Journal of Pathology
J Pathol 2011; **223:** 390–399
Published online 11 November 2010 in Wiley Online Library
(wileyonlinelibrary.com) DOI: 10.1002/path.2790

ORIGINAL PAPER

Mutant K-*ras* promotes carcinogen-induced murine colorectal tumourigenesis, but does not alter tumour chromosome stability

Feijun Luo,[1,2] George Poulogiannis,[1] Hongtao Ye,[1] Rifat Hamoudi,[1] Wenyan Zhang,[1] Gehong Dong[1] and Mark J Arends[1]*

[1] *University of Cambridge, Department of Pathology, Addenbrooke's Hospital, Hills Road, Cambridge, CB2 2QQ, UK*
[2] *University of Central South China, Xiangya School of Medicine, Institute of Cancer Research, Xiangya Road, Chansha, China*

**Correspondence to: Dr Mark J Arends, University of Cambridge, Department of Pathology, Box 235, Addenbrooke's Hospital, Hills Road, Cambridge, CB2 0QQ, UK e-mail: mja40@cam.ac.uk*

Abstract

K-*ras* (*KRAS*) mutations are observed in around 40% of human colorectal adenomas and carcinomas. Previously, we developed and characterized a strain of transgenic mice with inducible intestinal epithelial expression of K-ras^{Val12} via a *Cre/LoxP* system. To evaluate the influence of mutant K-*ras* on carcinogen-induced colorectal tumourigenesis, we induced neoplastic alterations in the large intestines of wild-type and K-ras^{Val12} mice using the colon-selective carcinogen 1,2-dimethylhydrazine (DMH), which has been widely used to induce colorectal tumours that are histopathologically similar to those observed in humans. K-ras^{Val12} expression significantly promoted DMH-induced colorectal tumourigenesis: the average lifespan of the mice decreased from 38.52 ± 1.97 weeks for 40 control mice to 32.42 ± 2.17 weeks for 26 K-ras^{Val12} mice (mean ± SEM, $p < 0.05$) and the abundance of large intestinal tumours increased from 2.27 ± 0.15 per control mouse to 3.85 ± 0.20 in K-ras^{Val12} mice (mean ± SEM, $p < 0.01$). Adenomas from DMH-treated K-ras^{Val12} mice showed significantly higher proportions of Ki-67-positive proliferating cells (10.9 ± 0.69%) compared with those from DMH-treated wild-type mice (7.77 ± 0.47%) (mean ± SEM, $p < 0.01$) and a mild increase in apoptotic nuclei staining for cleaved caspase-3 (1.94 ± 0.21% compared with 1.15 ± 0.14%, mean ± SEM, $p < 0.01$). In the adenomas from DMH-treated K-ras^{Val12} mice, K-ras^{Val12} transgene recombination and expression were confirmed, with immunohistochemical evidence of strong Erk/MapK and mild PI3K/Akt pathway activation compared with adenomas from DMH-treated wild-type mice. Microarray hybridization and clustering analysis demonstrated different expression profiles in adenomas from DMH-treated wild-type and DMH-treated K-ras^{Val12} mice, indicating involvement of different molecular mechanisms including Erk/MapK and PI3K/Akt signalling in K-ras^{Val12}-expressing adenomas. Array-comparative genomic hybridization analysis showed chromosome stability in both cohorts, with only a very few tiny alterations observed in one adenoma from a DMH-treated K-ras^{Val12} mouse. Taken together, these data show that mutant K-*ras* significantly promotes DMH-induced colorectal tumourigenesis, resulting in distinct changes in cell signalling and proliferation, but does not alter chromosome stability in the tumours.

Keywords: K-*ras*; colon; adenoma; mouse; 1,2-dimethylhydrazine (DMH); carcinogen; chromosome

Received 27 July 2010; Revised 8 September 2010; Accepted 16 September 2010

No conflicts of interest were declared.

Introduction

Colorectal cancers are amongst the most frequent carcinomas in Europe and North America [1]. K-*ras* mutations are found at all stages of colorectal tumour formation, including the earliest lesions such as dysplastic aberrant crypt foci/microadenomas and hyperplastic polyps [2–6]. K-*ras* mutations are associated with increased size and dysplasia in adenomas [7], suggesting that they may be permissive for growth disorder early in tumourigenesis. Mutation of the K-*ras* (*KRAS*) gene, typically affecting codons 12, 13, and 61, reduces or abolishes the protein's intrinsic GTPase activity after GAP binding, locking it in a GTP-bound conformation that is constitutively active [8]. This activation results in signalling to downstream effectors such as Erk/MAPkinase and PI3K/Akt pathways. Activation of *ras* has several effects on rodent cells *in vitro*, including the establishment of the transformed phenotype, anchorage-independent growth, focus formation, and tumourigenic potential when injected into animals [9–11]. Mutant K-*ras* containing human colorectal tumours are often aneuploid with many chromosome rearrangements, prompting us to ask whether selective induction of mutant K-*ras* could render a colorectal tumour genome unstable.

The colon-selective carcinogen 1,2-dimethylhydrazine (DMH) has been widely used to study chemically induced colorectal tumours [12–15]. DMH alkylates DNA, producing O^6-methylguanine (O^6-MeG), which

induces GC → AT transitions that are typically found in various genes linked to colonic cancer [16,17]. DMH treatment results in high frequency (~60%) mutation of K-*ras* in colorectal adenomas in the rat model [18], whereas there are very few K-*ras* mutations in adenomas of DMH-treated mice [17,19]. 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 transgenics and knockouts, whose cells may have different rates of proliferation, apoptosis, or DNA repair.

In a previous study, we developed a strain of transgenic mice with inducible intestinal epithelial expression of K-ras^{Val12} via conditional activation using a *Cre/LoxP* system [20]. The mice carry one copy of a human K-ras^{Val12} transgene, which was activated following β-naphthoflavone induction of *Cre* recombinase expression with subsequent *Cre*-mediated *LoxP* recombination, leading to intestinal expression of K-ras^{Val12} 4A and 4B transcripts and protein isoforms. Here, we induced neoplastic alterations in the colon and rectum of wild-type and K-ras^{Val12} mice using DMH to evaluate the influence of mutant K-*ras* on chemical carcinogen-mediated large intestinal tumourigenesis and to determine whether mutant K-*ras* affects chromosome stability in the tumours formed.

Materials and methods

Mice

The strain of K-ras^{Val12} mice was developed as reported previously [20], bred onto a C57Black6 background and crossed with *Ah-Cre* mice [21]. Mice were genotyped by polymerase chain reaction (PCR) as described previously [20,21]. All of the animals were fed a standard laboratory diet and water *ad libitum* and were maintained on a 12-h light–dark schedule. All mouse breeding and procedures were carried out under Home Office licence.

