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Intestinal adenoma formation and *MYC* activation are regulated by cooperation between MYB and Wnt signaling

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Aberrant Wnt signaling mediated by mutations affecting APC (adenomatous polyposis coli) or β-catenin initiates the majority of human colorectal cancers (CRC) and drives tumorigenesis through the activation of specific genes such as *MYC*. We report here a novel association whereby another oncogenic transcription factor, MYB/c-Myb, is necessary for intestinal adenoma development directed by activated Wnt signaling. $APC^{Min/+}$ mice in which *c-myb* is haploinsufficient survive longer than wild-type $APC^{Min/+}$ animals due to a delay in adenoma formation. Intestinal adenomas from $APC^{Min/+}$ mice were assessed and found to have high levels of *c-myc* gene expression. We explored the relationship between activated Wnt signaling and MYB in regulating *MYC* and found activated β-catenin in combination with MYB induces robust upregulation of *MYC* promoter activity, as well as endogenous *MYC* mRNA and protein expression, in human cells. This cooperation occurred through independent binding of MYB and β-catenin to the *MYC* promoter. These data highlight a cooperative function for MYB in the context of activated Wnt signaling and provide a molecular basis for the expression of *MYC* in CRC.
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Robust expression of the transcription factor MYB/c-Myb is principally restricted to immature cells in the hemopoietic system and colon epithelium. Experiments in *c-myb* mutant mice show that two copies of the *c-myb* gene are required for cell survival during emergency responses to cytotoxic damage in both these compartments.[1] In addition, colon-specific loss of *Myb* disrupts not only colon formation in the developing mouse, but also crypt homeostasis in adults.[2] Levels of MYB expression become progressively higher as human colonic epithelium undergoes transformation whereas persistent expression blocks cytodifferentiation in both mouse and human colon cells. A large number of colorectal cancer (CRC) cell lines and clinical samples show elevated levels of MYB, with altered transcriptional elongation control underpinning this increase.[1] Nevertheless, in the context of CRC development, the primary focus in both mouse and human cells has been on canonical Wnt signaling through β-catenin activation.

β-Catenin is normally targeted for degradation by a complex of proteins including APC, AXIN, and glycogen synthase kinase-3β.[3,4] Wnt binding to its receptor disrupts this complex and leads to the cytoplasmic stabilization of *de novo* synthesized and non-phosphorylated β-catenin.[5] β-Catenin subsequently translocates to the nucleus where it associates with TCF/LEF factors. This partnership converts β-catenin into a transcriptional activator and enhances the expression of Wnt target genes, such as *MYC* and *CYCLIND1*, which in turn promote colon transformation. A number of reports indicate that mutations in *APC* or *CTNNB1* (β-catenin) initiate the majority of CRC.[6–9]

Given that a significant proportion of CRC isolates over-express MYB and that this likely occurs in a background of activated Wnt signaling, we considered the contribution that MYB makes to CRC development initiated by Wnt pathway activation. We report here the convergence of MYB expression with Wnt signaling in regulating intestinal adenoma formation. *In vivo* mouse studies showed that reduced c-*myb* expression positively affects survival and that c-*myb* expression is a necessary feature of the carcinogenic process initiated by activated Wnt signaling. Further, to understand how c-Myb is contributing to CRC formation, expression profiling studies were undertaken, confirming a significant correlation between *c-myb* and *c-myc* expression in adenomas from $APC^{Min/+}$ mice and in human CRC. As the human *MYC* and mouse *c-myc* promoter landscapes are rich with previously characterized TCF/LEF and MYB binding sites, we

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Abbreviations: APC, adenomatous polyposis coli; FAP, familal adenomatous polyposis; ChIP, chromatin immunoprecipitation
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explored the possibility that MYB cooperates with β-catenin to activate *MYC* transcription directly. Molecular analysis confirmed that MYB and β-catenin are required in concert to enhance activation of reporters containing the human *MYC* promoter. This effect was mirrored when endogenous *MYC* gene expression was followed and occurred through the binding of both factors to the *MYC* promoter. These binding events induced distinct changes in histone acetylation. These findings thus further the paradigm of intestinal adenoma development through Wnt pathway activation by demonstrating the need for MYB as an accessory factor to drive *MYC* overexpression and subsequent colonic neoplasia.

