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ORIGINAL ARTICLE

Wild-type *K-ras* has a tumour suppressor effect on carcinogen-induced murine colorectal adenoma formation

Feijun Luo*, George Poulogiannis*,†, Hongtao Ye*,‡, Rifat Hamoudi*, Gehong Dong*, Wenyan Zhang*, Ashraf E. K. Ibrahim* and Mark J. Arends*,§

*Department of Pathology, University of Cambridge, Addenbrooke's Hospital, Cambridge, UK, †Division of Signal Transduction, Beth Israel Deaconess Medical Center and Department of Systems Biology, Harvard Medical School, Boston, MA, USA, ‡Department of Histopathology, Institute of Orthopaedics and Musculoskeletal Science, Royal National Orthopaedic Hospital NHS Trust, University College London, Stanmore, Middlesex, UK and §Division of Pathology, University of Edinburgh, Institute of Genetics & Molecular Medicine, Western General Hospital, Edinburgh, UK

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Correspondence
Dr Mark J. Arends
Division of Pathology
University of Edinburgh
Institute of Genetics & Molecular Medicine, Western General Hospital, Crewe Road Edinburgh EH4 2XR
UK
Tel.: 0131 651 8019
Fax: 0131 777 3520
E-mails: M.Arends@ed.ac.uk or mja40@cam.ac.uk

SUMMARY

K-ras mutations are found in ~40% of human colorectal adenomas and carcinomas and contribute to colorectal tumour formation at an early stage. Wild-type *K-ras* has been reported to be deleted in some tumours, but the consequences of changes in wild-type *K-ras* copy number for experimental colorectal carcinogenesis have not been investigated. To characterize the effects of K-*ras* copy number changes on formation of carcinogen-induced colorectal neoplasms in mice, wild-type (K-$ras^{+/+}$) and heterozygous K-*ras* exon 1 knockout (K-$ras^{+/-}$) mice were given 10 weekly treatments of 1, 2-dimethylhydrazine (DMH) to induce colorectal tumours. Colorectal expression levels of K-*ras* 4A and 4B transcripts in K-$ras^{+/-}$ mice were ~50% decreased compared with K-$ras^{+/+}$ mice. One year after DMH treatment, survival of K-$ras^{+/-}$ mice decreased from 88 to 82% compared with wild-type mice. Colorectal adenomas significantly increased from 0.52 ± 0.15 in K-$ras^{+/+}$ mice to 0.87 ± 0.14 in K-$ras^{+/-}$ mice (mean ± SEM per mouse, $P < 0.01$); total tumour volume increased 2.13-fold ($P < 0.05$). Comparing K-$ras^{+/+}$ with K-$ras^{+/-}$ murine adenomas, Ki-67-positive proliferating tumour cells significantly increased from 7.77 ± 0.64% to 9.15 ± 0.92% and cleaved caspase-3-positive apoptotic tumour cells decreased from 1.40 ± 0.37% to 0.80 ± 0.22% (mean ± SEM, $P < 0.05$ for both). No K-*ras* or B-*raf* mutations were detected in the adenomas. Immunohistochemical studies showed no significant changes in extracellular signal regulating kinase/mitogen-activated protein kinase (Erk/MapK) or PI3K/Akt pathway activation in the adenomas. In conclusion, the data collectively show that a 50% reduction in K-*ras* gene dosage and RNA expression promoted experimental colorectal tumourigenesis, consistent with wild-type K-*ras* having a tumour suppressor effect on carcinogen-induced murine colorectal adenoma formation.

Keywords
1, 2-dimethylhydrazine, adenoma, carcinogen, colorectal, K-*ras*, mouse, wild-type

K-Ras protein is critical in the regulation of cellular proliferation, apoptosis and differentiation, and is mutated in ~30% of all human tumours and ~40% colorectal adenomas and carcinomas (Malumbres & Barbacid 2003; Arends *et al.* 1993, 1994; Luo *et al.* 2011). Once activated by ligand-mediated extracellular stimuli, normal K-Ras protein activates multiple downstream pathways, including the Map-Kinase pathway (Raf, Mek, Erk), the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, the Ral GDP dissociation stimulator (RalGDS) pathway and others (Olson & Marais 2000; Markman *et al.* 2010; Campbell *et al.* 1998). *K-ras* activating mutations render K-Ras an oncoprotein that is constitutively active (locked in the GTP-bound form), leading to deregulated activation of Ras protein functions (Malumbres

& Barbacid 2003). The oncogenic alleles of *Ras* genes have been referred to as dominant alleles, because they demonstrate transforming ability in the presence of normal allele expression (Malumbres & Barbacid 2003; Marshall 1991), but this dogma has been challenged (Diaz *et al.* 2005).