Carcinogen treatments and intestinal examination

DMH· 2HCl (2.0 mg/ml) was dissolved in 0.90% w/v saline containing 1 mM EDTA and 10 mM sodium citrate, and the pH value was adjusted to 6.5 using 0.5 M NaOH. The protocol of DMH treatments consisted of weekly injections of 20.0 mg/kg, starting at age 7–8 weeks and continuing injections for 5 weeks (cumulative half dose = 100.0 mg/kg) or 10 weeks (cumulative full dose = 200.0 mg/kg DMH· 2HCl) [22]. Animals were weighed weekly and inspected regularly for signs suggestive of colonic tumour development (weight loss of 10% or more, inappetance, fur texture changes, diarrhoea, anaemia or rectal bleeding), at which time they were killed by cervical dislocation and examined grossly at necropsy. The entire intestine from the stomach to the anus was removed, and the stomach and small and large intestines were opened longitudinally, washed with cold phosphate-buffered saline (PBS), and the mucosal surface was examined for gross pathological abnormalities. Any polyps or tumours detected were measured, their location was noted, and polyp/tumour samples were dissected. The portion of intestine containing the polyp/tumour was taken for histological examination after fixation in neutral-buffered formalin. Sections were prepared for H&E staining and examined histologically for identification of tumour type, grade, and invasive stage, and also for immunohistochemistry. The separately dissected polyp/tumour sample and a section of macroscopically normal colonic tissue were frozen in liquid nitrogen and the samples stored at −70 °C for subsequent extraction of DNA, RNA, and proteins using standard methods.

Measurement of expression of K-ras^{Val12} in mice

In order to induce *Cre* expression via the *Ah* promoter, the K-ras^{Val12} × *Ah-Cre* mice were injected with 80.0 mg/kg β-naphthoflavone (β-NF) (Sigma, Dorset, UK) dissolved in corn oil, for 6 days as described previously [21]. As K-ras 4A and 4B isoforms can affect tumour formation, human K-ras^{Val12} 4A expression was determined using the primer pair K-*ras* 4A upstream primer (5′-AGT GCA ATG AGG GAC CAG TAC ATG AGG-3′), located in K-*ras* exon 3, and K-*ras* 4A downstream primer (5′-TTT GCT GAT GTT TCA ATA AAA GGA ATT-3′), located in K-*ras* exon 4A, whereas human K-ras^{Val12} 4B expression was determined using the K-*ras* 4B upstream primer (5′-GTA CCT ATG GTC CTA GTA GGA AAT AAA-3′), also located in human K-*ras* exon 3, and K-*ras* 4B downstream primer (5′-CTG ATG TTT CAA TAA AGG AAT TCC A-3′) in exon 4B [22–25]. The PCR product sizes for K-*ras* 4A and 4B were 248 bp and 159 bp, respectively. For quantitative RT-PCR, 100.0 ng of total RNA of different tissue samples was reverse-transcribed in a 25 µl volume using the iTaq SYBR Green RT-PCR Kit (Bio-Rad, Hercules, CA, USA). All RT-qPCR reactions were amplified starting with denaturation at 95.0 °C for 3 min and then 45 cycles of 95.0 °C for 15 s and 60.0 °C for 1 min.

Detection of apoptosis, proliferation, activated Erk, Akt, and β-catenin in intestinal adenomas

Immunohistochemical detection of cleaved caspase-3, Ki-67, phospho-Erk1/2, phospho-Akt-Ser-473, and β-catenin were performed as described previously [22]. The proportion of positive nuclei in 400 lesional cells per adenoma was counted in each of five adenomas from DMH-treated wild-type mice and five adenomas from DMH-treated K-ras^{Val12} mice for both cleaved caspase-3 and Ki-67.

Analysis of murine gene expression profiles by microarray hybridization

RNA isolation was carried out using the Qiagen RNeasy Mini Kit for animal tissues (Qiagen, Valencia, CA, USA). The RNA quality and quantity were checked using an Agilent 2100 bio-analyzer (Agilent Technologies, Inc., Santa Clara, CA, USA) and RNA 6000 nano-chips. Total RNA was used to generate biotin-labelled cRNA using the Illumina TotalPrep RNA Amplification Kit (Ambion, Austin, TX, USA). In brief, 0.5 μg of total RNA was first converted into single-stranded cDNA with reverse transcriptase using an oligo-dT primer containing the T7 RNA polymerase promoter site and then double-stranded cDNA molecules were produced. The double-stranded cDNA was cleaned and concentrated with the supplied columns and used in an overnight *in vitro* transcription reaction, where single-stranded RNA (cRNA) was generated and labelled by incorporation of biotin-16-UTP. A total of 0.75 μg of biotin-labelled cRNA was hybridized at 58 °C for 16 h to Illumina's Sentrix MouseRef-6 expression bead-chips arrays (Illumina, San Diego, CA, USA). The arrays were washed, blocked, and the labelled cRNA was detected by staining with streptavidin-Cy3. The arrays were scanned using an Illumina BeadStation 500× Genetic Analysis Systems scanner and the image data were extracted using Illumina BeadStudio software, version 3.0. Data files generated from Illumina BeadStudio software were imported into Genespring GX version 10.0 (Agilent, Santa Clara, CA, USA). The raw signals were thresholded to 1.0 and normalization was carried out using the percentile shift algorithm, followed by baseline transformation to the median of all the samples. The Illumina Sentrix MouseRef-6 expression bead-chips microarrays have 24 000 probes for well-annotated RefSeq transcripts with approximately 30-fold redundancy. The array data were filtered to identify those genes with expression changes outside the 95th percentile. Pathway analysis of the up-regulated and down-regulated genes in adenomas from the mutant K-*ras* and control mice was carried out using Ingenuity Pathway analysis (Ingenuity Systems, CA, USA) [26].

Analysis of murine genomic alterations by oligonucleotide microarray-comparative genomic hybridization

Array-comparative genomic hybridization (CGH) was performed using the Agilent Mouse Genome 244K CGH Microarray, with an average spatial resolution of 6.4 kb (Agilent). Labelling and hybridization were performed following the protocols provided by the manufacturer. Briefly, the test DNA from large intestinal adenomas and the reference DNA (normal mouse) were digested with AluI and RsaI (Promega, Madison, WI, USA), and purified with the QIAprep Spin Miniprep Kit (Qiagen, Germantown, MD, USA). Test DNA (1.0 μg) and reference DNA (1.0 μg; Promega) were labelled by random priming with either Cy3-dUTP or Cy5-dUTP using the Agilent Genomic DNA Labeling Kit Plus. Following the labelling reaction, the individually labelled test and reference samples were concentrated using Microcon YM-30 filters (Millipore, Billerica, MA, USA) and then combined. Following probe denaturation and pre-annealing with Cot-1 DNA, hybridization was performed at 65 °C with rotation for 40 h [27]. Four washing steps were performed with Agilent Oligo CGH washes: wash buffer 1 at room temperature for 5 min, wash buffer 2 at 37 °C for 1 min, an acetonitrile rinse at room temperature for 1 min, and a 30 s wash at room temperature in Agilent's Stabilization and Drying Solution. Slides were scanned using an Agilent DNA microarray scanner. Data were obtained by Agilent Feature Extraction software 9 and analysed with Agilent CGH Analytics software 3.4. We used the statistical algorithms z-score and ADM-2 according to sensitivity thresholds of 2.5 and 6.0 and a moving average window of 0.2 Mb. Genomic copy number alterations were mapped onto the murine genome sequence using the NCBI database build m37 and checked against the database of murine genomic copy number variants. Data were smoothed using an adaptive Hidden Markov Model written in R (version 2.8.0) [26,28] and the aberrations were plotted on a karyogram of schematic representations of mouse chromosomes.