Results

Maximum adenoma formation in $APC^{Min/+}$ mice is *c-myb* dependent. To explore the potential relationship between MYB and Wnt signaling in CRC development, we took a genetic approach whereby $APC^{Min/+}$ mice were crossed with $Myb^{+/-}$ mice, extending the observation that $APC^{Min/+}$ mice have a prolonged life span when crossed onto a $c\text{-}myc^{+/-}$ background.[10] Although $APC^{Min/+}$ mice started to show signs of distress by week 10, including wasting and bleeding from the anus, $APC^{Min/+}$ mice on a $c\text{-}myb^{+/-}$ background showed delayed onset of symptoms. When equal numbers of $APC^{Min/+}$ male and female mice were followed over time, $APC^{Min/+}$ mice on a $c\text{-}myb^{+/-}$ background survived significantly longer before developing symptoms severe enough to require mandatory killing ($P=0.01$; unpaired *t*-test) (Figure 1a). On average these mice survived beyond the death of all mice in the $APC^{Min/+}$ cohort ($P=0.03$; log-rank (Mantel–Cox) test) (Figure 1b). These results clearly demonstrate that c-Myb is a critical determinant of adenoma progression initiated through the deregulation of Wnt signaling.

Coordinate expression of *c-myb* and *c-myc* in adenomas from $APC^{Min/+}$ mice. It is evident that factors such as activated β-catenin and MYB promote tumorigenesis by regulating the activity of specific genes, particularly those having well-established functions in the carcinogenic process such as *CYCLIND1*, *MYC*, and *COX-2*.[4,11–13] Specifically, many studies point to the involvement of β-catenin in the activation of *MYC* in CRC, whereas activation of the human *MYC* promoter and endogenous gene by *MYB/c-Myb* has also been established.[14,15] To continue exploring the relationship between activated Wnt signaling and *MYB*, we used real-time PCR to assess expression of *c-myc* in intestinal adenomas developing in $APC^{Min/+}$ mice (Figure 1c). As expected from previous analyses, *c-myc* mRNA expression was markedly increased in adenomas from both the colon and small intestine (SI).[10,16] Furthermore, consistent with earlier reports and data from our own lab, *c-myb* mRNA expression was similarly elevated in adenomas from both sites (Figure 1d).

As *c-myb* haploinsufficiency significantly impeded the rate of adenoma formation in $APC^{Min/+}$ mice and *c-myc* expression concordantly increased in these adenomas, the relative levels *c-myc* mRNA in adenomas formed in $APC^{Min/+} \times c\text{-}myb^{+/-}$ mice were accordingly investigated. In a series of adenomas taken from both the SI and colon of double heterozygous mice, *c-myc* levels were significantly lower than in an equivalent series removed from $APC^{Min/+}$ mice ($P<0.0001$ and <0.006, respectively). Furthermore, *c-myb*

