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Original Paper

K-*ras* exon 4A has a tumour suppressor effect on carcinogen-induced murine colonic adenoma formation

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Abstract

K-*ras* encodes two isoforms, K-*ras* 4A and 4B, that are jointly affected by K-*ras* activating mutations, which are prevalent in colorectal cancer (CRC). CRC shows alterations in the expressed K-*ras* 4A : 4B isoform ratio in favour of K-*ras* 4B, in tumours both with and without K-*ras* mutations. The present study evaluated whether K-*ras* 4A expression can suppress colonic adenoma development in the absence of its oncogenic allele. Mice with homozygous targeted deletions of K-*ras* exon 4A (K-$ras^{tm\Delta 4A/tm\Delta 4A}$) that can express the K-*ras* 4B isoform only, along with heterozygous K-$ras^{tm\Delta 4A/+}$ and wild-type mice, were given ten weekly 1,2-dimethylhydrazine (DMH) treatments to induce colonic adenomas. There was a significant increase in both the number and the size of colonic adenomas in DMH-treated K-$ras^{tm\Delta 4A/tm\Delta 4A}$ mice, with reduced survival, compared with heterozygous and wild-type mice. No K-*ras* mutations were found in any of the 30 tumours tested from the three groups. Lack of expression of K-*ras* 4A transcripts was confirmed, whereas the relative expression levels of K-*ras* 4B transcripts were significantly increased in the adenomas of K-$ras^{tm\Delta 4A/tm\Delta 4A}$ mice compared with K-$ras^{tm\Delta 4A/+}$ and wild-type mice. Immunohistochemical studies showed that adenomas of K-$ras^{tm\Delta 4A/tm\Delta 4A}$ mice had significantly increased cell proliferation and significantly decreased apoptosis with evidence of activation of MapKinase and Akt pathways, with increased phospho-Erk1/2 and both phospho-Akt-Thr308 and phospho-Akt-Ser473 immunostaining, compared with adenomas from K-$ras^{tm\Delta 4A/+}$ and wild-type mice. In conclusion, following DMH treatment, K-*ras* exon 4A deletion promoted increased number and size of colonic adenomas showing increased K-*ras* 4B expression, increased proliferation, decreased apoptosis, and activation of MapKinase and Akt pathways, in the absence of K-*ras* mutations. Therefore, K-*ras* 4A expression had a tumour suppressor effect on carcinogen-induced murine colonic adenoma formation, explaining the selective advantage of the altered K-*ras* 4A : 4B isoform ratio found in human colorectal cancer.

Keywords: K-*ras*; exon 4A; colon; adenoma; mouse; 1,2-dimethylhydrazine; carcinogen

Introduction

K-*ras* is mutated in 40–50% of human colorectal adenomas and carcinomas, and mutated K-*ras* modulates tumour growth, proliferation, apoptosis, motility, and differentiation [1–9]. We previously demonstrated that wild-type K-*ras* exhibits tumour suppressor activity, as its absence promotes tumourigenesis in murine teratomas [5]. Furthermore, Zhang *et al* [10] showed that wild-type K-*ras* suppresses lung carcinogenesis in mice with an antagonistic effect to mutated K-*ras*, and we confirmed that mutationally activated K-*ras* 4A and 4B isoforms both mediate lung carcinogenesis in mice [11].

The K-*ras* gene encodes two protein isoforms, K-Ras 4A and K-Ras 4B, of 189 and 188 residues, respectively, by alternative splicing of the fourth coding exons 4A and 4B. The isoforms differ at their C-terminus, which is termed the hypervariable region. Post-translational modifications of residues within this

region target Ras proteins to the plasma membrane and are essential for their function. Both isoforms are farnesylated on their C-terminal cysteine residues, but K-Ras 4A is palmitoylated at additional upstream cysteine residues, whereas K-Ras 4B contains a polybasic domain essential for its plasma membrane localization [12]. The post-translational differences between K-Ras 4A and K-Ras 4B affect their membrane localization and there is evidence that this affects how they interact with different subsets of downstream effectors and their pathways. These differences appear to confer functional differences and indeed, the oncogenic isoforms differ in their ability to activate Raf-1 and induce transformed foci, with only K-Ras 4B promoting cell migration [13]. Other studies have shown that the K-Ras isoforms have different functions: only K-Ras 4B promotes expression of matrix metalloproteinase 2 in fibroblasts and has an important role in cardiovascular homeostasis [14,15]. Our previous studies showed that while K-*ras* is essential for mouse development, expression of the K-*ras* 4A splice variant is dispensable [16] and that the K-Ras 4A isoform promotes apoptosis but does not affect either the lifespan or spontaneous tumour incidence in ageing wild-type mice [17]. Similarly, K-Ras 4A proto-oncoprotein did not affect the incidence of adenomas in the small intestine in *Apc*-Min mice that were genetically predisposed to such tumours [18]. However, we showed that K-Ras 4A and 4B are co-expressed widely in human tissues and that the K-Ras 4A : 4B isoform ratio is altered in sporadic human colorectal cancer in favour of reduced K-Ras 4A and/or increased K-Ras 4B [19], and this has been confirmed by others [20].

1,2-Dimethylhydrazine (DMH) and its active metabolite azoxymethane (AOM) are colon-selective carcinogens that induce focal colonic tumours, mostly adenomas but sometimes adenocarcinomas, in susceptible murine strains. The guanine bases in genomic DNA are methylated by AOM, forming O^6-methylguanine adducts, which can either be repaired, trigger apoptosis, or lead to G-to-A mutations [21–24].