The array-based data files have been deposited with GEO under series accession number GSE24010.

Statistical analysis

The SPSS statistical package was used for all statistical analyses. The non-parametric Mann–Whitney U-test was used to compare adenoma numbers and immunostaining counts between the groups studied. Kaplan–Meier curves were used to estimate the time-related probabilities of survival and the log-rank test was used to estimate the significance of differences between survival distributions.

Results

K-ras^{Val12} promotes DMH-induced colorectal tumourigenesis in mice

To assess the effects of K-ras^{Val12} on DMH-induced intestinal tumourigenesis, intestinal K-ras^{Val12} expression was induced at 6–7 weeks after birth, by transient activation of Cre recombinase in the intestinal epithelium of the K-ras^{Val12} test mice (K-ras^{Val12} × *Ah-Cre*), by injection with β-naphthoflavone (80.0 mg/kg per day for 6 days). From the ninth or tenth week, both test and control (either wild-type or *Ah-Cre*) mice were injected once per week for 10 weeks with DMH (20.0 mg/kg). Mice (test and control groups) were killed after showing signs of intestinal tumour formation and necropsies were performed to examine

the full length of the gastro-intestinal tract. Intestinal tumours were counted and examined histopathologically. K-ras^{Val12} expression significantly promoted DMH-induced tumourigenesis, resulting in a significant decrease in the average lifespan of the mice from 38.52 ± 1.97 weeks (mean $\pm$ SEM, $n = 40$, control mice) to 32.42 ± 2.17 weeks ($n = 26$, K-ras^{Val12} mice) (log-rank test, $p < 0.05$; Figures 1A and 1B). No sex differences in lifespan were observed for either group. Almost all intestinal tumours in both groups occurred in the large intestines, where adenomas per mouse increased 1.7-fold in number from 2.27 ± 0.15 in control mice to 3.85 ± 0.20 in K-ras^{Val12} mice (mean $\pm$ SEM, $p < 0.01$) (Figure 1C). The effects of K-ras^{Val12} on chemical carcinogen-induced colorectal tumourigenesis were further evaluated using half the total dose of DMH (20.0 mg/kg injected over 5 weeks). This resulted in a two-fold increase in the number of adenomas per mouse from 0.42 ± 0.24 in control mice ($n = 20$) to 0.81 ± 0.24 in K-ras^{Val12} mice ($n = 20$, mean $\pm$ SEM, $p < 0.05$, Figure 1D). The large intestinal adenomas in the test and control groups showed very similar histopathological appearances, with moderate to severe dysplasia. There were three cases of invasive adenocarcinomas, all found in DMH-treated K-ras^{Val12} mice, and two cases of liver metastasis in DMH-treated K-ras^{Val12} mice, with no invasive or metastatic cancers in the control group (Figure 2 and Supporting information, Supplementary Figure 1). No evidence of metastasis was detected to the lungs, peritoneal surfaces or other organs. Analysis of the adenomas from DMH-treated K-ras^{Val12} mice confirmed both K-*ras* transgene recombination by PCR amplification across the deleted STOP cassette and expression of mutant K-*ras* 4A transcripts by RT-PCR of RNA (Figure 3). Expression of the transgenic K-*ras* 4B transcripts was also confirmed in these adenomas (data not shown).

Proliferation and apoptotic indices of the colorectal adenomas

The proportion of Ki-67 immunohistochemically-positive nuclei was markedly and significantly higher in adenomas from K-ras^{Val12} mice ($10.9 \pm 0.69\%$) than in adenomas from wild-type mice ($7.77 \pm 0.47\%$, mean $\pm$ SEM, $p < 0.01$) (Figures 4A and 4B). The proportion of apoptotic nuclei staining positively for cleaved caspase-3 was generally very low in all tumours from both genotypes, but adenomas from wild-type mice showed significantly fewer apoptotic cells ($1.15 \pm 0.14\%$) than adenomas from K-ras^{Val12} mice ($1.94 \pm 0.21\%$, mean $\pm$ SEM, $p < 0.01$) (Figures 4C and 4D). These data demonstrate that mutant K-ras^{Val12} expression leads to a marked increase in adenoma cell proliferation and a mild increase in apoptosis in DMH-induced colorectal adenomas.

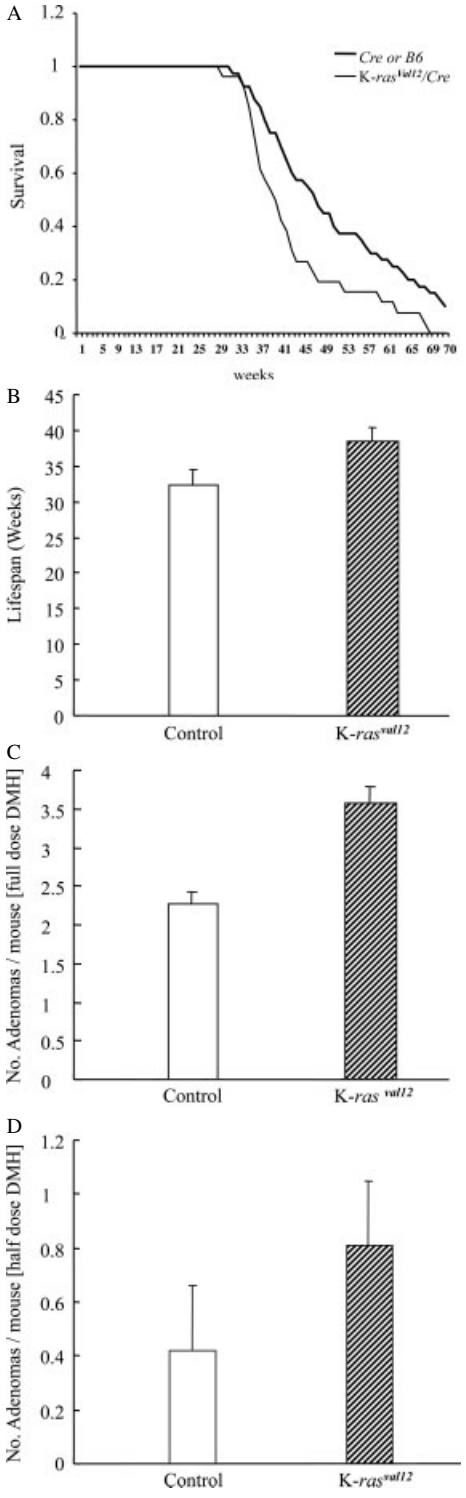

Figure 1. Survival and tumour load differences between wild-type and K-ras^{Val12} mice after DMH treatments. (A) Kaplan–Meier survival curves for DMH-treated control [*Ah-Cre* or Black6 (B6) wild-type] mice ($n = 40$) and DMH-treated K-ras^{Val12} mice ($n = 26$). Ages of the animals at death are given in weeks. (B) Average lifespan of DMH-treated control and K-ras^{Val12} mice (error bars = SEM). (C) Numbers of colorectal adenomas per mouse for full dose (10 weeks) DMH-treated control ($n = 40$) and K-ras^{Val12} mice ($n = 26$). (D) Numbers of colorectal adenomas per mouse for half dose (5 weeks) DMH-treated control and K-ras^{Val12} mice (both groups $n = 20$).