Spandidos and colleagues observed that the normal H-Ras protein could have some suppressive role over the mutated H-Ras protein (Spandidos *et al.* 1990). Our previous studies showed that expression of wild-type *K-ras* or mutant *K-ras* induce some overlapping and some different changes to the gene expression profiles in embryonic stem cells, including changes in expression of proliferation-, apoptosis- and differentiation-related genes (Luo *et al.* 2007a,b). We also demonstrated that murine wild-type K-*ras* exhibits tumour suppressor activity, as its absence promotes tumourigenesis in murine teratomas (James *et al.* 2003). Further, Zhang and colleagues showed that wild-type K-*ras* has tumour suppressor activity and is frequently lost during lung tumour progression (Zhang *et al.* 2001). Wild-type *K-ras* has been reported to be deleted in some human cancers, but so far, the role of reductions in copy number of wild-type *K-ras* in colorectal carcinogenesis has not been adequately investigated; hence, we studied this using a mouse model of carcinogen-induced colorectal tumour formation.

The colon-selective carcinogen 1, 2-dimethylhydrazine (DMH) has been widely used to study chemically induced colorectal tumours. DMH alkylates DNA, producing O^6-methylguanine (O^6-MeG), which induces GC→AT transitions that may be found in various genes linked to colorectal cancer (Jackson *et al.* 1997). DMH treatment results in high frequency mutation of K-*ras* in colorectal adenomas in the rat model (Jacoby *et al.* 1991), but for DMH-treated mice, K-*ras* mutations in adenomas are much less common or rare, although DMH is known to induce intestinal adenoma formation by either activating *Beta-Catenin* mutations or inactivating *Apc* mutations (Jackson *et al.* 1997; Deschner & Long 1977; Deschner *et al.* 1979; Diwan *et al.* 1977). DMH tends to induce more tumours in the distal colon and rectum with similar histopathological appearances to those observed in human sporadic colorectal tumours. DMH is metabolized to azoxymethane (AOM), which has also been used to similar effect in rodent models. Hence, treatment of rodents with these carcinogens provides a useful bioassay to assess colorectal tumour susceptibility in different murine strains, including those with gene knockouts that may influence rates of proliferation or apoptosis.

Mice lacking both K-*ras* alleles are not viable and die between 12 and 14 days of gestation due to foetal liver defects and anaemia (Johnson *et al.* 1997); therefore, they could not be tested. Here, we evaluated the effects of 2 or 1 alleles of wild-type K-*ras* on carcinogen-induced adenoma formation in the large intestine using K-*ras*$^{+/+}$ mice and K-*ras*$^{+/-}$ mice. K-*ras*$^{+/-}$ mice exhibit normal life expectancy and an overall similar spontaneous tumour incidence to wild-type mice (Johnson *et al.* 1997), indicating that K-*ras*$^{+/-}$ mice provide an excellent experimental model to explore the gene dosage effects of wild-type K-*ras* on colorectal tumourigenesis.

Materials and methods

Mice

The 129/Sv-K-*ras*$^{+/-}$ mice were previously acquired (Johnson *et al.* 1997; Luo *et al.* 2010; Patek *et al.* 2008). The heterozygous K-*ras* strain of mice (K-*ras*$^{+/-}$) has a deleted exon 1 of the K-*ras* gene, and this strain was crossed with C57BL/6J mice for >7 generations of crossing, to more than 99% C57BL/6J background. The K-*ras* genotype was determined by PCR assay as previously described (Johnson *et al.* 1997; Luo *et al.* 2010). K-*ras*$^{+/+}$ and K-*ras*$^{+/-}$ siblings from the same matings were used for carcinogen treatments.

Ethical approval

The animal model work was approved by the local ethical review process and was carried out under Home Office licence.

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 adjusted to 6.5 using 0.5 M NaOH. Previously validated DMH treatments consisted of 10 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) (Deschner *et al.* 1979; Luo *et al.* 2010).

Analysis of intestinal tumours

Mice were inspected daily for signs suggestive of colorectal tumour development at which time they were killed; otherwise they were observed for one year following carcinogen treatment and then killed. The small and large intestines were removed, 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% processed for DNA and RNA extraction by standard methods, whereas ~60–70% was formalin-fixed and paraffin-processed for histological examination and immunohistochemistry, as previously described using antibodies to Ki-67, cleaved caspase-3, phospho-Erk and phospho-Akt (Luo *et al.* 2009, 2010, 2011).