Here, we evaluate the role of unmutated K-*ras* 4A in carcinogen-induced adenoma formation in the large intestine. DMH was used to induce colonic tumours in wild-type, heterozygous K-$ras^{tm\Delta 4A/+}$ and homozygous K-$ras^{tm\Delta 4A/tm\Delta 4A}$ mice. Although activating K-*ras* mutations have sometimes been detected in DMH-induced bowel tumours in the rat model [25], they were not detected in colonic adenomas in mouse models investigated here or elsewhere [26–28]. K-$ras^{tm\Delta 4A/tm\Delta 4A}$ mice exhibit normal life expectancy and an overall similar spontaneous tumour incidence to that of wild-type mice [16], indicating that K-$ras^{tm\Delta 4A/tm\Delta 4A}$ mice provide an excellent experimental model to explore the effects of K-*ras* 4A and 4B isoforms on colonic tumourigenesis.

Materials and methods

Mice

The homozygous K-*ras* delta 4A mouse strain (K-$ras^{tm\Delta 4A/tm\Delta 4A}$), with targeted deletion of both K-*ras* exons 4A [16], was crossed with C57BL/6J mice. All mouse breeding and procedures were carried out under Home Office licence. The K-*ras* genotype was determined as previously described [16].

Carcinogen treatments

DMH·2HCl (2.0 mg/ml) was dissolved in 0.90% saline containing 1 mM EDTA and 10 mM sodium citrate, and the pH was adjusted to 6.5 using 0.5 M NaOH. Previously validated DMH treatments consisted of ten weekly injections of 20.0 mg/kg, starting at age 7–8 weeks (cumulative dose = 200.0 mg/kg DMH·2HCl or 145.0 mg/kg DMH) [26–28].

Analysis of intestinal tumours

Mice were inspected daily for signs suggestive of colonic tumour development, at which time they were killed; otherwise they were observed for 1 year following carcinogen treatment and then killed. The small and large intestines were removed, rinsed, opened, and examined. The numbers of intestinal polyps were counted and measured under a dissecting microscope at ×15 magnification, always by the same investigator (FL). Intestinal tumours were divided and ~30–40% was processed for DNA and RNA extraction by standard methods, whereas ~60–70% was formalin-fixed and paraffin-processed for histological examination and immunohistochemistry.

Immunohistochemistry

Immunoperoxidase detection was performed on 4 μm paraffin sections as previously described [29]. The anti-phospho-Erk1/2 primary antibodies (1 : 100 dilution), anti-phospho-Akt primary antibodies, both anti-p-Akt-(Ser473) and anti-p-Akt-(Thr308) (1 : 100 dilution), and rabbit polyclonal anti-cleaved caspase-3 (Asp175) antibody (1 : 200 dilution) (all from Cell Signaling Technology, Inc, Denver, CO, USA) were incubated with sections for 16 h at 4 °C. Biotinylated anti-rabbit IgG (dilution 1 : 200) (Vector Laboratories, Wertheim, Germany) served as a secondary antibody. For evaluation of proliferation, HRP-conjugated mouse monoclonal anti-Ki-67 antibody (1 : 1000 dilution) was incubated for 3 h at 25 °C. Following the washing of sections with PBS, antibody binding was detected using the Vectastain Elite ABC kit (Vector Laboratories Ltd, Peterborough, UK) as previously described [29]. No signal was detected in sections when the primary antibody was omitted as the negative control. Previously analysed colonic adenomas from another mouse model were used as known

positive controls [29]. The prevalence of either apoptosis or proliferating cells in the normal colon and colonic adenomas was measured by counting the proportion of either cleaved caspase-3 or Ki-67 positively stained brown cells per 500 normal or adenoma cells in tissue sections at ×400 magnification under the microscope from five to six age-matched mice of each genotype.

Mutation detection by PCR amplification and DNA sequencing of selected gene sequences

Enriched tumour tissue was obtained by macroscopic dissection of the tumour away from surrounding normal tissue, using tumours over 1.5 mm in diameter. Tissues were digested overnight at 55 °C in 500 µl of DNA lysis buffer, and genomic DNA was extracted by the QIAGEN genomic-tip kit (Qiagen, Crawley, UK) and used to detect mutations by PCR amplification and DNA sequencing of selected gene sequences, including mouse K-*ras* exons 1, 2, and 3; B-*raf* exon 5; and *Apc* exon 15. PCR primers, also used as DNA sequencing primers for standard dideoxy-sequencing (Department of Genetics, University of Cambridge, Cambridge, UK), were as follows: mouse K-*ras* exon 1, sense, 5′CGTCCTTTACAAGCGCACGCAGACT3′, and antisense, 5′CCATGTATTTTATTAAGTGTTGATGA3′; mouse K-*ras* exon 2, sense, 5′AGATCATGCAGGCATAACAATTAG3′, and antisense, 5′CTGTTTTGAATGGGGTCTTCTATT3′; mouse K-*ras* exon 3, sense, 5′GAAATGAAGATCAATGACGAACAC3′, and antisense, 5′GTGAAGACAATTTGGTAGGGTAGAA3′; mouse B-*raf* exon 5, sense, 5′GGCTTACAATGTTATTCCTGTGAGT3′, and antisense, 5′TTTTACCTGAAATCTTCAAAATGCT3′. For analysis of *Apc*, PCR primer sequences for three pairs of PCR primers were designed to cover three overlapping regions of mouse *Apc* exon 15: *Apc*-1 sense, 5′ATGAAATAAAGCAAAACGAGCAAAG3′, and antisense, 5′GCTGACCGAGTTACATCATTCTCTT3′; *Apc*-2 sense, 5′AAGAGAATGATGTAACTCGGTCAGC3′, and antisense, 5′TTTCAATATCATCGTCATCAGAATCA3′; *Apc*-3 sense, 5′TGATTCTGATGACGATGATATTGAAA3′, and antisense, 5′TGTATTCTTTCTCACACGCGTTCTA3′.