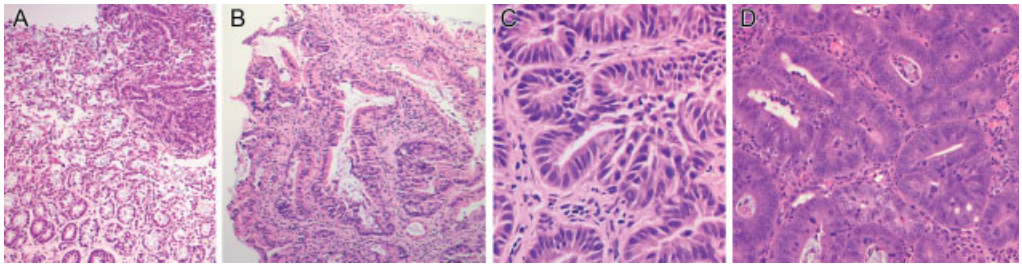

Figure 2. Histopathological appearances of large intestinal tumours in DMH-treated control and DMH-treated K-*ras*^{Val12} mice. (A) Colonic adenoma from a wild-type mouse (original magnification ×100; moderately dysplastic). (B) Colonic adenoma from a K-*ras*^{Val12} mouse (original magnification ×100; moderately dysplastic). (C) Colonic adenoma from a wild-type mouse (original magnification ×400; moderately dysplastic). (D) Colonic adenoma from a K-*ras*^{Val12} mouse (original magnification ×400; severely dysplastic).

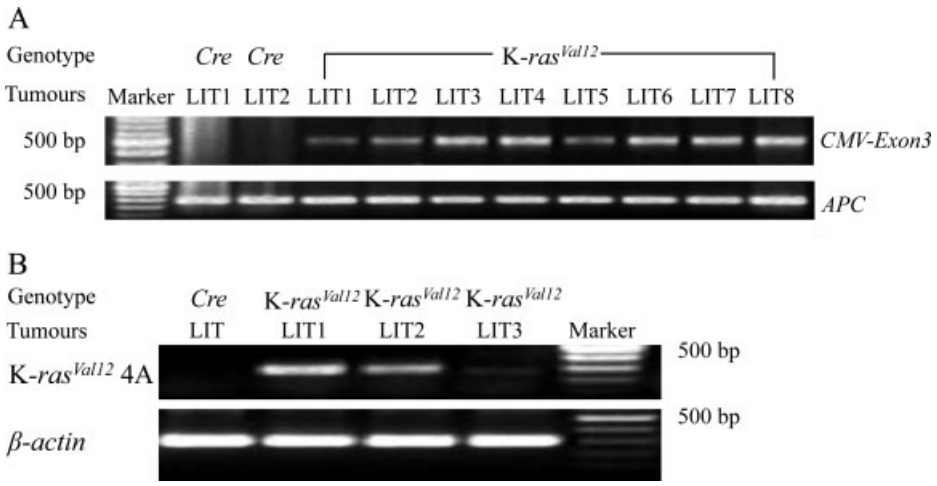

Figure 3. Transgene recombination and expression of mutant K-*ras* in large intestinal tumours. (A) Transgene recombination assay using PCR amplification of a 500 bp DNA fragment between the recombined CMV promoter and K-*ras*^{Val12} exon 3 (CMV-Exon 3) from genomic DNA of large intestinal tumours (LIT) from DMH-treated K-*ras*^{Val12} mice or DMH-treated *Ah-Cre* only (*Cre*) control transgenic mice, all treated with β-NF to induce Cre expression and transgene recombination (*Apc* gene amplification as control). (B) RT-PCR analysis of the expression of transgenic K-*ras*^{Val12} 4 A transcripts in large intestinal tumours (LIT) from DMH-treated K-*ras*^{Val12} mice or DMH-treated *Ah-Cre* only (*Cre*) control mice, after treatment with β-NF (β-*actin* RNA expression as normalization reference).

Effects of mutant K-*ras* expression on Erk/Mapk and PI3K/Akt pathway activation and β-catenin nuclear localization in the colorectal adenomas

Adenomas from DMH-treated K-*ras*^{Val12} mice showed increased expression of pErk1/2 (nuclear intensity and frequency) compared with that in adenomas from DMH-treated wild-type mice (Figures 5A and 5B). Similarly, adenomas from DMH-treated K-*ras*^{Val12} mice showed increased Akt phosphorylation to pAkt-Ser-473 (pAkt cytoplasmic intensity), but only to a mild degree relative to the pAkt staining seen in adenomas from DMH-treated wild-type mice (Figures 5C and 5D). Expression and subcellular localization of β-catenin, as a marker of Wnt pathway activation, were evaluated immunohistochemically using anti-β-catenin antibody, and this showed β-catenin nuclear staining in adenomas in a very similar pattern in both DMH-treated wild-type mice and K-*ras*^{Val12} mice, with no obvious differences in the staining pattern between the two groups (data not shown), indicating closely similar Wnt pathway activation in adenomas from both cohorts.

Expression profiling of adenomas by microarray hybridization and hierarchical clustering analysis

The mRNA expression profiles of adenomas from DMH-treated K-*ras*^{Val12} and wild-type mice were determined by microarray hybridization, with selection of those genes with an expression fold change ≥2 (25 genes showing up-regulation and 38 genes showing down-regulation) for hierarchical clustering analysis (Figure 6). The four adenomas from DMH-treated K-*ras*^{Val12} mice clustered tightly together and were clearly distinguished from the three adenomas from DMH-treated wild-type mice. Many of these genes are involved in Mapk, PI3K/Akt, Wnt, and apoptosis signalling pathways (Supporting information, Supplementary Figure 2). For example, *Dag1, Preb*, and *Jagn1* are all regulated by the Mapk pathway, whereas *Fen1* is a target of the PI3K/Akt pathway. *Pygo2* is a target gene of the Wnt pathway. Moreover, *Ddx18*, which encodes a protein with a DEAD box polypeptide motif, is involved in apoptosis. Interestingly, the up-regulated genes *Klf5* and *Slc38a2* also showed increased expression in embryonic stem cells transfected with mutant K-*ras*, whereas the down-regulated genes *Dcn, Mdm2*, metallothionein-1 (*Mt1*), and *Rab7* showed decreased

