DUSP5 is methylated in CIMP-high colorectal cancer but is not a major regulator of intestinal cell proliferation and tumorigenesis

Lars Tögel¹, Rebecca Nightingale¹, Rui Wu², Anderly C. Chüeh², Sheren Al-Obaidi², Ian Luk³, Mercedes Dávalos-Salas¹, Fiona Chionh¹, Carmel Murone¹, Daniel D. Buchanan³, Zac Chatterton³, Oliver M. Sieber³, Diego Arango⁵, Niall C. Tebbutt¹, David Williams³, Amardeep S. Dhillon¹ & John M. Mariadason¹,²

The ERK signalling pathway regulates key cell fate decisions in the intestinal epithelium and is frequently dysregulated in colorectal cancers (CRCs). Variations in the dynamics of ERK activation can induce different biological outcomes and are regulated by multiple mechanisms, including activation of negative feedback loops involving transcriptional induction of dual-specificity phosphatases (DUSPs). We have found that the nuclear ERK-selective phosphatase DUSP5 is downregulated in colorectal tumours and cell lines, as previously observed in gastric and prostate cancer. The DUSP5 promoter is methylated in a subset of CRC cell lines and primary tumours, particularly those with a CpG island methylator phenotype (CIMP). However, this epigenetic change alone could not account for reduced DUSP5 expression in CRC cells. Functionally, DUSP5 depletion failed to alter ERK signalling or proliferation in CRC cell lines, and its transgenic overexpression in the mouse intestine had minimal impact on normal intestinal homeostasis or tumour development. Our results suggest that DUSP5 plays a limited role in regulating ERK signalling associated with the growth of colorectal tumours, but that methylation the DUSP5 gene promoter can serve as an additional means of identifying CIMP-high colorectal cancers.

The ERK signalling pathway couples extracellular and oncogenic signals with molecular networks that control cell proliferation, differentiation, survival, and motility⁴,⁵. Activation of the pathway occurs when cell surface receptors such as the EGF receptor (EGFR) induce GTP-loading of RAS, which initiates sequential activation of RAF, MEK and ERK protein kinases. ERKs can phosphorylate a multitude of cytoplasmic and nuclear proteins, the combination of which depends on the duration, magnitude, and site of ERK activation⁶.

A key mechanism whereby cells control the dynamics of ERK signalling is by inducing negative feedback loops. This involves either direct ERK-mediated phosphorylation of RAF proteins, or transcriptional induction of upstream signalling inhibitors (eg. Sprouty2/4) and/or dual-specificity phosphatases (DUSPs) that directly target ERK⁴. These systems operate in a cell-specific manner and act at multiple steps of the pathway to maintain signalling flux at levels optimal for normal cell function.

DUSP proteins dephosphorylate tyrosine and serine/threonine residues required for activation of ERK, JNK and p38 mitogen-activated protein kinases (MAPKs)⁷,⁸. They contain a C-terminal catalytic domain, a N-terminal domain controlling subcellular localization, and a kinase interacting motif. DUSPs are broadly classified into 3

¹Olivia Newton-John Cancer Research Institute, Melbourne, Australia, La Trobe University School of Cancer Medicine, Melbourne, Australia. ²Ludwig Institute for Cancer Research, Melbourne, Australia. ³Colorectal Oncogenomics Group, Genetic Epidemiology Laboratory, Department of Pathology, The University of Melbourne, Parkville, Melbourne, Australia. ⁴Systems Biology and Personalised Medicine Division, The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia. ⁵Group of Biomedical Research in Digestive Tract Tumours, CIBBIM-Nanomedicine, Vall d’Hebron Research Institute (VHIR), Universitat Autonoma de Barcelona, Barcelona, Spain. Correspondence and requests for materials should be addressed to A.S.D. (email: amardeep.dhillon@onjcri.org.au) or J.M.M. (email: john.mariadason@onjcri.org.au)
groups based on their localization: group I DUSPs (DUSPs 1, 2, 4 and 5) are nuclear, group II DUSPs (DUSP 6, 7 and 9) are cytoplasmic, and group III DUSPs (DUSPs 8, 10 and 16) exist in both the nucleus and cytoplasm. Functionally, individual DUSPs often exhibit preference for specific MAPK substrates, with DUSPs 5, 6, and 7 being ERK-selective while DUSP9 prefers ERK over the p38 and JNK MAPKs.

