JNK signalling modulates intestinal homeostasis and tumourigenesis in mice

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Wnt signalling is a crucial signalling pathway controlling intestinal homeostasis and cancer. We show here that the JNK MAP kinase pathway and one of its most important substrates, the AP-1 transcription factor c-Jun, modulates Wnt signalling strength in the intestine. Transgenic gut-specific augmentation of JNK signalling stimulated progenitor cell proliferation and migration, resulting in increased villus length. In the crypt, c-Jun protein was highly expressed in progenitor cells and the absence of c-Jun resulted in decreased proliferation and villus length. In addition to several known c-Jun/AP-1 target genes, expression of Wnt target genes *Axin2* and *Lgr5* were stimulated by JNK activation, suggesting a cross talk of JNK to Wnt signalling. Expression of the Wnt pathway component TCF4 was controlled by JNK activity, and chromatin immunoprecipitation and reporter assays identified *tcf4* as a direct c-Jun target gene. Consequently, increased JNK activity accelerated tumourigenesis in a model of colorectal carcinogenesis. As *c-jun* is a direct target of the TCF4/ β -catenin complex, the control of *tcf4* expression by JNK/c-Jun leads to a positive feedback loop that connects JNK and Wnt signalling. This mechanism regulates the physiological function of progenitor cells and oncogenic transformation.

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Introduction

The intestinal tissue consists of adjoining villi and crypts. The villus protrudes into the intestinal lumen and contains terminally differentiated cells. Villi consist of three main epithelial cell types that fulfil the main functions of the gut. Enteroendocrine cells release gastrointestinal hormones, enterocytes absorb nutrients and goblet cells secrete a protective mucus barrier. The crypt is formed by an invagination of the epithelial sheet into the underlying connective tissue. Most of the cells present in the crypts are immature, with the notable exception of differentiated Paneth cells, which are located at the crypt base and secrete antibacterial peptides into the crypt lumen (Humphries and Wright, 2008).

Although it has been clear that intestinal crypts harbour the stem cells of this tissue, the exact location of these cells has remained controversial (Barker *et al*, 2008). Using long-term label retention, a classical assay for stem cells, the cell located at position +4 relative to the crypt bottom, above the terminally differentiated Paneth cells, was suggested to be the intestinal stem cell (Potten *et al*, 1974). The stem cell properties of +4 cells have recently been substantiated. Bmi-1 was identified as a marker for +4 stem cells and lineage tracing studies using a knock-in allele driving an inducible creER construct from the Bmi-1 promoter showed that +4 stem cells are capable of giving rise to all intestinal cell lineages (Sangiorgi and Capecchi, 2008).

In addition, using clonal marking techniques also the crypt base columnar (CBC) cells, which are located at the crypt bottom in between paneth cells, were suggested to have stem cell like properties (Cheng and Leblond, 1974a, b). CBC cells are easily identified due to their elongated shape and wedge shaped nuclei, in contrast to the +4 cells, which are difficult to distinguish morphologically from their neighbouring cells. An important finding that lends molecular support for the stem cell nature of CBC cells was the identification of *Lgr5*, also known as *Gpr49*, as a CBC cell marker gene. Moreover, elegant lineage-tracing experiments using a knock-in of an inducible creER allele showed that CBC cells could differentiate into all the intestinal cell lineages (Barker *et al*, 2007). Thus, there seem to co-exist two spatially separated stem cell compartments in the intestine. Whether the +4 and the CBC stem cells fulfill similar or different functions, or whether the molecular pathways regulating these two cell population are similar or distinct, is not clear.

The Wnt signalling pathway controls the proliferation and mediates developmental signals between cells. In the absence of Wnt signal, in unstimulated cells, a degradation complex consisting of the adenomatous polyposis coli (APC) tumour-suppressor protein, axin, and the glycogen synthase kinase, phosphorylates β -catenin marking it for subsequent ubiquitination and degradation. On Wnt ligand binding to its Frizzled receptor, a signalling cascade (termed canonical Wnt signalling) is triggered that destabilizes the degradation

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complex, allowing unphosphorylated β -catenin levels to accumulate and translocate to the nucleus where β -catenin functions as a co-factor for transcription factors of the T-cell factor/lymphoid-enhancing factor (TCF/LEF) family (Giles *et al*, 2003; Radtke and Clevers, 2005).

Wnt signalling can also activate β -catenin-independent pathways. An important pathway for non-canonical Wnt signal transduction is the activation of the c-Jun N-terminal kinases (JNK) (Veeman *et al*, 2003). JNKs are serine/threonine kinases that belong to the group of MAP kinases, which are activated by a plethora of extracellular signals and are essential mediators of signal transduction (Davis, 2000). JNK was originally identified, as the name suggests, as an activity that phosphorylated the c-Jun N-terminus (Derijard *et al*, 1994). The proto-oncoprotein c-Jun belongs to the AP-1 group of transcription factors, which is a crucial regulator of cellular proliferation, apoptosis and tumourigenesis (Shaulian and Karin, 2001; Eferl and Wagner, 2003). Using a leucine zipper interaction interface, c-Jun heterodimerizes and forms functional transcription factors with a number of interacting partners, including all members of the Fos and ATF families of proteins (Mechta-Grigoriou *et al*, 2001). AP-1 activity is strongly induced in response to numerous signals, including growth factors, cytokines and extracellular stresses (Davis, 2000). AP-1 stimulation is mediated, in part, through the phosphorylation of c-Jun by the JNKs (Davis, 2000). c-Jun N-terminal phosphorylation at the serine residues 63 and 73 and threonine residues 91 and 93 within its transactivation domain is thought to increase the transcription of target genes, one of which is the *c-jun* gene itself (Angel *et al*, 1988).

The Wnt signalling pathway is believed to be the major signalling pathway controlling intestinal homeostasis and cancer. *c-jun* is a well-characterized Wnt target gene (Mann *et al*, 1999; Staal *et al*, 2004) and absence of *c-jun* significantly delays tumourigenesis in mice heterozygous for a nonsense mutation at codon 850 of the Apc gene (*Apc*^{Min/+}), which develop multiple intestinal neoplasias due to excessive canonical Wnt signalling (Moser *et al*, 1993; Nateri *et al*, 2005). Although Wnt signalling seems to be essential for intestinal stem cell function and maintenance, little is known about the contribution and function of other signalling pathways. In this study, we show that the JNK MAP kinase signalling pathway and its main substrate, the c-Jun transcription factor, have an important role in intestinal progenitor cells and oncogenic transformation.

Results

Transgenic JNK pathway activation in the gut

Loss-of-function studies in the mouse have yielded important clues about JNK function (Yang *et al*, 1997; Kuan *et al*, 1999; Sabapathy *et al*, 1999; Chang *et al*, 2003), but the interpretation of the mutant phenotypes has been complicated by genetic redundancy as the JNK protein family is encoded by three genes *jnk1*, *jnk2* and *jnk3* (Gupta *et al*, 1996; Weston and Davis, 2002). It is conceivable that essential roles of MAP kinase signalling have been masked by redundancy and have remained undetected. Therefore, we devised a strategy that allows to reproducibly activate JNK signalling in mice.

To investigate the significance of JNK activation in the intestine, we generated transgenic mice allowing the overexpression of a constitutively active JNKK2-JNK1 fusion

protein (Zheng *et al*, 1999). A cassette encoding a fusion protein of β -galactosidase and NeoR (β -geo) flanked by loxP sites prevents the expression of constitutively active JNK1 (JNKK2-JNK1) before Cre-mediated recombination (β -geo-JNKK2-JNK1) (Figure 1A). Floxed single transgenic mice were crossed with Villin-cre transgenic mice previously shown to provide efficient gut-specific Cre activity (el Marjou *et al*, 2004).