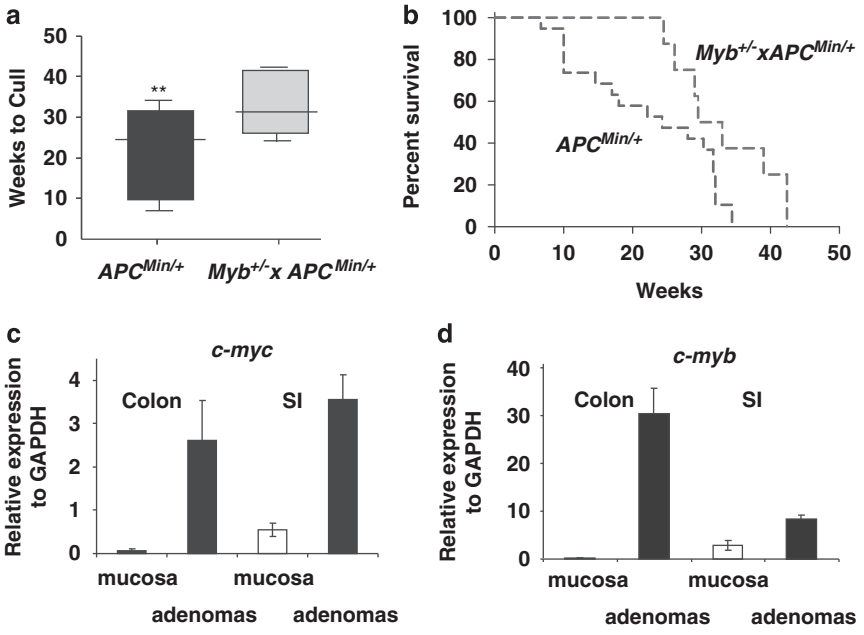

Figure 1 Two normal *c-myb* alleles are required for the rapid development of adenomas in $APC^{min/+}$ mice. When $APC^{min/+}$ mice were crossed onto a $c\text{-}myb^{+/-}$ background the weeks to killing was extended significantly ($P=0.01$) (**a**). Accordingly a Kaplan–Meier plot also indicates that survival is increased on a *c-myb* heterozygous background ($P=0.03$) (**b**). The analysis of mRNA levels for *c-myc* (**c**) and *c-myb* (**d**) for adenomas from $APC^{min/+}$ mice (35 colon, 8 mice; 44 SI, 6 mice) and histologically normal mucosa (3 colon; 4 SI) dissected from $APC^{min/+}$ mice was conducted and assessed by quantitative real-time RT-PCR

expression was also significantly decreased in these adenomas ($P > 0.0001$) (Supplementary Figure 1).

***MYC* expression correlates with *MYB* expression in human CRC.** To link these expression data from adenoma-prone $APC^{Min/+}$ mice to human CRC, mRNA expression analysis of 229 tumor samples belonging to 13 histological types, including colon, was performed focusing on *MYB* and *MYC* and standardized against an 11-cell-line set frequently used for this type of comparison.[17] These data, represented by a fold expression plot, emphasized consistently high *MYB* expression in CRC, as well as breast and gastric cancers (Figure 2a). When data for *MYB* and *MYC* were analyzed for each CRC alone and plotted for relative expression, an apparent association between *MYB* and *MYC* expression was evident (Figure 2b). The relative expression of *β-catenin* mRNA was also plotted to highlight the lack of correlation between the expression level of this gene and *MYC* (Figure 2b). This is consistent with literature showing that the mode of activation for β-catenin is typically at the level of protein abundance and/or localization rather than increases in mRNA expression levels.[3,4]

A correlation coefficient of $r = 0.51$ between *MYB* and *MYC* expression in CRC was calculated using the cDNA microarray data from 23 CRC specimens (Figure 2c, Pearson's correlation; $r = 0.51$, $P = 0.014$). By contrast, the value for

