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SHORT COMMUNICATION

Myc heterozygosity attenuates the phenotypes of APC deficiency in the small intestine

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The adenomatous polyposis coli (*APC*) gene encodes APC tumour suppressor protein, germline mutation of which causes familial adenomatous polyposis, an autosomal intestinal cancer syndrome. We have previously demonstrated that the proto-oncogene c-Myc is essential for all the phenotypes that occur after APC loss in the murine small intestine. One caveat to this study is that it was performed in the complete absence of c-Myc. In this study, we show that heterozygosity for *Myc* reduces the phenotypes of APC loss and Wnt target gene expression and slows tumourigenesis. Crucially, the levels of *Myc* are twofold higher than wild-type levels showing that the level of *Myc* induced by Wnt signalling is absolutely vital for the fate of APC-deficient cells. Taken together, this suggests that c-Myc inhibition may be a viable chemoprevention strategy for colorectal cancer.
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The adenomatous polyposis coli (*Apc*) gene is mutated in up to 80% of sporadic colorectal cancers and is thought to be the key initiating event (Kinzler *et al*., 1991; Bienz and Clevers, 2000). The predominant role of *Apc* gene as a tumour suppressor is as a negative regulator of the Wnt signalling pathway. APC forms part of the β-catenin destruction complex, along with glycogen synthase kinase-3 (GSK3), axin and casein kinase 1 (Bienz and Clevers, 2000). This complex allows the phosphorylation of β-catenin by GSK3, which targets β-catenin for degradation by the proteasome. In the absence of APC (or in the presence of Wnt signal), this destruction complex is dissociated and β-catenin accumulates and translocates into the nucleus, where it interacts with TCF/LEF transcription factors to drive transcription of TCF/LEF (or Wnt) target genes (Korinek *et al*., 1997).

Given the central nature of APC loss to colorectal cancer, we and others have previously investigated the phenotypes of APC loss within the murine intestinal epithelium and the associated Wnt target gene signature (Sansom *et al*., 2004; Andreu *et al*., 2005; Gregorieff and Clevers, 2005). These studies have shown that conditional deletion of *Apc* within the intestinal epithelium (using cre-lox technology) leads to a 'crypt progenitor cell-like' phenotype, with intestinal enterocytes showing hyperproliferation, perturbed differentiation and migration (Sansom *et al*., 2004; Andreu *et al*., 2005). Mechanistically, this phenotype is associated with concurrent nuclear β-catenin and transcriptional activation of a large number of Wnt target genes. Of these target genes, the proto-oncogene *Myc* (He *et al*., 1998) appears to be of central importance, as co-deletion of both *Apc* and *Myc* within the murine intestinal epithelium rescues this 'crypt progenitor cell-like' phenotype. Concordantly, many of the Wnt targets deregulated after APC loss revert back to wild-type levels in double knockout APC Myc intestinal enterocytes (Ignatenko *et al*., 2006; Sansom *et al*., 2007).

One of the potential caveats to this study is that c-Myc was completely ablated and given that c-Myc has a plethora of functions, including ribosome biosynthesis, metabolism, transcription activation and repression, it is possible that these dominate in an *Apc*-deficient background (Bommer and Fearon, 2007). Thus, it could be argued that the genetic rescue that was observed was due to the complete loss of function of c-Myc rather than the loss of β-catenin-dependent induction of c-Myc. Given the importance of the *Apc* gene loss in colorectal cancer and our data suggesting c-Myc as the major modifier, in this study we have investigated whether modifying (rather than removing) the levels of *Myc* after APC loss strongly impacts on the phenotypes of APC loss and tumourigenesis. To do this, we have investigated the phenotype of APC-deficient intestinal epithelium, which was also heterozygous for *Myc* inactivation.

