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Immortalized fibroblasts from NF-κB RelA knockout mice show phenotypic heterogeneity and maintain increased sensitivity to tumor necrosis factor α after transformation by v-Ras

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Activation of the NF-κB pathway can either promote or block apoptosis and oncogenesis in different cell types and circumstances. In this report, we show that independently derived immortalized mouse embryonic fibroblast cell lines prepared from RelA knockout mice have different phenotypes, based on their sensitivity to tumor necrosis factor α (TNFα)-induced apoptosis, morphology, ability to form colonies in soft agar, and the presence of distinct κB site-binding complexes. In addition, these RelA-deficient cell lines appear to have distinct alterations in the p53 pathway, which correlate with the normal vs transformed status of individual cell lines. We have also infected mouse embryonic fibroblasts lacking RelA, c-Rel or p50 with a retrovirus for the expression of v-Ha-Ras to determine whether individual NF-κB family members are required for Ras-mediated transformation. All three NF-κB-deficient cell types could be transformed by v-Ha-Ras. However, v-Ras-infected RelA-deficient cells formed colonies in soft agar at an approximately fourfold reduced efficiency compared to v-Ras-transformed control mouse 3T3 and p50-deficient cells. Ras transformation did not alter the sensitivity of RelA-deficient cells to TNFα-induced apoptosis, and Ras transformation did not affect the general resistance of 3T3, c-Rel-deficient, and p50-deficient cells to TNFα-induced apoptosis. However, TNFα specifically and dose-dependently decreased the ability of v-Ras-transformed RelA-deficient cells to form colonies in soft agar. These results suggest that RelA is a potential protein target for human tumors driven by oncogenic Ras mutations, but caution that inhibition of RelA may promote tumorigenesis in some circumstances.
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Introduction

NF-κB transcription factors regulate the expression of numerous genes, including several involved in controlling apoptosis and cell proliferation (reviewed in Loop and Pahl (2003); see also www.nf-kb.org). In mammalian cells, the NF-κB family includes five members: p50, p52, c-Rel, RelA, and RelB (reviewed in Hayden and Ghosh, 2004). These factors bind to related DNA target sites (κB sites), either as homodimers or heterodimers. NF-κB-regulated genes require distinct sets of dimers for transcriptional induction (Hoffmann *et al*., 2003), supporting the idea that distinct specificities of NF-κB dimers in gene regulation provide the molecular basis for NF-κB's ability to influence numerous physiological functions and cellular responses. Moreover, in different cell contexts, NF-κB dimers can have opposite effects: for example, as being pro- or antiapoptotic (reviewed in Kucharczak *et al*., 2003) or as being pro- or antioncogenic (reviewed in Baldwin, 2003). The molecular and cellular basis for these conflicting activities of NF-κB complexes is not understood.

NF-κB family members share a conserved region in their N-terminal halves, called the Rel homology (RH) domain, that contains sequences essential for DNA binding, dimerization, inhibitor (IκB) binding, and nuclear localization (Hayden and Ghosh, 2004). NF-κB transcription factors are regulated by their interaction with IκB proteins, which generally inhibit NF-κB complexes, in part by retaining them in a latent state in the cytoplasm. In the 'canonical' pathway, inducers of NF-κB act via the activation of the IκB kinase complex (IKK), which phosphorylates IκB, leading to its degradation (reviewed in Hayden and Ghosh, 2004). Degradation of IκB frees NF-κB to accumulate in the nucleus and regulate gene expression. Thus, mutants of IκBα that cannot undergo signal-induced phosphorylation/degradation act as stable 'super-repressors' of NF-κB complexes (Brown *et al*., 1995; DiDonato *et al*., 1996; Van Antwerp *et al*., 1996).

Ras proteins are a family of approximately 21 kDa membrane-localized G proteins that play important roles in the regulation of cellular processes such as growth, differentiation, and apoptosis (reviewed in

Lowy and Willumsen, 1993). Oncogenic mutations in *ras* genes are found in many human cancers and several highly oncogenic murine retroviruses have mutant *ras* genes. As such, the murine retroviral protein v-Ha-Ras is often used to study cellular and biochemical events involved in cellular transformation.

Oncogenic Ras has been reported to activate the NF-κB signaling pathway and this activation is thought to be required for Ras-induced transformation (Finco and Baldwin, 1993; Mayo *et al*., 2001). However, IKK-associated kinase activity is not induced by oncogenic Ras (Hanson *et al*., 2003), suggesting that Ras-induced activation of NF-κB does not occur through the canonical pathway. Expression of an IκBα super-repressor has been shown to block the ability of oncogenic Ras to induce focus formation in 3T3 cells (Finco *et al*., 1997). In addition, Jo *et al*. (2000) showed that inhibition of NF-κB activity by expression of an IκBα super-repressor decreases the growth rate of Ras-transformed rat fibroblast cells but has no effect on the growth of nontransformed cells. Moreover, activation of NF-κB appears to prevent Ras-induced cell death, in that induced expression of oncogenic Ha-Ras in cells overexpressing an IκBα super-repressor leads to cell death (Mayo *et al*., 1997).

NF-κB also plays a key role in preventing tumor necrosis factor α (TNFα)-induced apoptosis. For example, the embryonic lethality of *rela*$^{-/-}$ mice is due to increased sensitivity of hepatocytes to TNFα, and *rela* knockout mice survive if the gene encoding either TNF or TNF receptor 1 (TNFR-1) is also knocked out (Doi *et al*., 1999; Rosenfeld *et al*., 2000; Alcamo *et al*., 2001). Moreover, Beg and Baltimore (1996) showed that *rela* knockout fibroblasts grown in culture are more sensitive to TNFα-induced apoptosis. Furthermore, intratumoral administration of TNFα caused regression of tumors formed by human fibrosarcoma cells in which NF-κB activity was inhibited by expression of an IκB super-repressor (Wang *et al*., 1999).

To date, studies that have investigated the role of NF-κB transcription factors in Ras-induced transformation and tumorigenesis have generally used a super-repressor form of IκBα, which indiscriminately affects NF-κB complexes and may have effects on non-NF-κB pathways. In this study, we have characterized certain NF-κB-deficient mouse knockout cells and used these cells to determine the specific requirements of NF-κB family members for Ras-induced transformation. Our results indicate that RelA knockout fibroblasts show phenotypic heterogeneity and that, although RelA may play an antiapoptotic role in Ras transformation, RelA is not absolutely required for Ras-induced transformation.