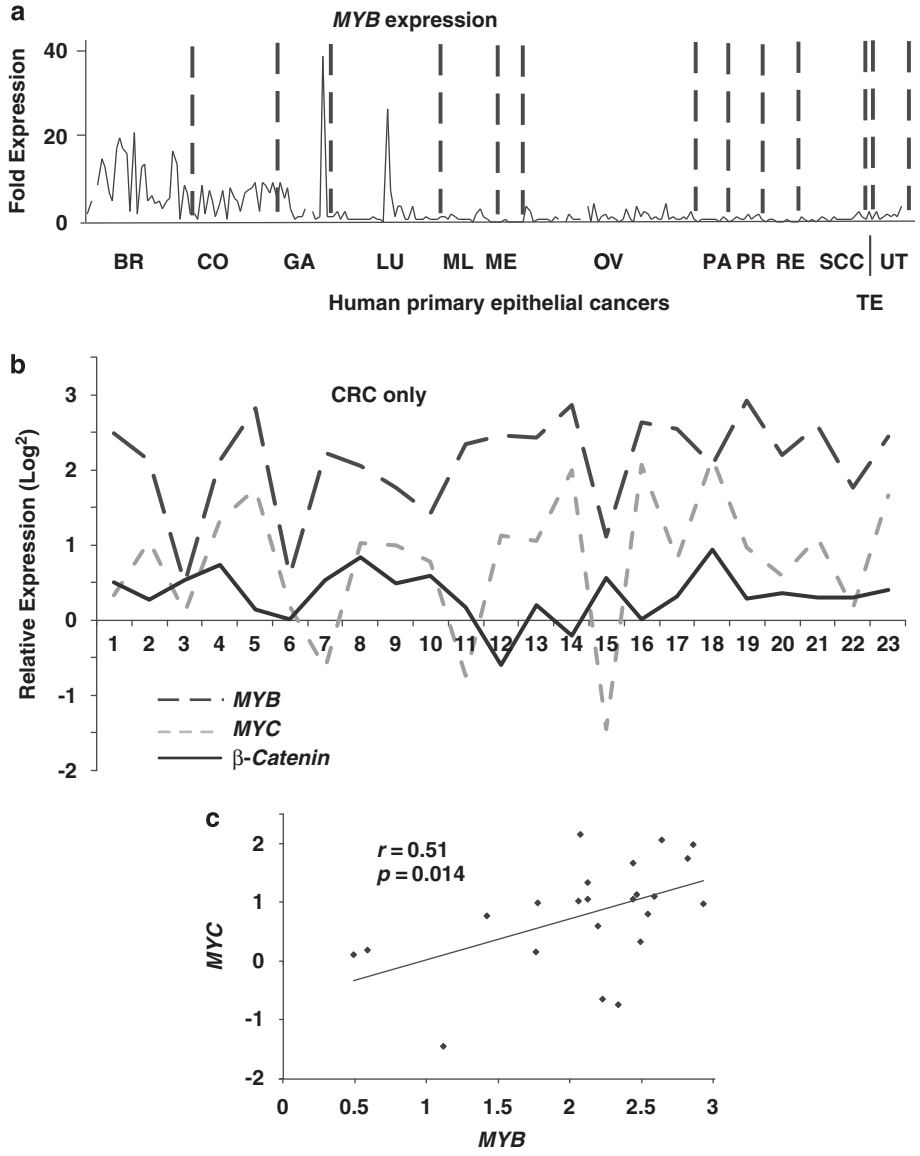

Figure 2 *MYB* is overexpressed in colorectal (CRC) and breast carcinomas. (**a**) A microarray gene expression analysis was performed for *MYB*. Fold expression of *MYB* in a range of epithelial cancers was compared to an 11-cell-line pooled control. The 13 tumor types are as follows: BR, breast; CO, colon; GA, gastric; LU, lung; ML, melanoma; ME, mesothelioma; OV, ovarian; PA, pancreas; PR, prostate; RE, renal; SCC, squamous-cell carcinoma; TE, testicular; UT, uterine. (**b**) The relative expression of *MYB*, *MYC*, and β-catenin for 23 CRC specimens was plotted to give an indication of whether there was an association between these three genes. (**c**) Using the cDNA microarray data, we calculated a Pearson's correlation ($r = 0.51$; $P = 0.014$) between *MYB* (median signal from two features on the array) and *MYC* (representing a single feature on the array) suggesting a there is a significant correlation between the two genes

coexpression of *MYB* and *MYC* in all other cancers was 17-fold lower ($r=0.03$, $P=0.651$), indicating that the observed correlation between *MYB* and *MYC* is highly significant in human CRC compared to 12 other cancer types.

siRNA knockdown of *MYB* reduces *MYC* expression in CRC cells. In view of the observations that *MYB* and *MYC* were coordinately upregulated in adenomas developing in $APC^{Min/+}$ mice and coexpressed in human CRC where mutations in APC prevail, we pursued the relationship between *MYB* and *MYC* in a context of activated Wnt signaling. Although *MYB* and *MYC* are commonly overexpressed in CRC cells, the potential dependency of *MYC* expression on *MYB* has not been explored in this cancer type. We therefore used lentivirus infection to generate stable pools of Colo201 CRC cells that expressed either a doxycycline-inducible shRNA against *MYB* or a scrambled sequence control. Colo201 CRC cells express robust levels of *MYB* and *MYC* and also harbor activating mutations in the Wnt pathway.[18,19] When *MYB* expression in this cell line is inhibited by induction of the shRNA (Figure 3a), we observed a corresponding decline in MYC expression (Figure 3b). Notably, the scrambled sequence control also led to some reduction in *MYB* expression when in the presence of doxycycline. The basis for this was unclear, but again emphasized that *MYC* expression levels in a background of activated Wnt signaling are influenced by the presence of MYB. Taken together, our results suggest involvement of *MYB* in the maintenance of MYC expression in CRC cells where canonical Wnt pathway mutations are also present.

β-Catenin does not induce *MYB* expression. The common upregulation of *MYB* and *MYC* in both CRC cells and murine adenomas harboring Wnt pathway activation suggested the possibility that in addition to *MYC*, activated β-catenin may also directly regulate *MYB*. This was particularly relevant in light of another study proposing that *MYB* is a target of the Wnt pathway.[20] However, despite the presence of prospective TCF/LEF binding sites in the human and mouse *MYB* promoter (Supplementary Figure 2a–c) we were unable to show upregulation of the *MYB* promoter in the presence of an activated form of β-catenin lacking the first 89 amino acids. Strikingly, high concentrations of activated β-catenin plasmid inhibited *MYB* promoter activity (Figure 4a). It is unclear whether this represents direct repression or the sequestration of transcription factors required for basal *MYB* promoter activity. In contrast, c-Myb induced its own promoter, consistent with previous findings.[21] Furthermore, endogenous c-Myb/MYB activity in HEK293 and NIH3T3 cells was unaffected by exogenous activated β-catenin (Figure 4b; Supplementary Figure 2d), although luciferase reporter assays with the archetypal β-catenin-responsive reporter pTOPFlash showed that the construct used for these assays had transactivation function. Taken together, these results show that the correlation between *MYB* and *MYC* expression in CRC is unlikely to be due to the common activation of both genes by deregulated Wnt signaling.