First, we intercrossed mice carrying the cytochrome P450-inducible $AhCre^{+}$ transgene to mice carrying loxp-inducible knockout Apc^{580S} (Shibata *et al*., 1997) (from here on called Apc^{fl}) and Myc^{fl} alleles (Baena *et al*., 2005) to generate experimental $AhCre^{+}Apc^{fl/fl}Myc^{fl/+}$ and control $AhCre^{+}Apc^{fl/fl}Myc^{+/+}$ mice. Mice were given three injections of β-naphthoflavone on a single day, which induces *cre* recombinase within the intestine, and killed and tissue harvested 4 days later. To investigate *Myc* expression in experimental $AhCre^{+}Apc^{fl/fl}Myc^{fl/+}$ and control $AhCre^{+}Apc^{fl/fl}Myc^{+/+}$ intestines, we per-

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formed quantitative PCR with *Myc*-specific primers on RNA from intestinal epithelial extracts (see supplementary information for protocols for epithelial tissue isolation, RNA extraction, cDNA synthesis and quantitative PCR analysis). Figure 1a shows a fourfold increase in expression between APC wild-type ($AhCre^+Apc^{+/+}Myc^{+/+}$) and APC-deficient intestine ($AhCre^+Apc^{fl/fl}Myc^{+/+}$). Importantly, APC-deficient intestines additionally heterozygous for *Myc* ($AhCre^+Apc^{fl/fl}Myc^{fl/+}$) showed only a twofold increase in *Myc* levels compared with those wild type for *Myc* ($AhCre^+Apc^{fl/fl}Myc^{+/+}$), and these *Myc* levels were significantly lower than those observed in *Apc*-deficient, *Myc* wild-type mice ($AhCre^+Apc^{fl/fl}Myc^{+/+}$). These data argue that *Myc* heterozygosity causes a reduction in the levels of c-Myc accumulation after *Apc* loss and thus this is an excellent system to test the effect of the reduction (rather than the ablation) of *Myc* on phenotypes of *Apc* loss.

Next, we investigated the phenotype of the intestinal epithelium in experimental $AhCre^+Apc^{fl/fl}Myc^{fl/+}$ and control $AhCre^+Apc^{fl/fl}Myc^{+/+}$ 4 days after induction. As previously reported, APC-deficient intestinal epithelium shows dramatically enlarged crypts compared with wild type (Figures 1b and c) and a clear induction of proliferation, as shown by 5-bromodeoxyuridine (BrdU) incorporation (Figures 1e and f). Despite having increased levels of *Myc* compared with wild-type intestines, $AhCre^+Apc^{fl/fl}Myc^{fl/+}$ intestinal crypts were significantly reduced compared with $AhCre^+Apc^{fl/fl}$