*Analysis of K-*ras *and other transcript expression levels*

Total RNA (100 ng) from large intestinal tumours or normal tissues from 10 wild-type and 10 K-*ras*$^{+/-}$ mice were reverse transcribed and amplified in 25 μl volume using the iTaq SYBR Green RT-PCR kit run on an iCycler RT-qPCR machine (Bio-Rad, Hemel Hempstead, UK) as described previously (Patek *et al.* 2008; Luo *et al.* 2009). All real-time reverse transcription quantitative polymerase chain reactions (RT-qPCR) were amplified starting with denaturation at 95 °C for 3 min 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, (as previously described; Patek *et al.* 2008; Luo *et al.* 2009), as well as for *pRB*, *p107/RBL1*, *p130/RBL2*, *cyclin D1*, c-*myc* and β-*actin* using the following primers: *p107/RBL1* sense primer: 5′ GGA GTG CTA AGA GGA GAC TGT TTG G 3′, *p107/RBL1* anti-sense primer: 5′ ATA GGA ACC AGT GTG ATT TCT CCA G 3′; *p130/RBL2* sense primer: 5′ GAC TAG CTC CTT AGC GCT CTT CTT T 3′, *p130/RBL2* anti-sense primer: 5′ AAG TTC GGT GCA CTG GAT TAT AGA G 3′; *cyclin* D1 sense primer: 5′ TTT CTT TCC AGA GTC ATC AAG TGT G 3′, *cyclin* D1 anti-sense primer: 5′ ACC AGC CTC TTC CTC CAC TTC 3′; *c-myc* sense primer: 5′ AAA TCC TGT ACC TCG TCC GAT TC 3′, *c-myc* anti-sense primer: 5′ ATC AAT TTC TTC CTC ATC TTC TTG C 3′; *pRB* sense primer: 5′ TTT TCT AGT TCA CCC TTA CGG ATT C 3′, *pRB* anti-sense primer: 5′ ATT TTC TGG AAC TTT TCA GAT GTC C 3′. RT-qPCRs were performed in triplicate, and the average relative expression levels normalized against β-*actin* levels were calculated as described previously (Patek *et al.* 2008; Luo *et al.* 2009).

Bioinformatic analysis

The expression microarray data of the Sanger Cell Line Project (SCLP) (n = 732, the cancer cell lines with mutation data of the common tumour suppressor genes and oncogenes) were downloaded from the Broad Institute server (www.broadinstitute.org/), and a Pearson's correlation coefficient analysis was performed to correlate mRNA levels of *KRAS* with those of *p107/RBL1* and *p130/RBL2* genes in cancer cell lines without (n = 648) or with (n = 84) *KRAS* point mutations. The P-values were computed using an asymptotic confidence interval based on Fisher's Z transform, and the samples were clustered using the Euclidean distance metric and the complete linkage algorithm. Mean tumour numbers, incidence and tumour volumes for the different animal groups were compared using the unpaired two-tailed Student's t-test.

Results

*Reduction from two alleles to one K-*ras *allele decreases expression levels of K-*ras *transcripts in the large intestine*

To explore the effects of K-*ras* hemizygosity on the expression levels of K-*ras* transcripts in the normal colons of the K-$ras^{+/-}$ mice, real-time reverse transcription quantitative PCR (real-time RT-qPCR) was used to analyse relative expression levels of both K-*ras* 4A and 4B isoform transcripts normalized against β-actin RNA levels. The relative expression levels of K-*ras* 4A transcripts decreased from 15.2 ± 1.9 (mean±SEM) in colons of wild-type mice to 7.1 ± 1.3 in colons from K-$ras^{+/-}$ mice ($P < 0.01$) (Figure 1). The relative expression levels of K-*ras* 4B transcripts decreased from 17.6 ± 2.0 (mean ± SEM) in colons of wild-type mice to 8.7 ± 1.6 in colons from K-$ras^{+/-}$ mice ($P < 0.01$) (Figure 1). K-*ras* 4A and 4B transcript expression levels were found to be reduced to approximately half in colons of K-$ras^{+/-}$ mice, consistent with the expected gene dosage effect.