Analysis of K-*ras* 4A and 4B transcript relative expression levels by real-time quantitative reverse transcription-polymerase chain reaction

Total RNA (100 ng) from normal lung, normal colon, or colonic adenomas was reverse-transcribed and amplified in 25 µl volume using the iTaq SYBR Green RT-PCR kit run on an iCycler qRT-PCR machine (Bio-Rad, Hemel Hempstead, UK) as described previously [11,18]. All real-time quantitative reverse transcription-polymerase chain reactions (RT-qPCRs) were amplified starting with denaturation at 95 °C for 3 min and then 45 cycles of 95 °C for 15 s and 60 °C for 1 min using exon-spanning PCR primers for mouse K-*ras* 4A, K-*ras* 4B, and *β-actin*. RT-qPCRs were performed in triplicate and the average relative expression levels normalized against *β-actin* levels were calculated as described previously [11,18].

Statistical analysis

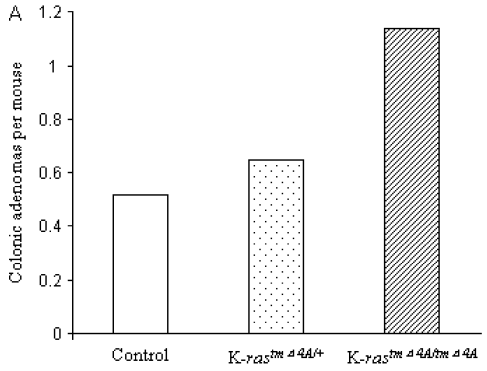
Mean tumour incidences, mean tumour volumes, mean proportions of Ki-67-positive and cleaved caspase-3-positive cells, and K-*ras* 4B transcript levels of tissues from different animal groups were compared using the unpaired two-tailed Student's *t*-test.

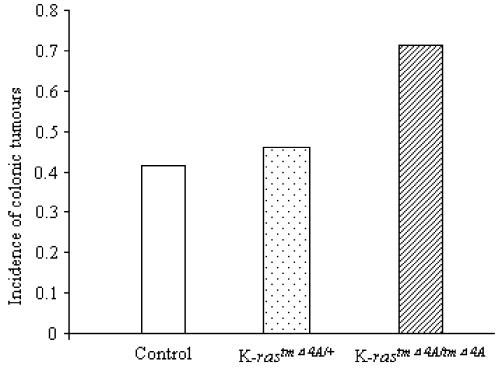
Results

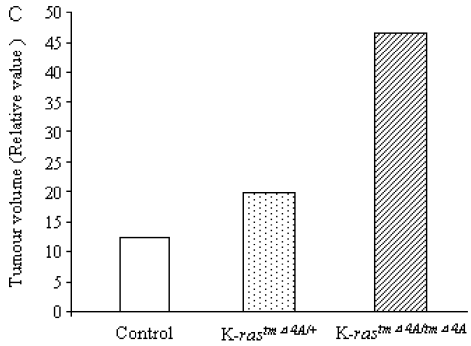
K-*ras* 4A deficiency increases the number and volume of DMH-induced colonic adenomas not bearing K-*ras* mutations

To assess the effects of K-*ras* 4A deficiency on large intestinal adenoma formation, 25 wild-type (control), 26 heterozygous K-$ras^{tm\Delta 4A/+}$, and 21 homozygous K-$ras^{tm\Delta 4A/tm\Delta 4A}$ mice were given ten weekly intraperitoneal injections of DMH. Their survival over 1 year was monitored, with the culling of mice that became ill with intestinal tumours. The remaining mice were then killed at 53 weeks after the start of the DMH treatment and all mouse small and large intestines were carefully examined. Most of the intestinal tumours were present in the distal colon and rectum, with very few in the small intestines, so only colorectal tumours were statistically analysed. The tumours were shown histopathologically to be adenomas, with three adenomas progressing to invasive adenocarcinomas in K-$ras^{tm\Delta 4A/tm\Delta 4A}$ mice (Figure 2D). The colonic tumour number increased significantly from 0.52 ± 0.77 adenomas per colon (mean ± standard deviation) in the wild-type control mice to 1.14 ± 0.96 adenomas per colon in the K-$ras^{tm\Delta 4A/tm\Delta 4A}$ mice ($p < 0.01$), but there was no significant change in the number of colonic adenomas (0.65 ± 0.87) in the heterozygous K-$ras^{tm\Delta 4A/+}$ mice compared with wild-type mice ($p = 0.28$) (Figure 1A).