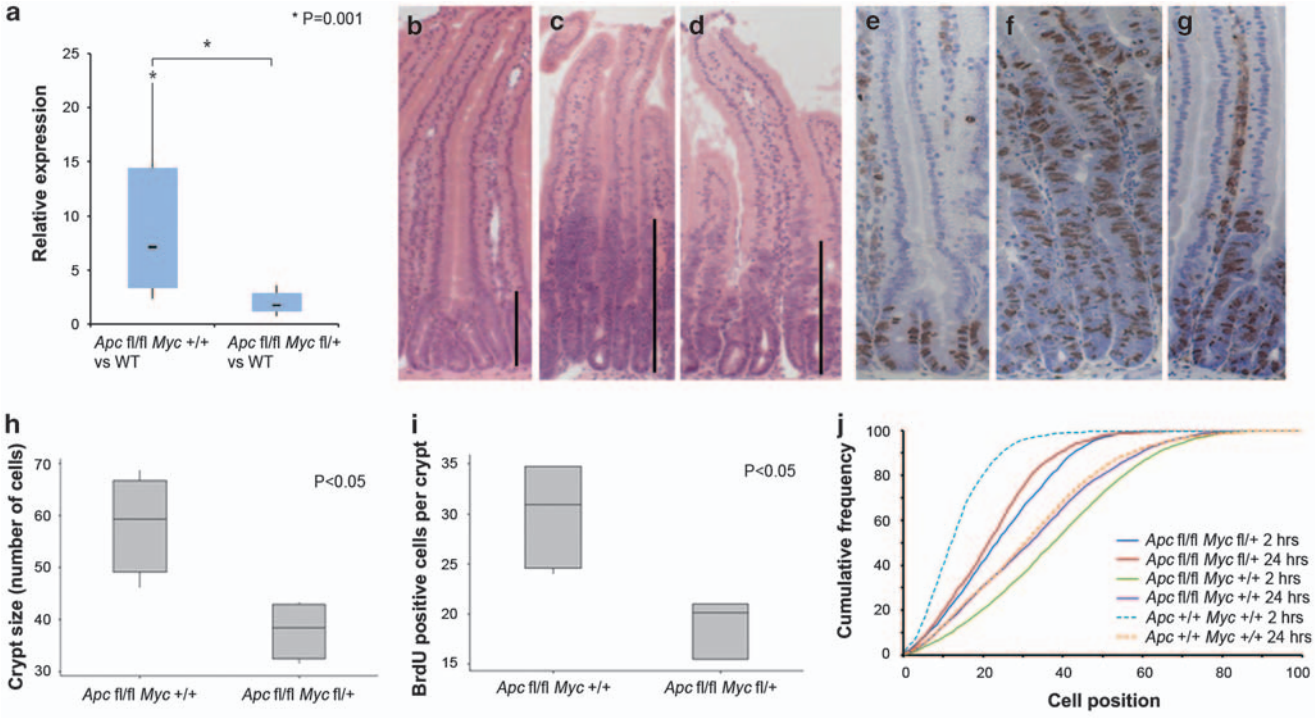
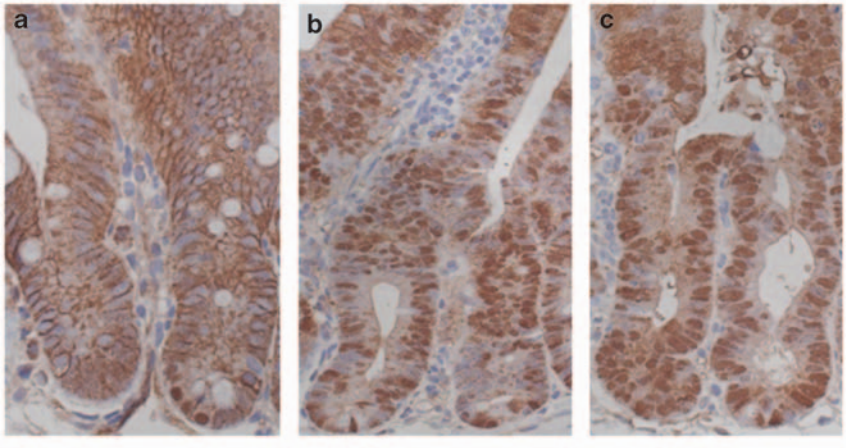