In the absence of Cre recombinase, JNKK2-JNK1 fusion protein was not detectable in the intestine of β -geo-JNKK2-JNK1 single transgenic mice (Figure 1B), but JNKK2-JNK1 expression was induced in β -geo-JNKK2-JNK1;Villin-cre + double transgenic mice (designated JNKK2-JNK1^{AG} mice). c-Jun N-terminal phosphorylation was stimulated in JNKK2-JNK1^{AG} mice, and presumably because of the autoregulation of the *c-jun* promoter by c-Jun, led to increased c-Jun protein levels (Figure 1B). JNKK2-JNK1^{AG} mice developed normally and were indistinguishable from their control littermates. Histological analysis showed that the gross morphology of the intestine was normal in JNKK2-JNK1^{AG} mice, and that all four differentiated cell types of the intestine (paneth cells, enterocytes, goblet cells and enteroendocrine cells) were present (Figure 1C and Supplementary Figure 1).

JNK signalling increases intestinal cell proliferation and villus length

Although intestinal tissue architecture and cell differentiation was unaffected by increased JNK signalling, we noted that the average villus length seemed to be increased in JNKK2-JNK1^{AG} mice, which was corroborated by morphometric quantification (Figure 1C, D; and Supplementary Figure 2). Average villus length was slightly but consistently increased throughout the intestine in JNKK2-JNK1^{AG} mice compared with control mice, albeit not to the same extent. This suggested that JNK signalling increased the total number of cells present per villus unit.

JNK/c-Jun signalling controls proliferation in a number of cell types (Johnson *et al*, 1993; Hess *et al*, 2004), therefore we determined whether the increase in villus length might be caused by a pro-proliferative role of JNK signalling. At the crypt-villus junction, rapidly proliferating transit-amplifying (TA) cells are present that are capable of differentiating towards the intestinal cell lineages. Both bromodeoxyuridine (BrdU) labelling (Figure 1E) and immunohistochemistry (IHC) for the proliferation marker Ki67 (data not shown) indicated that the number of cycling transit-amplifying cells per crypt was increased by augmented JNK signalling in the small intestine (Figure 1E black arrowheads and Figure 1F; Supplementary Figure 2) and also in the colon (Supplementary Figure 3). In contrast to several other stem cell populations, including the +4 cells, CBC cells are frequently cycling and incorporate BrdU (Figure 1E red arrowheads). Activation of the JNK pathway in JNKK2-JNK1^{AG} mice significantly increased the percentage of CBC cells incorporating BrdU by 50% (Figure 1G).

JNK signalling also has well-established functions in the regulation of cell death and cell migration (Shaulian and Karin, 2002; Xia and Karin, 2004). However, survival of intestinal cells was not altered in JNKK2-JNK1^{AG} mice (Supplementary Figure 4). In contrast, long-term BrdU labelling showed an increased rate of migration across the crypt-villus axis (Figure 1H, I). Thus, it seems that stimulation of

Figure 1 JNK signalling increases progenitor cell proliferation and villus length. (A) Scheme of the β -geo-JNKK2-JNK1 construct before and after Cre recombination. When these single transgenic mice are crossed to Cre transgenic mice, the β -geo cassette is excised and the JNKK2-JNK1 cDNA is expressed. (B) Protein lysates from unrecombined β -geo-JNKK2-JNK1 and JNKK2-JNK1^{ΔG} intestines were analysed for JNK1, c-Jun, ser73 phosphorylated c-Jun (p-c-Jun^{ser73}), total JNK and β -actin (loading control) expression. (C) Haematoxylin and eosin staining of duodenum epithelium from β -geo-JNKK2-JNK1 and JNKK2-JNK1^{ΔG} mice. Green shading denotes proliferative zone (crypt, C) and yellow shading marks the zone of differentiation (villus, V). All animals were killed between 8–12 weeks. Scale bar represents 50 μ m. (D) Quantification of the villus length from the base of the villus to the villus apex of β -geo-JNKK2-JNK1 and JNKK2-JNK1^{ΔG} intestines. Histogram represents the villus length as mean $\pm$ s.e.m. in different regions of the gut (D = duodenum, J = jejunum and I = ileum) (* $P \leq 0.05$; student's *t* test). (E) Immunohistochemistry for BrdU on representative crypts of β -geo-JNKK2-JNK1 and JNKK2-JNK1^{ΔG} intestines 2 h BrdU post-injection. Black arrowheads represent BrdU+ proliferative progenitors whereas red arrow heads indicate BrdU+ columnar base cells (CBC). Scale bar represents 30 μ m. (F) Quantification of BrdU+ and Ki67+ cells in β -geo-JNKK2-JNK1 and JNKK2-JNK1^{ΔG} crypts (* $P \leq 0.05$; student's *t* test). (G) BrdU+ CBC quantification in β -geo-JNKK2-JNK1 and JNKK2-JNK1^{ΔG} crypts (* $P \leq 0.05$; student's *t* test). (H) Immunohistochemistry for BrdU on representative sections of β -geo-JNKK2-JNK1 and JNKK2-JNK1^{ΔG} intestines 24 h BrdU post-injection. (I) Quantification of BrdU+ cells in the villi of β -geo-JNKK2-JNK1 and JNKK2-JNK1^{ΔG} intestines 24 h BrdU post-injection (* $P \leq 0.05$; student's *t* test). (*n* and *P* values are detailed in Supplementary Table S1).

JNK signalling leads to increased cell number and villus length by regulating proliferation and migration of progenitor cells.

JNK signalling controls both AP-1 and Wnt target genes

To investigate in more detail the function of JNK signalling in intestinal homeostasis, we carried out IHC for a crucial substrate of the JNK family of kinases, the c-Jun transcription factor. Staining for c-Jun protein was detected at the apex of the villi (data not shown), but in the crypt c-Jun protein was highly expressed in CBC cells (Figure 2A red arrowheads).

Moreover, phosphorylated c-Jun at serine 73 (p-c-Jun^{ser73}) as a readout for JNK activity was detected in both CBC and TA progenitor cells and was elevated in JNKK2-JNK1^{ΔG} mice compared with controls (Figure 2B).

Importantly, staining for phosphorylated c-Jun was absent in *junAA* homozygous mutant mice, in which the JNK phosphoacceptor serines 63 and 73 are mutated to alanines and which served as a negative control for the IHC (Behrens *et al*, 1999) (Supplementary Figure 5). The expression of c-Jun and p-c-Jun^{ser73} indicates that JNK activity is present in progenitor cells and increased in JNKK2-JNK1^{ΔG} mice.

To understand the molecular mechanism of how JNK signalling controls intestinal stem cell function, we investigated the expression of known target genes of the JNK/c-Jun

signalling pathway. c-Jun autoregulates the transcription of the *c-jun* gene and accordingly *c-jun* mRNA levels were increased in JNKK2-JNK1^{ΔG} intestine (Figure 2C) (Angel *et al*, 1988). The expression of *cyclinD1* (*ccnd1*) is regulated by both JNK and Wnt signalling (Tetsu and McCormick, 1999; Wisdom *et al*, 1999; Wulf *et al*, 2001), and *cyclinD1* expression was also increased in JNKK2-JNK1^{ΔG} mice. CD44 is expressed in the crypt and induced in intestinal tumours (Wielenga *et al*, 1999; Sansom *et al*, 2004). *cd44* is a c-Jun target gene both in the intestine and in neurons (Raivich *et al*, 2004; Nateri *et al*, 2005), and augmentation of JNK signalling significantly induced *cd44* mRNA expression (Figure 2C). IHC showed that CD44-protein expression was greatly augmented in CBC cells and transient amplifying cells in the crypts of JNKK2-JNK1^{ΔG} mice (Figure 2D arrowheads).