The proportion of mice in each group that developed colonic tumours, termed the incidence of colonic tumours, was significantly higher (71.4%) in K-$ras^{tm\Delta 4A/tm\Delta 4A}$ mice than that observed in the control group (44.0%) and K-$ras^{tm\Delta 4A/+}$ mice (46.2%) ($p = 0.02$ for both) (Figure 1B). The relative tumour volume increased significantly from 1.0 in control mice to 3.84-fold in K-$ras^{tm\Delta 4A/tm\Delta 4A}$ mice, as opposed to 1.64-fold in K-$ras^{tm\Delta 4A/+}$ mice ($p < 0.01$ and $p < 0.04$, respectively) (Figure 1C). There were no significant differences in tumour volume between male and female animals ($p > 0.05$). The pattern of survival of the three groups of mice over a period of 53 weeks showed a decrease from 88.0% in controls to 84.6% in K-$ras^{tm\Delta 4A/+}$ and 80.9% in K-$ras^{tm\Delta 4A/tm\Delta 4A}$ mice, but as the mice were killed at the 1 year time point, statistical testing of







D

Mouse survival after starting DMH treatment

Weeks	0	23	30	36	40	46
Control	25	25 (100%)	24 (96.0%)	23 (92.0%)	22 (88.0%)	22 (88.0%)
K-*ras*$^{tm\Delta4A/+}$	26	25 (100%)	25(100.0%)	23 (88.5%)	22 (84.6%)	22 (84.6%)
K-*ras*$^{tm\Delta4A/tm\Delta4A}$	21	20 (95.2%)	20 (95.2%)	19 (90.4%)	18 (85.7%)	17 (80.9%)

Figure 1. Differences in the number and volume of colonic adenomas and the survival of DMH-treated K-*ras*$^{tm\Delta4A/+}$ and K-*ras*$^{tm\Delta4A/tm\Delta4A}$ mice compared with control wild-type mice. (A) Mean numbers of large intestinal adenomas per mouse in DMH-treated control mice ($n = 25$), K-*ras*$^{tm\Delta4A/+}$ mice ($n = 26$), and K-*ras*$^{tm\Delta4A/tm\Delta4A}$ mice ($n = 21$). (B) Incidence of adenomas in the large intestine in DMH-treated control mice (11/25 mice), K-*ras*$^{tm\Delta4A/+}$ mice (12/26 mice), and K-*ras*$^{tm\Delta4A/tm\Delta4A}$ mice (15/21 mice). (C) Relative values of the tumour volume in DMH-treated control mice ($n = 25$), K-*ras*$^{tm\Delta4A/+}$ mice ($n = 26$), and K-*ras*$^{tm\Delta4A/tm\Delta4A}$ mice ($n = 21$). (D) Survival of DMH-treated control, K-*ras*$^{tm\Delta4A/+}$ and K-*ras*$^{tm\Delta4A/tm\Delta4A}$ mice, given as the proportions of mice surviving at various time periods in weeks, up to 1 year after starting DMH treatment

natural survival by log-rank analysis was not possible (Figure 1D).

Although all of the tumours in the three groups were intestinal adenomas that were moderately dysplastic, three adenomas showed progression to adenocarcinomas in K-*ras*$^{tm\Delta4A/tm\Delta4A}$ mice, whereas no adenocarcinomas were detected in the other two groups (Figure 2); however, such small numbers of adenocarcinomas preclude statistical analysis. Thirty tumours were analysed, ten adenomas from each group of mice, for the presence of mutations in K-*ras* exons 1, 2, and 3, and also in B-*raf* exon 5, but no mutations were found in either gene in any of the tumours tested. However, *Apc* exon 15 mutations were identified in three of ten adenomas from wild-type mice, four of ten adenomas from K-*ras*$^{tm\Delta4A/+}$ mice and three of ten adenomas from K-*ras*$^{tm\Delta4A/tm\Delta4A}$ mice.

Carcinogen-induced colonic adenomas in K-*ras* 4A-deficient mice show increased proliferation and decreased apoptosis

To assess whether K-*ras* 4A deficiency affects adenomatous cell proliferation and apoptosis, adenomas were immunohistochemically analysed for Ki-67 expression and positivity for cleaved caspase-3 using age-matched colonic adenomas from the three groups of mice (Figure 3). The proportion (in 500 cells counted from five to six mice) of Ki-67-positive cells in adenomas from K-*ras*$^{tm\Delta4A/tm\Delta4A}$ mice ($12.5 \pm 2.8\%$) was significantly higher than that in adenomas from wild-type mice ($7.59 \pm 1.1\%$, $p < 0.05$) and K-*ras*$^{tm\Delta4A/+}$ mice ($8.5 \pm 1.8\%$, $p < 0.05$), but the proportion of Ki-67-positive cells in adenomas from K-*ras*$^{tm\Delta4A/+}$ mice was not significantly different from that in adenomas from wild-type mice ($p > 0.05$). The proportion (in 500 cells counted from five to six mice) of adenoma nuclei that stained positively for cleaved caspase-3 varied, with adenomas from wild-type mice showing more cleaved caspase-3-positive apoptotic cells ($1.4 \pm 0.8\%$) than adenomas from K-*ras*$^{tm\Delta4A/+}$ mice ($1.1 \pm 0.8\%$, $p < 0.05$) and adenomas from K-*ras*$^{tm\Delta4A/tm\Delta4A}$ mice ($0.4 \pm 0.4\%$, $p < 0.05$), but the difference in apoptosis in adenomas between K-*ras*$^{tm\Delta4A/+}$ and K-*ras*$^{tm\Delta4A/tm\Delta4A}$ mice, although showing a trend towards significance, did not reach statistical significance ($p = 0.06$).