MYB and activated β-catenin cooperate to induce *MYC* reporter activation. Considering the mutual activation of β-catenin and MYB in CRC, in addition to *MYC* overexpression, we next considered whether MYB and activated β-catenin share *MYC* as a common gene target. This was assessed using chloramphenicol acetyltransferase (CAT) reporter assays with the human *MYC* promoter in HEK293 epithelial cells. HEK293 cells were used as they express very low levels of MYB while retaining a functional Wnt pathway. MYB conferred a dose-dependent increase in transcription from the *MYC* promoter, with a maximum level of 4.0-fold activation observed (Figure 5a). It is also notable that increasing c-Myb beyond this maximum level leads to a progressive decline in reporter activation.[14] When similar levels of activated β-catenin were co-transfected with the *MYC* promoter, there was a more modest effect on reporter activity compared with c-Myb alone, with a twofold increase observed at best, in keeping with previous analyses (Figure 5b).[11]

The prominence of binding sites for both MYB and the β-catenin transcriptional partner TCF/LEF within the *MYC* promoter sequence indicates a possible convergence of these pathways in the activation of *MYC*. As expected from the previous dose–response curves, transfection of

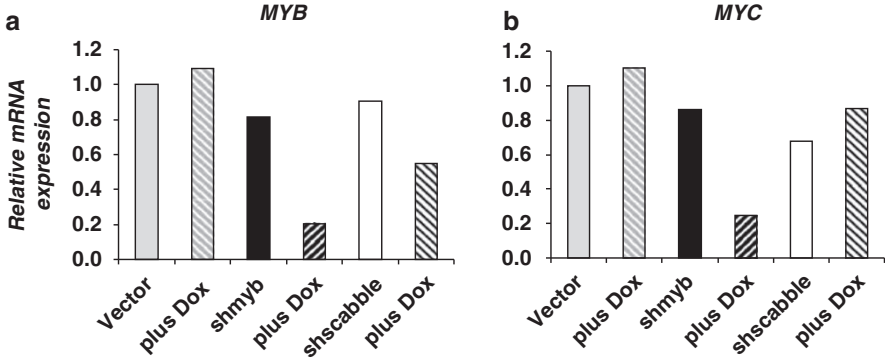

Figure 3 shRNA against *MYB* downregulates *MYC* expression in CRC cells. Stable cell line pools of Colo201 cells that expressed doxycycline-inducible shRNA against *MYB*, a scrabbled shRNA, or vector control were generated by lentivirus infection. Colo201 CRC cells express robust levels of both genes and when *MYB* expression is reduced (**a**) by shmyb following 24 h induction by doxycycline we observed a corresponding reduction in *MYC* expression (**b**). The mean expression levels based on two separate cDNA syntheses and real-time RT-PCRs conducted in triplicate in both cases are shown