DUSP5 is a nuclear ERK1/2-selective phosphatase induced by ERK signalling in mammalian cells7,8. It also controls ERK pathway activity by anchoring inactive ERK proteins in the nucleus, preventing MEK-mediated reactivation of ERK in the cytoplasm9. In mice, DUSP5 deficiency in the epidermis increases sensitivity to H-Ras-driven papilloma formation in a carcinogen-induced model of skin cancer9.

Consistent with this putative tumour suppressor role, DUSP5 expression is downregulated in prostate and gastric cancers where loss of expression is associated with poor prognosis10–12. In gastric cancer, DUSP5 downregulation was reported to correlate with promoter CpG island methylation, and its re-expression in gastric cancer cell lines reduced nuclear p-ERK levels and cell growth12.

Downregulation of DUSP5 was also recently reported in colorectal cancer (CRC), and shown to be associated with worse outcome16. However, the mechanisms underlying DUSP5 loss in this disease, and whether its loss contributes to aberrant ERK signalling or the initiation and/or progression colorectal cancers has not been established. In this study, we confirm that DUSP5 is significantly downregulated in CRC. Furthermore, we show that the DUSP5 promoter is methylated in a subset of CRCs, specifically those harboring the CpG island methylator phenotype (CIMP). However, promoter methylation alone did not correlate with altered DUSP5 expression in CRC cell lines or primary tumours, suggesting multiple mechanisms contribute to DUSP5 downregulation in this disease. Unexpectedly, knockdown of endogenous DUSP5 in CRC cell lines did not affect ERK signaling or proliferation, while transgenic overexpression of DUSP5 in the mouse intestine had little impact on normal intestinal homeostasis or tumorigenesis.

**Results**

**DUSP5 is downregulated in colorectal cancers (CRCs).** DUSP5 is a putative tumour suppressor gene that is aberrantly expressed in several cancers. To determine if the expression of DUSP5 is altered in CRC, we interrogated an in-house microarray dataset comprising 102 human CRCs and normal colonic tissue from 16 individuals (Fig. 1A), and RNA-seq data from the TCGA of 49 CRCs and matched adjacent normal tissue (Fig. 1B). Both datasets revealed significantly lower DUSP5 expression in CRC compared to normal tissue. This finding was further verified by qPCR analysis using an independent patient cohort consisting of 31 matched pairs of CRCs and normal colonic tissue (Fig. 1C). The tumour and normal colonic origins of the specimens is illustrated by higher levels of MYC in the tumour tissue (Fig. 1D).

**DUSP5 is induced by ERK signalling in CRC cell lines.** DUSP5 expression is induced by ERK signalling in several cell types6,7. To examine the dynamics of DUSP5 induction in CRC cells, we treated the KRAS/BRAF wild-type CRC cell line LIM1215 with epidermal growth factor (EGF), which induced a transient and robust increase in ERK phosphorylation (Fig. 2A). EGF also invoked a significant increase in DUSP5 protein levels but in a delayed and more prolonged manner compared to ERK phosphorylation, consistent with its role as a negative feedback regulator of this pathway. Examination of these effects at the transcriptional level demonstrated that EGF also induced a delayed but sustained induction of DUSP5, in contrast to the transient induction of the well-established ERK target, FOS (Fig. 2B). EGF induction of DUSP5 and FOS were also strongly attenuated by a MEK inhibitor (trametinib), demonstrating that DUSP5 is a bone fide target of ERK signaling in KRAS/BRAF wild-type CRC cells (Fig. 2C).

**The DUSP5 promoter is methylated in a subset of CRCs.** Downregulation of DUSP5 expression in gastric cancer is associated with hypermethylation of a CpG island in its promoter14. To determine if this epigenetic mechanism contributes to downregulation of DUSP5 in CRCs, we used bisulphite conversion and sequencing to interrogate 4 contiguous regions spanning the DUSP5 proximal promoter, exon 1, and the first part of intron 1, in a panel of 25 CRC cell lines (Fig. 3A). The 4 regions were found to be methylated in 5 cell lines (KM12, SW48, Co115, LIM2405 and RKO, Fig. 2B), all of which are microsatellite unstable (P = 0.046, Fisher’s Exact Test) and feature the CpG island methylator high phenotype (CIMP-H) (P = 0.009) (Fig. 3B). However, DUSP5 promoter methylation was not significantly associated with reduced DUSP5 mRNA levels across the cell line panel, with some heavily methylated cell lines (RKO) expressing high levels of DUSP5 (Fig. 3C).