Results

Four RelA knockout mouse fibroblast cell lines show differences in sensitivity to tumor necrosis factor and in morphological transformation

We have previously characterized one immortalized mouse fibroblast cell line derived from RelA knockout

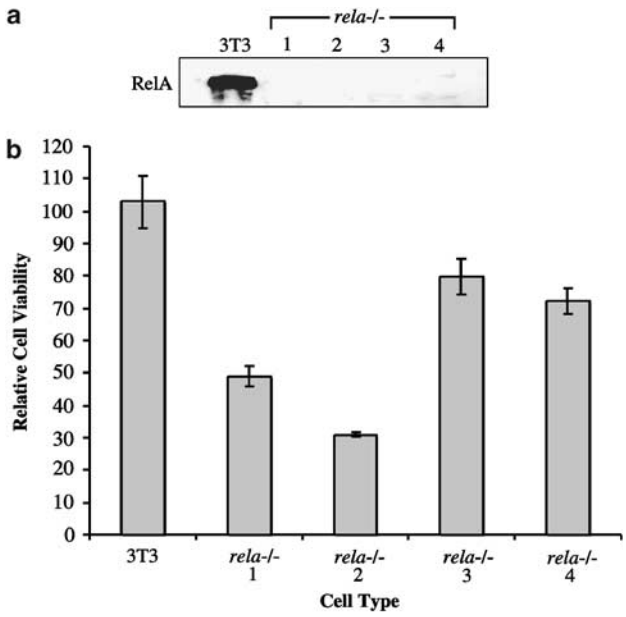
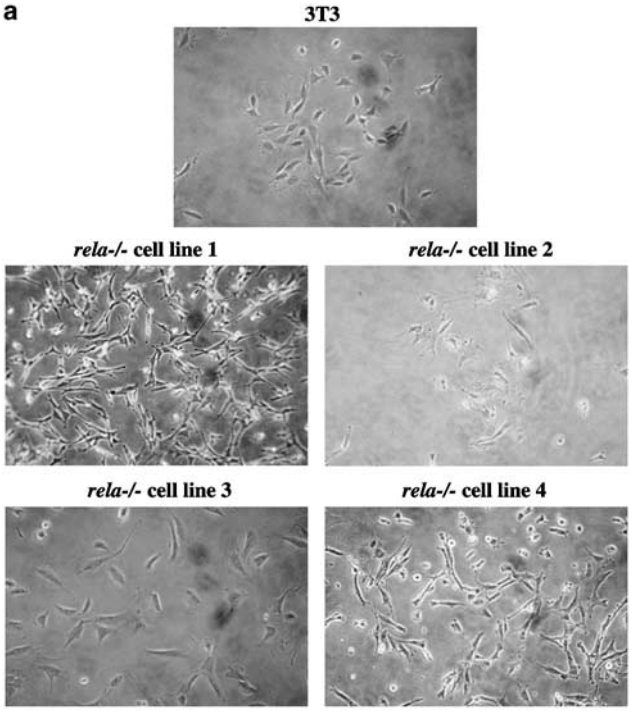


Figure 1 Characterization of four RelA knockout cell lines. (**a**) Cell extracts from 3T3 cells and *rela*$^{-/-}$ cell lines 1–4 were prepared, and lysates containing equal amounts of total protein were analysed by Western blotting with anti-RelA antiserum. RelA is indicated by the arrow. (**b**) Equal numbers of cells were plated for 3T3 cells and each *rela*$^{-/-}$ cell line. Cells were treated with 50 ng/ml of recombinant human TNFα for 16 h. The relative number of viable cells was then determined by staining the plates with crystal violet stain and the values are relative to the number of cells seen in untreated controls (100)

embryos (cell line 1, herein) that is weakly transformed (Gapuzan *et al*., 2002). In this study, three additional RelA-deficient cell lines have been characterized. As a control, we show that all four RelA-deficient cell lines do not express RelA (Figure 1a), and are more sensitive to killing by 50 ng/ml of TNFα than are wild-type 3T3 cells (Figure 1b). However, there are differences among the RelA-deficient cell lines in sensitivity to TNFα: cell lines 1 and 2 have approximately 50 and 70% cell killing, while cell lines 3 and 4 have approximately 20 and 25% cell killing, respectively, after TNFα treatment (Figure 1b).

The four RelA-deficient cell lines also differ in their cell morphologies (Figure 2a). Cell lines 1 and 4 have a spindled morphology, which is characteristic of transformed cells (see also Gapuzan *et al*., 2002). In contrast, RelA-deficient cell lines 2 and 3 have a flat morphology, which is similar to normal 3T3 cells. Consistent with their spindled morphologies, RelA-deficient cell lines 1 and 4 can form colonies in soft agar, although cell line 4 cells do so at a much lower frequency (25-fold reduced) than cell line 1 cells (Figure 2b). Cell lines 2 and 3, which have a flat morphology, do not have the ability to form colonies in soft agar (Figure 2b). Taken together, these results indicate that RelA-deficient cell lines 1 and 4 have a weakly transformed phenotype, whereas cell lines 2 and 3 do not. Thus, not all RelA-deficient cell lines have a transformed phenotype, but all do have an increased sensitivity to TNFα. However, the relative



b

	Relative Colony Formation
3T3	<0.05
rela-/- 1	100
rela-/- 2	<0.05
rela-/- 3	<0.05
rela-/- 4	4.0

Figure 2 Morphologies and soft agar colony-forming potential of RelA-deficient cell lines. (**a**) The indicated cell lines were photographed. The morphologies of RelA-deficient cell lines 2 and 3 are similar to normal 3T3 cells. In contrast, *rela*$^{-/-}$ cell lines 1 and 4 have a spindled morphology. (**b**) As described in Materials and methods, the indicated cells were seeded in soft agar. At 14 days following plating, soft agar colonies were counted and divided by the number of plated cells. Values are relative to the number of colonies seen with transformed *rela*$^{-/-}$ clone 1 cells (100) and are the averages of two independent assays