As Wnt signalling is a key pathway in intestinal stem cells, we also investigated the regulation of bona fide Wnt target genes. Unexpectedly, JNK signalling also substantially increased the expression of the classical Wnt target genes *axin2* and *lgr5* (also called *gpr49*) (Jho *et al*, 2002; Van der Flier *et al*, 2007). *Lgr5* is highly expressed in CBC cells and has been used as a marker gene to define the CBC cells (Barker *et al*, 2007). It was conceivable that the increased *lgr5* mRNA abundance was due to an increased number of CBC cells, or alternatively, activation of JNK signalling might

Figure 2 Wnt target gene activation in JNKK2-JNK1^{ΔG} mice. (A, B) Immunohistochemistry for c-Jun (A) or p-c-Jun^{ser73} (B) on representative crypts of β-geo-JNKK2-JNK1 and JNKK2-JNK1^{ΔG} intestines. Red arrowheads represent c-Jun positively labelled columnar base cells (CBC). Scale bar represents 30 μm. (C) qRT-PCR analysis of *c-jun*, *ccdn1*, *cd44*, *axin2*, *lgr5*, *tcf4* and *gapdh* transcripts in JNKK2-JNK1^{ΔG} intestines compared with β-geo-JNKK2-JNK1. The data are normalized to β-actin and represented as fold induction over β-geo-JNKK2-JNK1 mice. (D) Immunohistochemistry for CD44 on representative crypts of β-geo-JNKK2-JNK1 and JNKK2-JNK1^{ΔG} intestines. Red arrowheads indicate CD44 staining in CBC whereas black arrowheads indicate CD44+ proliferative progenitors. Scale bar represents 10 μm. (E) *Lgr5* *in situ* hybridization in comparable regions from β-geo-JNKK2-JNK1 and JNKK2-JNK1^{ΔG} intestines. (F) Quantification of CBCs in β-geo-JNKK2-JNK1 and JNKK2-JNK1^{ΔG} crypts. (G) Western blot analysis of protein lysates from unrecombined β-geo-JNKK2-JNK1 and JNKK2-JNK1^{ΔG} intestine and liver for cyclinD1, TCF4 and β-catenin expression.

change the expression pattern of *lgr5* and stimulate *lgr5* expression inappropriately in differentiated cell types. However, *in-situ* hybridization for *lgr5* mRNA showed that the cell type specificity of *lgr5* expression was not altered in JNKK2-JNK1^{ΔG} mice, which as in controls was largely confined to the bottom of the crypt. Instead, *lgr5* levels in CBC cells were noticeably increased in JNKK2-JNK1^{ΔG} mice (Figure 2E arrowheads). Moreover, quantification showed normal numbers of CBC cells in JNKK2-JNK1^{ΔG} mice (Figure 2F). Therefore, activation of JNK signalling

stimulates both AP-1 and Wnt target gene expression in progenitor cells.

Wnt target gene induction in the gut is mainly controlled by the TCF4 transcription factor. *tcf4* (encoded by the *tcf7l2* gene) mRNA levels were augmented in JNKK2-JNK1^{ΔG} mice (Figure 2C), suggesting a potential mechanism of JNK-mediated Wnt target gene induction. Western blot analysis showed higher levels of cyclinD1 and TCF4 protein in the gut of JNKK2-JNK1^{ΔG} mice (Figure 2F). The expression of cyclinD1 and TCF4 in the liver, where Villin-cre is not active

and the JNKK2-JNK1 fusion protein is not expressed (data not shown), was comparable (Figure 2G).

Decreased progenitor cell proliferation and villus length in the absence of c-Jun

The expression of c-Jun in progenitor cells indicated a potential role of c-Jun in this cell population. To directly test the importance of *c-Jun* in intestinal homeostasis, we used mice with a tissue-specific inactivation of *c-jun* in the gut (*c-jun^{fl/fl}*; villin-cre+ mice or *c-jun^{ΔG}* mice) (Behrens *et al*, 2002; el Marjou *et al*, 2004; Nateri *et al*, 2005). IHC staining for c-Jun was not detected in the intestine of *c-jun^{ΔG}* mice, confirming the deletion of the floxed *c-jun* gene in progenitor cells and the specificity of the IHC staining (Figure 3A arrowheads). As in JNKK2-JNK1^{ΔG} mice, the gross intestinal architecture and histological appearance of *c-jun^{ΔG}* intestine was normal. However, the average villus length in *c-jun^{ΔG}* mice was reduced (Figure 3B). Morphometric quantification showed that there was subtle, but reproducible and statistically significant reduction of villus length in the absence of *c-jun* (Figure 3C). Not all regions of the gut were affected to the same extent by increased JNK signalling, possibly because of the different histological structure and biology of distinct regions of the intestine. Inactivation of *c-jun* also led to an appreciable reduction in the percentage of cells incor-

porating BrdU (Figure 3D, E) whereas *c-jun* deletion had no significant effect on CBC cell number (Figure 3F, G). Moreover, quantitative PCR and western blot analysis showed diminished levels of cyclinD1, TCF4 and *Lgr5* mRNA, and TCF4 and cyclinD1 protein in *c-jun^{ΔG}* gut (Figure 3H, I). Thus, inactivation of *c-jun* leads to the opposite phenotype than augmentation of JNK signalling.

tcf4 is a direct c-Jun target gene

The deregulation of *tcf4* expression in JNKK2-JNK1^{ΔG} mice suggested a potential mechanism of cross talk between JNK and Wnt signalling. Therefore we examined the regulation of TCF4 by JNK signalling in more detail. Treatment of HCT116 colon cancer cells with the JNK activator anisomycin (Ans) stimulated c-Jun and TCF4 expression (Figure 4A), whereas a pharmacological JNK inhibitor (JNKi) reduced *c-jun* and *tcf4* mRNA and protein levels (Figure 4B, C). Thus, *tcf4* expression like *c-jun* expression, is controlled by JNK activity. c-Jun overexpression resulted in increased TCF4 protein levels (Figure 4D). Moreover, to investigate whether endogenous c-Jun is required for *tcf4* expression, we generated a stable polyclonal HCT116 cell line expressing a lentivirus-mediated *c-jun* knockdown shRNA hairpin construct and a matched control cell line expressing an irrelevant shRNA hairpin. *c-jun* depletion significantly reduced *tcf4* mRNA and TCF4 protein

Figure 3 Absence of c-Jun decreases crypt cell proliferation and villus length. (A) Immunohistochemistry for c-Jun on representative crypts from *c-jun^{fl/fl}* and *c-jun^{ΔG}* intestines. Red arrow heads represent c-Jun positively labelled columnar base cells (CBC), black arrow heads represent c-Jun negative CBCs. Scale bar represents 30 μm. (B) Haematoxylin and eosin staining of jejunum epithelium from *c-jun^{fl/fl}* and *c-jun^{ΔG}* mice. Green shading denotes proliferative zone (crypt, C) and yellow shading marks the zone of differentiation (villus, V). All animals were killed between 8–12 weeks. Scale bar represents 50 μm. (C) Quantification of the villus length from the base of the villus to the villus apex of *c-jun^{fl/fl}* and *c-jun^{ΔG}* intestines. Histogram represents the villus length as mean ± s.e.m. in different regions of the gut (D = duodenum, J = jejunum and I = ileum) (**P* ≤ 0.05; student's *t* test). (D) Immunohistochemistry for BrdU on representative crypts from *c-jun^{fl/fl}* and *c-jun^{ΔG}* intestines. Black arrowheads represent BrdU+ proliferative progenitors. Scale bar represents 25 μm. (E) Quantification of BrdU+ cells is represented in the histogram and expressed as % of positive cells per crypt, considering *c-jun^{fl/fl}* as 100% (**P* ≤ 0.05; student's *t* test). (F) *Lgr5* *in situ* hybridization in comparable crypts from *c-jun^{fl/fl}* and *c-jun^{ΔG}* intestines. (G) Quantification of CBCs in *c-jun^{fl/fl}* and *c-jun^{ΔG}* crypts. (H) qRT-PCR analysis of *c-jun*, *ccdn1* and *tcf4* transcripts in *c-jun^{ΔG}* intestines compared with *c-jun^{fl/fl}*. The data are normalized to β-actin and represented as fold induction over *c-jun^{fl/fl}* mice. (I) Western analysis of protein lysates from *c-jun^{fl/fl}* and *c-jun^{ΔG}* intestines for c-Jun, TCF4, cyclinD1 and β-actin (loading control). * indicates non-specific band. (*n* and *P* values are detailed in Supplementary Table S1).