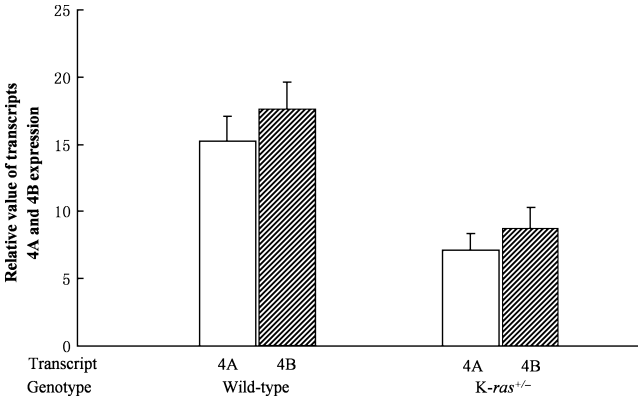
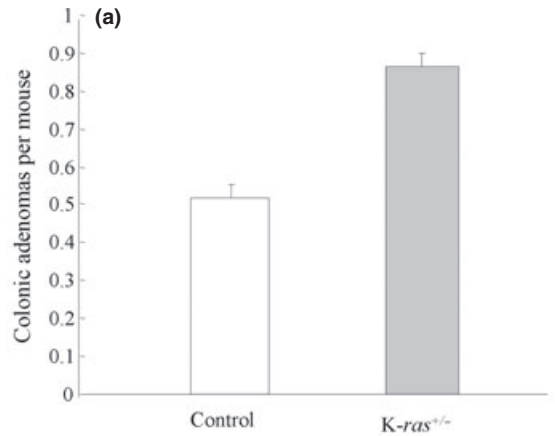
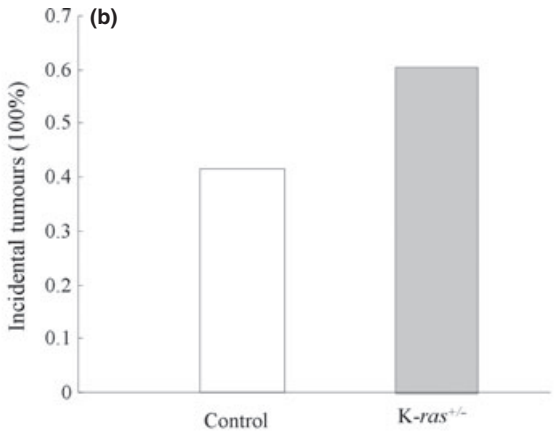


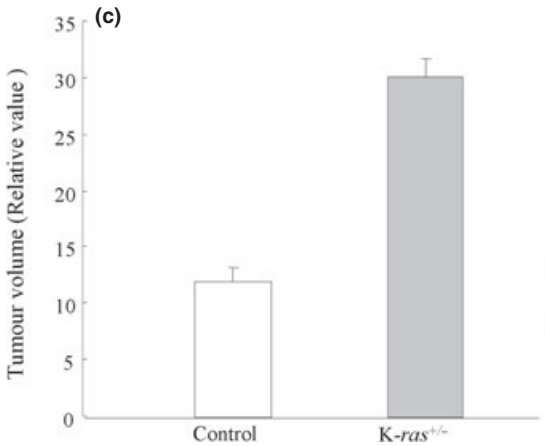
Figure 1 Relative expression levels of K-*ras* 4A and 4B transcripts in the normal colonic tissues from control wild-type K-$ras^{+/+}$ and K-$ras^{+/-}$ mice by real-time RT-qPCR (mean ± SEM). The relative expression levels of both K-*ras* 4A and 4B transcripts were significantly decreased in colons of K-$ras^{+/-}$ *vs.* wild-type mice ($P < 0.01$).

*K-*ras *hemizygosity promotes DMH-induced colorectal tumourigenesis in mice*

To assess the effects of K-*ras* hemizygosity on large intestinal tumour formation, 25 wild-type (control K-$ras^{+/+}$) and 28 hemizygous K-$ras^{+/-}$ mice were given 10 weekly intraperitoneal injections of DMH. Survival over one year was monitored with culling of mice that showed signs of intestinal tumour formation, and the remaining mice were killed at 53 weeks after the start of the DMH treatment. All murine small and large intestines were carefully examined for tumours. 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, all of which displayed low-grade dysplasia with no differences between the two cohorts, with no invasive adenocarcinomas found in either cohort. The average colorectal tumour numbers increased from 0.52 ± 0.15 adenomas/mouse (mean ± SEM) in the control K-$ras^{+/+}$ mice to 0.87 ± 0.14 adenomas/mouse in the K-$ras^{+/-}$ mice ($P < 0.01$) (Figure 2a). Proportions of mice in each group that developed colorectal tumours, termed incidence of colorectal tumours, was significantly higher (60%) in K-$ras^{+/-}$ mice compared with that observed in the control group (44%) (Figure 2b). The relative tumour volume significantly increased from 1.0 in control mice by 2.13-fold in K-$ras^{+/-}$ mice ($P < 0.05$) (Figure 2c). There were no significant differences in the tumour volume between male and female animals ($P > 0.05$). The pattern of survival differed between the two groups of mice over a period of 53 weeks, with a decrease from 88.0% in controls to 82.1% in K-$ras^{+/-}$ mice,