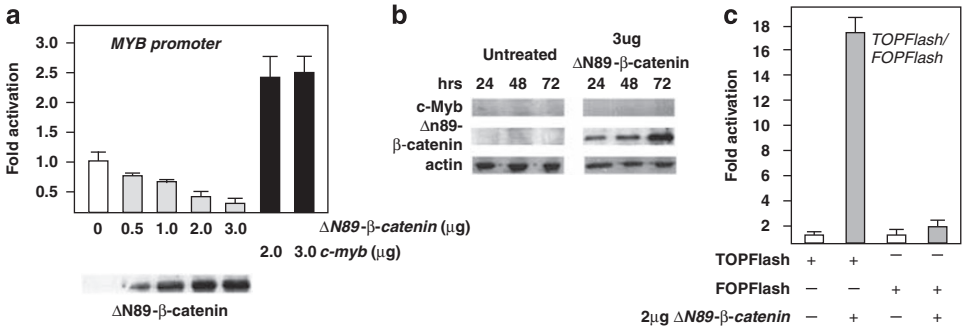

Figure 4 Activated β-catenin is unable to stimulate either *MYB* promoter activity or endogenous *MYB* expression in HEK293 epithelial cells. (**a**) Cells were transfected with the reporter pMBS-CAT (harboring the multiple c-Myb binding sites) and increasing amounts of either full-length c-Myb (*pACT-c-myb*) or activated β-catenin (*pcDNA3.1-ΔN89-β-catenin*). After 72 h, cells were harvested, lysed, and assessed for CAT enzyme activity. Mean ± S.E.M. of quadruplicate experiments is shown. Western blots depicting the expression of the effector proteins in the transfected cells are presented below the graphs from one of the replicate experiments. (**b**) HEK293 cells were transfected with activated β-catenin and 72 h later, harvested, lysed, and assessed for c-Myb and transfected β-catenin by western blot. Pan actin is shown as a loading control. (**c**) The ability of activated β-catenin to stimulate the luciferase reporters, pTOPFlash and pFOPFlash, is shown. HEK293 cells were transfected with 1 μg of either pTOPFlash or pFOPFlash reporter, along with 2 μg of activated β-catenin and the amount of luciferase activity in cells was measured 72 h after transfection. Mean ± S.E.M. of triplicate experiments is shown

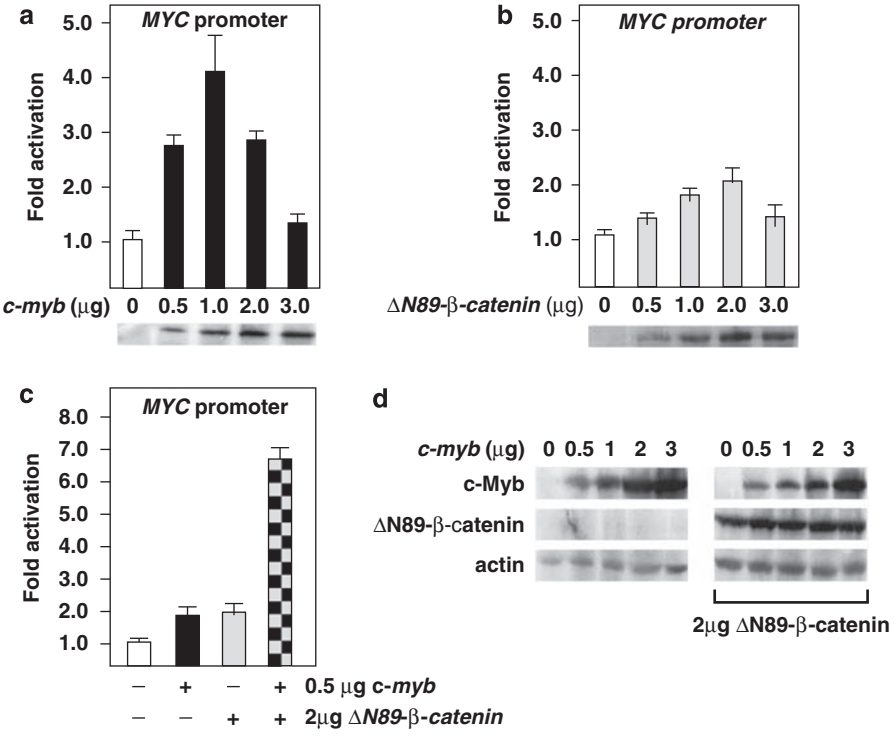

Figure 5 Suboptimal amounts of c-Myb cooperate with activated β-catenin to transactivate the *MYC* promoter in HEK293 cells. (**a**) c-Myb activates the *MYC* reporter to a maximum of approximately fourfold whereas (**b**) activated β-catenin achieves at best an approximately twofold increase in reporter activity. (**c**) When maximum levels of activated β-catenin and minimum levels of c-Myb are combined activation is now approximately sevenfold. Activation of the *MYC* promoter is significantly greater when c-Myb and activated β-catenin are combined compared to the individual effectors alone (*t*-test; $P < 1 \times 10^{-5}$). (**d**) Western blots showing the expression of the c-Myc-epitope-tagged ΔN89-β-catenin, c-Myb, and a loading control pan-actin

submaximal amounts of c-Myb/MYB and activated β-catenin provided only minimal stimulation of the reporter construct. In combination, however, these same levels of c-Myb and activated β-catenin gave a 6.5-fold increase in reporter stimulation, suggesting a cooperative activity exists between c-Myb and β-catenin in regulating the *MYC* promoter (Figure 5c). Furthermore, the pattern of activation observed in Figure 5c is maximal and sustainable, such that adding more c-Myb does not lead to the reduction in activity normally associated with excess c-Myb (Figure 5a; data not shown). Moreover, the levels of *MYB* expression are unchanged in the presence or absence of activated β-catenin (Figure 5d). These results thus demonstrate that MYB enhances the capacity of activated β-catenin to transactivate the Wnt target

MYC. In addition, the presence of activated β-catenin overcomes the apparent inhibitory effects of c-Myb on *MYC* promoter activity when c-Myb is present in excess.