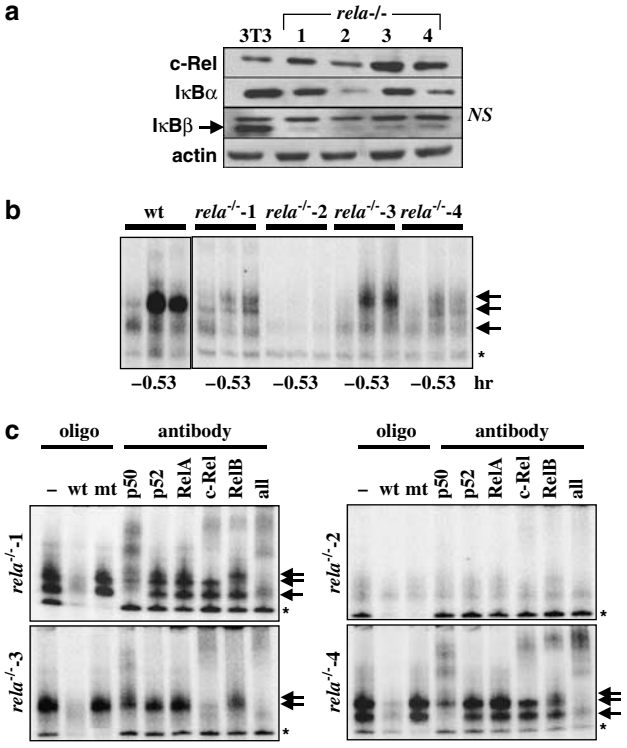


Figure 3 Molecular heterogeneity in *rela*$^{-/-}$ cells. (**a**) Equal amounts of total protein were analysed by Western blotting for the indicated proteins. NS, nonspecific band. (**b**) Equal numbers of cells were plated for wild-type 3T3 cells and each *rela*$^{-/-}$ cell line. An EMSA was performed using a double-stranded radiolabeled κB-site containing oligonucleotide and nuclear extracts made from the indicated cell lines following treatment with 10 ng/ml TNFα for the indicated times. Arrows indicate NF-κB complexes; the asterisk indicates a nonspecific protein–DNA complex. (**c**) κB-site complexes at 3 h of TNFα treatment were probed with cold competitor oligonucleotides or antibodies specific for the indicated NF-κB proteins. Arrows indicate specific NF-κB complexes whose identity is revealed by antibodies and are summarized in Table 1

sensitivity to TNFα is not correlated with whether the cell line has a transformed morphology. (Based on its relatively normal phenotype, RelA-deficient cell line 3 was used for most of the v-Ha-Ras transformation studies presented below.)

RelA knockout cell lines have different κB site-binding complexes

In an effort to understand the phenotypic differences between the immortalized RelA-deficient cell lines, we examined the status of certain aspects of the NF-κB pathway in these cells. Specifically, we looked at the levels of the RelA-related NF-κB family member c-Rel and the IκBα and IκBβ inhibitors of NF-κB (Figure 3a). With the exception of RelA-deficient cell line 2, the levels of c-Rel were higher in *rela*$^{-/-}$ cells than in wild-type 3T3 cells. In contrast, the levels of IκBα and particularly IκBβ were lower in all *rela*$^{-/-}$ cells as compared to wild-type 3T3 cells (Figure 3a). These results indicate that *rela*$^{-/-}$ cell lines 1, 3, and 4 have likely compensated for the lack of RelA by having increased levels of c-Rel, and that the loss of RelA leads to reduced levels of IκB proteins, no doubt because interaction with NF-κB complexes stabilizes IκB (Pando and Verma, 2000).

As a second measure of the NF-κB pathway in the *rela*$^{-/-}$ cells, we assessed the nuclear κB site-binding activity induced by TNFα in these cell lines (Figure 3b, Table 1). Consistent with our previous report (Hoffmann *et al*., 2003), *rela*$^{-/-}$-1 cells contain low, but readily detectable nuclear κB site-binding activity after treatment with TNFα (Figure 3b). Similarly, TNFα also induces easily detectable κB site-binding activity in *rela*$^{-/-}$-3 and -4 cell lines, albeit at a different mobility in native gel electrophoresis than cell line 1. Strikingly, *rela*$^{-/-}$-2 cells show little if any induced κB-site DNA-binding activity. Probing of the induced DNA–protein complexes in *rela*$^{-/-}$ cells with double-stranded oligonucleotides and antibodies revealed their specificity for the κB-site sequence and their molecular composition (Figure 3c): *rela*$^{-/-}$-1, -3, and -4 cells have κB

Table 1 Summary of TNFα response and transformed state of single NF-κB-gene-deficient cell lines

3T3 cell line	*TNF-induced dimers*	*TNF resistance*	*Basal transformation*
rela$^{-/-}$-1	p50 : p50; p50 : c-Rel; p50 : RelB	+	+ + + +
rela$^{-/-}$-2		−	−
rela$^{-/-}$-3	p50 : c-Rel; p50 : RelB	+ +	−
rela$^{-/-}$-4	p50 : p50; p50 : c-Rel; p50 : RelB	+ +	+
wild-type	p50 : p50; p50 : RelA; RelA : RelA	+ + +	−
c-rel$^{-/-}$	p50 : p50; p50 : RelA; RelA : RelA	+ + +	−
nfkb1$^{-/-}$	p52 : RelA; RelA : RelA	+ + +	+

Cell lines (column 1) gave rise to the indicated nuclear NF-κB complexes (column 2) in response to treatment with TNFα. Their relative resistance to TNFα-induced apoptosis (column 3) is summarized as follows: + + + = > 80% viability, + + = 60–80% viability, + 40–60% viability, − = < 40% viability. Basal transformation rates (column 4) are indicated by + + + + = 100% (by definition; Gapuzan *et al*., 2002), + = < 10% but detectable, − = below detectable limit

site-binding activity consisting of p50 : c-Rel and p50 : RelB heterodimers, while p50 : p50 homodimers are only found in *rela*$^{-/-}$-1 and -4 cells.

These results indicate that immortalized *rela*$^{-/-}$ fibroblasts contain different amounts of 'compensating' NF-κB family members, but the expression and activity of these compenstating proteins does not correlate with the morphological phenotype of the different *rela*$^{-/-}$ cell lines.

p53 status is distinct in different rela$^{-/-}$ *cell lines and correlates with the transformed vs normal state of the cells*

There are distinct mutations to inactivate p53 that often occur during the immortalization of mouse fibroblasts (Lundberg *et al*., 2000). Usually alterations either occur directly in the *p53* gene (deletion, mutation or silencing) or the gene encoding Arf is silenced, causing Mdm2 to become a constitutive inhibitor of p53. By Western blotting and immunoprecipitation, we find that the basally transformed *rela*$^{-/-}$ cell lines express mutant (cell line 1) or reduced (cell line 4) levels of p53 and high levels of the Mdm2 inhibitor p19-Arf; on the other hand, nontransformed *rela*$^{-/-}$ cell lines (2 and 3) express levels of p53 protein that are similar to those found in 3T3 cells, but express little detectable Arf protein (Figure 4a). Cell lines with mutant p53 are known to express reduced levels of Mdm2 (Alarcon-Vargas and Ronai, 2002). Consistent with their mutant and reduced levels of p53, respectively, cell lines 1 and 4 have low levels of the p53 inhibitor Mdm2 (Figure 4b).