(d) Survival of mice after DMH treatment

Weeks	0	30	37	42	47	53
Control	25	25 (100.0%)	24 (96.0%)	23 (92.0%)	22 (88.0%)	22 (88.0%)
K-*ras*$^{+/-}$	28	28 (100.0%)	26 (96.42%)	25 (89.2%)	24 (85.7%)	23 (82.1%)

Figure 2 Differences in number, incidence and volume of colorectal adenomas and survival of DMH-treated control wild-type K-*ras*$^{+/+}$ mice compared with DMH-treated K-*ras*$^{+/-}$ mice. (a) Mean numbers (±SEM) of large intestinal adenomas per mouse in DMH-treated mice were significantly higher in the K-*ras*$^{+/-}$ mice ($n = 28$; $P < 0.01$) than in control K-*ras*$^{+/+}$ mice ($n = 25$). (b) Incidence of adenomas in the large intestine in DMH-treated control mice (11/25 mice) and K-*ras*$^{+/-}$ mice (17/28 mice). (c) Mean relative values of tumour volume in DMH-treated mice were significantly higher in the K-*ras*$^{+/-}$ mice ($n = 28$; $P < 0.05$) than in control mice ($n = 25$). (d) Survival of DMH-treated control and K-*ras*$^{+/-}$ mice, given as proportions of mice surviving at various time periods in weeks, up to one year after starting DMH treatment.

but as the mice were killed at the 1-year timepoint, statistical testing of natural survival by log-rank analysis was not possible (Figure 2d). Twenty tumours were analysed by DNA sequencing, 10 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. The *Beta-Catenin* gene was not examined for mutations. However, *Apc* exon 15 mutations were identified in 4 of 10 adenomas from wild-type mice and in 5 of 10 adenomas from K-*ras*$^{+/-}$ mice.

*Carcinogen-induced colorectal adenomas in hemizygous K-*ras *mice show increased proliferation and decreased apoptosis*

To assess whether K-*ras* hemizygosity affects adenomatous cell proliferation and apoptosis, adenomas were immunohistochemically analysed for Ki-67 expression and positivity for cleaved caspase-3 using age-matched colorectal adenomas from the two groups of mice (Figure 3). The proportion (in 500 cells counted from 5 to 6 mice) of Ki-67 positive cells in adenomas from K-*ras*$^{+/-}$ ($9.15 \pm 0.92\%$, mean ± SEM) was significantly higher than that in adenomas from wild-type mice ($7.77 \pm 0.64\%$, $P < 0.05$) (Figure 3a,b). The proportion (in 500 cells counted from 5 to 6 mice) of adenoma apoptotic nuclei stained positively for cleaved caspase-3 was reduced from $1.40 \pm 0.37\%$ (mean±SEM) in wild-type mice to $0.80 \pm 0.22\%$ in K-*ras*$^{+/-}$ mice ($P < 0.05$) (Figure 3c,d).

*Investigation of possible mechanisms of how K-*ras *hemizygosity may affect carcinogen-induced colorectal adenoma formation in mice*

To investigate possible mechanisms of how K-*ras* may exert a gene dosage effect on colorectal tumourigenesis, evidence of activation of the Erk/MapKinase and PI3K/Akt pathways was sought by immunostaining the adenomas with

