# Harvey-*ras* Gene Expression and Epidermal Cell Proliferation in Dibenzo[*a,l*]Pyrene-Treated Early Preneoplastic SENCAR Mouse Skin

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**Topical application of dibenzo[*a,l*]pyrene (DB[*a,l*]P) to the dorsal skin of SENCAR mice induces codon 61 ($CAA^{Gln}$ to $CTA^{Leu}$) mutations in the Harvey (H)-*ras* gene within 12 h after treatment. Between days 1 and 3, the frequency of these mutations increases rapidly, suggesting that skin cells carrying the codon 61 mutations proliferate in this period. We have investigated DB[*a,l*]P-treated mouse skin (12 h–7 d) for further evidence of H-*ras* expression and epidermal cell proliferation. Two waves of cell proliferation were observed: the first wave (1–2 d) correlated with the clonal proliferation of codon 61-mutated cells, and the second wave (3–7 d) correlated with DB[*a,l*]P-induced hyperplasia. DB[*a,l*]P-induced early preneoplastic cell proliferation correlated with H-*ras* and specific G1 cyclin expression. Total H-*ras* protein and cyclin D1 were found to increase during DB[*a,l*]P-induced hyperplasia, but the levels of guanosine triphosphate-bound (active) H-*ras* protein and cyclin E were increased during the putative clonal proliferation of codon 61-mutated cells. These results suggest that DB[*a,l*]P-induced oncogenically mutated cells proliferate in early preneoplastic skin. As this proliferation occurs in the absence of any promoting treatment, we propose that this phenomenon is a tumor initiation event.**

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Mutations in three ras genes, H-, K-, and N-*ras*, are frequently associated with cancer (Bos, 1989; Barbacid, 1990; van der Schroeff *et al*, 1990). K- and N-*ras* gene mutations at codons 12 and 13 are the most common (Bos, 1989; Barbacid, 1990), but codon 61 mutations in all three *ras* genes have also been reported (Grendys *et al*, 1997; Shiraishi *et al*, 1998; Esapa *et al*, 1999; Semczuk *et al*, 2001). Environmental carcinogens may have a major role in inducing oncogenic *ras* mutations (Vahakangas *et al*, 2001; Li *et al*, 2002).

The SENCAR mouse skin carcinogenesis model has been extensively used to examine the induction of H-*ras* mutations by environmental carcinogens such as polycyclic aromatic hydrocarbons (PAH) (Chakravarti *et al*, 1995). Two lines of evidence suggest that mutated H-*ras* is involved in tumor initiation in mouse skin. First, PAH such as dibenzo[*a,l*]pyrene (DB[*a,l*]P) and 7,12-dimethylbenz[*a*]anthracene (DMBA) induce oncogenic H-*ras* mutations very early in tumorigenesis (Chakravarti *et al*, 1998, 2000 and unpublished results). Second, cells carrying the mutated H-*ras* gene can form benign tumors (Roop *et al*, 1986). The mutated H-*ras* gene is, however, found at all stages of tumorigenesis (Kemp *et al*, 1994) and is also involved in tumor progression (Rodriguez-Puebla *et al*, 1999a; Nagase *et al*, 2003).

Evidence suggests that non-cycling cells play a major role in tumor initiation. For example, DMBA induces 3-fold (males) and 13-fold (females) more skin tumors when the dorsal skin of mice is treated in the resting phase than in the proliferative phase (Andreasen, 1953). In another study, mouse skin was treated with 5-fluorouracil (5-FU) either 1 d before or 1 d after treatment with DMBA (Morris *et al*, 1997). These results showed that 5-FU only marginally reduced the number of benign tumors and did not affect the yield of carcinomas. As pre-treatment with 5-FU stopped DNA synthesis for 2 d and specifically killed the cycling cells, the marginal reduction in the yield of papillomas suggests that quiescent cells contribute more to tumor initiation than cycling cells (Morris *et al*, 1997). DNA-damaged quiescent cells would be expected to induce mutations by erroneous repair. Our studies in mouse skin suggest that erroneous DNA repair of DB[*a,l*]P-induced apurinic sites generates preneoplastic H-*ras* mutations (Chakravarti *et al*, 1998, 2000). Thus, erroneous repair of damaged DNA in quiescent cells is a major mechanism of tumor initiation.

Relatively little is known about the fate of the cells that have acquired oncogenic *ras* mutations. Although PAH

Abbreviations: 5-FU, 5-fluorouracil; DB[*a,l*]P, dibenzo[*a,l*]pyrene; DMBA, 7-12-dimethylbenz[*a*]anthracene; FACS, fluorescence-activated cell sorter; GTP, guanosine triphosphate; IP, immunoprecipitation; PAH, polycyclic aromatic hydrocarbons; PCNA, proliferating cell nuclear antigen

induce DNA adducts in the entire epidermis, most of the adducted cells are lost during epidermal renewal, and presumably do not contribute to tumor formation (Morris *et al*, 1986). Tumors are thought to arise from the remaining "initiated" cells that escape epidermal turnover and subsist as slowly cycling cells in the basal epidermis, as well as in the hair follicles of skin (Bickenbach *et al*, 1986; Morris *et al*, 1986, 2000). The slow-cycling cells retain a stem cell-like high proliferative potential (Morris and Potten, 1994; Morris, 2000). The slow-cycling cells of the early preneoplastic period, however, have not been shown to carry oncogenic *ras* gene mutations.

DB[*a,l*]P-treated SENCAR mouse skin (12 h–1 d) showed a high frequency of mutations (oncogenic codon 61 and other mutations) in the H-*ras* gene (Chakravarti *et al*, 1998, 2000). In this period, the mutations were found to be in the form of mismatched heteroduplexes as would be expected from erroneous repair. Consistent with this idea, recent studies indicate that DB[*a,l*]P treatment induces base excision repair genes during the mutagenic period (4–24 h) (Chakravarti *et al*, 2004). Following the initial period of erroneous repair-induced mutagenesis, there was a transient (1–3 d) increase in the frequency of oncogenic codon 61 mutations. In this period, the mutations did not appear as mismatched heteroduplexes, but were present as "fixed" mutations, as would be expected from a round of replication that would synthesize complementary daughter strands and separate the DNA into two molecules. Finally, between 4 and 7 d, the skin rapidly lost a majority of the H-*ras* mutations. Based on these results, we hypothesize that in DB[*a,l*]P-treated skin, cells that acquire the oncogenic H-*ras* codon 61 mutations transiently proliferate, but subsequently, only a small subpopulation of these cells persists in skin to form tumors, whereas a majority of the mutated cells are lost. We speculate that not all of the codon 61-mutated cells were stem cells. Indeed, the rapid regression of mutations (days 4–7) suggests that the partially differentiated mature or "transit" cells, which represent the majority of cells in the epidermis, also acquire the codon 61 mutation, divide once, and terminally differentiate to lose their nuclei and are shed from skin (Dover, 1994).