Figure 4 *tcf4* is a direct c-Jun target gene. (A) Western blot analysis of c-Jun and TCF4 protein levels in HCT116 cells treated with anisomycin (Ans) for the indicated time periods. (B) qRT-PCR analysis of *c-jun* and *tcf4* transcripts in HCT116 cells treated with SP600125 (JNKi) for the indicated time periods. (C) Western analysis of protein lysates from HCT116 cells treated with SP600125 (JNKi) for c-Jun, TCF4 and β-actin (loading control). (D) Western blot analysis of c-Jun and TCF4 protein levels in HCT116 transfected with an expression plasmid for c-Jun (c-Jun-Ires-gfp) or empty vector (Ires-gfp). (E) qRT-PCR analysis of *c-jun*, *tcf4*, *axin2* and *ccdn1* transcripts in HCT116-si-c-jun (stable cell line that express an shRNA against c-jun) or HCT116-si-control (expressing an irrelevant shRNA) (F) Western blot analysis of c-Jun, TCF4, cyclinD1 and β-actin (loading control) in HCT116-si-c-jun and HCT116-si-control cell lines. (G) Schematic representation of TCF4 promoter. Black rectangles denote AP1 consensus binding sites. Arrows indicate approximate positions of qPCR primers used in ChIPs. (H) ChIP was carried out using HCT116 cells treated for 2 h with JNK inhibitor (JNKi) or Ans and c-Jun binding to the TCF4-1 region was determined by qPCR. Data is represented as IP/INPUT (%). Rabbit IgG antibody was used as an isotype control. (I) Schematic representation of pGL3-TCF4-1. (J) HCT116 cells were transfected with pGL3-TCF4-1, pGL3-uPA or pGL3-TATA together with c-Jun, c-Jun~ATF2 and c-Jun~Fos overexpression vector (or empty vector as control). Data represent luciferase activity relative to pGL3-TATA+empty-vector-transfected cells. (K–M) Double immunofluorescence for c-Jun (red; K) and TCF4 (green; L) and the merge image (M) on paraffin section from Apc^{Min/+} tumour.

(Figure 4E, F). Moreover, also *cyclinD1* and, importantly, *axin2* mRNA levels were noticeably reduced (Figure 4E). Consequently the JNK/c-Jun pathway was required for efficient proliferation of HCT116 tumour cells as both *c-jun*

knock down and pharmacological JNK inhibition reduced growth of these cells (Supplementary Figure 6).

To investigate the regulation of *tcf4* expression, we screened the *tcf4* promoter for the presence of AP-1/c-Jun

binding sites. Computational transcription factor binding site predictions identified two clusters of AP-1/c-Jun binding sites. At position –996 to –833 bp relative to the TATA box, three AP-1 sites were predicted closely adjacent to each other, and a second cluster of binding sites within the first exon of the *tcf4* gene (Figure 4G). To investigate the relevance of these sites, c-Jun chromatin immunoprecipitation (ChIP) assays were carried out. c-Jun ChIP showed efficient binding to the cluster of AP-1 sites in the *tcf4* promoter, but not to the cluster in the *tcf4* coding region (Supplementary Figure 7). These findings are in agreement with a genome-wide ChIP-on-chip analysis, which identified the *tcf4* promoter region as a target for phosphorylated c-Jun (Hayakawa *et al*, 2004). c-Jun autoregulates its transcription through two proximal binding sites (Stein *et al*, 1992; Nateri *et al*, 2005) and *c-jun* is probably the best characterized gene regulated by JNK signalling (see also Figure 4A–C). The regulation of c-Jun binding to the *tcf4* promoter was similar to c-Jun interaction with the *c-jun* promoter. JNK activation by Ans-stimulated c-Jun binding to both promoters whereas pharmacological JNK inhibition (JNKi) reduced it (Figure 4H; Supplementary Figure 7).

To further analyse the regulation of the *tcf4* promoter, a 375 bp fragment containing the three AP-1/c-Jun sites of the *tcf4* promoter was fused to a luciferase reporter gene (*tcf4*-luc, Figure 4I). AP-1 is a dimeric transcription factor that is made up of the members of the Jun, Fos and ATF protein families. Different dimer combinations have different DNA-binding specificities: c-Jun/ATF2 dimers (and c-Jun homodimers) preferentially bind to the 8-bp CRE site, whereas c-Jun/c-Fos dimers preferentially bind to 7-bp TRE sites. To investigate potential AP-1 dimer specificity in *tcf4*-luciferase regulation, we made use of tethered single-chain AP-1 dimers (Bakiri *et al*, 2002). We carried out a side-by-side comparison in HCT116 cells of the *tcf4*-luc construct with a classical AP-1 reporter construct, urokinase plasminogen activator-1 (uPA)-luciferase, that contains a well-characterized CRE AP-1 site, to have a benchmark for the extent of activation that can be achieved. c-Jun overexpression activated uPA-luciferase 3-fold, compared with 1.5-fold for the *tcf4*-luciferase construct (Figure 4J). In agreement with the presence of a CRE site in the uPA-luciferase, c-Jun/ATF2 dimers activated this construct to similar degree as c-Jun, but a c-Jun/c-Fos dimer was less efficient. Interestingly, Jun/ATF2 dimers were very inefficient on *tcf4*-luciferase, but activation through c-Jun/c-Fos dimer was substantial and comparable to uPA-luciferase activation by Jun/ATF2 dimer. Thus, the *tcf4* promoter fragment containing the c-Jun binding site identified by ChIP does mediate transcriptional induction in response to AP-1 activation. In addition, strong activation of Wnt signalling in adenomas of aged *Apc^{Min/+}* heterozygous mice resulted in c-Jun expression, and TCF4 was found to be highly expressed in c-Jun-expressing tumour cells (Figure 4K–M). These data indicate that *tcf4* is a direct target gene of JNK/c-Jun.

Activation of JNK signalling accelerates colitis-induced tumourigenesis, but not tumourigenesis triggered by mutant APC

Chronic inflammation (colitis) increases the risk of colon-cancer development in humans (Itzkowitz and Yio, 2004). In mice, administration of azoxymethane (AOM) followed by

dextran sodium sulphate (DSS) ingestion causes severe colonic inflammation and the subsequent development of colitis-associated tumours (Figure 5A). AOM/DSS-induced tumours most frequently show mutations of the β -catenin gene in its GSK-3 β phosphorylation consensus motif, thereby leading to constitutive activation of the Wnt signalling pathway (Takahashi and Wakabayashi, 2004). Using this protocol, control mice develop tumours in the colon approximately 20 weeks after the treatment (Neufert *et al*, 2007). However, even at 13 weeks, some JNKK2-JNK1^{AG} mice showed distress and weight loss. Quantification showed that both tumour incidence and tumour number was increased in AOM/DSS-treated JNKK2-JNK1^{AG} mice compared with controls (Figure 5B, C). Tumour cell proliferation was significantly increased by augmented JNK signalling (Figure 5D). In adjacent colonic tissue, β -catenin was mainly detected at the cell membrane (Figure 5E, F), but tumours in both control and JNKK2-JNK1^{AG} mice contained cells showing nuclear accumulation of β -catenin protein (Figure 5G, H arrowheads). c-Jun levels were increased in JNKK2-JNK1^{AG} adenomas compared with control tumours (Figure 5I, J). Co-staining for β -catenin and c-Jun showed a tendency that c-Jun protein expression was increased in tumour cells with nuclear β -catenin, but the percentage of tumour cells showing nuclear accumulation of β -catenin was comparable in JNKK2-JNK1^{AG} mice and control tumours (Supplementary Figure 8). Biochemical analysis of tumour material showed that the JNKK2-JNK1 transgene was expressed in adenomas and the levels of phosphorylated c-Jun and TCF4 were augmented (Figure 5K). Presumably as a consequence, the transcription of both AP-1 target genes like *c-jun* and *cd44* and of the Wnt target genes *axin-2* and *lgr5* was increased (Figure 5L). Thus, activation of JNK/c-Jun signalling leads to increased susceptibility to colitis-induced tumourigenesis.

To analyse JNK function in intestinal tumourigenesis in more detail, we analysed the effect of JNK activation in tumours triggered by mutant *Apc*. Unexpectedly, the presence of the JNKK2-JNK1^{AG} transgene did neither alter tumour number nor tumour cell proliferation in an *Apc^{Min/+}* genetic background (Figure 6A, B). Moreover, c-Jun protein levels in *Apc^{Min/+}*:JNKK2-JNK1^{AG} compound-mutant tumours were comparable to *Apc^{Min/+}* tumours (Figure 6C). Thus, augmentation of JNK signalling has differential effects in two different models of intestinal tumourigenesis.