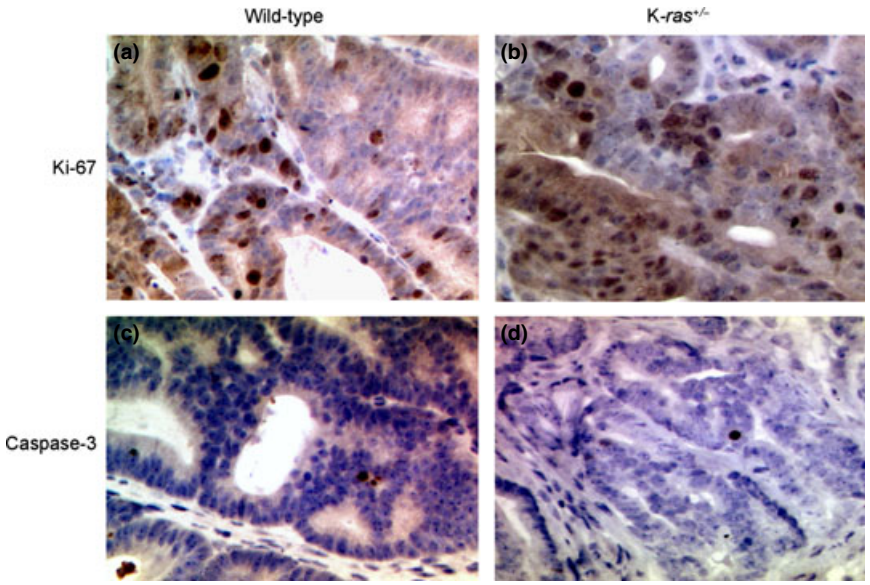


Figure 3 Immunohistochemical staining of the proliferation marker Ki-67 and the apoptosis marker cleaved caspase-3 in colorectal adenomas from age-matched DMH-treated wild-type and K-*ras*$^{+/-}$ mice (a–d, all at magnification ×200): Ki-67 expression in a colorectal adenoma from a DMH-treated wild-type mouse (a) and a K-*ras*$^{+/-}$ mouse (b). Ki-67 positivity in adenomas was significantly higher in the K-*ras*$^{+/-}$ mice than in wild-type mice ($P < 0.05$). Immunohistochemical staining of cleaved caspase-3 detection in a colorectal adenoma from a DMH-treated wild-type mouse (c) and a K-*ras*$^{+/-}$ mouse (d). Cleaved caspase-3 positive cells in adenomas were significantly lower in frequency in the K-*ras*$^{+/-}$ mice than in wild-type mice ($P < 0.05$).

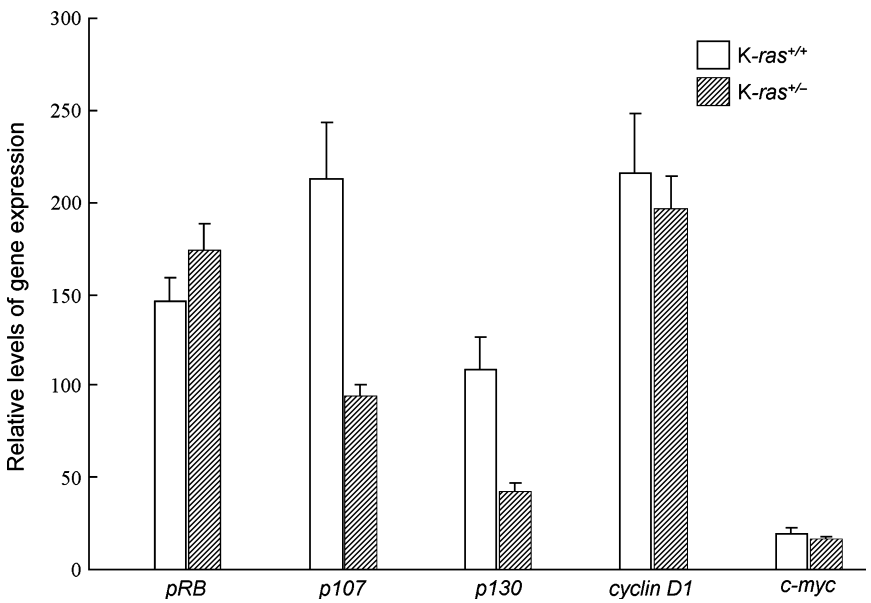


Figure 4 Relative RNA expression levels in colorectal adenomas from DMH-treated wild-type K-*ras*$^{+/+}$ and K-*ras*$^{+/-}$ mice, of *pRB*, *p107/RBL1*, *p130/RBL2*, *cyclin D1* and c-*myc* transcripts (mean ± SEM), determined by real-time RT-qPCR. Adenomas from K-*ras*$^{+/-}$ mice showed significantly lower levels of *p107/RBL1* and *p130/RBL2*; ($P < 0.05$ for both) than wild-type mice.

anti-phospho-Erk1/2 and anti-phospho-Akt (both anti-p-Akt-Thr-308 and anti-p-Akt-Ser-473) antibodies. There was no evidence of a detectable change in immunopositivity for either phospho-Erk1/2 or phospho-Akt (at either position p-Akt-Ser-308 or p-Akt-Ser-473) in adenomas from DMH-treated K-*ras*$^{+/-}$ mice compared with adenomas from wild-type mice (data not shown). Real-time RT-qPCR was used to evaluate the transcript expression levels of RB family members *pRB*, *p107/RBL1* and *p130/RBL2*, as well as *cyclin D1* and c-*myc*. K-*ras* hemizygosity was associated

with significantly decreased levels of *p107/RBL1* and *p130/RBL2* transcripts in the colorectal adenomas from K-*ras*$^{+/-}$ mice (93.38 ± 7.92 for *p107/RBL1*; and 42.90 ± 4.66 for *p130/RBL2*; mean ± SEM) compared with adenomas from wild-type K-*ras*$^{+/+}$ mice (212.53 ± 31.44 for *p107/RBL1*; and 109.34 ± 16.77 for *p130/RBL2*; $P < 0.05$ for both comparisons), but the transcript expression levels of *pRB*, *cyclin D1* and c-*myc* showed no differences between K-*ras* hemizygous and wild-type murine adenomas (Figure 4).