We describe this phenomenon of putative transient proliferation and regression of codon 61-mutated cells as transient clonoplasia (Chakravarti *et al*, 2000). As these events occur without any promoting treatments (e.g. with phorbol ester) to the skin, we propose that these events are part of the process of tumor initiation. In this paper, we provide further evidence for unpromoted early-preneoplastic proliferation. In addition, we report an apparent correlation between proliferation, H-*ras* expression, and signaling.

## Results

**Expression of H-*ras* protein in DB[*a,l*]P-treated mouse skin** Our previous studies suggested that DB[*a,l*]P treatment of SENCAR mouse skin results in rapid induction (within 12 h) of oncogenic H-*ras* codon 61 ($CAA^{Gln}$ to $CTA^{Leu}$) mutations (Chakravarti *et al*, 1998, 2000). Following mutagenesis, the frequency of the codon 61 mutations in skin clonally increased for a brief period (days 1–4) and then regressed rapidly (days 4–7). These results suggested that cells that acquire the codon 61 mutation clonally proliferate for a brief period (days 1–4) and then are eliminated (days 4–7) from the skin. The rapid regression of codon 61 mutations coincided with DB[*a,l*]P-induced hyperplasia that approximately doubles the skin layer (Casale *et al*, 2000).

We studied the expression of H-*ras* protein in DB[*a,l*]P-treated mouse skin to examine whether these preneoplastic events are correlated with H-*ras* mutation. Immunoprecipitation (IP) western blot analysis (Fig 1*A* and *B*) showed that DB[*a,l*]P treatment induced increased expression of the

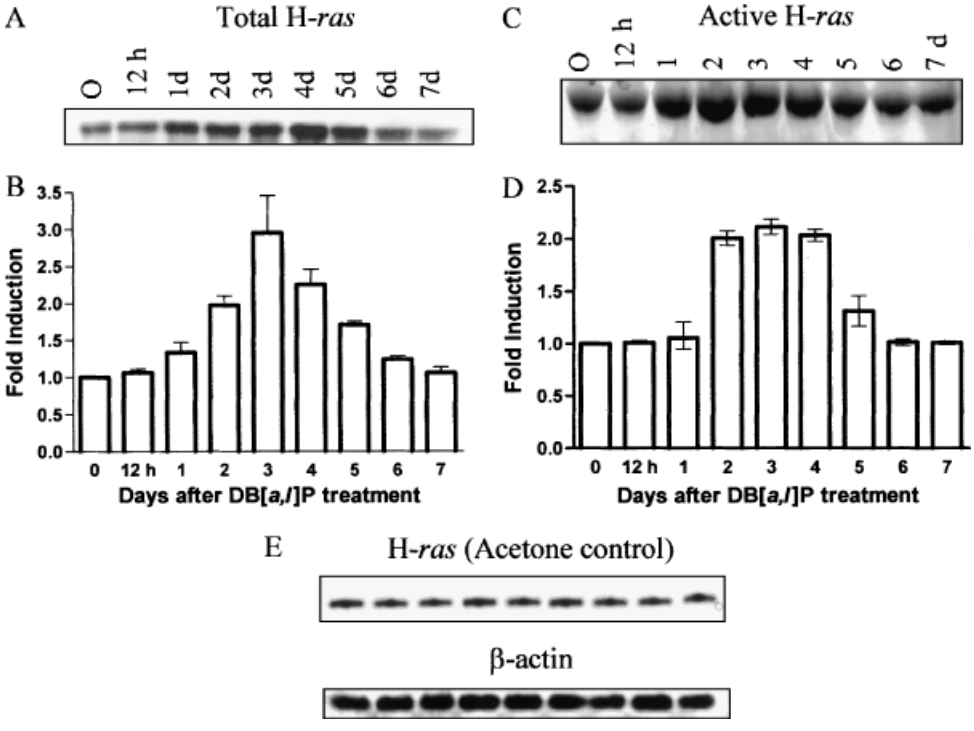


**Figure 1**
**Levels of total and guanosine triphosphate (GTP)-bound H-*ras* protein in dibenzo[*a,l*]pyrene (DB[*a,l*]P)-treated SENCAR mouse skin.** (*A*, *B*) Changes in the levels of total H-*ras* protein in the early preneoplastic period (12 h–7 d). (*C*, *D*) Changes in the levels of GTP-bound H-*ras* protein. The error bars in the bar charts (*B*, *D*) show the variability of results obtained from three independent immunoprecipitation western experiments with skins from two different mice/time point. (*E*) Levels of H-*ras* remained unchanged by the single treatment with acetone. The levels of β-actin in pre-immunoprecipitated extracts are shown as loading control.

H-*ras* gene that became evident at day 1, peaked between days 2 and 5, became highest at day 3 ( $\sim$ 3-fold over untreated skin), and then declined to near-untreated levels by day 7. The levels of guanosine triphosphate (GTP)-bound H-*ras* also increased, essentially following the expression patterns of total H-*ras*, except that its level showed a plateau between days 2 and 4 (Fig 1*C* and *D*). For total H-*ras*, the slope of time (0.5724; $p<0.0001$) and time squared ($-0.0821$; $p<0.0001$) were significantly different from zero, indicating that total H-*ras* tends to follow a quadratic curve with expected peak activation at $\sim$ 3.5 d. For active H-*ras*, the slope of time (0.4642; $p<0.0001$) and time squared ($-0.0687$; $p<0.0001$) were significantly different from zero, with expected peak activation at $\sim$ 3.4 d.

The early plateau of GTP-bound H-*ras* suggests that skin cells contained a higher fraction of active H-*ras* at day 2 than at day 3, when the highest level of H-*ras* is observed. These results are consistent with the idea that cells carrying H-*ras* codon 61 mutations briefly proliferate (days 1–2), express the mutated H-*ras* gene, forming constitutively GTP-bound H-*ras* protein (Krengel *et al*, 1990; Scheffzek *et al*, 1997; Ahmadian *et al*, 1999), and then are eliminated, as suggested by the mutational data (12, 13). These results also raise the possibility that mutated H-*ras* might drive the proliferation of these cells.