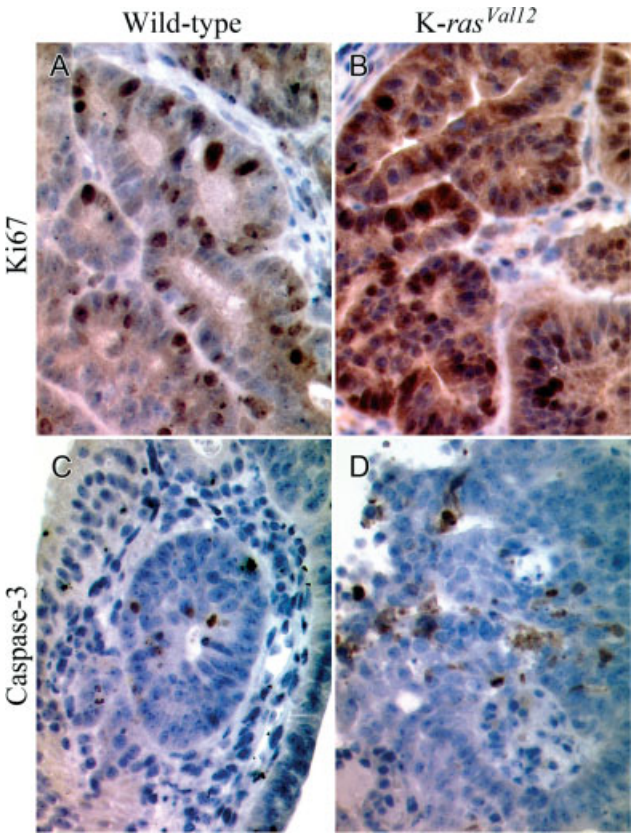

Figure 4. Immunohistochemical staining of proliferation marker Ki-67 (A, B) and apoptosis marker cleaved caspase-3 (C, D) in adenomas from either DMH-treated wild-type mice (A, C) or DMH-treated K-*ras*^{Val12} mice (B, D) (all original magnification ×400).

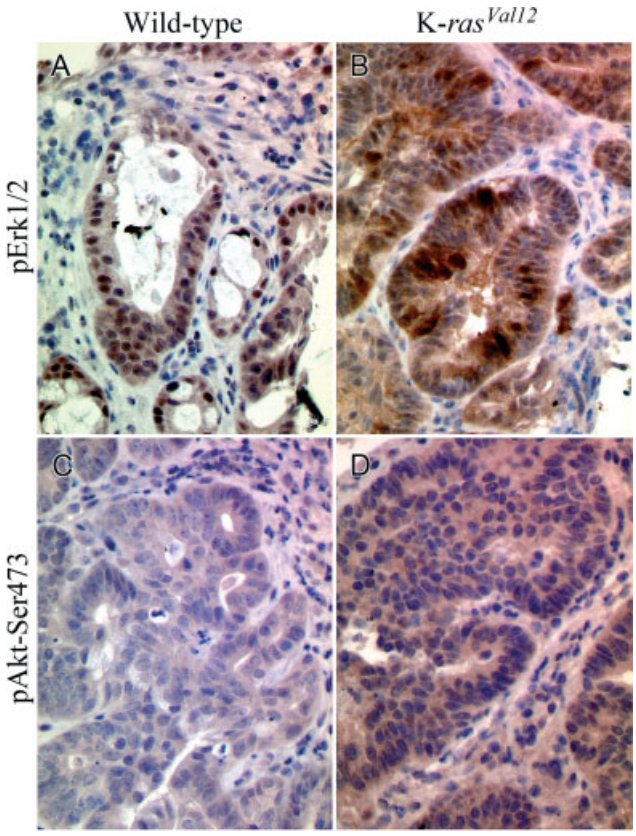

Figure 5. Immunohistochemical staining of phospho-Erk1/2 (A, B) and phospho-Akt-Ser-473 (C, D) in adenomas from either DMH-treated wild-type mice (A, C) or DMH-treated K-*ras*^{Val12} mice (B, D) (all original magnification ×400).

expression in embryonic stem cells transfected with mutant K-*ras* [29]. Hence, there is evidence that these differentially expressed genes relate to the presence of mutant K-*ras* and are consistent with Mapk and PI3K/Akt signalling pathway activation. The different expression profiles of the two groups shown by the heatmap (Figure 6) indicate different molecular mechanisms of adenoma formation in these two groups, with mutant K-*ras* modulating the Mapk and PI3K/Akt pathways, and this was supported by pathway analysis of the up-regulated and down-regulated genes (Supporting information, Supplementary Figure 2).

Genomic copy number changes in adenomas detected by oligonucleotide microarray comparative genomic hybridization

To identify any genomic copy number alterations in the tumours, DNA was extracted from five adenomas each from DMH-treated K-*ras*^{Val12} and wild-type mice, labelled, and hybridized to Agilent mouse genome oligonucleotide microarrays. In nine of ten adenomas analysed, there were no detectable chromosome alterations. However, one adenoma from a DMH-treated K-*ras*^{Val12} mouse showed very small regions of DNA copy number loss on murine chromosomes 4, 6, and 14, and very small regions of DNA copy number gain mapping to murine chromosomes 5 and 14 (Supporting information, Supplementary Figure 3).

Discussion

K-*ras* mutations can be found early in adenomagenesis and have been associated with increased size and dysplasia of colorectal adenomas [4,5,7]. Here, murine intestinal expression of mutant K-*ras*^{Val12} was demonstrated to promote DMH-induced colorectal tumourigenesis in mice, resulting in significantly reduced survival and increased numbers of adenomas, confirmed at two doses of DMH. This is consistent with our previous mouse model studies, which have shown that mutant K-*ras* accelerates intestinal adenomagenesis on both a mutant *Apc* background and an *Msh2*-null background [20,30]. Analysis of the adenomas induced by DMH in this *in vivo* model demonstrated recombination of the K-*ras*^{Val12} transgene and expression of the transgenic mutant K-*ras* in the adenomas. This resulted in a significant increase in adenoma cell proliferation and a mild increase in apoptosis. This is broadly consistent with a previous investigation that showed that mutant K-*ras* enhanced apoptosis in embryonic stem cells [31]. Mutant *ras* has been shown to modulate both apoptosis and necrosis, influencing net tumour growth [10,11]. Thus, mutant K-*ras*