To assess DUSP5 promoter methylation in primary CRCs, we developed a high-resolution melt (HRM) assay applicable to DNA extracted from formalin-fixed paraffin-embedded (FFPE) tissue samples. The assay interrogates a 51 base pair fragment harboring 9 CpG dinucleotides (Fig. 3A, grey shaded region) within the proximal DUSP5 promoter. After validating its efficacy in discriminating DUSP5 promoter methylation states in cell lines (Fig. S1), we used the HRM assay to evaluate DUSP5 promoter methylation status in 47 CRCs, which included 14 with a CIMP-Intermediate/High (CIMP-I/H) phenotype. DUSP5 promoter methylation was identified in 8/47 cases (17%). As observed in CRC cell lines, the methylation was more frequent in CIMP I/H tumours (6/14) compared to CIMP-Low (2/33) cases (<0.005, Fisher’s exact test) (Fig. 3D), however, was not associated with reduced DUSP5 mRNA levels (Fig. 3E, p = 0.189, unpaired t-test). Collectively, these data indicate that DUSP5 promoter methylation represents a novel marker of CIMP status in CRC but is not the predominant mechanism of DUSP5 repression in these tumours.

**DUSP5 is not a major regulator of ERK signalling in CRC cells.** The major functions of DUSP5 described to date are to dephosphorylate nuclear ERK1/2 proteins and to sequester their inactive forms in the nucleus6,8. We therefore assessed whether basal DUSP5 expression correlates with the levels of nuclear p-ERK in
a panel of 25 CRC cell lines. Basal levels of DUSP5 varied significantly among the cell lines but displayed no clear relationship with the ratio of phosphorylated to total ERK1/2 in the nucleus (Fig. 4A,B).

DUSP5 levels have been reported to be elevated in some cancers, particularly in the context of BRAF mutations. Studies in DUSP5-deficient MEFs suggest that these high levels of DUSP5 serve to dampen ERK pathway hyperactivation to facilitate transformation by mutant BRAF. To test if endogenous DUSP5 plays a similar role in CRC cells, we used the BRAF mutant RKO cell line, which expresses high levels of DUSP5 (Fig. 4A) and is dependent on ERK signalling for proliferation (Fig. S2). DUSP5 knockdown in RKO cells did not alter nuclear or cytoplasmic phospho-ERK1/2 levels or phosphorylation of the nuclear ERK substrate FRA1 (Fig. 4C). DUSP5 knockdown also had no effect on ERK-dependent transcriptional induction of FOS or FOSL1 (Fig. 4D).

Finally, DUSP5 depletion did not affect proliferation of RKO cells grown under standard or low-serum conditions (Fig. 4E). These results indicate that endogenous DUSP5 plays a limited role in regulating basal levels of ERK signalling in BRAF mutant CRC cells.

Next, we examined if induction of DUSP5 following EGF stimulation regulates the magnitude or duration of EGF-induced ERK signalling in CRC cells. Knockdown of DUSP5 expression in KRAS/BRAF wild-type LIM1215 cells had no effect on the dynamics of EGF-induced ERK phosphorylation or transcriptional induction of FOS or FOSL1 (Fig. 5A–C). Collectively, our data suggest that although DUSP5 is induced upon ERK pathway activation, it is not a major regulator of ERK signalling in CRC cells.

Figure 1. Downregulation of DUSP5 mRNA expression in CRC. DUSP5 mRNA expression in normal colonic mucosa and colorectal tumours determined by analysis of (A) an in-house Affymetrix microarray dataset and (B) RNA-Seq data available from the TCGA. (C) DUSP5 and (D) MYC mRNA expression in colon tumour/normal pairs normalised to GAPDH levels.
DUSP5 overexpression in the mouse intestinal epithelium does not alter ERK signalling, intestinal homeostasis or adenoma formation. To complement our cell line studies and test the growth suppressive effects of DUSP5 in vivo, we generated a mouse strain in which expression of a human Myc-tagged DUSP5 transgene is driven by the intestinal-specific villin promoter (Vil1-DUSP5Tg). The functionality of Myc-tagged human DUSP5 has previously been demonstrated in mouse embryo fibroblasts and in vivo studies involving transgene expression in the lymphoid compartment of mice15,18. Overexpression of Myc-DUSP5 specifically in the small intestine was confirmed at the mRNA and protein level by detection of human DUSP5 mRNA and the Myc-tag, respectively (Fig. 6A,B). Vil1-DUSP5Tg mice were viable, fertile and lacked a discernible phenotype, including no difference in body weight at 1, 3 or 12 months (Fig. 6C). We were unable to detect overexpression of the Myc-DUSP5 protein in the colonic epithelium, in agreement with previous reports that...
transgene expression driven by the villin promoter is more robust in the mouse small intestine. All subsequent studies were therefore performed on this tissue. To determine if DUSP5 overexpression alters ERK signalling in this tissue, we assessed p-ERK1/2 levels by immunohistochemistry. No difference in the intensity and pattern of p-ERK1/2 staining was evident in the crypt-villus axis between WT and Vil1-DUSP5 Tg mice (Fig. 6D). Similarly, analysis of intestinal epithelial cells isolated from WT and Vil1-DUSP5 Tg mice revealed no difference in expression of the ERK signalling-regulated genes Fos, Jun, Atf3, Egr1, Egr3, Myc, Dusp5 and Dusp6 (Fig. 6E).