The IP western experiments show relative changes in the levels of H-*ras* protein, but did not provide the knowledge of the actual levels of total and GTP-bound H-*ras* proteins. To quantify the levels of GTP-bound and total H-*ras* protein, we conducted quantitative ELISA experiments. A typical standard curve for these experiments is shown in Fig 2. The results indicate that the fraction of GTP-bound H-*ras* protein in DB[*a,l*]P-treated mouse skin was approximately 3%–6% of the total H-*ras* protein. A change in the total H-*ras* protein in the early preneoplastic period was clearly observable. For total H-*ras*, the slope of time (0.3255; $p<0.0001$) and time squared ($-0.0348$; $p<0.0001$) were also significantly different from zero, again indicating that total H-*ras* tended to follow a quadratic curve, with expected peak activation at $\sim$ 4.7 d. For active H-*ras*, the coefficient for time (0.1218; $p=0.02$) was significant; however, the slope for time squared ($-0.0132$; $p=0.07$) was not significantly different from zero.

Two periods of increased H-*ras* expression were discernible (1 and 3–6 d), corresponding to the two putative periods of preneoplastic cell proliferation, namely, clonal proliferation of codon 61-mutated cells and DB[*a,l*]P-induced hyperplasia (Casale *et al*, 1997, 2000) (Fig 2*B*). The hyperplastic response is known to increase the number of epidermal cells (from the single-layer epidermis in untreated skin to a bilayer at day 7 and multilayer at day 10 after treatment with 200 nmol DB[*a,l*]P) (Casale *et al*, 2000). The peak level of H-*ras* protein by the ELISA experiments was $\sim$ 2.5-fold over control (see Table S1).

Both IP western experiments (Fig 1) and the ELISA experiments (Fig 2) showed increased H-*ras* levels. The ELISA experiments, however, did not trace an identical profile as in the IP western experiments. Presumably, these differences are because of the enrichment of H-*ras* in the IP western experiments. The ELISA analysis was not sufficiently sensitive to track DB[*a,l*]P-induced changes relative to control levels of GTP-bound H-*ras* protein (see Table S1).

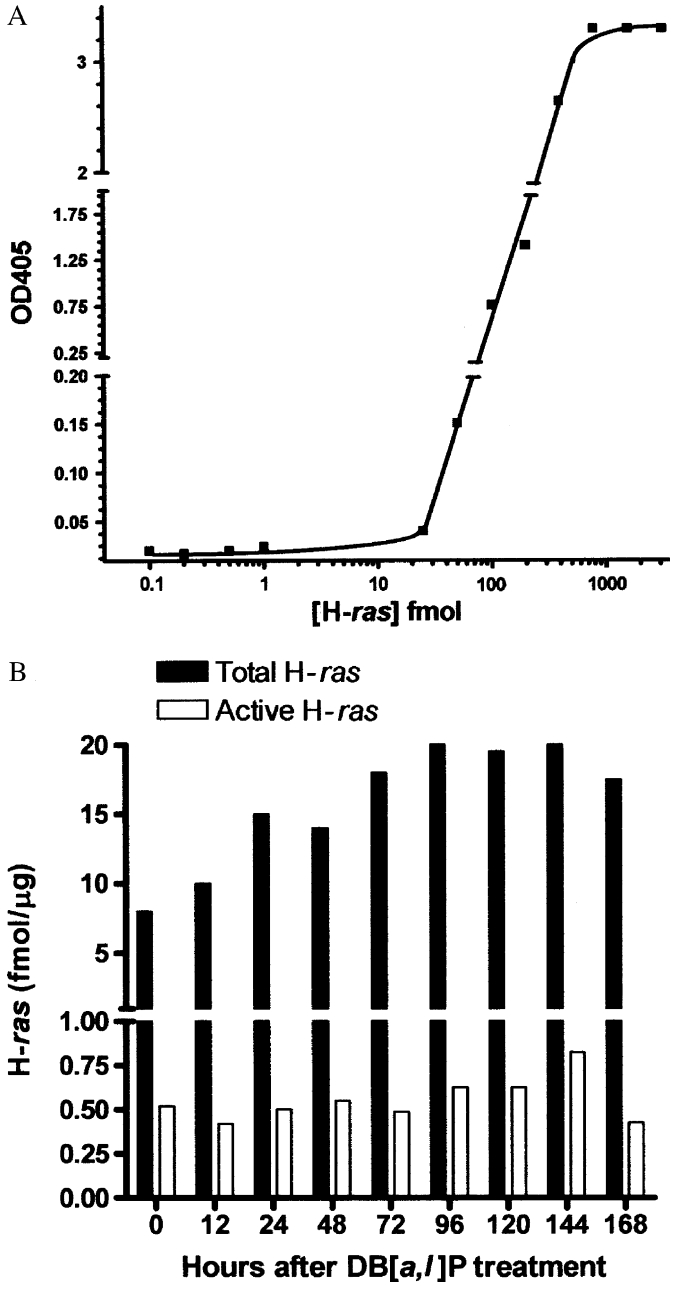


**Figure 2**
**Quantification of total and guanosine triphosphate (GTP)-bound H-*ras* protein levels in dibenzo[*a,l*]pyrene (DB[*a,l*]P)-treated mouse skin.** (*A*) Representative standard curve constructed with pure H-*ras* protein. (*B*). Comparative levels of total and GTP-bound H-*ras* protein in DB[*a,l*]P-treated mouse skin. Results of two ELISA experiments from two different mice/time point are shown.

**Epidermal cell kinetics in DB[*a,l*]P-treated mouse skin**

The results of fluorescence-activated cell sorter (FACS) analysis of nuclear DNA content in propidium iodide-stained epidermal cells isolated from DB[*a,l*]P-treated dorsal skin are shown in Fig 3*A*. Two waves of DNA synthesis are observed: the first correlated with the putative clonal proliferation of the codon 61-mutated cells (1–2 d) and the second correlated with epidermal hyperplasia (3–6 d). Epidermal cells isolated from untreated control mice, as well as epidermal cells isolated from the belly skin of the treated mice, did not show these changes in cell kinetics (data not shown).