To gain insights into the differential function of JNK signalling in intestinal tumourigenesis, we considered the possibility that there might be differences in JNK activity in different regions of the gut. Colitis-induced tumours arise in the colon, whereas most of the adenomas in *Apc^{Min/+}* mice form in the small intestine. However, the levels of active JNK and phosphorylated c-Jun were similar throughout the intestine (Figure 6D). In contrast, there was a striking difference in JNK activity between colitis-induced and *Apc^{Min/+}* tumours. Different JNK isoenzymes were expressed and activated in *Apc^{Min/+}* tumours and colitis-induced tumours. Presumably, as a consequence, the levels of phosphorylated c-Jun were very high in *Apc^{Min/+}* tumours compared with colitis-induced tumours. (Figure 6E). Expression of the JNKK2-JNK1^{AG} transgene increased the low levels of c-Jun phosphorylation in colitis-induced tumours, but had no appreciable effect in *Apc^{Min/+}* tumours (Figure 6E). These observations suggest that the differential effects of JNK

Figure 5 JNK overexpression accelerates tumourigenesis in the AOM/DSS model of colon carcinoma. **(A)** Schematic representation of the experimental design followed. **(B)** Quantification of incidence of tumours. Data represent the % of mice with tumours (JNKK2-JNK1 ($n = 9$) and JNKK2-JNK1^{ΔG} ($n = 7$)). **(C)** Quantification of number of AOM/DSS-induced tumours in β-geo-JNKK2-JNK1 and JNKK2-JNK1^{ΔG} mice ($*P \leq 0.05$; student's t test). **(D)** Quantification of BrdU+ cells per tumour area. **(E–J)** Immunohistochemistry for β-catenin in large bowel crypts adjacent to tumours (E, F) and β-catenin (G,H) and c-Jun (I,J) staining on AOM/DSS-induced tumours of β-geo-JNKK2-JNK1 and JNKK2-JNK1^{ΔG} mice. Black arrowheads indicate some nuclear β-catenin or c-Jun positively labelled cells. Scale bar represents 50 μM. **(K)** Western blot analysis of c-Jun, p-c-Jun^{ser73}, TCF4, JNK, JNKK2-JNK1 and β-actin as control in JNKK2-JNK1^{ΔG} and β-geo-JNKK2-JNK1 AOM/DSS-induced colorectal tumours. **(L)** qRT-PCR analysis of *c-jun*, *cd44*, *tcf4*, *axin2*, *lgr5* and *gapdh* transcripts in JNKK2-JNK1^{ΔG} and β-geo-JNKK2-JNK1 AOM/DSS-induced colorectal tumours. The data are normalized to β-actin and represented as fold induction over β-geo-JNKK2-JNK1 mice tumour. (n and P values are detailed in Supplementary Table S1).

activation in the two intestinal tumour models might be because of relative differences in endogenous JNK pathway activation status.

Discussion

Cross talk of JNK and Wnt signalling

In this study, we show that JNK signalling regulates intestinal homeostasis and tumourigenesis, and we propose that this at

least in part is due to a cross talk of JNK to Wnt signalling. Recently, it has been shown that JNK2, and to a lesser extent the related JNK1 isoenzyme, can induce nuclear translocation of β-catenin by directly phosphorylating the protein (Wu *et al*, 2008). We have not obtained evidence for the increased nuclear localization of β-catenin in JNKK2-JNK1^{ΔG} intestine (Figure 5E, F; Supplementary Figure 8). A potential explanation for this could be the fact that we used an activated form of JNK1, but not JNK2, in our study.

Figure 6 JNK overexpression in the APC^{Min/+} model of intestinal tumorigenesis. (A) Quantification of number of tumours in β -geo-JNKK2-JNK1 and JNKK2-JNK1 ΔG mice in an APC^{Min/+} background. (B) Quantification of BrdU+ cells per area in tumours found in different intestinal regions. (C) Immunohistochemistry for c-Jun staining on APC-induced tumours of β -geo-JNKK2-JNK1 and JNKK2-JNK1 ΔG mice. Scale bar represents 50 μ m. (D) Western blot analysis of p-c-Jun^{ser73}, p-JNK, JNK and β -actin in different intestinal regions in wild-type mouse (D: duodenum, J: jejunum, I: ileum and C: colon). (E) Western blot analysis of p-c-Jun^{ser73}, p-JNK, JNK and β -actin in JNKK2-JNK1 ΔG and β -geo-JNKK2-JNK1 tumours obtained in the two models used: AOM/DSS and APC^{Min/+}. (F) Summary table with the proposed mechanism for tumour promotion/tumour suppression mediated by JNK/cJun pathway modulation in different intestinal tumorigenic models.

Our data suggest that in addition to β -catenin, JNK signalling also regulates TCF4, the second component of this transcription factor co-activator complex. TCF4 and c-Jun are the key transcription factors that mediate the effects of Wnt and JNK signalling, respectively. TCF/ β -catenin strongly activates *c-jun* transcription in both hematopoietic cells and colon-cancer cells (Mann *et al*, 1999; Staal *et al*, 2004; Nateri *et al*, 2005). We show that the interaction between Wnt and JNK signalling is reciprocal, and that the JNK/c-Jun pathway regulates the expression of the *tcf4* gene. Thus, the mutual regulation of c-Jun and TCF4 form a positive feedback loop that links Wnt and JNK signalling. This mechanism seems to operate both in physiological and pathological conditions. High JNK activity not only stimulated the expression of AP-1 target genes, but also increases the strength of Wnt signalling, and caused augmented progenitor-cell proliferation and longer villi. Similarly, JNKK2-JNK1 ΔG mice were more susceptible to inflammation-induced colon cancer, presumably because of the concomitant activation of both AP-1 and Wnt target genes by JNK signalling. TCF4 protein levels were significantly reduced in the absence of c-Jun, suggesting a mechanism for the reduction of intestinal cancer development

observed in Apc^{Min/+} mice lacking *c-jun* (Nateri *et al*, 2005). It is noteworthy that an important role for JNK signalling has recently been described in *Drosophila* gut. Also in this organism JNK signalling controls the proliferation of intestinal stem cells (Biteau *et al*, 2008).

Function of JNK in intestinal cancer

The function of JNK signalling in cancer is ambiguous. On one hand, JNK activity is strongly activated by oncogenes and JNK activity is elevated in certain tumour types (Derijard *et al*, 1994; Engelberg, 2004). However, loss-of-function mutations in JNK-pathway components (JNK3 and MKK4) are found in human tumours, indicating a tumour suppressing role for JNK (Teng *et al*, 1997; Yoshida *et al*, 1999, 2001). The genetic and mechanistic basis for these different roles of JNK in tumour biology is a key question that remains unresolved. The realization that JNK inhibition may be of therapeutic benefit has led to the development of small molecule inhibitors of JNK (Manning and Davis, 2003; Karin and Gallagher, 2005), but an improved understanding of the apparently contradictory functions of JNK will be required to validate JNK inhibition as a therapeutic strategy for cancer.

This study suggests that JNK signalling can have a pro-tumourigenic function in certain forms of intestinal cancer, however, another study found that mice lacking *jnk1* develop spontaneous intestinal tumours (Tong *et al*, 2007). The reasons for these apparent discrepancies are not clear. As in this study we used intestine-specific gene activation and inactivation, respectively, the phenotypes observed are due to a cell-autonomous function of JNK/c-Jun in intestinal epithelial cells. In contrast, *jnk1*-mutant mice are conventional knockouts that lack JNK1 in all cells. It is therefore conceivable that JNK1 function in non-intestinal cell types, for example in immune cells by causing inflammation or reduced immune surveillance, contributes to tumour development. In line with this, *jnk1*-mutant mice have been reported to show reduced immunity and *jnk1*-deficient T cells show abnormal T helper cell differentiation and cytokine production (Dong *et al*, 1998; Constant *et al*, 2000). Moreover, there is cross talk between JNK isoenzymes. Fibroblasts lacking *jnk2* unexpectedly show higher JNK activity and c-Jun phosphorylation (Sabapathy and Wagner, 2004; Sabapathy *et al*, 2004). As neither JNK activity nor the level of c-Jun phosphorylation was analysed in *jnk1*-deficient intestines, it is unclear whether a similar phenomenon occurs in the absence of JNK1 (Tong *et al*, 2007).

c-Jun was reported to be expressed only at the tip of the villus (Hasselblatt *et al*, 2008). However, it is unlikely that the expression of c-Jun in differentiated intestinal cells contributes to its function in intestinal tumour formation. We have confirmed c-Jun expression at the tip of the villus (data not shown), but our analysis shows that c-Jun is also highly expressed in CBC stem cells and it is likely that the function of c-Jun in intestinal homeostasis and tumourigenesis are because of c-Jun function in stem cells. It should be noted that c-Jun was recently identified as one of the 95 genes that form the core transcriptome of Lgr5-positive CBC stem cells (van der Flier *et al*, 2009), thereby providing an independent confirmation for the stem cell specific c-Jun expression described in this study.