Carcinogen-induced colonic adenomas in K-*ras* 4A-deficient mice show evidence of activation of the MapKinase and Akt pathways

Activation of Erk to phospho-Erk (p-Erk) was determined by immunostaining the adenomas with anti-phospho-Erk1/2 antibody, as Erk activity is directly regulated by phosphorylation of the activation loop,

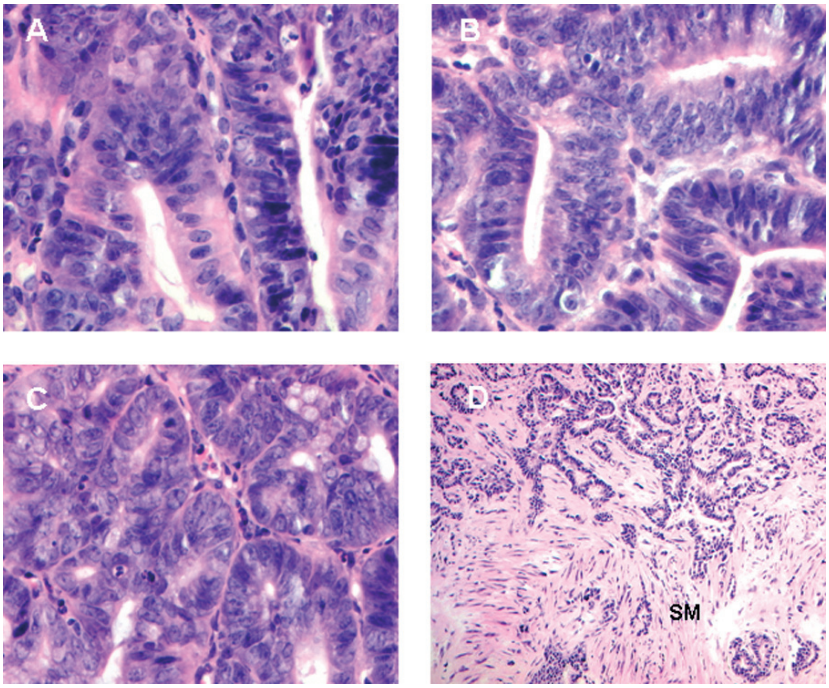


Figure 2. Histopathological appearances of the large intestinal tumours in DMH-treated wild-type, K-*ras*$^{tm\Delta 4A/+}$, and K-*ras*$^{tm\Delta 4A/tm\Delta 4A}$ mice. (A–C) Moderately dysplastic adenomas showing no invasion (original magnification ×400) in a wild-type mouse (A), in a K-*ras*$^{tm\Delta 4A/+}$ mouse (B), and in a K-*ras*$^{tm\Delta 4A/tm\Delta 4A}$ mouse (C). (D) Moderately differentiated colonic adenocarcinoma in a K-*ras*$^{tm\Delta 4A/tm\Delta 4A}$ mouse (original magnification ×100), showing invasion into the smooth muscle (SM) of the muscularis propria

which is recognized by the anti-phospho-Erk1/2 antibody. There was an inverse correlation between K-*ras* 4A transcript expression and p-Erk1/2 immunoreactivity, with increased p-Erk1/2 staining, in terms of both nuclear and cytoplasmic intensity and the proportion of adenoma cells staining positively, in adenomas from K-*ras*$^{tm\Delta 4A/tm\Delta 4A}$ mice compared with adenomas from either K-*ras*$^{tm\Delta 4A/+}$ mice or wild-type mice (Figures 4A–4C). Evaluation of Akt activation in adenomas from K-*ras*$^{tm\Delta 4A/tm\Delta 4A}$ mice showed a widespread increase in cytoplasmic staining for phosphorylated Akt at Thr-308 by anti-p-Akt-Thr308 immunohistochemistry, compared with adenomas from either K-*ras*$^{tm\Delta 4A/+}$ mice or wild-type mice, which showed occasional nuclear positivity but little or no cytoplasmic positivity (Figures 4D–4F). The antibody against p-Akt-Ser473 showed similar changes to the antibody against p-Akt-Thr308 (data not shown). There were no obvious differences in either p-Akt-Thr308 or p-Akt-Ser473 staining when adenomas from wild-type mice were compared with those from K-*ras*$^{tm\Delta 4A/+}$ mice.