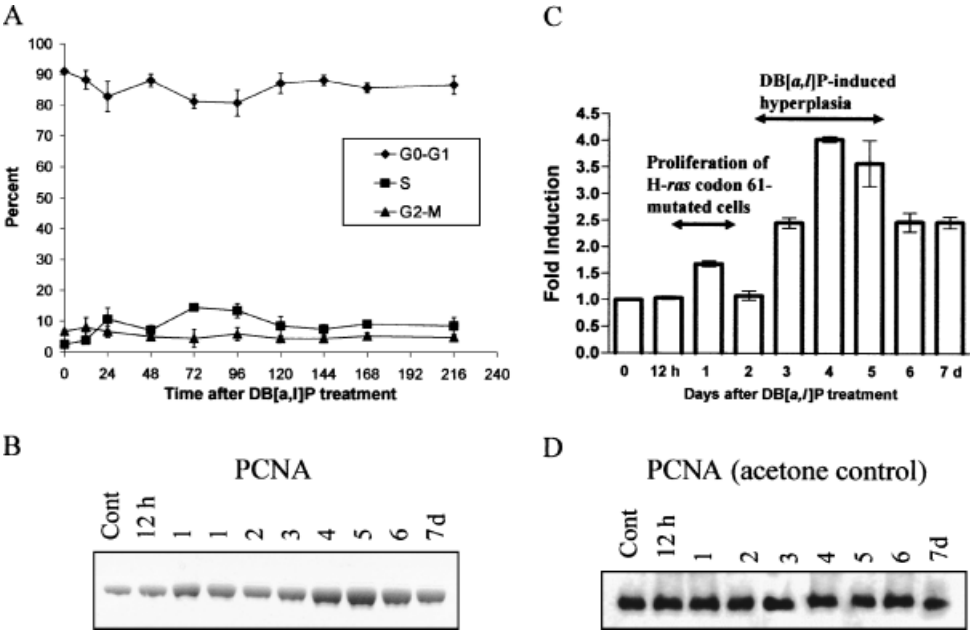


**Figure 3**
**Epidermal cell proliferation in dibenzo[*a,l*]pyrene (DB[*a,l*]P)-treated mouse skin.** (*A*) Cell kinetics (fluorescence-activated cell sorter analysis of propidium iodide-stained nuclei) of keratinocytes isolated from DB[*a,l*]P-treated skin. Each time point shows the cell kinetic analysis of pooled keratinocytes from four to five SENCAR mice, determined in triplicate. (*B*, *C*) Induction of proliferating cell nuclear antigen (PCNA) levels (as a marker of DNA replication) in DB[*a,l*]P-treated skin. Immunoprecipitation western experiments indicate that the levels of PCNA increased in two waves, corresponding to the cell kinetics. The correlations between the two putative proliferation events with proliferation of codon 61-mutated cells and DB[*a,l*]P-induced hyperplasia are shown. The error bars in the bar chart show the variability of results obtained from three independent experiments obtained from two to three mice/time point. (*D*) Expression of PCNA in acetone-treated mouse skin.

Epidermal cell kinetics in DB[*a,l*]P-treated skin was also examined by following the levels of proliferating cell nuclear antigen (PCNA) (Fig 3*B* and *C*). DB[*a,l*]P treatment resulted in increased PCNA levels at two time periods, between days 1 and 2 ($\sim$ 1.6-fold) and, again, between days 3 and 7 ($\sim$ 4-fold). The slope of time (0.6273; $p < 0.0001$) and time squared ($-0.0663$; $p < 0.0001$) were significantly different from zero, indicating that like H-*ras*, PCNA also tends to follow a quadratic curve with expected peak activation at $\sim$ 4.7 d. Therefore, these results are consistent with the idea that epidermal cells in DB[*a,l*]P-treated skin undergo two distinct waves of replicative DNA synthesis that correspond to the unpromoted clonal proliferation of codon 61-mutated cells and the DB[*a,l*]P-induced hyperplasia.

**Induction of G1 cyclins** Previous studies with the two-stage carcinogenesis model suggest that cylin D1 is not overexpressed in normal or hyperproliferative skin, but overexpressed in premalignant lesions and carcinomas (Bianchi *et al*, 1993; Robles and Conti, 1995). These and other studies suggested that oncogenic H-*ras* and cyclin D1 overexpression play a major role in tumor formation (Robles *et al*, 1998; Rodriguez-Puebla *et al*, 1998, 1999b; Miliani De Marval *et al*, 2004). It was, however, reported that v-H-*ras* induces cyclins D1, D2, and E in primary mouse keratinocytes (Robles *et al*, 1998). Changes in the levels of G1 cyclins (cyclins D1, D2, and E) in DB[*a,l*]P-treated mouse skin are shown in Fig 4. Cyclin D1 was found to increase during DB[*a,l*]P-induced hyperplasia (maximal induction at day 5), but not during the putative unpromoted proliferation of codon 61-mutated cells (Fig 4*A* and *B*). The cyclin D2 protein level did not change significantly (Fig 4*C* and *D*) and cyclin D3 protein could not be detected (data not shown). An earlier study also reported poor cyclin D3 expression in mouse skin (Robles *et al*, 1998). The levels of cyclin E protein, on the other hand, were found to increase (days 1–3) during the putative clonal proliferation of codon 61-mutated cells (Fig 4*E* and *F*).

## Discussion

Our previous studies (Chakravarti *et al*, 1998, 2000) and the present data are consistent with the idea that epidermal cells carrying H-*ras* codon 61 mutations briefly undergo clonal proliferation. An increase in the levels of GTP-bound H-*ras* protein was observed during this period (1–4 d, Fig 1). These profiles resemble our previous observations on the induction of codon 61 mutations in DB[*a,l*]P-treated mouse skin (Chakravarti *et al*, 1998, 2000). But changes in the levels of GTP-bound H-*ras* protein were under the limit of sensitivity of quantification by the ELISA procedure (Fig 2). To our knowledge, these are the first studies about the early preneoplastic profiles of H-*ras* gene expression and epidermal cell proliferation in PAH-treated skin. A previous study indicated that DMBA-treated Long–Evans rats produce preleukemic cells carrying the codon 61-mutated N-*ras* gene within 48 h after treatment (Osaka *et al*, 1996). It was later found that N-*ras* mRNA is overexpressed at this time, but whether the increased expression was because of the codon 61-mutated N-*ras* gene was not examined (Gyongyi *et al*, 2002).

We observed a small but significant increase in the S-phase population of epidermal keratinocytes and a corresponding increase in PCNA levels during the putative clonal proliferation of H-*ras* codon 61-mutated cells (Fig 3). A second wave of cell proliferation occurred during DB[*a,l*]P-induced epidermal hyperplasia. Further evidence for two waves of cell proliferation was obtained from the profiles of G1 cyclins. Cyclin E levels were increased during the putative clonal proliferation of H-*ras* codon 61-mutated cells whereas cyclin D1 levels were increased during hyperplasia.