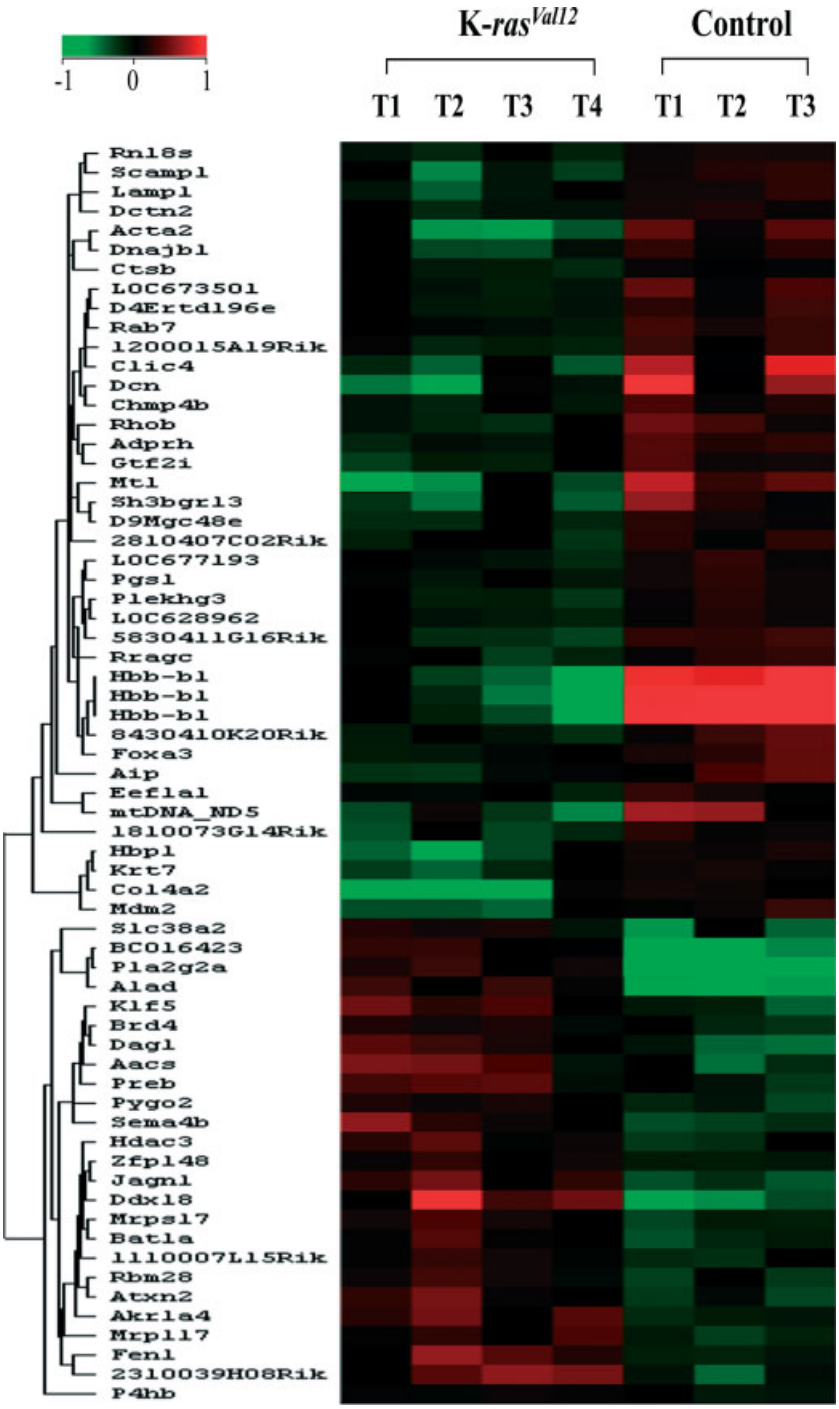

Figure 6. Hierarchical clustering analysis heatmap of discriminant genes (listed on left) from RNA microarray hybridization gene expression profiles comparing adenomas from DMH-treated K-*ras*^{Val12} mice (four tumours, T1–T4, on the left) with those from DMH-treated wild-type mice (three tumours, T1–T3, on the right) (green = decreased expression; red = increased expression, scale provided).

confers tumour cell growth advantage via increased proliferation despite some modulation of cell death.

Induction of colorectal tumours in mice by DMH is a well-established model of chemical carcinogenesis, usually generating adenomas with few or no mutations of K-*ras* in mice, but with tumour initiation due to mutation of β-*catenin* leading to nuclear localization of β-catenin protein, resulting in activation of the Wnt signalling pathway. This was confirmed here by β-catenin immunohistochemistry showing nuclear localization in adenoma cells in both groups of DMH-treated mice. Takahashi et al [32,33] discovered highly frequent mutations of the β-catenin (*Ctnnb1*) gene in AOM-induced mouse colonic tumours. These data suggest that mutational activation of K-*ras* is not an essential requirement for the initiation of DMH-induced colorectal tumours in mice, but may promote tumour progression, consistent with 60% absence (40% prevalence) of K-*ras* mutations in human colorectal neoplasms. In this study, K-*ras*^{Val12} was shown by immunohistochemistry and microarray expression profiling to strongly activate the Erk/Mapk pathway and to mildly

activate the PI3K/Akt pathway, in the presence of Wnt/β-catenin pathway activation. Our previous study discovered that mutated K-*ras* accelerated tumourigenesis in $Apc^{Min/+}$ mice more in the large than in the small intestines, with synergistic effects between the K-*ras*/Erk/Mapk and Wnt pathways [30]. In agreement with this, activation of both Erk/Mapk and PI3K/Akt pathways has been described in *Wnt3a*-induced cell proliferation [34,35].

The microarray expression profiling with clustering analysis demonstrated two distinct groups with different molecular mechanisms involved in adenoma formation in the DMH-treated K-ras^{Val12} mice compared with adenomas from DMH-treated wild-type mice. Expression profiling showed increased expression of Krüppel-like factor 5 (*Klf5*) in the DMH-induced tumours in mutant K-*ras* mice. *Klf5* encodes a zinc finger transcription factor that regulates cell proliferation and is involved in carcinogenesis. Elevated levels of Klf5 protein have been strongly correlated with activating *K-ras* mutations in intestinal tumours *in vitro* and *in vivo*, and in a previous study we found that mutant K-*ras* up-regulated *Klf5* expression in embryonic stem cells [29]. *Klf5* is a mediator of intestinal tumourigenesis in mice harbouring combined Apc^{Min} and K-ras^{Val12} mutations [36]. Inhibition of *Klf5* expression in tumour cells resulted in significantly reduced rates of proliferation and transforming activities [37]. Similarly, the tumour suppressor gene decorin (*Dcn*) was found to be down-regulated in the DMH-induced tumours in mutant *K-ras* mice. Previously, we found decreased expression levels of *Dcn* in mutant *K-ras* transfected embryonic stem cells [29]. Further, activating K-*ras* mutations have been correlated with significantly reduced expression of *Dcn* in colorectal cancer [38].