Figure 1 c-Myc heterozygosity suppresses the phenotypes of Apc loss. (**a**) Quantitative PCR (qPCR) showing reduced levels of c-Myc in epithelial extracts from $AhCre^+Apc^{fl/fl}Myc^{fl/+}$ and $AhCre^+Apc^{fl/fl}Myc^{+/+}$ intestines (see Supplementary information for the qPCR protocol and primers and for the epithelial cell isolation). (**b**–**d**) Haematoxylin and eosin-stained sections of wild-type ($AhCre^+Apc^{+/+}Myc^{+/+}$) (**b**), Apc-deficient ($AhCre^+Apc^{fl/fl}Myc^{+/+}$) (**c**) and Apc-deficient Myc heterozygote ($AhCre^+Apc^{fl/fl}Myc^{fl/+}$) (**d**) intestines. Note that the enlarged crypts in Apc-deficient mice (**c**) are reduced in the Myc heterozygote crypts (**d**). The black vertical bars indicate the size of the crypt compartment. (**e**–**g**) Immunohistochemistry (IHC) for BrdU (Sansom *et al*., 2004) performed on wild-type ($AhCre^+Apc^{+/+}Myc^{+/+}$) (**e**), Apc-deficient ($AhCre^+Apc^{fl/fl}Myc^{+/+}$) (**f**) and Apc-deficient Myc heterozygote ($AhCre^+Apc^{fl/fl}Myc^{fl/+}$) (**g**) intestines, 4 days after cre induction and 2 h after BrdU injection. Note that Apc-deficient Myc heterozygote (**g**) intestines are much smaller than the Apc-deficient intestine (**f**). BrdU IHC was performed using an anti-BrdU antibody (BD Pharmingen Oxford, UK, cat. no. 347580) at a 1:100 dilution. (**h**, **i**) Boxplots of crypt size and proliferation of Apc-deficient ($AhCre^+Apc^{fl/fl}Myc^{+/+}$) and Apc-deficient Myc heterozygote ($AhCre^+Apc^{fl/fl}Myc^{fl/+}$) intestines. A significant reduction of crypt size and proliferation was observed between Apc-deficient and Apc-deficient Myc heterozygous intestines (Mann–Whitney $P<0.05$, $n\leqslant4$). Boxplots represent the average number of cells per crypt (**h**) and average number of BrdU-positive cells per crypt (**i**). The median value for each genotype is indicated by a horizontal line in the boxplots. (**j**) Intestinal enterocyte migration is still perturbed in Apc-deficient Myc heterozygotes. Graphs show the position of BrdU-labelled cells 2 (blue and green lines) and 24 h (red and purple lines) after labelling. Within Apc-deficient ($AhCre^+Apc^{fl/fl}Myc^{+/+}$) crypts, cells label throughout the crypt–villus axis at 2 h and there is no movement 24 h later, indicated by the cumulative frequency curves overlying each other (green and purple lines, Kolmogorov–Smirnov test, $P=0.8$). Within Apc-deficient Myc heterozygotes ($AhCre^+Apc^{fl/fl}Myc^{fl/+}$), labelling occurs in a much smaller zone (consistent with less proliferation), but there is still no movement between 2 and 24 h (red and blue lines) unlike wild-type intestines (see Sansom *et al*., 2004). For comparison, graphs from wild-type ($AhCre^+Apc^{+/+}Myc^{+/+}$) mice are also included (dashed lines). Here, labelling occurs only in the crypt at 2 h (light blue line), but the cells migrate into the villus at the later time point (orange line). All Experiments were performed under the UK Home Office guidelines. Outbred male mice from 6 to 12 weeks of age were used, which were segregating for the C57BLJ and S129 genomes.

$Myc^{+/+}$ crypts (Figures 1c, d and h), and this corresponded with a strong reduction of proliferation as scored by BrdU incorporation (Figures 1f, g and i). In both cases, crypt size and proliferation were reduced to ~50% (Figures 1h and i). Interestingly, not all of the phenotypes of *Apc* deficiency were modified; for example, failed intestinal enterocyte movement up the crypt–villus axis was still evident in $AhCre^{+}Apc^{fl/fl}Myc^{fl/+}$ intestinal crypts (Figure 1j). In addition, levels of apoptosis in $AhCre^{+}Apc^{fl/fl}Myc^{fl/+}$ mice showed a decrease compared with $AhCre^{+}Apc^{fl/fl}Myc^{+/+}$ mice (data not shown). This could represent the moderately increased levels of *Myc*, which are still twofold over levels in *Apc* wild-type intestine, arguing that the highest levels of *Myc* are required to drive proliferation and probably apoptosis after *Apc* loss, although lower levels (yet still increased compared with wild type) perturb migration within the intestinal crypt. These data are consistent with a recent study showing that the precise levels of *Myc* overexpression *in vivo* govern the biological output (Murphy *et al*., 2008). In this study, a clear twofold difference in *Myc* levels could drive differences in proliferation in a number of tissues.