Our observations that JNK signalling is differentially activated in colitis-induced and *Apc*^{Min/+} tumours highlights the different molecular mechanisms of tumour formation in these two models of intestinal cancerogenesis. Moreover, it may provide a molecular explanation for the discrepancies that have been reported with regard to the function of c-Jun during intestinal tumourigenesis. c-Jun is required for *Apc*^{Min/+}-triggered tumourigenesis (Nateri *et al*, 2005), but mice lacking *c-jun* show normal colitis-induced intestinal-tumour formation (Hasselblatt *et al*, 2008). The differential requirement for c-Jun is directly opposite to the phenotypes observed for JNKK2-JNK1^{ΔG} mice. During colitis-induced tumourigenesis, in which JNK/c-Jun signalling is low, JNK activation accelerates tumourigenesis, whereas in *Apc*^{Min/+} tumours, in which the JNK/c-Jun pathway is highly active, transgenic JNK activation has no effect. This suggests that loss-of-function of the JNK/c-Jun pathway only reduces tumour formation in tumours with high pathway activity, whereas gain-of-function of the JNK/c-Jun pathway only accelerates tumourigenesis in tumours with low JNK activity (Figure 6F). We speculate that the different status of JNK/c-Jun pathway activation in colitis-induced and *Apc*^{Min/+}-induced intestinal tumours may explain the differential

requirements for JNK activity and c-Jun in different model of intestinal cancer.

Materials and methods

Mouse lines

The β-geo-JNKK2-JNK1 transgenic construct was electroporated into embryonic stem cells, stable transfectants were selected and mice generated according to standard protocols (Behrens *et al*, 1999). β-gal staining was used to ensure high transgene expression in ES cells and Southern blot analysis was used to confirm single-copy integration (data not shown). *c-jun*^{fl/fl} and Villin-cre mice have been described before (Behrens *et al*, 2002; el Marjou *et al*, 2004; Nateri *et al*, 2005).

Immunohistochemical staining and immunofluorescence

Mice were injected intraperitoneally with 100 mg/kg BrdU (Sigma), 1.5 h (or 24 h where indicated) before killing. Mice were killed by cervical dislocation and the small intestines were dissected out into ice-cold PBS and the faecal contents flushed out with ice-cold PBS. The intestines were cut longitudinally into pieces of similar size, opened out and fixed overnight in 10% neutral buffered formalin, briefly washed with PBS and transferred into 70% ethanol, rolled, processed and embedded into paraffin. Sections of the size of 4 μm were cut for haematoxylin and eosin staining, immunohistochemistry and immunofluorescence. For immunohistochemistry, antibodies against BrdU (Becton Dickinson), c-Jun (Becton Dickinson), CD44 (Chemicon) and β-catenin (Becton Dickinson) were used. Anti-TCF4 (Abcam) and anti-c-Jun (Santa Cruz Biotechnology) were used for immunofluorescence as described before (Nateri *et al*, 2005). For the morphometric analysis of the villi, the distance between the base of the villus and the villus apex was measured using AxioVision digital image processing software. A minimum of 40 crypt-villus units in comparable intestinal regions were quantified for at least four mice of each genotype. To quantify the BrdU, Ki67 or CBC-BrdU positive cells per crypt, 100 full crypts were scored from at least three mice of each genotype. Data are represented as mean ± s.e.m. ($P \leq 0.05$ was considered statistically significant in student's *t* test).

In situ hybridization

In situ hybridization was carried out as previously described (Barker *et al*, 2007). All ISH were carried out by the CRUK *in situ* hybridization service at LRI.

Western blot analysis

Mice were killed by cervical dislocation and the intestines were removed and flushed extensively with cold PBS. The first 3 cm of duodenum was taken and frozen in liquid nitrogen. Cell lysates were homogenized in RIPA lysis buffer (NEB) supplemented with protease inhibitor (Sigma). Immunoblots were carried out as previously described (Nateri *et al*, 2005). Antibodies against monoclonal anti-human-JNK1 (Becton Dickinson), c-Jun (Becton Dickinson), p-c-Jun^{ser73} (Cell Signalling), SAPK/JNK (Cell Signalling), cyclinD1 (Cell Signalling), TCF4 (Upstate), β-catenin (Becton Dickinson) and β-actin (Sigma) were used. The proteins on the gels were transferred to nitrocellulose membranes, and the membranes were immunoblotted with various antibodies as indicated. HCT116 western-blot analysis was carried out as described above.

qRT-PCR analysis

For quantitative real-time-PCR analysis, total mRNA was isolated from dissected intestines or HCT116 cells using RNeasy Mini-kit according to the manufacturer's instructions (Qiagen). For quantitative real-time-PCRs, cDNA was synthesized using Invitrogen Superscript reagents according to the manufacturer's instructions. Quantitative real-time PCR was accomplished with SYBR Green incorporation (Platinum Quantitative PCR SuperMix-UDG w/ROX, Invitrogen) using an ABI7900HT real-time PCR system (Applied Bioscience), and the data were analysed using the SDS 2.3 software. Results were normalized to those obtained with β-actin and results are presented as fold induction over control mice. The following primers were used for mouse tissue qRT-PCR analysis:

F-c-JUN: 5'-TGAAAGCTGTGTCCCTGTC-3'
R-c-JUN: 5'-ATCACAGCACATGCCACTTC-3'

F-CD44: 5'-CTCTGGGCACTGGCTCTGA-3'
R-CD44: 5'-CTGCCCACCTTCTCTACTATT-3'
F-CCDN1 : 5'-GTGCGTGAGAGGAGATTGT-3'
R-CCDN1: 5'-CTCACAGACCTCCAGCATCCA-3'
F-TCF7L2: 5'-GAGAGTGCAGCCATCAACCAG-3'
R-TCF7L2: 5'-GTGATCGGAGGAGCGAAAGG-3'
F-AXIN2: 5'-GGTTCGGCTATGCTTTTGC-3'
R-AXIN2: 5'-CAGTGCCTCGCTGGATAACTC-3'
F-GRP49: 5'-CGGAGGAAGCGCTACAGAAT-3'
R-GRP49: 5'-CTGGGTGGCAGTAGCTGAT-3'
F- β -actin: 5'-ATGCTCCCGGGCTGTAT-3'
R- β -actin: 5'-CATAGGAGTCCTTCTGACCCATTC-3'
F- GAPDH: 5'-TGAAGCAGGCATCTGAGGG-3'
R-GAPDH: 5'-CGAAGGTGGAAGAGTGGGAG-3'
The following primers sequences were used for HCT116 qRT-PCR analysis:
F-TCF7L2-h: 5'-TGAAGGCAGCTGCCTCAGC-3'
R-TCF7L2-h: 5'-GTGGGTGGCCTCAGCGAGC-3'
F-c-JUN-h: 5'-TCGACATGGAGTCCAGGA-3'
R-c-JUN-h: 5'-GGCGATTCTCTCCAGCTTCC-3'
F- β -actin-h: 5'-GGATGCAGAAGGAGATCACTG-3'
R- β -actin-h: 5'-CGATCCACACGGAGTACTTG-3'

Chromatin Immunoprecipitation

ChIP analysis was carried out as described previously (Nelson *et al*, 2006). Cells were treated 2 h before collection with 50 μ M JNKi (SP600125, Calbiochem) or 25 ng/ml Ans (Sigma). Immunoprecipitations were carried out with conjugated agarose beads for anti-c-Jun (Santa-Cruz Biotechnology) or rabbit IgG as isotype control. The oligonucleotide sequences used to amplify the *tcf4* DNA fragments by qPCR were:

Ch-dist-TCF4-H (FW): 5'-TCATTGTAGATGACCAGGAACCTTTG-3'
Ch-dis-TCF4-H (RV): 5'-AAAGATCAACCTCCACTTTCAGA-3'

Reporter gene assay

The region containing the distal 3xAP1 consensus binding sites of the *tcf7l2* promoter (from -996 to -833 bp relative to the TATA box) was cloned in pGL3-enhancer (Promega). Expression plasmid for c-Jun has been described before (Nateri *et al*, 2005). Expression

plasmid for c-Jun ~ ATF2 or c-Jun ~ Fos have been described before (Bakiri *et al*, 2002). HCT116 cells were transfected using Lipofectamine reagent (Invitrogen). Transient transfections of the experimental samples and controls of firefly and renilla luciferase reporters were carried out and measured using the Dual-Luciferase Reporter Assay System, 36 h post-transfection (Promega). Data are expressed as fold induction after being normalized using tk-renilla luciferase (mean $\pm$ s.e.m.; $n = 3$).