To assess the ability of the *rela*$^{-/-}$ cell lines to respond to an inducer of the p53 pathway, we treated cells with the DNA-damaging agent doxyrubicin, which is known to stabilize p53 and enable p53 to induce its target gene *p21* (Waldman *et al*., 1995). *rela*$^{-/-}$ cell lines 2 and 3 showed robust increases in p53 after treatment with doxyrubicin, which were greater than the p53 increase seen in the wild-type 3T3 cell line (Figure 4c). The enhanced induction of p53 in the absence of RelA is consistent with the increased induction of p53 seen in mouse cell lines lacking NF-κB due to inactivation of IKK (Tergaonkar *et al*., 2002). In contrast, cell line 1 has high levels of p53 both with and without doxyrubicin treatment (Figure 4c), again suggesting the presence of a dominant-negative mutation in p53;

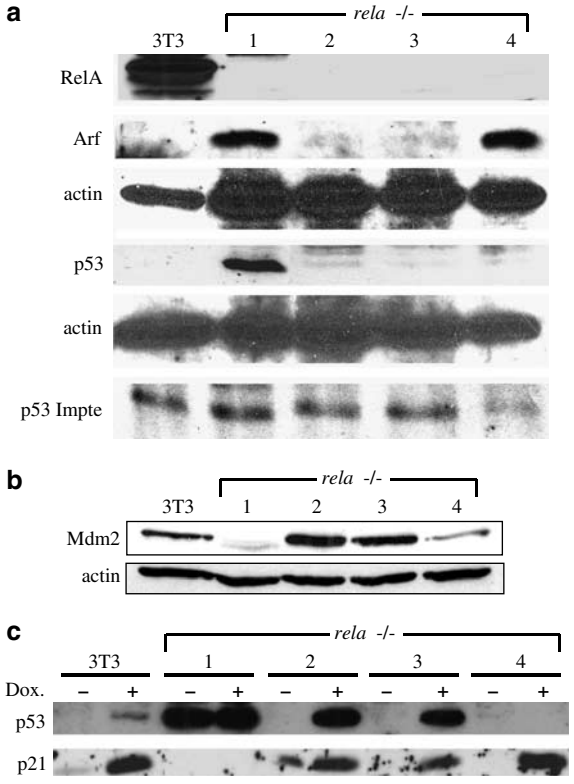


Figure 4 Different alterations in the p53 pathway in *rela*$^{-/-}$ cell lines. (**a**) The expression of the indicated proteins was monitored by Western blotting in the indicated cell lines. The high expression of p53 in cell line 1, as judged by Western blotting, is likely to be due to a dominant-negative mutation in p53, as such mutations stabilize p53 protein (reviewed in Sigal and Rotter, 2000). Due to its short half-life, wild-type p53 protein is difficult to detect by Western blotting (N Rosenberg, personal communication); therefore, p53 protein levels were also assessed by immunoprecipitation of ^{35}S-labeled cell extracts (bottom panel), which demonstrates that cell line 4 expresses lower levels of p53 protein than the other cell lines. (**b**) As in (**a**), the expression of Mdm2 and actin was analysed by Western blotting. (**c**) The indicated cell types were treated with doxyrubicin (+) or were untreated (−) for 16 h as described in Materials and methods. Western blotting for p53 and p21 was then performed

moreover, we could not detect induction of p53 in cell line 4, which has low steady-state levels of p53. Wild-type 3T3 cells and *rela*$^{-/-}$ cell lines 2 and 3 showed doxyrubicin-induced increases in the levels of the

product of the p53 target gene *p21*, whereas cell line 1 (expressing a dominant-negative p53) did not. Surprisingly, *rela*$^{-/-}$ cell line 4, which showed no detectable induction of p53 following doxyrubicin treatment, still showed increased levels of p21 in response to doxyrubicin; this result suggests that *rela*$^{-/-}$ cell line 4 has a p53-independent mechanism for inducing p21 following treatment with doxyrubicin.

Taken together, these results indicate that the type of mutation that has occurred in the p53 pathway to immortalize the cells correlates with whether the *rela*$^{-/-}$ cells are transformed (p53 mutations in cell lines 1 and 4) or normal (low Arf expression in cell lines 2 and 3). However, there is no clear correlation between the transformed state in *rela*$^{-/-}$ cell lines and the ability of doxyrubicin to induce the p53 target gene *p21*.

Expression of v-Ha-Ras in RelA-deficient, c-Rel-deficient, and p50-deficient cells can induce colony growth in soft agar

To determine whether individual NF-κB family members are required for Ras-induced transformation, we used knockout cell lines that were deficient for RelA, c-Rel, or p50, and that appeared to have a normal morphology. We used RelA-deficient and p50-deficient cell lines because Ras-induced NF-κB activation is due to an increased transactivation function of RelA (Finco *et al*., 1997) and the NF-κB complex in the nucleus of Ras-transformed 3T3 cells is a p50–RelA heterodimer (Jeay *et al*., 2003). c-Rel-deficient cells were used because c-Rel is the only directly oncogenic NF-κB family member (reviewed in Gilmore *et al*., 2004), suggesting the possibility that compensation by c-Rel mediates oncogenic transformation by Ras.

Control 3T3 cells and mouse fibroblasts lacking RelA, p50, or c-Rel were infected with a retroviral vector for the expression of v-Ha-Ras, and the cells were then placed directly in soft agar to measure transformation efficiency. Infection of all cell lines with the v-Ras retroviral vector enabled the cells to form colonies in soft agar; however, v-Ras-infected RelA-deficient cells (cell line 3) formed colonies at an approximately fourfold reduced efficiency as compared to v-Ras-infected 3T3 cells or p50-deficient cells. c-Rel-deficient cells showed a small reduction in soft agar colony formation after v-Ras infection, approximately 30% lower than that seen with control 3T3 cells (Figure 5a).

To ensure that the level of v-Ras expression was comparable among the different cell lines, anti-v-Ras Western blotting was performed on lysates from all four v-Ras virus-infected cell cultures. v-Ha-Ras was expressed at similar levels in all infected cell cultures (Figure 5b).