Increased expression of K-*ras* 4B transcripts in carcinogen-induced adenomas of K-*ras* 4A-deficient mice

To explore the effects of heterozygous and homozygous K-*ras* 4A deficiency on the expression levels of K-*ras* 4B transcripts in the normal colon (with normal lung as a comparator) and colonic adenomas of these mice, real-time reverse transcription-quantitative PCR (real-time RT-qPCR) was used to analyse the relative expression levels of both K-*ras* 4A and K-*ras* 4B transcripts normalized against *β-actin* RNA levels. In normal untreated lung tissue, the K-*ras* 4A : 4B splice variant ratio was about 1 : 10, whereas in normal colon, the ratio was almost 1 : 1 (Figure 5A), suggesting that the K-*ras* 4A isoform may play a more prominent role in the colon, potentially including the development of colon adenomas or cancers relative to lung cancers. K-*ras* 4A transcript expression levels in normal untreated colons of K-*ras*$^{tm\Delta 4A/+}$ mice were found to be reduced to less than half of those in wild-type mouse normal colons, whereas levels were undetectable in K-*ras*$^{tm\Delta 4A/tm\Delta 4A}$ mice as expected (Figure 5A). Following DMH treatment, the relative expression levels of K-*ras* 4B transcripts increased from 17.3 ± 3.5 (mean ± SD) in normal colon to 57.0 ± 31.3 (3.29-fold increase) in adenomas from wild-type mice ($p < 0.01$) (Figure 5B). In DMH-induced colonic adenomas, the expression of K-ras 4B transcripts increased from 57.0 ± 31.3 in wild-type to 88.6 ± 23.4 in K-*ras*$^{tm\Delta 4A/+}$ and to 134 ± 54.8 in K-*ras*$^{tm\Delta 4A/tm\Delta 4A}$ mouse adenomas, respectively (both comparisons were significantly different with $p < 0.05$, Figure 5B).

Discussion

The effects of Ras proteins on the regulation of apoptosis, proliferation, and tumour formation are complex as they vary between Ras family members and with

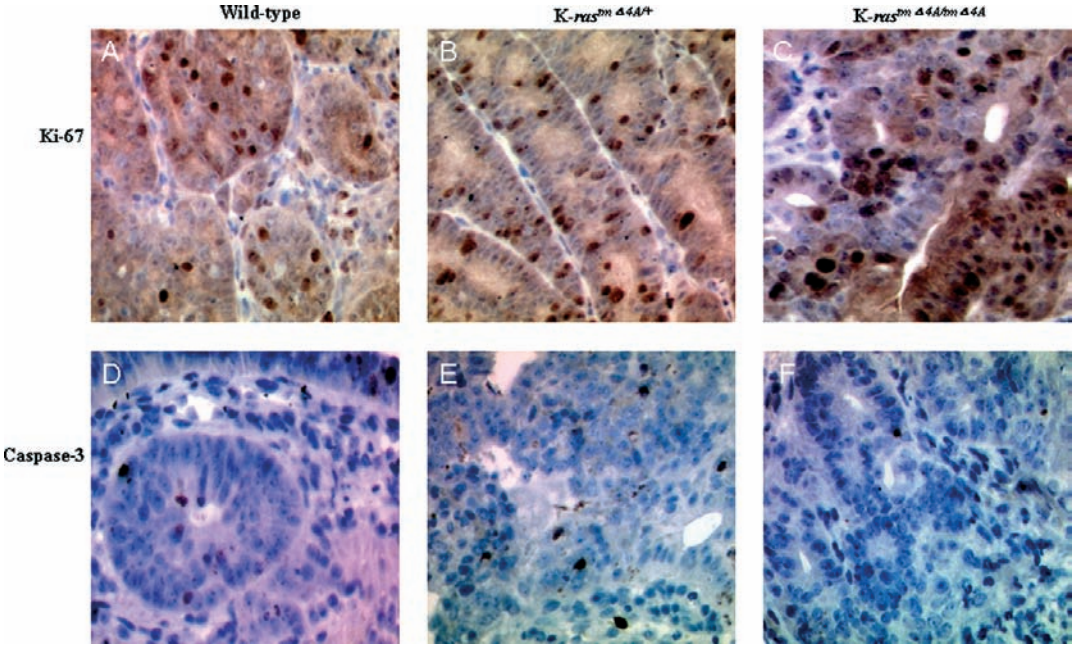


Figure 3. Immunohistochemical staining of the proliferation marker Ki-67 and the apoptosis marker cleaved caspase-3 in colonic adenomas from age-matched wild-type, K-*ras*$^{tm\Delta 4A/+}$, and K-*ras*$^{tm\Delta 4A/tm\Delta 4A}$ mice (all at original magnification ×400). Ki-67 expression in a colonic adenoma from a wild-type mouse (A), a K-*ras*$^{tm\Delta 4A/+}$ mouse (B), and a K-*ras*$^{tm\Delta 4A/tm\Delta 4A}$ mouse (C). Cleaved caspase-3 detection in a colonic adenoma from a wild-type mouse (D), a K-*ras*$^{tm\Delta 4A/+}$ mouse (E), and a K-*ras*$^{tm\Delta 4A/tm\Delta 4A}$ mouse (F)