The concurrence of *ras* mutations and genomic instability in some tumours prompted us to test whether expression of activated K-*ras* in colorectal tumours results in chromosome instability. Denko *et al* [39] observed that the human Ha-*ras* oncogene (*HRAS*) induced genomic instability in murine fibroblasts, which is associated with abnormal mitotic figures. Saavedra *et al* [40] showed that v-*ras* induced micronuclei in NIH3T3 cells, with evidence of the Mapk pathway mediating *ras*-induced chromosome instability. Some of the instability observed was due to aberrant mitotic events, including the formation of mitotic chromosome bridges. We hypothesized that DMH-induced colorectal tumours expressing K-ras^{Val12} might show evidence of chromosome breaks and rearrangements. Unexpectedly, we did not find any large-scale chromosome alterations and no significant evidence of chromosome instability in the adenomas from either DMH-treated K-ras^{Val12} or wild-type mice, with only a few very small DNA copy number changes in a single adenoma. Additional mutations may be required to trigger chromosome instability in colorectal tumour formation. Kasiappan *et al* [41] and Shivapurkar *et al* [4,42,43] reported that inactivation of *p53* and activation of oncogenes including *ras* can synergistically promote chromosome instability in rodent and human colorectal tumours. Therefore, we sequenced the mutational hotspot regions (high frequency mutation susceptible exons 5–8) of the *p53* gene and found no mutations in ten tumours tested (data not shown). These data suggest that K-*ras* mutation alone is insufficient to generate tumour aneuploidy, whereas cooperation with mutant or null *p53* or other genes may be required to promote genomic instability.

In conclusion, we have shown that intestinal expression of mutant K-ras^{Val12} promoted DMH-induced tumourigenesis in the large rather than the small intestines in a mouse model that more closely recapitulates human colorectal neoplasia. DMH-treated K-ras^{Val12} mice showed a higher number of tumours compared with DMH-treated wild-type mice, including three adenomas that progressed to invasive adenocarcinomas with two occurrences of metastasis to liver, which was consistent with a significantly poorer survival. The adenomas from DMH-treated K-ras^{Val12} mice showed a prominent increase in proliferation associated with strong activation of the Erk/Mapk pathway, on a background of Wnt/β-catenin pathway activation and mild activation of the PI3K/Akt pathway. Expression profiling identified distinct molecular mechanisms in the two groups of adenomas, with evidence of activation of Mapk and PI3K/Akt pathways, but genomic analyses showed no significant evidence of tumour cell chromosome instability as a result of mutant K-*ras* expression.

Acknowledgment

We are grateful to Maggie Green and Ian Purvis (Department of Pathology, University of Cambridge) for technical assistance. We would like to thank Dr Ian McFarlane of the Microarray CoreLab, National Institute of Health Research, Cambridge Comprehensive Biomedical Research Centre for his technical assistance. This work was supported by grants from Cancer Research UK and the Wellcome Trust.

Abbreviations

ACF	aberrant crypt foci
AOM	azoxymethane
DMH	1,2-dimethylhydrazine (dichloride)
Erk	extracellular signal regulating kinase
Mapk	mitogen-activated protein kinase
PBS	phosphate-buffered saline
H&E	haematoxylin and eosin
SDS	sodium dodecyl sulphate

Author contribution statement

FL, HY, WZ, and GD performed the experimental work; RH and GP carried out the microarray data

analysis and bioinformatics; FL and MJA designed the experiments, interpreted the histopathological data, and wrote most of the manuscript, with some contributions from all of the other authors.