Cell clones of Ras-transformed 3T3, c-Rel-deficient, RelA-deficient, and p50-deficient cells express v-Ha-Ras and show a transformed morphology

Soft agar colonies induced by v-Ras virus infection of 3T3, c-Rel-deficient, RelA-deficient, and p50-deficient

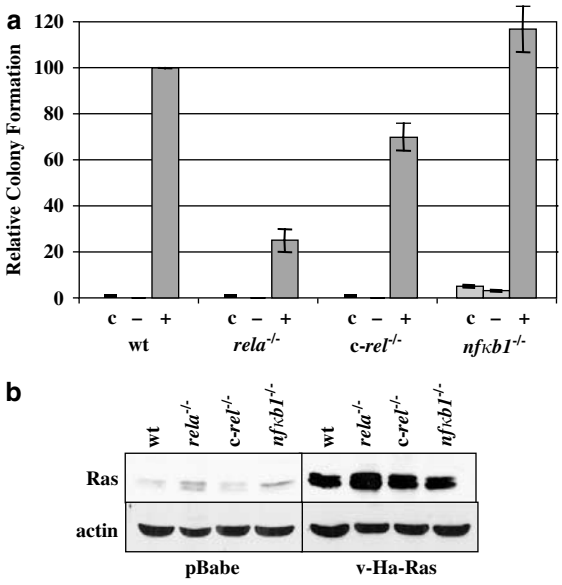


Figure 5 Transforming activity of v-Ha-Ras in cell lines missing RelA, p50 (*nfkb1* gene), or c-Rel. (**a**) Soft agar colony formation by v-Ras-transformed fibroblasts. The indicated cell types were infected with the indicated virus stocks, and were then plated in soft agar as described in Materials and methods. Values are relative to the number of colonies seen with v-Ras-infected wild-type 3T3 cells (100) and are the averages (with standard errors) of five independent assays with duplicate samples. c, uninfected cells; −, pBabe-infected cells; +, pBabe-v-Ras-infected cells. (**b**) Expression of v-Ha-Ras in Ras-transformed cell lines deficient in RelA, p50, or c-Rel. Extracts were prepared from pools of the indicated cells infected with a v-Ha-Ras virus or an empty virus (pBabe). Equal amounts of protein in cell extracts were analysed by Western blotting using antibodies specific for v-Ha-Ras and actin

cells were picked and propagated in tissue culture plates. We chose two clones from each transformed cell type to characterize further. All of these cell clones showed a transformed morphology as compared to their non-transformed counterparts (Figures 6a and b) and expressed v-Ha-Ras (Figure 6c). However, Ras-transformed RelA-deficient cell clones (Ras/RelA-1 and Ras/RelA-2) expressed v-Ha-Ras at a slightly higher level than the Ras-transformed 3T3, c-Rel-deficient, and p50-deficient cell clones.

Ras transformation also increased the expression of c-Rel and p50 in wild-type 3T3 cells (Ras/wt-1 and Ras/wt-2), but not in p50- and RelA-deficient cells, which already have elevated levels of c-Rel (Figure 6c; see also Gapuzan *et al*., 2002). Both c-Rel and p50 are encoded by NF-κB response genes (Loop and Pahl, 2003). As a control, we show that actin expression did not vary among the cell lines (Figure 6c).

The sensitivity of RelA-deficient fibroblasts to TNFα-induced cell death is not affected by overexpression of v-Ha-Ras

We next determined whether v-Ras transformation affected the sensitivity of knockout cells to cell killing by TNFα. Consistent with previous results, nontransformed 3T3, c-Rel-deficient, and p50-deficient cells are

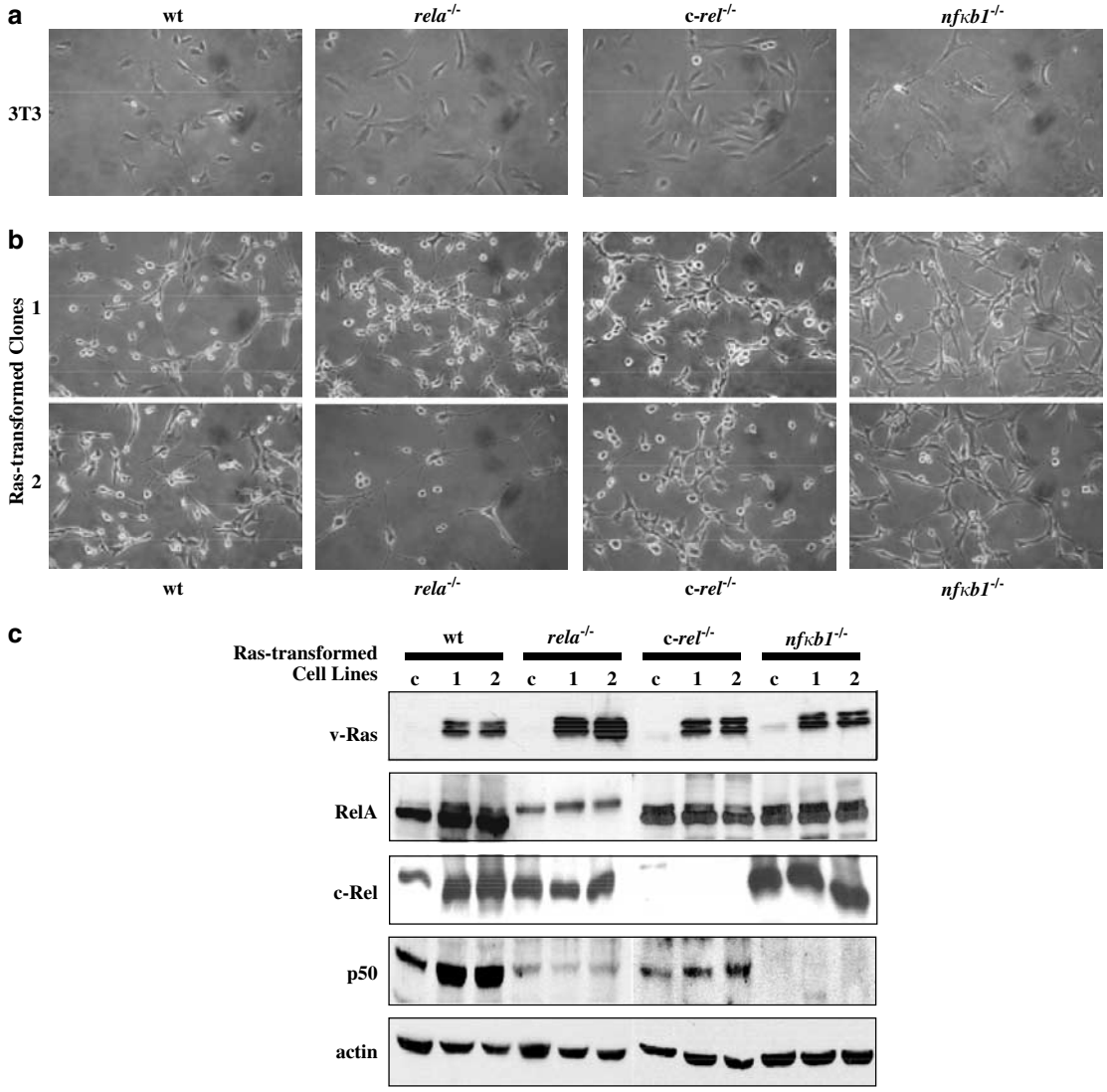


Figure 6 Ras-transformed NF-κB knockout cells show a transformed morphology compared to their wild-type counterparts. The indicated uninfected, parental cell types (**a**), or Ras-transformed cell clones (**b**) growing on tissue culture plates were photographed at × 200 magnification. (**c**) Expression of v-Ha-Ras and NF-κB proteins in cell clones of v-Ras-transformed 3T3, RelA-deficient, c-Rel-deficient, and p50-deficient cells. As indicated, uninfected and Ras-transformed cell clones of each were analysed. Equal amounts of protein in cell extracts of the indicated cell clones were analysed by Western blotting for the expression of the indicated proteins