To assess mechanistically how *Myc* haploinsufficiency was affecting the phenotypes of Apc loss, we first stained for levels of nuclear β-catenin. As expected with c-Myc being a transcriptional target of β-catenin, we found nuclear β-catenin in the crypt cells of both $AhCre^{+}Apc^{fl/fl}Myc^{+/+}$ and $AhCre^{+}Apc^{fl/fl}Myc^{fl/+}$ mutant intestines (Figures 2a-c). This argues that *Myc* heterozygosity did not have an effect upstream of β-catenin signalling. Given that the major function of c-Myc is as a transcriptional activator and repressor, we next investigated the expression of a number of Wnt target genes



* P≤0.05	Apc fl/fl Myc +/+	Apc fl/fl Myc fl/+
Ascl2	**3.2**	1.1*
Axin2	**6.0**	4.3
Ccnd2	**5.4**	2.5*
Cd44	**11.4**	2.0*
Cdk4	**2.7**	**1.9**
Ephb3	**3.4**	1.4*
Lgr5	**6.9**	2.4*
Nkd1	**41.3**	22.0
Tcf7	**6.4**	3.2*
Tiam1	**8.8**	3.1*
Tnfrsf12a (Fn14)	**3.0**	1.2*
Tnfrsf19 (Troy)	**25.8**	13.3*

Figure 2 Reduction in a number of transcriptional targets deregulated after Apc loss in c-Myc heterozygous intestines. (**a**) Immunohistochemistry (IHC) for β-catenin showing that β-catenin is localized at the cell edge of nearly all intestinal enterocytes of wild-type crypts ($AhCre^{+}Apc^{+/+}Myc^{+/+}$ mice) 4 days after cre induction. (**b**) IHC for β-catenin showing nuclear localization of β-catenin in every cell within Apc-deficient crypts ($AhCre^{+}Apc^{fl/fl}Myc^{+/+}$ mice). (**c**) IHC for β-catenin showing nuclear localization of β-catenin in every cell within Apc-deficient Myc heterozygote ($AhCre^{+}Apc^{fl/fl}$ $Myc^{fl/+}$) crypts. B-catenin IHC was performed as described by Sansom *et al*. (2004), using the BD Transduction Laboratories antibody (Oxford, UK, cat. no. 610154) at 1:50 dilution. (**d**) Quantitative PCR (qPCR) analysis for a number of Wnt target genes deregulated after Apc loss that have been shown to be dependent on c-Myc (graphical representation of *Ascl2* shown as an example). Details of qPCR primers are given in Supplementary information. The numbers represent differential expression compared with wild-type intestine. Where numbers are in bold, it means they are significantly higher than in wild-type intestinal epithelium. Asterisks denote significant downregulation ($P \leqslant 0.05$) of $AhCre^{+}Apc^{fl/fl}Myc^{fl/+}$ intestine versus $AhCre^{+}Apc^{fl/fl}Myc^{+/+}$ intestine.

that are deregulated after APC loss (Sansom *et al*., 2004, 2007). Importantly, we have shown that the expression of these targets are returned to wild-type levels in double knockout *Apc* and *Myc* intestines (Sansom *et al*., 2007). Figure 2d shows the expression of 12 Wnt target genes in $AhCre^{+}Apc^{fl/fl}Myc^{+/+}$ and $AhCre^{+}Apc^{fl/fl}Myc^{fl/+}$ intestines four days after Cre induction. Of the 12 genes, 12 were reduced and 9 showed a significant reduction ($P \leqslant 0.05$) in $AhCre^{+}Apc^{fl/fl}Myc^{fl/+}$ compared with $AhCre^{+}Apc^{fl/fl}Myc^{+/+}$ intestines (indicated with an asterisk in the table). Once again, the reduction was not as dramatic as complete Myc deletion, as target gene expression was still increased in 9 out of 12 genes compared with wild-type intestines. These data highlight that the reduced levels of c-Myc activation after APC loss leads to lower activation of target genes deregulated after APC loss and a reduced phenotype in the intestine *in vivo*. Of the genes downregulated, *Ascl2* was reduced back to wild-type levels. Recent studies have shown that *Ascl2* is a Wnt target gene and overexpression of this alone is sufficient to cause increased intestinal enterocyte proliferation and crypt size (Jubb *et al*., 2006; van der Flier *et al*., 2009). This reduction of *Ascl2*, in conjunction with reduction of *Cyclin D2*, provides a ready mechanism for reduced crypt proliferation and size in c-Myc heterozygotes.