AOM/DSS model of colon carcinoma

The AOM/DSS model used has been previously described by Neufert *et al* (2007). Mice were injected intraperitoneally with 10 mg/kg body weight of AOM (Sigma) dissolved in physiological saline. Seven days later, 2% DSS was given in the drinking water over 7 days, followed by regular water until the end of the experiment. Body weight was measured every week, and the animals were killed 13 weeks after AOM injection for histological analysis by staining with c-Jun, β -catenin and BrdU antibodies. The number of tumours, incidence of tumours and BrdU-positive cells per area were determined. For biochemical characterization dissected tumours were snap frozen in liquid nitrogen and used for western blot and qRT-PCR analysis.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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References

- Angel P, Hattori K, Smeal T, Karin M (1988) The *jun* proto-oncogene is positively autoregulated by its product, Jun/AP1. *Cell* **55**: 875–885
- Bakiri L, Matsuo K, Wisniewska M, Wagner EF, Yaniv M (2002) Promoter specificity and biological activity of tethered AP-1 dimers. *Mol Cell Biol* **22**: 4952–4964
- Barker N, van de Wetering M, Clevers H (2008) The intestinal stem cell. *Genes Dev* **22**: 1856–1864
- Barker N, van Es JH, Kuipers J, Kujala P, van den Born M, Cozijnsen M, Haegebarth A, Korving J, Begthel H, Peters PJ, Clevers H (2007) Identification of stem cells in small intestine and colon by marker gene *Lgr5*. *Nature* **449**: 1003–1007
- Behrens A, Sibilio M, David JP, Mohle-Steinlein U, Tronche F, Schutz G, Wagner EF (2002) Impaired postnatal hepatocyte proliferation and liver regeneration in mice lacking c-jun in the liver. *EMBO J* **21**: 1782–1790
- Behrens A, Sibilio M, Wagner EF (1999) Amino-terminal phosphorylation of c-Jun regulates stress-induced apoptosis and cellular proliferation. *Nat Genet* **21**: 326–329
- Biteau B, Hochmuth CE, Jasper H (2008) JNK activity in somatic stem cells causes loss of tissue homeostasis in the aging *Drosophila* gut. *Cell Stem Cell* **3**: 442–455
- Chang L, Jones Y, Ellisman MH, Goldstein LS, Karin M (2003) JNK1 is required for maintenance of neuronal microtubules and controls phosphorylation of microtubule-associated proteins. *Dev Cell* **4**: 521–533
- Cheng H, Leblond CP (1974a) Origin, differentiation and renewal of the four main epithelial cell types in the mouse small intestine. I. Columnar cell. *Am J Anat* **141**: 461–479
- Cheng H, Leblond CP (1974b) Origin, differentiation and renewal of the four main epithelial cell types in the mouse small intestine. V. Unitarian Theory of the origin of the four epithelial cell types. *Am J Anat* **141**: 537–561
- Constant SL, Dong C, Yang DD, Wysl M, Davis RJ, Flavell RA (2000) JNK1 is required for T cell-mediated immunity against *Leishmania* major infection. *J Immunol* **165**: 2671–2676
- Davis RJ (2000) Signal transduction by the JNK group of MAP kinases. *Cell* **103**: 239–252
- Derijard B, Hibi M, Wu IH, Barrett T, Su B, Deng T, Karin M, Davis RJ (1994) JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. *Cell* **76**: 1025–1037
- Dong C, Yang DD, Wysl M, Whitmarsh AJ, Davis RJ, Flavell RA (1998) Defective T cell differentiation in the absence of Jnk1. *Science* **282**: 2092–2095
- Eferl R, Wagner EF (2003) AP-1: a double-edged sword in tumorigenesis. *Nat Rev Cancer* **3**: 859–868
- el Marjou F, Janssen KP, Chang BH, Li M, Hindie V, Chan L, Louvard D, Chambon P, Metzger D, Robine S (2004) Tissue-specific and inducible Cre-mediated recombination in the gut epithelium. *Genesis* **39**: 186–193
- Engelberg D (2004) Stress-activated protein kinases-tumor suppressors or tumor initiators? *Semin Cancer Biol* **14**: 271–282
- Giles RH, van Es JH, Clevers H (2003) Caught up in a Wnt storm: Wnt signaling in cancer. *Biochim Biophys Acta* **1653**: 1–24
- Gupta S, Barrett T, Whitmarsh AJ, Cavanagh J, Sluss HK, Derijard B, Davis RJ (1996) Selective interaction of JNK protein kinase isoforms with transcription factors. *EMBO J* **15**: 2760–2770
- Hassellblatt P, Gresh L, Kudo H, Guinea-Viniegra J, Wagner EF (2008) The role of the transcription factor AP-1 in colitis-associated and beta-catenin-dependent intestinal tumorigenesis in mice. *Oncogene* **27**: 6102–6109
- Hayakawa J, Mittal S, Wang Y, Korkmaz KS, Adamson E, English C, Ohmichi M, McClelland M, Mercola D (2004) Identification of promoters bound by c-Jun/ATF2 during rapid large-scale gene activation following genotoxic stress. *Mol Cell* **16**: 521–535