ERK signalling plays an important role in regulating normal cell proliferation and maturation in the mouse intestine, particularly differentiation along the secretory cell lineage. No difference in the intensity and pattern of p-ERK1/2 staining was evident in the crypt-villus axis between WT and Vil1-DUSP5 Tg mice (Fig. 6D). Similarly, analysis of intestinal epithelial cells isolated from WT and Vil1-DUSP5 Tg mice revealed no difference in expression of the ERK signalling-regulated genes Fos, Jun, Atf3, Egr1, Egr3, Myc, Dusp5 and Dusp6 (Fig. 6E).

Finally, previous work has shown that DUSP5 exhibits tumour suppressive activity in a mouse model of carcinogen-induced skin tumorigenesis. To determine the tumour suppressive effects of DUSP5 in the intestinal...
Figure 4. DUSP5 regulation of basal ERK signaling in CRC cells. (A) Relationship between DUSP5 expression and levels of phosphorylated and total ERK1/2 in nuclear extracts of CRC cell lines. HDAC3 was used as loading control. (B) Analysis of the correlation between DUSP5 expression normalized to HDAC3 and the p-ERK2/ERK2 ratio following densitometric analysis of the data shown in (A). (C) Effect of DUSP5 knockdown in RKO cells on cytoplasmic and nuclear levels of phosphorylated and total ERK1/2, and ERK-mediated phosphorylation of the transcription factor FRA1 (FOSL1). BRAF was used as cytoplasmic marker. (D) Effect of DUSP5 knockdown on expression of the ERK-regulated genes FOSL1 and FOS in RKO cells. (E) Effect of DUSP5 knockdown on proliferation of RKO cells grown under normal (10% FCS) or low (1% FCS) serum conditions. *P < 0.05.

Figure 5. DUSP5 regulation of EGF-induced ERK signalling in CRC cells. Effect of DUSP5 knockdown on induction of (A) ERK1/2 phosphorylation, and mRNA levels of (B) DUSP5, (C) FOS, and (D) FOSL1 following treatment of LIM1215 cell with EGF (50 ng/ml).
epithelium in vivo, we crossed Vil1-DUSP5Tg mice with ApcMin/+ mice. Adenoma formation in ApcMin/+ mice requires ERK signalling and this model is widely used to investigate mechanisms regulating Apc mutation-initiated intestinal adenoma formation. ApcMin/+ mice developed the expected number of intestinal adenomas at 4 months of age, which was not affected by DUSP5 overexpression (Fig. 8A). Similarly, DUSP5 overexpression had no impact on tumour size (Fig. 8B) or the pattern and intensity of pERK staining in the adenomas (Fig. 8C). These findings indicate that DUSP5 overexpression does not exert tumour suppressive activity in a mouse model of intestinal tumorigenesis.

Discussion
In this study we provide clear evidence of DUSP5 downregulation in CRCs compared to normal colonic tissue. We show that the DUSP5 promoter is methylated in both CRC cell lines and patient tumours, but in contrast to previous findings in gastric cancer, our data indicate that this epigenetic mechanism alone is insufficient to account for downregulation of DUSP5. Notably, DUSP5 promoter methylation occurs predominantly in CIMP-high CRCs, where multiple genes and loci are coordinately methylated. Thus, DUSP5 may be a novel addition to the panel of genes preferentially methylated in this tumour subset.