not killed by treatment with 50 ng/ml TNFα (Figure 7). In contrast, as described above, RelA-deficient cell lines – including one weakly transformed (cell line 1) and one 'normal' (cell line 3) – showed increased cell killing by this concentration of TNFα: that is, approximately 50% of cell line 1 RelA-deficient ('basally transformed') cells were killed by TNFα and approximately 25% of the cell line 3 RelA-deficient ('normal') cells were killed by TNFα treatment (Figure 7).

Overexpression of v-Ha-Ras did not affect cell killing in response to TNFα. v-Ras-transformed 3T3 (Ras/3T3-1 and Ras/3T3-2), c-Rel-deficient (Ras/c-Rel-1 and Ras/c-Rel-2), and p50-deficient (Ras/p50-1 and Ras/p50-2) cells exhibit little TNFα-induced cell killing (Figure 7). Moreover, the 'normal' RelA-deficient cell line (cell line 3) transformed by v-Ha-Ras (Ras/RelA-1 and Ras/RelA-2) and the weakly transformed RelA-deficient cell line (cell line 1) overexpressing v-Ha-Ras (Ras/RelA-3) had similar levels of TNFα-induced cell death as their uninfected counterparts (Figure 7).

v-Ras-transformed RelA-deficient cells have a reduced ability to form colonies in soft agar in the presence of TNFα

We next sought to determine whether TNFα could selectively affect the ability of v-Ras-transformed RelA-deficient cells to form colonies in soft agar. Therefore, we compared the abilities of v-Ras-transformed 3T3 and RelA-deficient cells (clone 3) to form colonies in soft agar in the presence of increasing concentrations of TNFα. As shown in Figure 8, v-Ras-transformed

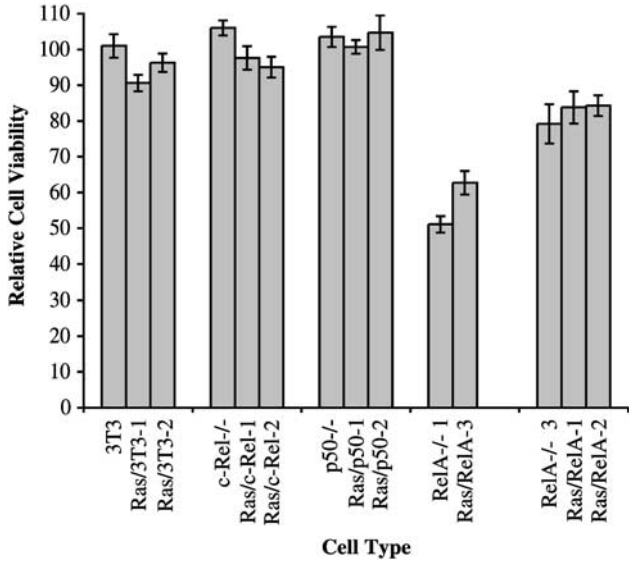


Figure 7 TNFα sensitivity of v-Ras-transformed cell clones of 3T3, c-Rel-deficient, p50-deficient, and RelA-deficient cells. Equal numbers of cells were plated for each cell line. Cells were treated with 50 ng/ml of TNFα for 16 h. The relative number of viable cells was then determined by staining the plates with crystal violet stain and is relative to the number of cells seen in untreated controls (100). Ras-transformed 3T3, c-Rel-deficient, and p50-deficient clones are generally not killed by TNFα treatment. However, RelA-deficient clones transformed by v-Ha-Ras are still sensitive to TNFα-induced cell killing

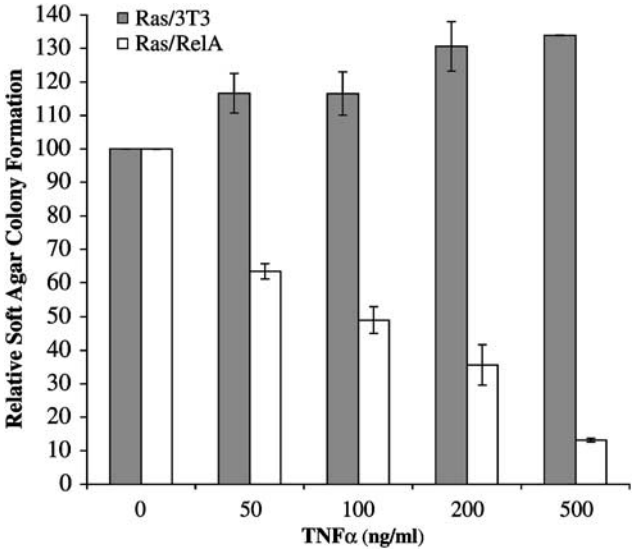


Figure 8 Effect of TNFα on soft agar colony formation by v-Ras-transformed *rela*$^{-/-}$ fibroblasts and v-Ras-transformed 3T3 cells. Duplicate dishes were prepared, containing 10^5 cells in DMEM containing 0.3% agar, 10% FBS, and the indicated concentrations of TNFα. For each cell type, values are relative to the number of colonies seen with no TNFα (100). Values are the averages of three (0, 50, and 100 ng/ml), two (200 ng/ml), and one (500 ng/ml) assay(s) performed with duplicate samples

RelA-deficient cells formed reduced numbers of colonies in soft agar in a dose-dependent manner in the presence of TNFα. (The high levels of TNFα required to induce nearly complete inhibition of soft agar colony formation by v-Ras-transformed RelA-deficient cells may be a consequence of reduced diffusion of TNF in agar or due to inactivation of TNF over the course of the 2-week experiment.) In contrast, the ability of v-Ras-transformed wild-type 3T3 cells to form colonies in soft agar was not decreased over the concentration range used in these studies (in fact, in repeated assays, the addition of TNFα increased soft agar colony formation by v-Ras-transformed 3T3 cells). Thus, while TNFα, which activates NF-κB, increases the transforming ability of v-Ras in wild-type 3T3 cells in a soft agar assay, it reduces the transforming ability of v-Ras in RelA-deficient cells, which exhibit greater sensitivity to TNFα-induced apoptosis.