Finally, we wanted to assess the downstream consequences of *Myc* heterozygosity to intestinal tumourigenesis after *Apc* loss. We have previously shown that $AhCre^{+}Apc^{fl/+}$ mice develop intestinal adenomas associated with the loss of the remaining *Apc* allele and a clear nuclear accumulation of β-catenin within the adenomas (Sansom *et al*., 2006). To assess the consequences of *Myc* heterozygosity on this phenotype, we generated cohorts of experimental $AhCre^{+}Apc^{fl/+}Myc^{fl/+}$ and control $AhCre^{+}Apc^{fl/+}Myc^{+/+}$ mice. The mice were injected with 3×80 mg/kg β-naphthoflavone in a single day to induce maximal cre-mediated recombination within the intestinal epithelium (Sansom *et al*., 2006) and aged until they developed symptoms of intestinal disease. These included paling of feet because of anaemia caused by intestinal polyposis, hunching and weight loss. When mice showed these symptoms, they were killed and the intestines removed and intestinal tumour size and number scored. *Myc* heterozygosity strongly suppressed intestinal tumourigenesis in these mice (Figure 3a) with $AhCre^{+}Apc^{fl/+}Myc^{+/+}$ mice having a median age of 265 days at death and

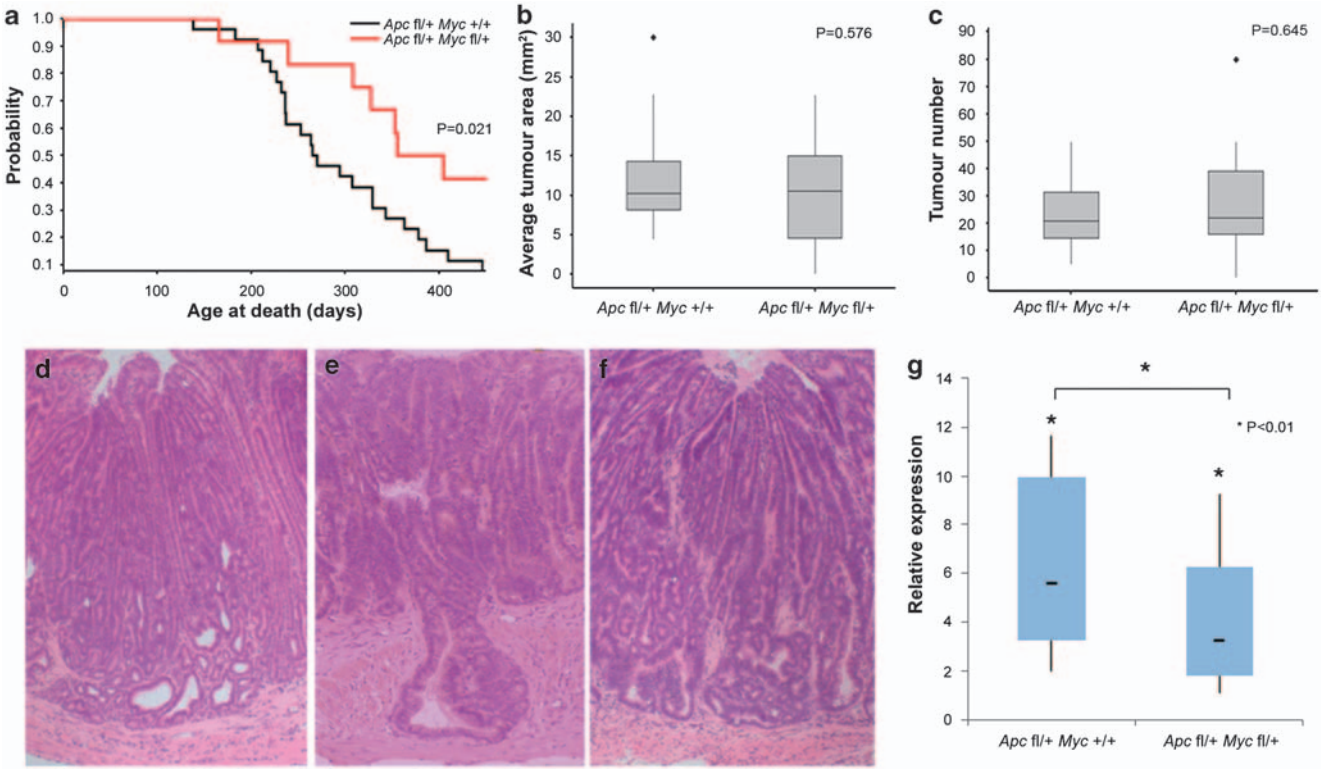


Figure 3 c-Myc haploinsufficiency slows intestinal tumourigenesis. (**a**) Kaplan–Meier curve showing time to intestinal tumourigenesis in $AhCre^{+}Apc^{fl/+}Myc^{+/+}$ (black line) and $AhCre^{+}Apc^{fl/+}Myc^{fl/+}$ (red line) mice. $AhCre^{+}Apc^{fl/+}Myc^{fl/+}$ mice lived significantly longer than $AhCre^{+}Apc^{fl/+}Myc^{+/+}$ mice (Kaplan–Meier log-rank, $P=0.021$, $n=15$ and 25, respectively). (**b**) Boxplots showing average tumour area (in mm²) at death in $AhCre^{+}Apc^{fl/+}Myc^{+/+}$ and $AhCre^{+}Apc^{fl/+}Myc^{fl/+}$ mice. No significant difference was observed between the two groups (Mann–Whitney, $P=0.58$, $n=25$ and 15, respectively). (**c**) Boxplots showing tumour number at death in $AhCre^{+}Apc^{fl/+}Myc^{+/+}$ and $AhCre^{+}Apc^{fl/+}Myc^{fl/+}$ mice, no significant difference was observed between the two groups (Mann–Whitney, $P=0.64$, $n=25$ and 15, respectively). (**d**–**f**) Haematoxylin and eosin-stained sections showing representative histological photos from intestinal tumours from $AhCre^{+}Apc^{fl/+}Myc^{+/+}$ (**d**, **e**) and $AhCre^{+}Apc^{fl/+}Myc^{fl/+}$ (**f**) mice. (**g**) Quantitative PCR of *Myc* expression showing elevated *Myc* levels in adenomas from both $AhCre^{+}Apc^{fl/+}Myc^{+/+}$ and $AhCre^{+}Apc^{fl/+}Myc^{fl/+}$ mice compared with wild-type epithelium.

$AhCre^+Apc^{fl/+}Myc^{fl/+}$ a median age of 355 days at death (Kaplan—Meier, $P=0.021$). At death, mice had a similar tumour burden and location (Figures 3b and c, Supplementary Figure 1), but showed delayed progression to carcinoma. Only 4 out of 15 $AhCre^+Apc^{fl/+}Myc^{fl/+}$ mice had invasive intestinal tumours (determined by microscopic analysis of haematoxylin and eosin-stained sections, representative examples of which can be seen in Figures 3d–f), compared with 15 out of 25 of $AhCre^+Apc^{fl/+}Myc^{+/+}$ mice (χ^2, $P=0.04$). This was due to a smaller percentage of adenomas in $AhCre^+Apc^{fl/+}Myc^{fl/+}$ mice progressing to carcinoma (2.3% in $AhCre^+Apc^{fl/+}Myc^{fl/+}$(4 adenocarcinomas/172 adenomas), compared with 7.2% in $AhCre^+Apc^{fl/+}Myc^{+/+}$ mice (22 adenocarcinomas/285 adenomas; χ^2, $P=0.022$)), suggesting that c-Myc levels have a vital role in driving tumour progression/invasion as well as tumour growth.