While the lack of correlation between DUSP5 promoter methylation and gene expression was unexpected, this observation is consistent with genomic analyses indicating that the majority of genes methylated in CRC are not altered in expression. In fact, we found that some cell lines with extensive methylation of the DUSP5 promoter, such as RKO, feature high DUSP5 expression, likely due to strong ERK pathway activation driven by mutant BRAF. DUSP5 overexpression has been documented in several tumour types and cell lines harboring BRAF mutations. Recent studies using DUSP5-deficient MEFs indicate that the elevated DUSP5 levels function to prevent senescence by limiting ERK pathway activation to a level that promotes cell proliferation and transformation. In contrast to these data, we found that knockdown of endogenous DUSP5 did not alter proliferation, p-ERK levels or phosphorylation of the nuclear-localised ERK substrate FRA1 in BRAF mutant CRC cells. Furthermore, transgenic overexpression of DUSP5 in the mouse intestinal epithelium did not alter basal p-ERK levels or ERK-regulated gene expression, cell differentiation, proliferation or adenoma formation, processes that require activation of this pathway. Importantly, our findings are consistent with the lack of any discernible phenotype of DUSP5 knockout mice that has been previously reported, other than the differential response to DMBA challenge.
Our findings raise the possibility that other DUSP family members or negative feedback regulators, play more dominant roles in regulating the ERK pathway on the normal intestinal epithelium and in CRC. Indeed, previous studies have identified an important role for DUSP4 in regulating nuclear ERK phosphorylation in intestinal epithelial cells and in CRC cell lines. It was also reported that DUSP6 induction by p53 reduces global phospho-ERK levels in HCT116 CRC cells.

Collectively, our data indicate that DUSP5 is not a major negative regulator of ERK signalling in the intestinal epithelium and has limited tumour suppressive activity in CRC. Promoter methylation of DUSP5 in these cancers may be a passenger event, which differs from its role in gastric cancer cells. It however remains possible that DUSP5 may regulate the functions of the pathway in contexts other than those examined in the present study. Notably, DUSP5 downregulation in CRC has been shown to be adversely prognostic in advanced but not early stage disease, where it is associated with metastasis and markers of epithelial-mesenchymal transition. Further studies are thus needed to determine if DUSP5 contributes to regulation of the ERK pathway in more advanced stages of colorectal tumorigenesis.

**Figure 7.** Impact of DUSP5 overexpression on intestinal phenotype. Effect of DUSP5 overexpression on cell differentiation as determined by staining for (A) Goblet cell (Alcian Blue) and (B) Paneth cell (lysozyme) markers, and measuring mRNA levels of (C) villin (Vil1) and (D) alkaline phosphatase (Alpi). Effect of DUSP5 overexpression on (E,F) small intestinal length, (G) cell proliferation and (H) crypt cell number (10 crypts per mouse; 3 mice per genotype).
Methods

Cell lines and treatments. Colorectal cancer cell lines were maintained at 37 °C and 5% CO₂ in Dulbecco's Modified Eagle's Minimal Essential Medium Nutrient Mixture F-12 (DMEM/F-12) supplemented with 10% foetal calf serum (FCS) and penicillin (100 units/mL)/streptomycin (100ug/mL), all supplied by Invitrogen (Carlsbad, CA, USA).

Quantitative real-time PCR. RNA was extracted using the High Pure RNA Isolation Kit from Roche (Penzberg, Germany) according to the manufacturer's instructions. The quality, purity and amount of RNA isolated was analysed using the Nanodrop 1000 Spectrophotometer from Thermo Fischer Scientific (Waltham, MA, USA). The High Pure Paraffin Kit (Roche) was used to extract total RNA from FFPE human specimens. RNA Samples were reverse transcribed into cDNA using the Transcriptor first strand cDNA Synthesis Kit (Roche). Gene expression was measured by quantitative real time PCR (qPCR) using the 7500 Fast and Viia 7 Real-Time PCR Systems from Life Technologies (Carlsbad, CA, USA). Gene expression changes were compared relative to expression of a house keeping gene (GAPDH or β-actin) using the 2^−ΔΔCt method.