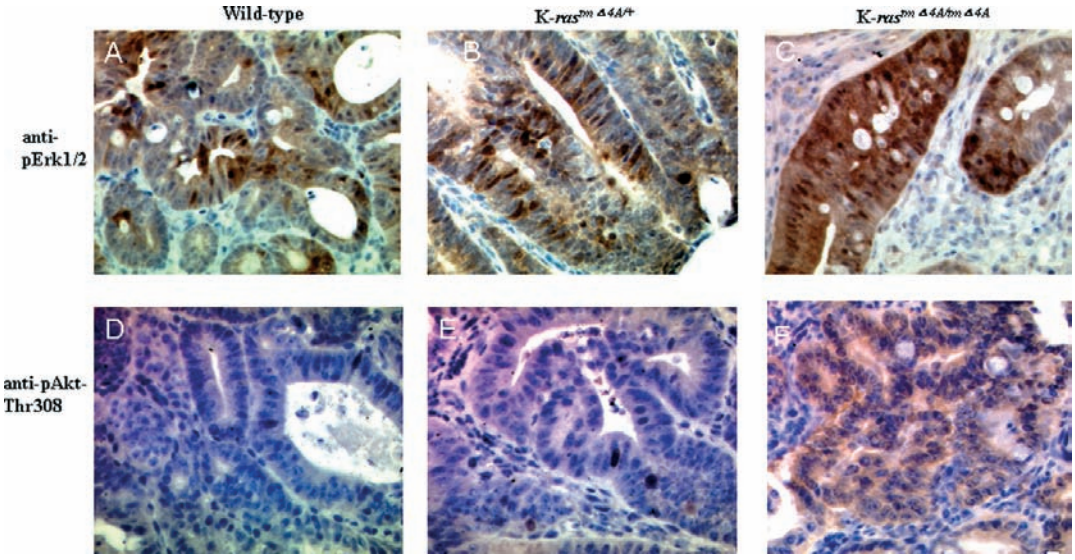


Figure 4. Immunohistochemical staining of phospho-Erk1/2 (A–C, original magnification ×400) and phospho-Akt-Thr308 (D–F, original magnifications ×400, ×400, and ×200, respectively) in colonic adenomas from age-matched wild-type (A, D), K-*ras*$^{tm\Delta 4A/+}$ (B, E), and K-*ras*$^{tm\Delta 4A/tm\Delta 4A}$ mice (C, F)

their mutational status, expression levels, and the isoforms expressed [1–9,11,16–19,29,30]. The C57BL/6 mice used in this study do not usually develop spontaneous colonic tumours but have previously been shown to develop moderate numbers of colonic adenomas following DMH treatment [28]. The present study found that K-*ras* 4A deficiency promotes DMH-induced colonic tumourigenesis *in vivo*, with significant increases in both the number and the tumour volume of DMH-induced colonic adenomas, despite the finding that these adenomas do not bear activating K-*ras* mutations but do have increased expression levels of K-*ras* 4B transcripts. DMH-treated homozygous K-*ras*$^{tm\Delta 4A/tm\Delta 4A}$ mice formed adenomas almost entirely in their large intestines and these showed significantly reduced apoptosis and increased proliferation compared with adenomas from wild-type mice, indicating that the absence of K-*ras* 4A, together with increased K-*ras*4B expression, exerts an anti-apoptotic effect but a pro-proliferative effect *in vivo*, consistent with previously published data showing that K-Ras 4A and 4B proteins exhibit pro- and anti-apoptotic actions, respectively, but anti- and pro-proliferative effects, respectively [11,17,18]. Since K-Ras 4A and