To further investigate the correlation between *KRAS* and *p107/RBL1* or *p130/RBL2* transcript levels in human neoplastic samples, a correlation analysis in the largest up-to-date collection of human cancer cell line expression microarray data ($n = 732$, Sanger Cell Line Project) was performed. There was a weak but statistically significant positive correlation in the expression of both *p107/RBL1* and *p130/RBL2* with *KRAS* (Pearson's correlation, adjusted *P*-value <0.01 for both) in cell lines without *KRAS* point mutations ($n = 648$), indicating that tumour cells with low *KRAS* expression appear to have low levels of *p107/RBL1* and *p130/RBL2* expression (Figure S1). When cancer cell lines with mutant *KRAS* were analysed in the same way, there was no significant correlation between *KRAS* and either *p107/RBL1* or *p130/RBL2* expression (data not shown).

Discussion

The effects of Ras proteins on regulation of proliferation, apoptosis and tumour formation are complex, as they vary between Ras family members and with their mutational status, expression levels and isoforms (K-*ras* 4A and 4B) expressed (Malumbres & Barbacid 2003; Luo *et al.* 2007a, b, 2009, 2011; Johnson *et al.* 1997; Patek *et al.* 2008). Here, we show that reduction in gene dosage from two wild-type K-*ras* alleles to one was associated with a 50% reduction in expression levels of wild-type K-*ras* transcripts in the K-*ras*$^{+/-}$ colons. This correlated with formation of significantly increased number, incidence and size of colorectal adenomas that do not bear K-*ras* or B-*raf* mutations, following treatment with the bowel-selective carcinogen DMH. DMH is known to induce intestinal adenoma formation by either activating *Beta-Catenin* mutation or inactivating *Apc* mutation, as found here. These adenomas in K-*ras*$^{+/-}$ mice showed significantly increased proliferation and decreased apoptosis.

The K-*ras*$^{+/-}$ murine colorectal adenomas showed no clear immunohistochemical evidence of increased activation of either the Erk/MapKinase pathway or the PI3K/Akt pathway, as well as no evidence of changes to the expression levels of c-*myc* or *cyclin D1* transcripts by RT-qPCR, compared with those from controls. However, there was a significant reduction in the expression levels of *p107/RBL1* and *p130/RBL2* transcripts in the K-*ras*$^{+/-}$ colorectal adenomas compared with those from K-*ras*$^{+/+}$ mice, consistent with a previously described relationship between *ras* and *p107/RBL1* and *p130/RBL2* expression in other contexts (Diaz *et al.* 2005; Mason *et al.* 2004). This finding is of an association with differential gene expression, and this should be confirmed by future functional studies in the relevant cell type.

The RB family of pocket proteins (pRB, p107/RBL1, and p130/RBL2) has been shown to regulate the activity of members of the E2F family of heterodimeric transcription factors by binding directly to E2F family proteins and repressing E2F-mediated transcription (Hurford *et al.* 1997; Sun *et al.* 2007; Classon *et al.* 2000). Others have shown *ras* signalling either associates with or mediates regulation of the levels of expression of *p107/RBL1* and/or *p130/RBL2* (Diaz *et al.* 2005; Luo *et al.* 2007a,b; Mason *et al.* 2004). Thus, modulation of E2F family protein activity may link K-*ras*-mediated transcriptional changes to the control of cell cycle progression, with promotion of DMH-induced adenoma formation. A similar but weak correlation between *KRAS* and *p107/RBL1* and/or *p130/RBL2* transcript levels was also seen in a series of 648 human cancer cell lines with wild-type *KRAS,* but not in cell lines with oncogenic mutations in *KRAS*, in keeping with the previous observation that the transcriptional effects of *KRAS* are different depending on its mutation status (Luo *et al.* 2007a,b), and the proposed mechanism that the tumour suppressor activity of wild-type *KRAS* appears to involve changes in *p107/RBL1* and *p130/RBL2* levels. Activating mutations in *KRAS* have been shown to exert their main oncogenic activity through the canonical MAPK and PI3K/AKT pathways (Malumbres & Barbacid 2003; Luo *et al.* 2007a,b, 2009, 2010, 2011; Patek *et al.* 2008), which showed little or no activation in DMH-induced adenomas from mice with either heterozygous deletion or wild-type *K-ras*, with no mutations in K-*ras* in the adenomas.