MYB and β-catenin converge in regulating endogenous *MYC* expression. We next examined whether cooperation between activated β-catenin and MYB extended to the regulation of endogenous *MYC*. In a result mirroring those from the reporter studies, transfection of submaximal amounts of c-Myb led to a slight increase in the level of *MYC* mRNA (Figure 6a). Activated β-catenin was also able to induce endogenous *MYC* mRNA modestly (Figure 6a). Combining the two transcription factors however yielded a more immediate, greater and sustainable increase

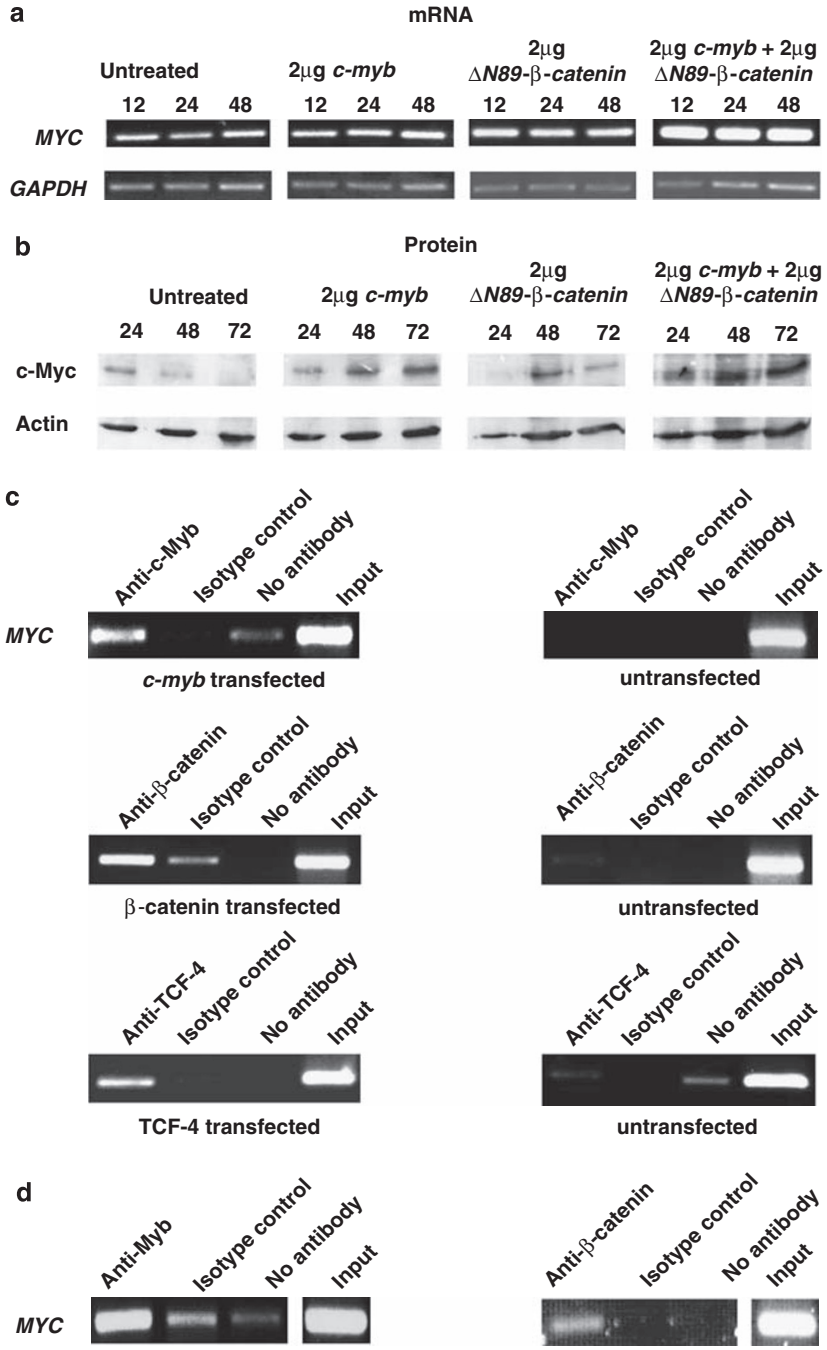

Figure 6 c-Myb and activated β-catenin cooperate in activating endogenous MYC and bind to the endogenous *MYC* promoter. HEK293 cells were transfected with constructs for c-Myb, activated β-catenin, or a combination of both and then harvested for total RNA at the times indicated after transfection. (**a**) Total RNA was reverse-transcribed, then subsequently used in PCR reactions to detect changes in the level of *MYC* mRNA. Housekeeping gene *GAPDH* was used to control for loading of cDNA for the PCR reactions. (**b**) Proteins were extracted from HEK293 cells transfected as in (**a**) and probed for changes in the level of c-Myc. Pan-actin was used to control for the loading of protein. (**c**) c-Myb, TCF4, and β-catenin are recruited to the *MYC* promoter in transfected HEK293 epithelial cells and (**d**) c-Myb and β-catenin in the case of Colo201 colorectal cancer cells as determined by ChIP assays

(approximately sixfold; determined by densitometry scans) in *MYC* mRNA expression than that achieved by the individual effectors alone. Changes in *MYC* mRNA levels associated with the expression of *MYB* and activated β-catenin were also reflected as an increase in c-Myc protein (Figure 6b). It is apparent from these results that MYB and activated β-catenin also cooperate to support robust expression of endogenous MYC.

MYB and activated β-catenin bind to the endogenous *MYC* promoter. c-Myb and activated β-catenin may cooperate in regulating *MYC* by associating together at the cellular target gene promoter. To evaluate this, chromatin immunoprecipitation (ChIP) assays were carried out in HEK293 cells transfected with expression constructs for c-Myb and β-catenin. These experiments showed that both proteins were recruited to the *MYC* promoter (Figure 6c). In the absence of transfected proteins, very little *MYC* promoter was detected in ChIP lysates, consistent with the minimal c-Myb/*MYB* expression and low amounts of endogenous nuclear β-catenin in this cell line.