Ras can transduce a variety of proliferative, apoptotic, senescent, and differentiation signals (Crespo and Leon, 2000; Malumbres and Barbacid, 2003). In particular, growth signaling by oncogenically mutated *ras* is important for tumor formation. For example, a number of transcription factors, including Ets, c-jun, c-myc, NF-$\kappa$B, and C/EBP$\beta$,

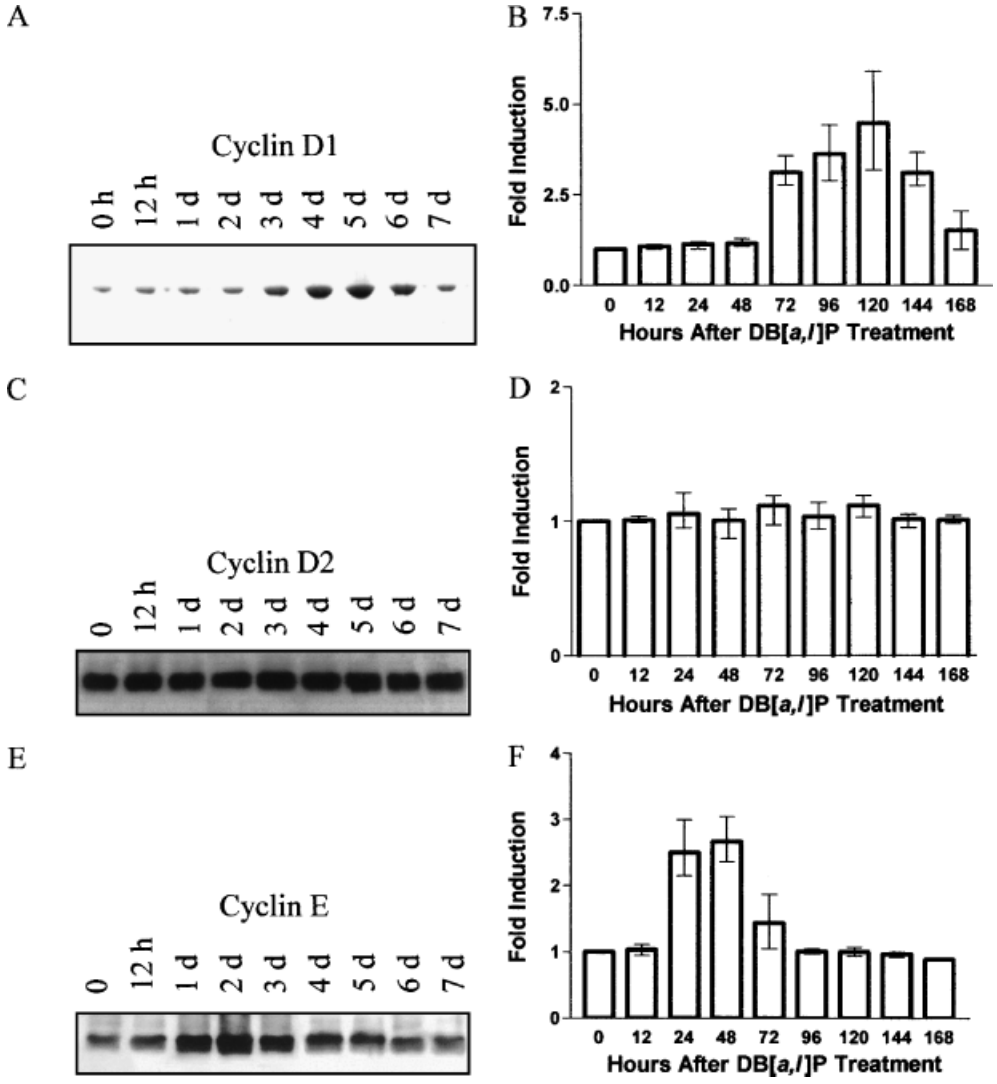


**Figure 4**
**Expression of G1 cyclins in dibenzo[*a,l*]pyrene (DB[*a,l*]P)-treated mouse skin.** (*A*, *B*) Changes in the levels of cyclin D1 protein. (*C*, *D*) Changes in the levels of cyclin D2 protein. (*E*, *F*). Changes in the levels of cyclin E protein. The error bars in the bar charts show the variability of results obtained from immunoprecipitation western analyses of three to four independent determinations with skin harvested from two to three different mice/time point. Similar experiments with a single treatment with 100 μL acetone (vehicle) did not show changes in the levels of G1 cyclins (not shown).

have been reported to have roles in oncogenic *ras*-mediated cellular transformation (Sklar *et al*, 1991; Langer *et al*, 1992; Johnson *et al*, 1996; Finco *et al*, 1997; Zhu *et al*, 2002). Earlier studies suggest that oncogenic H-*ras* utilizes cyclin D1 as a primary downstream signal for G1–S transition, but cyclins D2 and E may also have significant roles (Robles *et al*, 1998).Our results indicate that cyclin E levels are increased during the putative clonal proliferation of codon 61-mutated cells, and cyclin D1 levels are increased during the hyperplastic period. These results are correlative, and do not prove that the putative unpromoted proliferation of codon 61-mutated cells occurs through a cyclin E-mediated mechanism. As DB[*a,l*]P-treated skin cells carry various mutations between days 1 and 2 (Chakravarti *et al*, 2000), a direct correlation between the increment in the level of cyclin E and the expression of codon 61-mutated H-*ras* protein is not possible from these results. These results could also be confounded by DB[*a,l*]P-induced toxicity and homeostatic proliferation of skin cells. A role of cyclin E in oncogenic H-*ras* signaling, however, has been indicated in other systems (Ludde *et al*, 2001; Starkel *et al*, 2001; Geisen and Moroy, 2002).

In summary, these results suggest that an unpromoted clonal proliferation of oncogenically mutated cells in early preneoplastic mouse skin is an event of tumor initiation. This clonal proliferation may be correlated with the expression of activated H-*ras* and induction of cyclin E protein. We are conducting further studies to examine whether codon 61 mutations are associated with these events of tumor initiation.

## Materials and Methods

**DB[*a,l*]P treatment of dorsal skin of SENCAR mice** Eighteen female SENCAR mice (8 wk old; two mice each for nine time points: 0 h (solvent control), 12 h, 1, 2, 3, 4, 5, 6, and 7 d) were treated with 200 nmol of DB[*a,l*]P in 100 μL acetone on an area of shaved dorsal skin, as described previously (Chakravarti *et al*, 1998). At this dose, the tumor yield is five to seven per animal and significant skin toxicity is observed (Casale *et al*, 1997, 2000). This dose was chosen for the current experiments because, at DB[*a,l*]P doses lower than 200 nmol, a diminished frequency of early H-*ras* codon 61 mutations is observed (unpublished results). Mice were sacrificed at indicated times, and treated dorsal skin was harvested, flash-frozen in liquid $N_2$, and stored at −80°C until use. The medical ethical committee of the University of Nebraska Medical Center approved all described studies.