Discussion

In this study, we demonstrate that there is phenotypic, and probably genetic, heterogeneity among immortalized mouse fibroblasts derived from RelA knockout mice. In addition, we have used NF-κB knockout cell lines to show that cells deficient in either RelA, p50 or c-Rel can be transformed by v-Ha-Ras. However, the transforming activity of v-Ras is specifically reduced in RelA-deficient cells, as compared to normal 3T3 cells. Also, TNFα can inhibit the ability of v-Ras-transformed RelA-deficient cells to form colonies in soft agar.

A requirement for NF-κB activity in Ras-induced cellular transformation has been shown previously, generally by using the IκBα super-repressor (Finco *et al*., 1997; Mayo *et al*., 1997; Jo *et al*., 2000). However, IκBα super-repressor expression does not allow for distinguishing between NF-κB complexes, may not block NF-κB activity completely, and has been reported to have effects on non-NF-κB signaling pathways (Chang, 2002; Li *et al*., 2003; Zhou *et al*., 2003; Aguilera *et al*., 2004). Thus, in this report, we show that genetically altered cells missing either RelA, p50, or c-Rel can be transformed by v-Ha-Ras: however, the transforming efficiency of v-Ras in RelA-deficient cells is fourfold lower than control 3T3 cells, whereas cells missing either p50 or c-Rel alone are more similar in their v-Ras susceptibility to wild-type 3T3 cells. These results suggest that RelA plays a more critical role in Ras-induced transformation than either p50 or c-Rel. This result is consistent with previous results showing that activation of κB-dependent reporter plasmids in response to Ras was inefficient in RelA-deficient cells (Finco *et al*., 1997). Moreover, while this work was in progress, Hanson *et al*. (2004) also showed that mouse cells missing c-Rel, RelA, or both can be transformed by a human oncogenic Ras protein.

Our results demonstrate that no individual NF-κB subunit – RelA, p50 or c-Rel – is absolutely required for v-Ras-induced transformation. Thus, these factors may have redundant or compensatory roles in Ras-induced transformation. As the absence of RelA, and to a lesser

extent c-Rel, moderately reduces Ras-induced transformation, it is likely that c-Rel and RelA are the members performing the overlapping functions. Indeed, c-Rel/RelA double-deficient fibroblasts show an even more drastically reduced ability to be transformed by human oncogenic Ras protein c-RasV12G than cells missing either subunit alone (J Wiezorek, personal communication). Nevertheless, it is clear that RelA and c-Rel are not completely redundant in Ras transformation, because RelA-deficient cells are less efficiently transformed than wild-type or c-Rel-deficient cells and exhibit greater sensitivity to cell killing by TNFα following transformation (Figure 7).

We also noticed that the expression of v-Ha-Ras was slightly increased in the two Ras-transformed RelA-deficient cell clones as compared to Ras-transformed cell clones of 3T3, c-Rel-deficient, and p50-deficient cells (Figure 6c). Of course, this could simply represent clonal variation among cell lines. However, based on the reduced ability of Ras to transform RelA knockout cells, it is also possible that RelA-deficient cells require higher levels of v-Ha-Ras expression for the cells to undergo stable transformation. It is thought that oncogenic Ras activates NF-κB-dependent genes to block Ras-induced apoptosis (Mayo *et al*., 1997). Therefore, for transformation to occur in cells lacking RelA, higher levels of v-Ras may be required to increase the activity of other antiapoptotic or proliferative pathways to compensate for the loss of RelA activity. Of note, oncogenic Ras effects cellular transformation by acting on multiple signaling pathways, including also Raf kinase, phosphatidylinositol 3 kinase (PI3-kinase), and RalGDS pathways, and these pathways, like NF-κB, also mediate proproliferative and antiapoptotic signals (Joneson and Bar-Sagi, 1997; Campbell *et al*., 1998).

There are contrasting reports on the effect of Ras transformation on TNFα-induced cell death. Fernandez *et al*. (1994) found that transformation of C3 H mouse embryo fibroblasts by oncogenic c-Ha-Ras made them sensitive to TNFα-induced cell death. However, Jo *et al*. (2000) found that c-Ha-Ras-transformed rat fibroblasts exhibit little sensitivity to TNFα-induced cell killing. Similarly, Wang *et al*. (1999) demonstrated that TNFα could specifically induce regression of tumors formed by fibrosarcoma cells only if they were expressing the IκBα super-repressor.

Previously, one RelA-deficient cell line (cell line 1, herein) was shown to be more sensitive to TNFα-induced cell killing than the corresponding wild-type cells (Beg and Baltimore, 1996; Gapuzan *et al*., 2002). This RelA-deficient cell line is also weakly transformed, but tumors induced by these cells regress in *scid* immunodeficient mice, which still can mount a TNF response to tumors (Gapuzan *et al*., 2002). In contrast, tumors induced by a v-Ras-transformed cell clone of this RelA-deficient cell line, Ras/RelA(1)-3, did not regress (data not shown). This suggests that the level of circulating TNFα in *scid* mice is not sufficient to kill the *rela*$^{-/-}$ cell line 1 tumor cells in the presence of an oncogenic Ras signal. Therefore, if endogenous TNFα were responsible for inducing apoptosis and regression of tumors formed by the weakly transformed RelA-deficient cell line (cell line 1) in previous studies (Gapuzan *et al*., 2002), this effect was overcome by Ras transformation.