Bisulfite sequencing. Genomic DNA was extracted from colorectal cancer cell lines using the DNeasy Blood & Tissue Kit from QIAGEN (Hilden, Germany). Genomic DNA was extracted from formalin-fixed paraffin-embedded (FFPE) tumour tissue from patients with CRC using the QIAmp DNA FFPE Tissue Kit (QIAGEN, Hilden). Extracted DNA (500–1000ng) was bisulfite treated and purified using the EpiTect Bisulfite Kit (QIAGEN)). Converted DNA was eluted in 20 μl, of which 4μl was used to amplify 4 individual regions of the DUSP5 CpG island spanning the proximal promoter and 5′ intragenic region for PCR using Platinum Taq DNA polymerase (Invitrogen). PCR products were Sanger-sequenced by the Australian Genome Research Facility (AGRF, Melbourne, Australia). Sequences of converted DNA were evaluated using the BiQ Analyzer software28. Primers used were as follows: F1, 5′-TTAAATATATAGAAAAATATAGT-3′ and R1, 5′-ACACAAAAAATACCTTTC-3′, to amplify a region spanning −574 to −346 nucleotides upstream of the TSS. F2, 5′-GATTTTTTTGGGTATTGT-3′ and R2, 5′-TATAACAAAAAATACCTTTC-3′ to amplify a region spanning −395 to −26nt upstream of the TSS. F3, 5′-GGGAAATTTTATTTGTTTATTATTCTTTC-3′ to amplify a region spanning +512 to +726 nucleotides downstream of the TSS. R3, 5′-CCTCTTCTTGGAAATACCTTAC-3′ to amplify a region spanning −49 to +309nt of the TSS. F4, 5′-GGGAAATTTTATTTGTTTATTATTCTTTC-3′ to amplify a region spanning +512 to +726 nucleotides downstream of the TSS. R4, 5′-AACCCAAAAATACACCTCCTCAGA-3′ to amplify a region spanning +512 to +726 nucleotides downstream of the TSS.
High-resolution melting methylation analysis. Bisulfite-converted genomic DNA extracted from CRC cell lines or from cores of FFPE tumour tissue from patients with CRC were subjected to HRM using the MeltDoctor HRM Master Mix (Thermo Fischer Scientific) according to the manufacturer’s instructions. A sequence spanning nucleotides −396 to −303 upstream of the TSS was analyzed. The primers used were: DUSP5-1845.2005 (2005). The use of human CRC samples was performed in accordance with relevant guidelines and regulations of the Austin Health Human Research Ethics Committee. All patients gave written informed consent for use of their tumour material for research purposes.

Western blotting. Total protein was extracted from cells in RIPA buffer (50 mM Tris-HCL pH 7.5, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 0.1% SDS, 5 mM EGTA). Cytosolic protein was extracted in hypotonic lysis buffer (10 mM Tris-HCL pH 7.5, 10 mM KCl, 1.5 mM MgCl₂), following which nuclei were lysed in RIPA buffer. All buffers were supplemented with a cocktail of proteases (complete, Roche) and phosphatase (PhosSTOP, Roche) inhibitors. Protein concentrations were determined using the Bradford protein assay from Bio-Rad (Hercules, CA, USA). Samples were mixed with Reducing Agent and NuPAGE LDS Sample Loading Buffer (both from Invitrogen), and sections were counterstained with hematoxylin, scanned using a ScanScope XT system (Aperio), and analyzed using ImageScope ver.12.1.0.5029 (Aperio).

Generation of Vil1-DUSP5<sup>tg</sup> mice. The myc-tagged coding sequence of human DUSP5 was amplified from the expression plasmid pSG5-DUSP5<sup>5</sup>, kindly provided by SM Keyse) using the forward primer BsiWI-DUSP5: TACGTACGCGGCAATTCTGAAGGTC and the reverse primer MluI-DUSP5: TACACGCCGTAGATCTGGATCCTTACAG, which introduced a BsiW I and a Mlu I restriction site at the 5′ and 3′ end of the PCR product, respectively. These sites were used to clone the PCR product into the expression vector pBSKsVillinSV40polA (kindly provided by Sylvie Robine), which contains a 9 kb fragment of the murine villin gene promoter to generate pBSKs-Vil1-DUSP5. This construct was used for pronuclear injections (Transgenic Animal Service, University of Queensland). Transgenic founder animals were bred with C57Bl/6 mice to generate heterozygous Vil1-DUSP5<sup>tg</sup> mice. All animal experiments were carried out in accordance with protocols approved by the Austin Health Animal Ethics Committee.

Immunohistochemistry. Immunohistochemistry was performed on 5 μm FFPE sections. Antigen was retrieved using Dako Target Retrieval Citrate buffered solution (Agilent Technologies, CA, USA), and sections incubated with anti-pERK1/2 (4370, Cell Signalling Technology, 1:100) and anti-Ki67 (MA5–14520, Thermo Scientific) antibodies used were fluorescent-labelled goat anti-mouse (IRdye680CW, LI-COR, 1:1500) and goat anti-rabbit (IRDy800CW, LI-COR, 1:15000). Signal was detected using the Odyssey imaging system (Li-Cor), and fluorescent intensities quantified using the Odyssey Infrared Imaging System software.