**Protein extraction from mouse skin** All procedures were conducted at 4°C. One milligram of skin tissue was finely chopped and

homogenized in a mortar and pestle in 2.5 mL ice-cold RIPA buffer (50 mM Tris, 5 mM EDTA, 150 mM NaCl, 0.25% sodium-deoxycholate, and 1% NP-40, pH 7.5), and centrifuged at 13,000 r.p.m. (15,700 *g*) for 15 min to collect the supernatant. To extract protein from larger sections of skin samples, this protocol was scaled up. Protein concentration was determined by the Comassie Plus reagent (Pierce Biotechnology, Rockford, Illinois). The extracts were used for immuoprecipitation western blots and ELISA assays.

**IP western blotting** The expression of PCNA, G1 cyclins, and H-*ras* protein in untreated or DB[*a,l*]P-treated SENCAR mouse skin was examined by this protocol. Unless otherwise stated, all procedures were conducted at 4°C. In these experiments, 0.1–1 mg aliquots of the extracted protein were diluted with phosphate-buffered saline (PBS) (pH 7.4, Sigma Biochemicals, St Louis, Missouri) to 100 μL, precleared by incubating for 30 min with 10 μL of Protein A sepharose (Amersham Biosciences, Piscataway, New Jersey), and centrifuged at 13,000 r.p.m. for 15 min. The supernatants were incubated with 5 μL of undiluted primary antibody for 3 h with shaking. Into these mixtures, 20 μL of protein A sepharose was added and incubated overnight with shaking. After centrifugation (13,000 r.p.m. for 15 min), the immunoprecipitated pellets were harvested and washed six times with PBS, resuspended in 10 μL of 2 × SDS-PAGE sample loading buffer (Laemmli, 1970), and boiled for 5 min at 100°C. Boiled supernatants were harvested by centrifugation (13,000 r.p.m. for 5 min) and electrophoresed in a 12% SDS-PAGE gel (Laemmli, 1970).

The gels were electroblotted onto PVDF membranes (Millipore Corporation, Bedford, Massachusetts) at 400 mA for 2 h. The membranes were blocked overnight with TBST (50 mM Tris, 150 mM NaCl, pH 7.5, supplemented with 0.1% Tween-20) containing 2% BSA (Sigma Biochemicals), and then washed five times with TBST. The blots were incubated with appropriate dilutions of the primary antibodies for 1 h at room temperature, washed, and incubated with secondary antibody (HRP-linked IgG) (Santa Cruz Biotechnology, Santa Cruz, California), and developed with the ECL kit according to vendor (Amersham Biosciences). Antibodies against PCNA (sc-56), H-*ras* (sc-520), and cyclins D1 (sc-246), D2 (sc-452), D3 (sc-6283), and E (sc-481) were obtained from Santa Cruz Biotechnology. A Raf-1 RBD-agarose conjugate (14–278) was obtained from Upstate Biotechnology (Charlottsville, Virginia).

**Quantitative ELISA of total and GTP-bound H-*ras* protein** An ELISA technique (Engvall and Perlmann, 1972) was adapted for the quantification of total or GTP-bound (active) H-*ras* protein levels in untreated and DB[*a,l*]P-treated SENCAR mouse skin.

*Standard curves* A purified H-*ras* protein (Cytoskeleton, Denver, Colorado) was diluted in bicarbonate buffer (pH 9.0) (Engvall and Perlmann, 1972) generating standard curves for the measurement of total (200 fmol–3.2 pmol) or active (0.2 fmol–3.2 pmol) H-*ras* proteins.

To generate the standard curves, various amounts of H-*ras* protein (diluted to an uniform volume of 100 μL with bicarbonate buffer) were spotted in the wells of a microtiter plate (Immplate MaxiSRP, Nalge NUNC, Rochester, New York), and incubated overnight at 4°C. The plate was washed 4 × with a PBST wash buffer (10 mM PBS, pH 7.4, with 0.05% Tween 20), blocked for 1 h with 150 μL of PBST containing 1% BSA (blocking buffer) at room temperature, washed again, and incubated with 100 μL of blocking buffer containing 1:100 dilution of anti-H-*ras* antibody for 1 h at room temperature. The plate was washed 4 × with PBST as described above, and blocked with 100 μL of blocking buffer containing a 1:5000 dilution of the secondary antibody (anti-goat IgG-Biotin, Sigma Biochemicals) and added to the wells and incubated for 1 h at room temperature. Again, the plate was washed 4 × with PBST as above, and incubated for 1 h at room temperature with 100 μL of a 1:100 dilution of avidin–alkaline phosphatase (Sigma Biochemicals) in blocking buffer. The plate was washed 4 × with PBST as above and color developed with 100 μL of *p*-nitrophenyl phosphate solution (1 mg per mL) (Sigma Biochemicals) for 15 min at room temperature. Reactions were stopped with 100 μL of 1 M NaOH, and the color was measured at 405 nm.

*Quantification of total and GTP-bound H-ras in untreated or DB[a,l]P-treated SENCAR mouse skin* Total H-*ras* protein in untreated or DB[*a,l*]P-treated mouse skin samples was determined using 80 μg protein extracts. The procedures were similar to that for the generation of standard curves, except that a 1:500 dilution of the anti-H-*ras* antibody was used. To determine the GTP-bound *ras* levels in these samples, 200 μg of protein extracts were used. The GTP-bound *ras* in the extract was immunoprecipitated with 10 μL Raf-1 *ras*-binding domain (RBD)–agarose conjugate for 30 min at 4°C in a rotary shaker. The precipitated protein conjugate was harvested by centrifugation (13,000 r.p.m. for 15 min at 4°C), the pellet was washed 3 × with ice-cold bicarbonate buffer, and then resuspended in 100 μL of bicarbonate buffer. GTP-bound *ras* was released from the conjugate by heating the slurry at 80°C for 3 min and harvested by centrifugation (13,000 r.p.m. for 5 min at room temperature). The resulting supernatants were analyzed by ELISA with anti-H-*ras* antibody, as described above.

*Statistical methods* A generalized linear model assuming a log link and Poisson's distribution allowing for underdispersion was used to examine differences over time (days). The dispersion parameter was estimated as the deviance divided by the degrees of freedom (McCullagh and Nelder, 1989). Separate analyses for total H-*ras*, active H-*ras*, and PCNA were conducted using time and time squared as covariates (see Table S1). Wald $\chi^2$ tests were used to test whether estimated coefficients were significantly different from zero. A p-value $<0.05$ was considered to be significant.