Compared with K-*ras* hemizygosity promoting methylnitrosourea (MNU)-induced lung tumour formation (Patek *et al.* 2008), the present study found that K-*ras* hemizygosity also significantly promotes DMH-induced colorectal tumourigenesis *in vivo*, but to a lesser degree. This difference in the degree of tumour promoting effect of K-*ras* hemizygosity may be due to the single K-*ras* allele in the K-*ras*$^{+/-}$ mice usually becoming mutated (K-*ras* c.35G≥A (p.Gly12Asp) or G12D mutation) in the MNU-induced lung tumours, but rarely or not becoming mutated in the DMH-induced colorectal tumours on the C57BL/6J background. Here, the tumour promoting effect relates to the gene dosage change being manifested as a ~50% reduction in K-*ras* transcript expression levels with decreased *p107/RBL1* and *p130/RBL2* expression, which is hypothesized to act via E2F proteins to modulate proliferation and tumour susceptibility.

This study adds to the accumulating evidence that proto-oncogenic *ras* can have a tumour suppressor effect. In separate experiments, overexpression of either wild-type K-*ras*, H-*ras* or N-*ras* genes have been reported to partially or completely suppress the transformed phenotype induced by their oncogenic counterparts (Diaz *et al.* 2005; Spandidos

et al. 1990; Luo *et al.* 2007a,b; Spandidos & Wilkie 1988; Singh *et al.* 2005). K-*ras* null embryonic stem cells, when stably transfected with wild-type K-*ras*, induce smaller and more differentiated teratomas than those expressing oncogenic K-*ras*, indicating the oncosuppressive effect of the wild-type form (James *et al.* 2003). Loss of wild-type *ras* or allelic imbalance at *ras* loci in animal tumours is consistent with the oncosuppressive nature of wild-type *ras* (Zhang *et al.* 2001; Guerrero *et al.* 1985). Cancers of human origin, such as lung adenocarcinomas (Takeuchi *et al.* 1996), prostate cancer (Kibel *et al.* 1998), pancreatic cancer (Mahlamaki *et al.* 1997), breast cancer (Sourvinos *et al.* 1997) and acute lymphoblastic leukaemia (Baccichet & Sinnett 1997) , have been found to show loss of heterozygosity around or near to the K-*ras* locus on chromosome 12p in some cases, consistent with a tumour-suppressive effect of wild-type K-*ras*.

In conclusion, following DMH treatment, K-*ras* hemizygosity promotes formation of increased number, incidence and size of colorectal adenomas that do not bear K-*ras* mutations. These adenomas showed increased proliferation, decreased apoptosis, along with evidence of an association with decreased transcript expression of *p107/RBL1* and *p130/RBL2* members of the RB family. Collectively, these data are consistent with expression of wild-type K-*ras* having a tumour suppressor effect on carcinogen-induced adenoma formation in the murine large bowel.

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Author contributions

Feijun Luo, Hongtao Ye, Wenyan Zhang and Gehong Dong performed the experimental work; Rifat Hamoudi and George Poulogiannis carried out the bioinformatics analyses; Feijun Luo and Mark Arends designed the experiments, interpreted the histopathological and expression data and wrote most of the manuscript with some contributions from all of the other authors.

Conflict of interest

There are no disclaimers and no conflicts of interest. The experimental work was supported by a project grant from Cancer Research United Kingdom, and the mouse work was carried out under Home Office Licence.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Correlation of either *p107/RBL1* or *p130/RBL2* with *KRAS* transcript expression in human cancer cell lines with wild-type *KRAS*. (A) Scatter plots comparing the expression levels of *p107/RBL1* (left), or *p130/RBL2* (right) with *KRAS,* both show a significant correlation ($P = 5.3 \times 10^{-7}$ for *p107/RBL1* and $P = 0.008$ for *p130/RBL2*, Pearson's correlation coefficient analysis) with wild-type *KRAS* expression levels (includes those cell lines with no *KRAS* point mutation, $n = 648$, Sanger Cell Line Project). (B) Heatmap of *KRAS* and *p107/RBL1* (top) or *p130/RBL2* (bottom) expression levels across the 648 cancer cell lines.