Statistical methods. All values shown are mean ± standard error of mean. For all analyses, significance level was set at p < 0.05.

Data availability. Additional data and reagents relating to the manuscript will be made available upon request.

References
1. Shaul, Y. D. & Seger, R. The MEK/ERK cascade: from signaling specificity to diverse functions. Biochim Biophys Acta 1773, 1213–1226, https://doi.org/10.1016/j.bbamcr.2006.10.005 (2007).
2. Dhillon, A. S., Hagan, S., Rath, O. & Kolch, W. MAP kinase signalling pathways in cancer. Oncogene 26, 3279–3290, https://doi.org/10.1038/sj.onc.1210421 (2007).
3. Murphy, L. O. & Blenis, J. MAPK signal specificity: the right place at the right time. Trends Biochem Sci 31, 268–275, https://doi.org/10.1016/j.tibs.2006.03.009 (2006).
4. Lake, D., Correa, S. A. & Muller, J. Negative feedback regulation of the ERK1/2 MAPK pathway. Cell Mol Life Sci 73, 4397–4413, https://doi.org/10.1007/s00018-016-2297-8 (2016).
5. Camps, M., Nichols, A. & Arkinstall, S. Dual specificity phosphatases: a gene family for control of MAP kinase function. FASEB J 14, 6–16 (2000).
6. Kidger, A. M. & Keyse, S. M. The regulation of oncogenic Ras/ERK signalling by dual-specificity mitogen activated protein kinase phosphatases (MKPs). Semin Cell Dev Biol 10, 125–132, https://doi.org/10.1016/j.semcdb.2001.01.009 (2016).
7. Kucharska, A., Rushworth, L. K., Staples, C., Morrice, N. A. & Keyse, S. M. Regulation of the inducible nuclear dual specificity phosphatase DUSP5 by ERK MAPK. Cell Signal 21, 1794–1805, https://doi.org/10.1016/j.cellsig.2009.07.015 (2009).
8. Mandl, M., Slack, D. N. & Keyse, S. M. Specific inactivation and nuclear anchoring of extracellular signal-regulated kinase 2 by the inducible dual-specificity protein phosphatase DUSP5. Mol Cell Biol 25, 1830–1845, https://doi.org/10.1128/MCB.25.5.1830-1845.2005 (2005).
9. Rushworth, L. K. et al. Dual-specificity phosphatase 5 regulates nuclear ERK activity and suppresses skin cancer by inhibiting mutant Harvey-Ras (H RasQ61L)-driven SerpinB2 expression. *Proc Natl Acad Sci USA* **111**, 18267–18272, https://doi.org/10.1073/pnas.1420159112 (2014).

10. Yan, X. et al. Dual specificity phosphatase 5 is a novel prognostic indicator for patients with advanced colorectal cancer. *Am J Cancer Res* **6**, 2325–2333 (2016).

11. Cai, C. et al. Down-regulation of dual-specificity phosphatase 5 predicts poor prognosis of patients with prostate cancer. *Int J Clin Exp Med* **8**, 4186–4194 (2015).

12. Shin, S. H., Park, S. Y. & Kang, G. H. Down-regulation of dual-specificity phosphatase 5 in gastric cancer by promoter CpG island hypermethylation and its potential role in carcinogenesis. *Am J Pathol* **182**, 1275–1285, https://doi.org/10.1016/j.ajpath.2013.01.004 (2013).

13. Montero-Conde, C. et al. Relief of feedback inhibition of HER2 transduction by RAF and MEK inhibitors attenuates their antitumor effects in BRAF-mutant thyroid carcinomas. *Cancer Discov* **3**, 520–533, https://doi.org/10.1158/2159-8290.CD-12-0531 (2013).

14. Pratilas, C. A. et al. V600E BRAF is associated with disabled feedback inhibition of RAF-MEK signaling and elevated transcriptional output of the pathway. *Proc Natl Acad Sci USA* **106**, 4519–4524, https://doi.org/10.1073/pnas.0900780106 (2009).

15. Kidger, A. M. et al. Dual-specificity phosphatase 5 controls the localized inhibition, propagation, and transforming potential of ERK signaling. *Proc Natl Acad Sci USA* **114**, E317–E326, https://doi.org/10.1073/pnas.1614641114 (2017).