References

1. Kinzler KW, Vogelstein B. Lessons from hereditary colorectal cancer. *Cell* 1996; **87**: 159–170.
2. Bos JL, Fearon ER, Hamilton SR, *et al*. Prevalence of *ras* gene mutations in human colorectal cancers. *Nature* 1987; **327**: 293–297.
3. Bird R, McLellan E, Bruce W. Aberrant crypts, putative precancerous lesions, in the study of the role of diet in the aetiology of colon cancer. *Cancer Surv* 1989; **8**: 189–200.
4. Shivapurkar N, Huang L, Ruggeri B, *et al*. K-*ras* and p53 mutations in aberrant crypt foci and colonic tumours from colon cancer patients. *Cancer Lett* 1997; **115**: 39–46.
5. Otori K, Oda Y, Sugiyama K, *et al*. High frequency of K-*ras* mutations in human colorectal hyperplastic polyps. *Gut* 1997; **40**: 660–663.
6. Andreyev HJ, Norman AR, Cunningham D, *et al*. Kirsten *ras* mutations in patients with colorectal cancer: the 'RASCAL II' study. *Br J Cancer* 2001; **85**: 692–696.
7. Ohnishi T, Tomita N, Monden T, *et al*. A detailed analysis of the role of K-*ras* gene mutation in the progression of colorectal adenoma. *Br J Cancer* 1997; **75**: 341–347.
8. Barbacid M. Ras genes. *Annu Rev Biochem* 1987; **56**: 779–827.
9. Spandidos DA, Wilkie NM. Malignant transformation of early passage rodent cells by a single mutated human oncogene. *Nature* 1984; **310**: 469–475.
10. Arends MJ, McGregor AH, Toft NJ, *et al*. Susceptibility to apoptosis is differentially regulated by c-*myc* and mutated Ha-*ras* oncogenes and is associated with endonuclease availability. *Br J Cancer* 1993; **68**: 1127–1133.
11. Arends MJ, McGregor AH, Wyllie AH. Apoptosis is inversely related to necrosis and determines net growth in tumours bearing constitutively expressed *myc, ras*, and HPV oncogenes. *Am J Pathol* 1994; **144**: 1045–1057.
12. Deschner EE, Long FC. Colonic neoplasms in mice produced with six injections of 1,2-dimethylhydrazine. *Oncology* 1977; **34**: 255–257.
13. Deschner EE, Long FC, Maskens AP. Relationship between dose, time and tumor yield in mouse dimethylhydrazine-induced colon tumorigenesis. *Cancer Lett* 1979; **8**: 23–28.
14. Diwan BA, Meier H, Blackman KE. Genetic differences in the induction of colorectal tumors by 1,2-dimethylhydrazine in inbred mice. *J Natl Cancer Inst* 1977; **59**: 455–458.
15. Diehl D, Oesterle D, Elmlinger MW, *et al*. IGF-II transgenic mice display increased aberrant colon crypt multiplicity and tumor volume after 1,2-dimethylhydrazine treatment. *J Carcinog* 2006; **5**: 24–38.
16. James JT, Autrup H. Methylated DNA adducts in the large intestine of ICR/Ha and C57BL/Ha mice given 1,2-dimethylhydrazine. *J Natl Cancer Inst* 1983; **70**: 541–546.
17. Jackson PE, Hall CN, O'Connor PJ, *et al*. Low O^6-alkylguanine DNA-alkyltransferase activity in normal colorectal tissue is associated with colorectal tumours containing a GC → AT transition in the K-*ras* oncogene. *Carcinogenesis* 1997; **18**: 1299–1302.
18. Jacoby RF, Llor X, Teng B-B, *et al*. Mutations in the *K-ras* oncogene induced by 1,2-dimethylhydrazine in preneoplasic and neoplastic rat colonic mucosa. *J Clin Invest* 1991; **87**: 624–630.
19. Jackson PE, Cooper DP, Hall CN, *et al*. The relationship between 1,2-dimethylhydrazine dose and the induction of colon tumours: tumour development in female SWR mice does not require a K-*ras* mutation event. *Carcinogenesis* 1999; **20**: 509–513.
20. Luo F, Brooks DG, Ye H, *et al*. Conditional expression of mutated K-*ras* accelerates intestinal tumourigenesis in *Msh2*-deficient mice. *Oncogene* 2007; **26**: 4415–4427.
21. Ireland H, Kemp R, Houghton C, *et al*. Inducible Cre-mediated wild-type of gene expression in the murine gastrointestinal tract: effect of loss of β-catenin. *Gastroenterology* 2004; **126**: 1236–1246.
22. Luo F, Hamoudi R, Dong G, *et al*. K-*ras* exon 4A has a tumour suppressor effect on carcinogen-induced murine colonic adenoma formation. *J Pathol* 2010; **220**: 542–550.
23. Plowman SJ, Williamson DJ, O'Sullivan MJ, *et al*. While K-*ras* is essential for mouse development, expression of the K-*ras* 4A splice variant is dispensable. *Mol Cell Biol* 2003; **23**: 9245–9250.
24. Plowman SJ, Arends MJ, Brownstein DG, *et al*. The K-Ras 4A isoforms promotes apoptosis but does not affect either lifespan or spontaneous tumour incidence in ageing mice. *Exp Cell Res* 2006; **312**: 16–26.
25. Plowman SJ, Berry RL, Bader SA, *et al*. K-*ras* 4A and 4B are co-expressed widely in human tissues, and their ratio is altered in sporadic colorectal cancer. *J Exp Clin Cancer Res* 2006; **25**: 259–267.
26. Hamoudi RA, Appert A, Ye H, *et al*. Differential expression of NF-κB target genes in MALT lymphoma with and without chromosome translocation: insights into molecular mechanism. *Leukemia* 2010; **24**: 1487–1497.
27. Poulogiannis G, Ichimura K, Hamoudi R, *et al*. Prognostic relevance of DNA copy number changes in colorectal cancer. *J Pathol* 2010; **220**: 338–347.
28. Chanudet E, Ye H, Ferry J, *et al*. A20 deletion is associated with copy number gain at the TNFA/B/C locus and occurs preferentially in translocation-negative MALT lymphoma of the ocular adnexa and salivary glands. *J Pathol* 2009; **217**: 420–430.
29. Luo F, Hamoudi R, Brooks DG, *et al*. Stem cell gene expression changes induced specifically by mutated K- *ras*. *Gene Expr* 2007; **14**: 101–115.
30. Luo F, Brooks DG, Ye H, *et al*. Mutated K-ras^{Asp12} promotes tumourigenesis in $Apc^{Min/+}$ mice more in the large than small intestines, with synergistic effects between K-*ras* and *Wnt* pathway. *Int J Exp Pathol* 2009; **90**: 558–574.
31. Brooks DG, James RM, Patek CE, *et al*. Mutant K-*ras* enhances apoptosis in embryonic stem cells in combination with DNA damage and is associated with increased levels of p19 ARF. *Oncogene* 2001; **20**: 2144–2152.
32. Takahashi M, Fukuda K, Sugimura T, *et al*. β-Catenin is frequently mutated and demonstrates altered cellular location in azoxymethane-induced rat colon tumours. *Cancer Res* 1998; **58**: 42–46.
33. Takahashi M, Nakatsugi S, Sugimura T, *et al*. Frequent mutations of the beta-catenin gene in mouse colon tumours induced by azoxymethane. *Carcinogenesis* 2000; **21**: 1117–1120.
34. Yun MS, Kim SE, Jeon SH, *et al*. Both ERK and Wnt/beta-catenin pathways are involved in Wnt3a-induced proliferation. *J Cell Sci* 2005; **118**: 313–322.
35. Kim SE, Lee WJ, Choi KY. The PI3 kinase–Akt pathway mediates Wnt3a-induced proliferation. *Cell Signal* 2007; **19**: 511–518.
36. Nandan MO, McConnell BB, Ghaleb AM, *et al*. Krüppel-like factor 5 mediates cellular transformation during oncogenic KRAS-induced intestinal tumorigenesis. *Gastroenterology* 2008; **134**: 120–130.
37. Nandan MO, Ghaleb AM, McConnell BB, *et al*. Krüppel-like factor 5 is a crucial mediator of intestinal tumorigenesis in mice harboring combined ApcMin and KRASV12 mutations. *Mol Cancer* 2010; **9**: 63–77.

38. Mlakar V, Berginc G, Volavsek M, *et al*. Presence of activating KRAS mutations correlates significantly with expression of tumour suppressor genes DCN and TPM1 in colorectal cancer. *BMC Cancer* 2009; **9**: 282–301.
39. Denko D, Giaccia AJ, Stringer JR, *et al*. The human Ha-*ras* oncogene induces genomic instability in murine fibroblasts within one cell cycle. *Proc Natl Acad Sci U S A* 1994; **91**: 5124–5128.
40. Saavedra HI, Fukasawa K, Conn CW, *et al*. MAPK mediates Ras-induced chromosome instability. *J Biol Chem* 1999; **274**: 38083–38090.
41. Kasiappan R, Shih HJ, Chu KL, *et al*. Loss of *p53* and MCT-1 overexpression synergistically promote chromosome instability and tumorigenicity. *Mol Cancer Res* 2009; **7**: 536–548.
42. Shivapurkar N, Tang Z, Ferreira A, *et al*. Sequential analysis of K-*ras* mutations in aberrant crypt foci and colonic tumours induced by azoxymethane in Fischer-344 rats on high risk diet. *Carcinogenesis* 1994; **15**: 775–778.
43. Shivapurkar N, Belinsky SA, Wolf DC, *et al*. Absence of *p53* gene mutations in rat colon carcinomas induced by azoxymethane. *Cancer Lett* 1995; **96**: 63–70.

SUPPORTING INFORMATION ON THE INTERNET

The following supporting information may be found in the online version of this article.

Figure S1. Macroscopic appearances of metastatic tumour in the liver of a DMH-treated K-*ras*^{Val12} mouse.

Figure S2. Schematic representations of Ingenuity Pathway analysis to show pathway alterations resulting from changes in gene expression affected by either down-regulated genes (A) or up-regulated genes (B), identified in adenomas from DMH-treated K-*ras*^{Val12} mice (the grey-coloured molecules represent proteins found to be up-regulated or down-regulated).

Figure S3. Karyogram using schematic representations of murine chromosomes to portray genomic copy number alterations detected by array-comparative genomic hybridization of DNA from a single adenoma from a DMH-treated K-*ras*^{Val12} mouse (green = regions of loss, located in A5 band of chromosome 4, B3 band of chromosome 6 and D2 band of chromosome 14; red = regions of gain, located in G3 band of chromosome 5 and E2.1 band of chromosome 14).