**Cell kinetic analysis of epidermal keratinocytes from adult SENCAR mice** Epidermal keratinocytes from untreated or DB[*a,l*]P-treated SENCAR mouse skin were isolated as described before (Morris and Fischer, 1994). In these experiments, a total of 50 mice (8 wk-old; five each for 10 time points: 0 h (solvent control), 12 h, 1, 2, 3, 4, 5, 6, 7, and 9 d) were used. Briefly, dorsal skins from five mice were floated on 0.25% trypsin (Invitrogen, Carlsbad, California) and incubated for 2 h at 32°C. The loosened epidermis was scraped off with paddles and suspended in 30 mL DMEM containing 10% FBS and 0.2 mg per 100 mL gentamycin (Invitrogen). Cells were gently stirred for 20 min and passed through a 40 μm filter and centrifuged. The cell pellet was resuspended in 5 mL of the above culture medium by trituration. This procedure yielded 1–2 million epidermal cells, of which >90% are keratinocytes.

Cells were added dropwise to a vortexing solution of 70% ethanol for alcohol fixing, centrifuged and washed once with PBS (Sigma Biochemicals), suspended in 1 mL Telford reagent (Telford *et al*, 1991), and incubated for 30 min at 4°C for FACS analysis. Each sample was divided into three parts and analyzed as replicates. The results were compared with epidermal cell preparations from the belly skins of the treated animals to determine whether the skin of these mice was in the resting or proliferating phases.

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## Supplementary Material

The following material is online for this article.
**Table S1** Statistical Analysis

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## References


Ahmadian MR, Zor T, Vogt D, Kabsch W, Selinger Z, Wittinghofer A, Scheffzek K: Guanosine triphosphatase stimulation of oncogenic Ras mutants. Proc Natl Acad Sci USA 96:7065–7070, 1999

Andreasen E: Cyclic changes in the skin of the mouse. Acta Pathol Scand 32:157–164, 1953

Barbacid M: Ras oncogenes: Their role in neoplasia. Eur J Clin Invest 20:225–235, 1990

Bianchi AB, Fischer SM, Robles AI, Rinchik EM, Conti CJ: Overexpression of cyclin D1 in mouse skin carcinogenesis. Oncogene 8:1127–1133, 1993

Bickenbach JR, McCutecheon J, Mackenzie IC: Rate of loss of tritiated thymidine label in basal cells in mouse epithelial tissues. Cell Tissue Kinet 19:325–333, 1986

Bos JL: Ras oncogenes in human cancer: A review. Cancer Res 49:4682–4689, 1989

Casale GP, Cheng Z, Liu J, Cavalieri EL, Singhal M: Profiles of cytokine mRNAs in the skin and lymph nodes of SENCAR mice treated epicutaneously with dibenzo[*a,l*]pyrene or dimethylbenz[*a*]anthracene reveal a direct correlation between carcinogen-induced contact hypersensitivity and epidermal hyperplasia. Mol Carcinogen 27:125–140, 2000

Casale GP, Higginbotham S, Johansson SL, Rogan EG, Cavalieri EL: Inflammatory response of mouse skin exposed to the very potent carcinogen dibenzo[*a,l*]pyrene: A model for tumor promotion. Fundam Appl Toxicol 36:71–78, 1997

Chakravarti D, Bhattacharya G, Mailander PC: Repression of replication and induction of base excision repair in dibenzo[*a,l*]pyrene-treated SENCAR mouse skin. Proc Am Assoc Cancer Res 45:1560, 2004

Chakravarti D, Mailander P, Cavalieri EL, Rogan EG: Evidence that error-prone DNA repair converts dibenzo[*a,l*]pyrene-induced depurinating lesions into mutations: Formation, clonal proliferation and regression of initiated cells carrying H-ras oncogene mutations in early preneoplasia. Mutation Res 456:17–32, 2000

Chakravarti D, Mailander P, Franzen J, Higginbotham S, Cavalieri EL, Rogan EG: Detection of dibenzo[*a,l*]pyrene-induced H-*ras* codon 61 mutant genes in preneoplastic SENCAR mouse skin using a new PCR-RFLP method. Oncogene 16:3203–3210, 1998

Chakravarti D, Pelling JC, Cavalieri EL, Rogan EG: Relating aromatic hydrocarbon-induced DNA adducts and c-H-ras mutations in mouse skin papillomas: The role of apurinic sites. Proc Natl Acad Sci USA 92:10422–10426, 1995

Crespo P, Leon J: Ras proteins in the control of the cell cycle and cell differentiation. Cell Mol Life Sci 57:1613–1636, 2000

Dover R: Cell kinetics of keratinocytes. In: Leigh IMLEB, Watt FM (eds). The Keratinocyte Handbook. Cambridge: Cambridge University Press, 1994; p 203–234

Engvall E, Perlmann P: Enzyme-linked immunosorbent assay (ELISA): Quantitation of specific antibodies by enzyme-labeled anti-immunoglobulin in antigen-coated tubes. J Immunol 109:129–135, 1972

Esapa CT, Johnson SJ, Kendall-Taylor P, Lennard TW, Harris PE: Prevalence of Ras mutations in thyroid neoplasia. Clin Endocrinol (Oxford) 50:529–535, 1999

Finco TS, Westwick JK, Norris JL, Beg AA, Der CJ, Baldwin AS Jr: Oncogenic Ha-*ras*-induced signaling activates NF-kappaB transcriptional activity, which is required for cellular transformation. J Biol Chem 272:24113–24116, 1997

Geisen C, Moroy T: The oncogenic activity of cyclin E is not confined to Cdk2 activation alone but relies on several other, distinct functions of the protein. J Biol Chem 277:39909–39918, 2002

Grendys EC Jr, Barnes WA, Weitzel J, Sparkowski J, Schlegel R: Identification of H, K, and N-*ras* point mutations in stage IB cervical carcinoma. Gynecol Oncol 65:343–347, 1997

Gyongyi Z, Grama L, Nadasi E, *et al*: Flow cytometric analysis of DMBA-induced early *in vivo* ras expression. *In Vivo* 16:323–326, 2002

Johnson R, Spiegelman B, Hanahan D, Wisdom R: Cellular transformation and malignancy induced by *ras* require c-jun. Mol Cell Biol 16:4504–4511, 1996

Kemp CJ, Burns PA, Brown K, Nagase H, Balmain A: Transgenic approaches to the analysis of *ras* and p53 function in multistage carcinogenesis. In: Cold Spring Harbor Symposia on Quantitative Biology. Cold Spring Harbor, NY: Cold Spring Harbor Press, 1994; p 427–434