16. Casalino, L., De Cesare, D. & Verde, P. Accumulation of Fra-1 in ras-transformed cells depends on both transcriptional autoregulation and MEK-dependent posttranslational stabilization. *Mol Cell Biol* **23**, 4401–4415 (2003).

17. Baboush, J., Chalbos, D., Hipskind, R., Jariel-Encontre, I. & Piechaczyk, M. Ubiquitin-independent proteasomal degradation of Fra-1 is antagonized by Erk1/2 pathway-mediated phosphorylation of a unique C-terminal destabilizer. *Mol Cell Biol* **27**, 3936–3950, https://doi.org/10.1128/MCB.01776-06 (2007).

18. Kovanen, P. E. et al. T-cell development and function are modulated by dual specificity phosphatase DUSP5. *J Biol Chem* **283**, 17362–17369, https://doi.org/10.1074/jbc.M709887200 (2008).

19. Madison, B. B. et al. Cis elements of the villin gene control expression in restricted domains of the vertical (crypt) and horizontal (duodenum, cecum) axes of the intestine. *J Biol Chem* **277**, 33275–33283 (2002).

20. de Jong, P. R. et al. ERKS signalling rescues intestinal epithelial turnover and tumour cell proliferation upon ERK1/2 abrogation. *Nat Commun* **7**, 11551, https://doi.org/10.1038/ncomms11551 (2016).

21. Heuberger, J. et al. Shp2/ MAPK signaling controls goblet/paneth cell fate decisions in the intestine. *Proc Natl Acad Sci USA* **111**, 3472–3477, https://doi.org/10.1073/pnas.1309342111 (2014).

22. Lee, S. H. et al. ERK activation drives intestinal tumorigenesis in Apc(min−/−) mice. *Nat Med* **16**, 665–670, https://doi.org/10.1038/nm.2143 (2010).

23. Jackstadt, R. & Sansom, O. J. Mouse models of intestinal cancer. *J Pathol* **238**, 141–151, https://doi.org/10.1002/path.4645 (2016).

24. Lao, V. V. & Grady, W. M. Epigenetics and colorectal cancer. *Nat Rev Gastroenterol Hepatol* **10**, 686–700, https://doi.org/10.1038/nrgastro.2011.173 (2011).

25. Moarri, M., Boeva, V., Vert, J. P. & Reyal, F. Changes in correlation between promoter methylation and gene expression in cancer. *BMC Genomics* **16**, 873, https://doi.org/10.1186/s12864-015-1994-2 (2015).

26. Cagnol, S. & Rivard, N. Oncogenic KRAS and BRAF activation of the MEK/ERK signaling pathway promotes expression of dual-specificity phosphatase 4 (DUSP4/MKP2) resulting in nuclear ERK1/2 inhibition. *Oncogene* **32**, 564–576, https://doi.org/10.1038/onc.2012.88 (2013).

27. Pya, S. et al. DUSP6 is a novel transcriptional target of p53 and regulates p53-mediated apoptosis by modulating expression levels of Bcl-2 family proteins. *FEBS Lett* **586**, 4233–4240, https://doi.org/10.1111/1742-4658.13489 (2012).

28. Bock, C. et al. BiQ Analyzer: visualization and quality control for DNA methylation data from bisulfite sequencing. *Bioinformatics* **21**, 4067–4068, https://doi.org/10.1093/bioinformatics/bti652 (2005).

Acknowledgements
Funding for this project was provided by the National Health and Medical Research Council (NHMRC) of Australia (1026555), an Australian Research Council Future Fellowship (FT0992234) to JMM, a NHMRC Senior Research Fellowship (1046092) to JMM, Ludwig Cancer Research, and the Operational Infrastructure Support Program, Victorian Government, Australia. Fiona Chionh was supported by a NHMRC PhD scholarship.

Author Contributions
J.M., L.T. and A.D. conceived and coordinated the study. Study design: L.T., R.N., J.M., A.D., D.A. and N.T. Experimental procedures and data processing: L.T., R.N., R.W., A.C., S.O., I.L., M.D., F.C., D.B., O.S. Manuscript writing: L.T., J.M. and A.D. All authors read and approved the final manuscript. Identification and provision of BRAF mutant colorectal cancer cases: D.W., C.M.

Additional Information
Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-018-20176-9.

Competing Interests: The authors declare that they have no competing interests.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2018