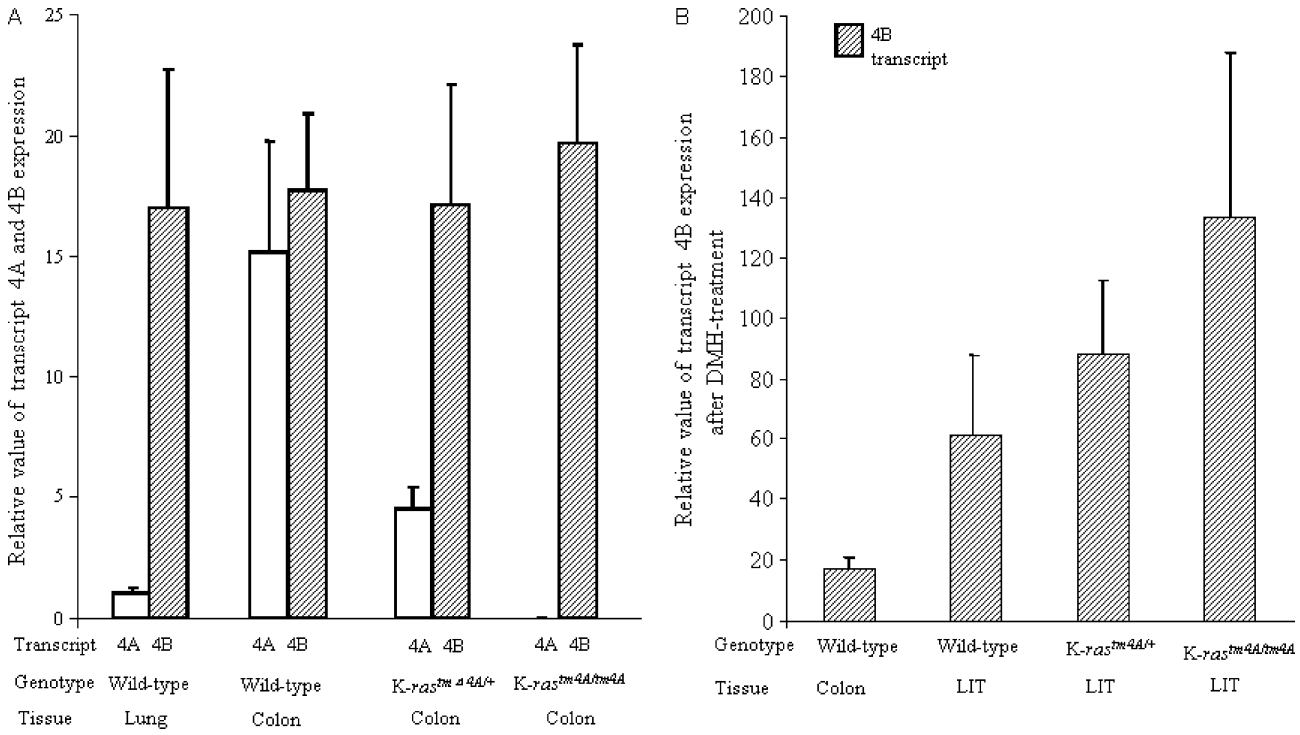


Figure 5. Real-time reverse transcription-quantitative PCR analysis (real-time RT-qPCR) showing the relative expression levels of K-*ras* 4A and 4B transcripts. (A) Real-time RT-qPCR analysis of K-*ras* 4A and 4B transcripts in the normal lung tissue from wild-type mice and normal colons from age-matched wild-type, K-$ras^{tm\Delta 4A/+}$, and K-$ras^{tm\Delta 4A/tm\Delta 4A}$ mice (mean ± SEM). (B) Real-time RT-qPCR analysis showing the relative expression levels of K-*ras* 4B transcripts in the normal colons from wild-type mice and large intestinal tumours (LIT — all adenomas) from age-matched wild-type, K-$ras^{tm\Delta 4A/+}$, and K-$ras^{tm\Delta 4A/tm\Delta 4A}$ mice following DMH treatment (mean ± SEM)

4B oncoproteins affect Raf-1 differentially, activation of different signalling pathways may explain these effects [4,13]. Thus, the effects of K-*ras* on apoptosis and proliferation may depend ultimately on the overall K-*ras* 4A : 4B isoform balance and, consequently, the levels of activation of effector pathways, as suggested for other cancer-related genes [31].

Here, we showed that deletion of K-ras exon 4A, leading to loss of expression of K-ras 4A transcripts, increased the relative expression levels of K-ras 4B transcripts in colonic adenomas and this may have contributed to the promotion of DMH-induced colonic tumourigenesis. The effects of such increased expression levels of K-ras 4B on tumourigenesis are likely to relate mainly to activation of the two major Ras effector pathways, the MapKinase and the Akt signalling pathways. PI3K/Akt/Rac signalling has an anti-apoptotic effect, whereas Raf/Mek/Erk signalling can have either anti-apoptotic or pro-apoptotic effects depending on context [4]. Although there was widespread increased cytoplasmic positivity for phospho-Akt immunostaining in adenoma cells, this pathway showed less evidence of activation than the MapKinase pathway by virtue of phospho-Erk1/2 positivity. In the present study, K-ras 4A transcript expression showed an inverse relationship with activation of Erk1/2 and Akt to their phosphorylated forms. Activation of Erk1/2 in the MapKinase signalling pathway can promote cell proliferation and inhibit cell apoptosis, consistent with the effects shown here.

Approximately 30–40% of adenomas tested from all three groups of mice showed *Apc* exon 15 mutations, suggesting that *Wnt* pathway activation is involved to some extent in DMH-induced colonic adenoma formation, in keeping with previous studies [32]. Increased accumulation and nuclear localization of β-catenin represent evidence of *Wnt* pathway activation and this was shown to occur in DMH-induced colonic adenomas in this mouse model (data not shown). This interaction of K-*ras* 4A deficiency and *Wnt* signalling in DMH-induced colonic adenomas was not identified in a previous study of ours that showed that K-*ras* 4A deficiency did not affect genetically predisposed intestinal adenoma formation in the *Apc*-Min mouse [18], which may reflect the different mechanisms of adenoma formation in these two models.

The K-*ras* 4A : 4B splice variant ratio has been shown to be altered (reduced 4A and/or increased 4B) in both human colon cancer cell lines and sporadic human colorectal cancers [19,20,33]. Therefore the findings presented here, that K-*ras* 4A and 4B promote and inhibit apoptosis, respectively, and that K-*ras* 4A deficiency increases tumour cell proliferation and promotes colonic tumourigenesis, provide a mechanistic explanation for the altered K-*ras* 4A : 4B isoform ratio found in colorectal cancers that favours K-*ras* 4B, as this would contribute to tumourigenesis of the colon, at least in part, by increased proliferation and decreased apoptosis.

We conclude that deletion of K-*ras* exon 4A promotes both increased number and size of colonic adenomas that do not bear K-*ras* mutations, following treatment with the bowel-selective carcinogen DMH. These adenomas showed higher relative levels of K-*ras* 4B expression with immunohistochemical evidence of increased activation of the MapKinase and Akt pathways, together with increased adenoma cell proliferation and decreased apoptosis. Thus, expression (from one or two gene copies) of K-*ras* 4A has a tumour suppressor effect on carcinogen-induced adenoma formation of the colon. This may explain, at least in part, the selective growth advantage conferred by the observed reduction in K-*ras* 4A expression relative to K-*ras* 4B expression in human colorectal cancers, including some that lack K-*ras* mutations [19,33]. A greater understanding of the role(s) played by each K-*ras* isoform in colonic tumourigenesis, including the signal transduction pathways affected, may be important in the development of more effective anti-*ras* therapeutic approaches to cancer treatment, which include the use of drugs that target *ras* isoform-specific post-translational modifications and the use of antisense oligonucleotides to modulate alternative K-*ras* splicing [34].

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