- Hess J, Angel P, Schorpp-Kistner M (2004) AP-1 subunits: quarrel and harmony among siblings. *J Cell Sci* **117**: 5965–5973
- Humphries A, Wright NA (2008) Colonic crypt organization and tumorigenesis. *Nat Rev Cancer* **8**: 415–424
- Itzkowitz SH, Yio X (2004) Inflammation and cancer IV. Colorectal cancer in inflammatory bowel disease: the role of inflammation. *Am J Physiol Gastrointest Liver Physiol* **287**: G7–G17
- Jho EH, Zhang T, Domon C, Joo CK, Freund JN, Costantini F (2002) Wnt/beta-catenin/Tcf signaling induces the transcription of Axin2, a negative regulator of the signaling pathway. *Mol Cell Biol* **22**: 1172–1183
- Johnson RS, van Lingen B, Papaioannou VE, Spiegelmann BM (1993) A null mutation at the *c-jun* locus causes embryonic lethality and retarded cell growth in culture. *Genes Dev* **7**: 1309–1317
- Karin M, Gallagher E (2005) From JNK to pay dirt: jun kinases, their biochemistry, physiology and clinical importance. *IUBMB Life* **57**: 283–295
- Kuan CY, Yang DD, Samanta Roy DR, Davis RJ, Rakic P, Flavell RA (1999) The Jnk1 and Jnk2 protein kinases are required for regional specific apoptosis during early brain development. *Neuron* **22**: 667–676
- Mann B, Gelos M, Siedow A, Hanski ML, Gratchev A, Ilyas M, Bodmer WF, Moyer MP, Riecken EO, Buhr HJ, Hanski C (1999) Target genes of beta-catenin-T cell-factor/lymphoid-enhancer-factor signaling in human colorectal carcinomas. *Proc Natl Acad Sci USA* **96**: 1603–1608
- Manning AM, Davis RJ (2003) Targeting JNK for therapeutic benefit: from junk to gold? *Nat Rev Drug Discov* **2**: 554–565
- Mechta-Grigoriou F, Gerald D, Yaniv M (2001) The mammalian Jun proteins: redundancy and specificity. *Oncogene* **20**: 2378–2389
- Moser AR, Mattes EM, Dove WF, Lindstrom MJ, Haag JD, Gould MN (1993) ApcMin, a mutation in the murine Apc gene, predisposes to mammary carcinomas and focal alveolar hyperplasias. *Proc Natl Acad Sci USA* **90**: 8977–8981
- Nateri AS, Spencer-Dene B, Behrens A (2005) Interaction of phosphorylated c-Jun with TCF4 regulates intestinal cancer development. *Nature* **437**: 281–285
- Nelson JD, Denisenko O, Bomsztyk K (2006) Protocol for the fast chromatin immunoprecipitation (ChIP) method. *Nat Protoc* **1**: 179–185
- Neufert C, Becker C, Neurath MF (2007) An inducible mouse model of colon carcinogenesis for the analysis of sporadic and inflammation-driven tumor progression. *Nat Protoc* **2**: 1998–2004
- Potten CS, Kovacs L, Hamilton E (1974) Continuous labelling studies on mouse skin and intestine. *Cell Tissue Kinet* **7**: 271–283
- Radtke F, Clevers H (2005) Self-renewal and cancer of the gut: two sides of a coin. *Science* **307**: 1904–1909
- Raivich G, Bohatschek M, Da Costa C, Iwata O, Galiano M, Hristova M, Nateri AS, Makwana M, Riera-Sans L, Wolfer DP, Lipp HP, Aguzzi A, Wagner EF, Behrens A (2004) The AP-1 transcription factor c-Jun is required for efficient axonal regeneration. *Neuron* **43**: 57–67
- Sabapathy K, Hochedlinger K, Nam SY, Bauer A, Karin M, Wagner EF (2004) Distinct roles for JNK1 and JNK2 in regulating JNK activity and c-Jun-dependent cell proliferation. *Mol Cell* **15**: 713–725
- Sabapathy K, Jochum W, Hochedlinger K, Chang L, Karin M, Wagner EF (1999) Defective neural tube morphogenesis and altered apoptosis in the absence of both JNK1 and JNK2. *Mech Dev* **89**: 115–124
- Sabapathy K, Wagner EF (2004) JNK2: a negative regulator of cellular proliferation. *Cell Cycle* **3**: 1520–1523
- Sangiorgi E, Capecchi MR (2008) Bmi1 is expressed *in vivo* in intestinal stem cells. *Nat Genet* **40**: 915–920
- Sansom OJ, Reed KR, Hayes AJ, Ireland H, Brinkmann H, Newton IP, Battle E, Simon-Assmann P, Clevers H, Nathke IS, Clarke AR, Winton DJ (2004) Loss of Apc *in vivo* immediately perturbs Wnt signaling, differentiation, and migration. *Genes Dev* **18**: 1385–1390
- Shaulian E, Karin M (2001) AP-1 in cell proliferation and survival. *Oncogene* **20**: 2390–2400
- Shaulian E, Karin M (2002) AP-1 as a regulator of cell life and death. *Nat Cell Biol* **4**: E131–E136
- Staal FJ, Weerkamp F, Baert MR, van den Burg CM, van Noort M, de Haas EF, van Dongen JJ (2004) Wnt target genes identified by DNA microarrays in immature CD34+ thymocytes regulate proliferation and cell adhesion. *J Immunol* **172**: 1099–1108
- Stein B, Angel P, van Dam H, Ponta H, Herrlich P, van der Eb A, Rahmsdorf HJ (1992) Ultraviolet-radiation induced c-jun gene transcription: two AP-1 like binding sites mediate the response. *Photochem Photobiol* **55**: 409–415
- Takahashi M, Wakabayashi K (2004) Gene mutations and altered gene expression in azoxymethane-induced colon carcinogenesis in rodents. *Cancer Sci* **95**: 475–480
- Teng DH, Perry 3rd WL, Hogan JK, Baumgard M, Bell R, Berry S, Davis T, Frank D, Frye C, Hattier T, Hu R, Jammulapati S, Janneck T, Leavitt A, Mitchell JT, Pero R, Sexton D, Schroeder M, Su PH, Swedlund B *et al* (1997) Human mitogen-activated protein kinase kinase 4 as a candidate tumor suppressor. *Cancer Res* **57**: 4177–4182
- Tetsu O, McCormick F (1999) Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. *Nature* **398**: 422–426
- Tong C, Yin Z, Song Z, Dockendorff A, Huang C, Mariadason J, Flavell RA, Davis RJ, Augenlicht LH, Yang W (2007) c-Jun NH2-terminal kinase 1 plays a critical role in intestinal homeostasis and tumor suppression. *Am J Pathol* **171**: 297–303
- Van der Flier LG, Sabates-Bellver J, Oving I, Haegebarth A, De Palo M, Anti M, Van Gijn ME, Suijkerbuijk S, Van de Wetering M, Marra G, Clevers H (2007) The Intestinal Wnt/TCF Signature. *Gastroenterology* **132**: 628–632
- van der Flier LG, van Gijn ME, Hatzis P, Kujala P, Haegebarth A, Stange DE, Begthel H, van den Born M, Guryev V, Oving I, van Es JH, Barker N, Peters PJ, van de Wetering M, Clevers H (2009) Transcription factor achaete scute-like 2 controls intestinal stem cell fate. *Cell* **136**: 903–912
- Veeman MT, Axelrod JD, Moon RT (2003) A second canon. Functions and mechanisms of beta-catenin-independent Wnt signaling. *Dev Cell* **5**: 367–377
- Weston CR, Davis RJ (2002) The JNK signal transduction pathway. *Curr Opin Genet Dev* **12**: 14–21
- Wielenga VJ, Smits R, Korinek V, Smit L, Kielman M, Fodde R, Clevers H, Pals ST (1999) Expression of CD44 in Apc and Tcf mutant mice implies regulation by the WNT pathway. *Am J Pathol* **154**: 515–523
- Wisdom R, Johnson RS, Moore C (1999) c-Jun regulates cell cycle progression and apoptosis by distinct mechanisms. *EMBO J* **18**: 188–197
- Wu X, Tu X, Joeng KS, Hilton MJ, Williams DA, Long F (2008) Rac1 activation controls nuclear localization of beta-catenin during canonical Wnt signaling. *Cell* **133**: 340–353
- Wulf GM, Ryo A, Wulf GG, Lee SW, Niu T, Petkova V, Lu KP (2001) Pin1 is overexpressed in breast cancer and cooperates with Ras signaling in increasing the transcriptional activity of c-Jun towards cyclin D1. *EMBO J* **20**: 3459–3472
- Xia Y, Karin M (2004) The control of cell motility and epithelial morphogenesis by Jun kinases. *Trends Cell Biol* **14**: 94–101
- Yang DD, Kuan CY, Whitmarsh AJ, Rincón M, Zheng TS, Davis RJ, Rakic P, Flavell RA (1997) Absence of excitotoxicity-induced apoptosis in the hippocampus of mice lacking the Jnk3 gene. *Nature* **389**: 865–870
- Yoshida BA, Dubauskas Z, Chekmareva MA, Christiano TR, Stadler WM, Rinker-Schaeffer CW (1999) Mitogen-activated protein kinase kinase 4/stress-activated protein/Erk kinase 1 (MKK4/SEK1), a prostate cancer metastasis suppressor gene encoded by human chromosome 17. *Cancer Res* **59**: 5483–5487
- Yoshida S, Fukino K, Harada H, Nagai H, Imoto I, Inazawa J, Takahashi H, Teramoto A, Emi M (2001) The c-Jun NH2-terminal kinase3 (JNK3) gene: genomic structure, chromosomal assignment, and loss of expression in brain tumors. *J Hum Genet* **46**: 182–187
- Zheng C, Xiang J, Hunter T, Lin A (1999) The JNK2-JNK1 fusion protein acts as a constitutively active c-Jun kinase that stimulates c-Jun transcription activity. *J Biol Chem* **274**: 28966–28971