Krengel U, Schlichting L, Scherer A, *et al*: Three-dimensional structures of H-*ras* p21 mutants: Molecular basis for their inability to function as signal switch molecules. Cell 62:539–548, 1990

Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685, 1970

Langer SJ, Bortner DM, Roussel MF, Sherr CJ, Ostrowski MC: Mitogenic signaling by colony-stimulating factor 1 and *ras* is suppressed by the ets-2 DNA-binding domain and restored by myc overexpression. Mol Cell Biol 12:5355–5362, 1992

Li D, Firozi PF, Zhang W, *et al*: DNA adducts, genetic polymorphisms, and K-*ras* mutation in human pancreatic cancer. Mutat Res 513:37–48, 2002

Ludde T, Kubicka S, Plumpe J, Liedtke C, Manns MP, Trautwein C: Ras adenoviruses modulate cyclin E protein expression and DNA synthesis after partial hepatectomy. Oncogene 20:5264–5278, 2001

McCullagh P, Nelder JA: Generalized Linear Models. London: Chapman & Hall, 1989

Malumbres M, Barbacid M: Ras oncogenes: The first 30 years. Nat Rev Cancer 3:459–465, 2003

Miliani de Marval PL, Macias E, Conti CJ, Rodriguez-Puebla ML: Enhanced malignant tumorigenesis in Cdk4 transgenic mice. Oncogene 23:1863–1873, 2004

Morris RJ: Keratinocyte stem cells: Targets for cutaneous carcinogens. J Clin Invest 106:3–8, 2000

Morris RJ, Coulter K, Tryson K, Steinberg SR: Evidence that cutaneous carcinogen-initiated epithelial cells from mice are quiescent rather than actively cycling. Cancer Res 57:3436–3443, 1997

Morris RJ, Fischer SM: A defined culture system for epidermal keratinocytes from adult mice. In: IM Leigh, Lane EB, Watt FM (eds). The Keratinocyte Handbook. Cambridge: Cambridge University Press, 1994; p 53–69

Morris RJ, Fischer SM, Slaga TJ: Evidence that a slowly cycling subpopulation of adult murine epidermal cells retains carcinogen. Cancer Res 46:3061–3066, 1986

Morris RJ, Potten CS: Slowly cycling (label-retaining) epidermal cells behave like clonogenic stem cells *in vitro*. Cell Prolif 27:279–289, 1994

Morris RJ, Tryson KA, Wu KQ: Evidence that the epidermal targets of carcinogen action are found in the interfollicular epidermis of infundibulum as well as in the hair follicles. Cancer Res 60:226–229, 2000

Nagase H, Mao JH, Balmain A: Allele-specific H-*ras* mutations and genetic alterations at tumor susceptibility loci in skin carcinomas from interspecific hybrid mice. Cancer Res 63:4849–4853, 2003

Osaka M, Matsuo S, Koh T, Sugiyama T: Specific N-*ras* mutation in bone marrow within 48 h of 7, 12-dimethylbenz[*a*]anthracene treatment in Huggins–Sugiyama rat leukemogenesis. Mol Carcinogen 16:126–131, 1996

Robles AI, Conti CJ: Early overexpression of cyclin D1 protein in mouse skin carcinogenesis. Carcinogenesis 16:781–786, 1995

Robles AI, Rodriguez-Puebla ML, Glick AB, *et al*: Reduced skin tumor development in cyclin D1-deficient mice highlights the oncogenic ras pathway *in vivo*. Genes Dev 12:2469–2474, 1998

Rodriguez-Puebla ML, LaCava MM, Bolontrade F, Russell J, Conti CJ: Increased expression of mutated Ha-*ras* during premalignant progression in SENCAR mouse skin. Mol Carcinogen 26:150–156, 1999a

Rodriguez-Puebla ML, LaCava M, Gimenez-Conti IB, Johnson DG, Conti CJ: Deregulated expression of cell cycle proteins during premalignant progression in SENCAR mouse skin. Oncogene 17:2251–2258, 1998

Rodriguez-Puebla ML, Robles AI, Conti CJ: Ras activity and cyclin D1 expression: An essential mechanism of mouse skin tumor development. Mol Carcinogen 24:1–6, 1999b

Roop DR, Lowy DR, Tambourin PE, *et al*: An activated Harvey-*ras* oncogene produces benign tumours on mouse epidermal tissue. Nature 323:822–824, 1986

Scheffzek K, Ahmadian MR, Kabsch W, Wiesmuller L, Lautwein A, Schmitz F, Wittinghofer A: The Ras–RasGAP complex: Structural basis for GTPase activation and its loss in oncogenic Ras mutants. Science 277:333–338, 1997

Semczuk A, Schneider-Stock R, Berbec H, Marzec B, Jakowicki JA, Roessner A: K-*ras* exon 2 point mutations in human endometrial cancer. Cancer Lett 164:207–212, 2001

Shiraishi T, Muneyuki T, Fukutome K, Ito H, Kotake T, Watanabe M, Yatani R: Mutations of *ras* genes are relatively frequent in Japanese prostate cancers: Pointing to genetic differences between populations. Anticancer Res 18:2789–2792, 1998

Sklar MD, Thompson E, Welsh MJ, *et al*: Depletion of c-myc with specific antisense sequences reverses the transformed phenotype in ras oncogene-transformed NIH 3T3 cells. Mol Cell Biol 11:3699–3710, 1991

Starkel P, Lambotte L, Sempoux C, De Saeger C, Saliez A, Maiter D, Horsmans Y: After portal branch ligation in the rat, cellular proliferation is associated with selective induction of *c*-Ha-*ras*, p53, cyclin E, and Cdk2. Gut 49: 119–130, 2001

Telford WG, King LE, Fraker PJ: Evaluation of glucocorticoid-induced DNA fragmentation in mouse thymocytes by flow cytometry. Cell Prolif 24:447–459, 1991

Vahakangas KH, Bennett WP, Castren K, *et al*: p53 and K-*ras* mutations in lung cancers from former and never-smoking women. Cancer Res 61:4350–4356, 2001

van der Schroeff JG, Evers LM, Boot AJ, Bos JL: Ras oncogene mutations in basal cell carcinomas and squamous cell carcinomas of human skin. J Invest Dermatol 94:423–425, 1990

Zhu S, Yoon K, Sterneck E, Johnson PF, Smart RC: CCAAT/enhancer binding protein-beta is a mediator of keratinocyte survival and skin tumorigenesis involving oncogenic Ras signaling. Proc Natl Acad Sci USA 99:207–212, 2002