There is emerging evidence that the absence of RelA can sometimes contribute to oncogenesis, at least in basal cell carcinomas and possibly colon cancer (van Hogerlinden *et al*., 1999; Deng *et al*., 2002; Dajee *et al*., 2003; Zhang *et al*., 2004). Thus, it is important to note that two of four RelA-deficient immortalized mouse cell lines that we have characterized have a malignantly transformed phenotype (Gapuzan *et al*., 2002; Figure 2, herein). This phenotypic variation could reflect an outcome of the culturing methods used to develop these cell lines, in that mouse fibroblasts can undergo spontaneous transformation in tissue culture. Alternatively, it may reflect a probabilistic set of genetic or heritable epigenetic events in cultured embryonic cells that in combination with the lack of RelA determines whether the resultant cell lines are normal or transformed. Indeed, the 'compensating' κB site-binding complexes in these cells are different (Figure 3b and c). Moreover, the transformed vs normal RelA knockout cell lines appear to have different mutations that contribute to their immortalization (Figure 4). In the most simplistic terms, our results suggest that inactivation of p53 or enhanced expression of Arf (as seen in cell lines 1 and 4) in combination with a lack of RelA can promote transformation (see Table 2), or that reduction in Arf activity in a *rela*$^{-/-}$ genetic background (cell lines 2 and 3) promotes a normal, nontransformed state in these cells. Interestingly, Arf has recently been shown to repress the transcriptional activity of RelA through a mechanism that does not involve p53 pathway (Rocha *et al*., 2003, 2005). Moreover, Arf has other p53-independent functions, including the ability to bind to and repress the transcriptional activity of c-Myc (Qi *et al*., 2004) and to bind to chromatin (Ayrault *et al*., 2004). Similar to the variable phenotype that we see with *rela*$^{-/-}$ fibroblasts, mouse fibroblasts from c-*abl* knockout mice show a transformed phenotype when p53 and Rb activities are also inactivated (Suzuki *et al*., 2004); of

Table 2 Summary of properties of *rela*$^{-/-}$ cell lines

Rela$^{-/-}$ *line*	*Transfomed*	*κB-site binding*	*p53 protein*	*Arf*	*Mdm2*	*p21 inducibility*
1	Yes	+	Mutant	High	Low	−
2	No	−	Normal	Low	Normal	+
3	No	+	Normal	Low	Normal	+
4	Yes	+	Low	High	Low	+

note, transformation of cells by Bcr–Abl requires activation of NF-κB (Reuther *et al*., 1998).

Taken together, the results presented herein suggest that activation of RelA-dependent genes may be an important, but not essential, step in oncogenic Ras-induced transformation and tumorigenesis. Therefore, compounds that target both oncogenic Ras and RelA may be sufficient to induce regression of certain human tumors. We also found that Ras-induced transformation was further inhibited in RelA-deficient cells when Ras was expressed in the presence of TNFα (Figure 8), supporting the hypothesis that RelA has a role in counteracting Ras-induced proapoptotic signaling (Mayo *et al*., 1997). Therefore, our results also suggest that therapeutics that specifically target RelA may enable the use of clinically tolerable levels of TNFα or other apoptosis-inducing agents whose activity is countered by RelA. However, our finding that two (out of four) RelA-deficient cell lines have a transformed phenotype cautions that inhibition of RelA, perhaps in certain genetic backgrounds, such as p53 deficiency, could also contribute to enhanced oncogenesis; thus, this possible conflicting consequence should be considered in the development and application of anti-RelA reagents.

Materials and methods

Knockout cell lines and cell culture

Spontaneously immortalized fibroblast cell lines were derived from E12.5–E14.5 mouse embryo fibroblasts by repeated passage according to the 3T3 protocol previously described (Aaronson and Todaro, 1968). Cell line *rela*$^{-/-}$-1 was described previously (Beg and Baltimore, 1996; Hoffmann *et al*., 2003), whereas cell lines *rela*$^{-/-}$-2, -3, and -4 were generated from different embryos but were passaged synchronously. All cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated bovine calf serum (BCS; Hyclone, Logan, UT, USA) and 50 U/ml penicillin and 50 μg/ml streptomycin (Invitrogen, Carlsbad, CA, USA) at 37°C with 5% CO_2. Where indicated (Figure 4c), cells were treated with 600 ng/ml doxyrubicin (Sigma, St Louis, MO, USA) for 24 h.

Plasmids

Plasmid pBabe/puro-v-Ha-Ras was constructed by subcloning a *Bam*HI fragment containing v-Ha-*ras* (Feig *et al*., 1986) into pBabe/puro (Morgenstern and Land, 1990) that was also digested with *Bam*HI.

Soft agar assays

For soft agar colony formation, virus stocks were prepared as described previously (Gapuzan *et al*., 2002), and the virus was then used to infect cells. After 2 days, infected cells were trypsinized, counted, and duplicate plates containing different dilutions of cells were placed into DMEM containing 10% fetal bovine serum (FBS; Biologos, Montgomery, IL, USA), penicillin, streptomycin, and 0.3% bacto-agar. For uninfected cells, cells were trypsinized, counted, and then placed directly into soft agar. Macroscopic colonies were counted 14–16 days later. Where indicated (Figure 8), TNFα was added to the soft agar mixture just prior to plating.

To select for cell clones transformed by v-Ha-Ras, individual colonies were picked from soft agar, propagated on tissue culture plates, and the expression of v-Ha-Ras was confirmed by Western blotting.

Assay for sensitivity to tumor necrosis factor

TNFα sensitivity was quantified using a crystal violet staining assay as described previously (Zong *et al*., 1998; Gapuzan *et al*., 2002).

EMSA, Western blotting, and immunoprecipitation

For electrophoretic mobility shift assays, extracts and assay procedures were as previously described (Hoffmann *et al*., 2003).

For Western blotting, extracts from subconfluent, exponentially growing cell cultures were prepared and analysed as described previously (Gapuzan *et al*., 2002; Hoffmann *et al*. 2003). The following primary antibodies (dilution; source) were used: anti-v-H-Ras (2.5 μg/ml; Zymed Laboratories, South San Francisco, CA, USA); anti-c-Rel (1 : 500; Santa Cruz Biotechnology, sc-71); anti-RelA (1 : 1000; Santa Cruz Biotechnology sc-372; and 1 : 2000; antiserum against the C-terminal residues of RelA, a kind gift of Nancy Rice); anti-p50 (1 : 500; Santa Cruz Biotechnology, sc-7178); anti-p19Arf (1 μg/ml; Novus Biologicals, Littleton, CO, USA, NB200-106); anti-p53 (2.5 μg/ml; Calbiochem, San Diego, CA, USA, AB-3); anti-p21 (1 : 200; Santa Cruz Biotechnology, sc-756); anti-actin (1 : 1000; Santa Cruz Biotechnology, sc-1616). Immunoreactive proteins were detected using horseradish peroxidase-conjugated secondary antibodies and chemiluminescence (Pierce, Rockford, IL, USA or Santa Cruz Biotechnology).

For immunoprecipitation of p53, subconfluent cells in 35 mm dishes were starved in media lacking methionine and cysteine for 1 h, and were then radiolabeled with 200 μCi/ml of Tran ^{35}S-label (ICN, Irvine, CA, USA) for 1 h. Cells were lysed and samples containing equal numbers of precipitable cpm were immunoprecipitated with anti-p53 antiserum (Oncogene Research Products/Calbiochem, OP03L) according to standard techniques. p53 bands were then detected by autoradiography.

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