One question that remained was whether the tumours that formed in the $AhCre^+Apc^{fl/+}Myc^{fl/+}$ mice had grown with low levels of c-Myc or if these 'escapee' tumours had upregulated c-Myc by another mechanism. To investigate this we performed quantitative PCR analysis on tumours from $AhCre\ Apc^{fl/+}Myc^{fl/+}$ and $AhCre\ Apc^{fl/+}Myc^{+/+}$ mice. As expected, adenomas from $AhCre\ Apc^{fl/+}Myc^{+/+}$ showed a 5.6-fold increase in *Myc* compared with normal intestinal epithelium, although adenomas from $AhCre\ Apc^{fl/+}Myc^{fl/+}$ showed a lower, but still significant, 3.3-fold increase in *Myc* levels (Figure 3g). Given that AhCre deletes specifically within the intestinal epithelium, it suggests that the mechanism that underlies delayed tumour growth is inefficient target gene activation. Interestingly, a previous study has suggested that Myc may affect tumour growth within the intestinal epithelium by allowing tumour angiogenesis (Yekkala and Baudino, 2007). Therefore, both studies underline the importance of c-Myc levels for intestinal tumour progression and there may be multiple key tumour-promoting mechanisms that c-Myc impinges on. This link between Myc and angiogenesis had been observed previously in pancreatic islet tumour growth driven by c-Myc overexpression and appears dependent on changes in c-Myc levels within the epithelial cells (Shchors *et al*., 2006).

Taken together, our results here show that the level of *Myc* upregulation is absolutely critical for transformation after *Apc* loss *in vivo* and thus suggests that levels of c-Myc will be crucial for colorectal cancer in humans. Two recent studies highlight that this may be the case (Pomerantz *et al*., 2009; Tuupanen *et al*., 2009). These studies identified within humans that a polymorphism in 8q24, rs6983267, which is associated with increased colorectal cancer risk, is an enhancer region within the *TCF7L2* (TCF4) gene, with the tumour-prone allele more strongly activating Wnt signalling. Both studies showed that c-Myc could bind to this promoter and one suggested a direct interaction with c-Myc (Pomerantz *et al*., 2009). These studies also highlight the co-operation between Wnt and c-Myc in colorectal cancer. It should be noted that an activation of c-Myc alone is not sufficient to drive the intestinal phenotypes equivalent to APC loss, instead in APC-deficient cells c-Myc is essential (Sansom *et al*., 2007; Murphy *et al*., 2008). Indeed, a recent study (Finch *et al*., 2009) shows that very high expression of c-Myc could only recapitulate a subset of the phenotypes of Apc loss and target gene activation. Therefore, this shows the requirement for co-operation between nuclear β-catenin and c-Myc in driving the phenotypes after Apc loss. In this study, we show that there is an optimal level of c-Myc activation after Apc loss, so reducing the deregulation of c-Myc by just twofold is sufficient to suppress tumour growth, invasion and modify target gene activation.

There has been much debate over whether c-Myc is a therapeutic target for colorectal cancer and the difficulties regarding targeting c-Myc using small molecules (Soucek *et al*., 2008; Wilkins and Sansom, 2008). Our study here suggests that reduction of c-Myc (as well as complete ablation) may be a rationale for chemoprevention of colorectal cancer. This is consistent with a recent study in the mouse which displayed that haploinsufficiency for MYB caused reduced c-Myc levels and slowed intestinal tumourigenesis in the $Apc^{Min/+}$ mouse (Ciznadija *et al*., 2009). Thus, Myc or proteins that regulate c-Myc expression levels may be potential therapeutical targets in the future.

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)