989b). Here, we show using jB-Tg fibroblasts that elevated JunB expression greatly reduces both *ras*- and *src*-mediated cell transformation. Since p16 has been shown to play a major inhibitory function in *ras*-mediated oncogenic transformation (Serrano, 1997), increased p16 levels are likely to account for the impaired *ras*-mediated transformation in jB-Tg cells. Although p16 involvement in *src*-mediated oncogenic transformation is not as well documented, increased p16 levels are also likely to account for the impaired *src*-induced transformation. Furthermore, the suppressive effect of JunB on both *ras*- and *src*-mediated transformation does not occur in *INK4a*^{-/-} fibroblasts. However, since the respective contribution of p16 and p19^{ARF} to oncogenic transformation is not yet understood, this experiment does not allow us to clearly attribute to p16 the absence of a JunB-mediated effect in *INK4a*^{-/-} fibroblasts. Finally, we show that elevated JunB expression also suppresses *ras*- and *src*-induced tumour growth *in vivo*, thereby identifying JunB as an anti-oncogene. Additional *in vivo* evidence for such a tumour suppressive function of JunB comes from the analysis of mice specifically lacking *junB* expression in the myeloid lineage, since absence of JunB leads to increased myeloid cell proliferation and to the development of a myeloid leukaemia (E.Passegué, W.Jochum and E.F.Wagner, manuscript in preparation). Future experiments will address whether the JunB/p16 pathway is important in suppressing tumour growth in other murine cancer models and whether the suspected tumour suppressive function of JunB can be extended to human cells and disease.

Materials and methods

Cell cultures and proliferation assays

All wild-type and jB-Tg MEFs were isolated from individual E12.5 fetuses obtained from intercrosses between *junB* transgenic mice

(Schorpp *et al.*, 1996). The *junB*^{-/-} 3T3 clone was derived from a single E8.5 fetus obtained from intercrosses between *junB*^{+/-} mice and all *junB*^{-/-} MEFs from individual E12.5 rescued fetuses obtained by micro-injection of *junB*^{-/-} ES cells into tetraploid wild-type blastocysts (Schorpp-Kistner *et al.*, 1999). All cell lines were immortalized from individual primary cultures according to the 3T3 protocol (Todaro and Green, 1963). *INK4a*^{-/-} fibroblasts were obtained from C.Sherr (Howard Hughes Medical Institute, Memphis, TN). Fibroblasts were grown at 37°C in 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, 2 mM glutamine, 1% penicillin/streptomycin and 0.1 mM β-mercaptoethanol. For fibroblast proliferation, 3.75 × 10⁵ cells were plated per 25 cm² flask and passaged every 3–4 days at the same density to determine the cumulative cell number. Senescence-associated β-galactosidase staining was performed as previously described (Dimri *et al.*, 1995).

Retroviral infections

Viral supernatants were generated by standard methods. Fibroblasts were plated at 3 × 10⁵ per 10 cm dish and infected the next day with viral supernatant containing 8 µg/ml polybrene. The selection was started 48 h post-infection using 2.5 µg/ml puromycin or 1 mg/ml G418. For transformation assays, the following retroviral supernatants were used: Ψ2-she-ras [LTR-Ha-ras], SR1 [LTR-neo TK-v-src] and N2/Ψ2 [LTR-neo] (Boulter and Wagner, 1988). For focus formation assays, cells were cultured in the same dishes without selection for 2 weeks, washed with PBS, fixed with methanol and stained with 0.2% methylene blue. For tumorigenicity assays, 1 × 10⁶ exponentially growing G418-resistant cells were subcutaneously injected into the scapular region of 5-week-old anaesthetized nude mice (two injection sites per mouse).

Flow cytometry analysis

Propidium iodide staining and propidium iodide exclusion were performed according to standard procedures. TUNEL staining (In Situ Cell Death Detection Kit, Boehringer Mannheim) and anti-BrdU-FITC staining (Becton Dickinson) were carried out according to the manufacturer's instructions. Data were collected and analysed with a Becton Dickinson FACScan and CellQuest software.

Protein and RNA analysis

Immunoblot analyses were performed according to standard procedures using 20 µg of nuclear proteins or 30 µg of whole cell extracts. For kinase assays, 500 µg of whole cell extracts were immunoprecipitated with cyclin D1- or cyclin E-agarose conjugate antibodies, and immune complexes were incubated with glutathione S-transferase (GST)-Rb (C-pocket 769–921; Santa Cruz) or histone H1 (Boehringer Mannheim) substrates as described (Matsushime *et al.*, 1994). The JunB monoclonal antibody and the p19^{ARF} polyclonal antibody were generous gifts from D.Lallemand (Pasteur Institute, Paris) and C.Sherr (Howard Hughes Medical Institute, Memphis, TN), respectively. The p53, pRb and actin antibodies were obtained from Medac, PharMingen and Sigma, respectively. All the other antibodies were obtained from Santa Cruz. Total RNA was isolated using Trizol reagent (Gibco-BRL) and northern blot analyses were performed using 20 µg of total RNA according to standard procedures.

Reporter gene assays

A 1.2 kb 5'-fragment of the mouse p16 promoter was cloned by PCR and inserted into the pGL3 plasmid to generate the reporter p16-Luc. Site-directed mutagenesis (QuickChange™ Site-directed Mutagenesis kit; Stratagene) was performed to mutate the individual AP-1-like binding sites A (5'-TGACTGA-3' to 5'-aaACTGA-3'), B (5'-TGACTTCA-3' to 5'-aaACTTCA-3') and C (5'-TGACACA-3' to 5'-aaACACA-3'). Transfections were performed in 24-well plates using LipofectaminePlus (Gibco BRL). Cells were harvested 24 h post-transfection and assayed for luciferase and β-galactosidase activities according to standard procedures.

Gel-shift experiments

Binding reactions were performed for 1 h on ice in the presence of 10 µg of nuclear extracts, 1 µg of poly(dI-dC), 15 000 c.p.m. of ³²P-end-labelled oligonucleotide and competitor where specified. For super-shift experiments, nuclear extracts were pre-incubated with the antibody for 1 h on ice prior to addition of the ³²P-labelled probe. Analyses of oligonucleotide bound complexes were performed according to standard procedures.

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