To determine whether endogenous c-Myb and β-catenin bind to the genomic *MYC* promoter in human CRC cells, ChIP assays were performed in Colo201 cells. These cells express abundant levels of *MYB*[18] and *MYC*[22] and have an activated Wnt pathway through *APC* mutation.[6] As shown in Figure 6c, both c-Myb and β-catenin are enriched at the *MYC* promoter in these CRC cells. Numerous attempts were made to examine whether c-Myb and β-catenin, with or without TCF4, are able to form an endogenous complex in CRC cells or in HEK293 cells when overexpressed. However, no evidence for such complexes was obtained, although binding of β-catenin to TCF4 was readily established (data not shown). These observations indicate that that both transcription factors most likely engage the *MYC* promoter independently of each other. To further understand the mechanism by which cooperation between the two factors occurs, we assessed acetylation at the *MYC* promoter using an antibody directed toward a motif correlated with transcriptional activation, acetylated lysine 27 of histone H3. We found increased acetylated histone H3 (lysine 27) associated with the binding of both factors, but in particular c-Myb (data not shown). In a broader context, these data provide additional support for the proposition that the activation of *MYC* expression by deregulated Wnt signaling is more readily achieved in the presence of c-Myb.

Discussion

The Wnt pathway has an early and prominent function in CRC genesis through mutations in *APC* or β-catenin.[3,4,23] Analysis of $APC^{Min/+}$ mice and their corresponding predisposition to intestinal adenoma formation has allowed the cellular and molecular aspects of human familial adenomatous polyposis (FAP) to be understood.[24]

The biology of adenoma formation needs to be considered in the context of how crypts are generated and maintained. In this regard, the Wnt pathway,[25–27] *c-Myb*,[2] and *MYC*[28,29] are particularly important. We observed that the normal kinetics of adenoma formation in $APC^{Min/+}$ mice is correspondingly and significantly delayed when *c-myb* is haploinsufficient. This is likely because of the need to drive strong expression of important gene targets such as *MYC*, as shown in the current study. It has been reported that intestine-specific knockout of *c-myc* reduces adenoma formation in $APC^{Min/+}$ mice.[30] We found a similar phenomenon in $APC^{Min/+}$ mice when one allele of *c-myb* is deleted. This is an especially important observation given that *MYC* is a common target for both MYB and the Wnt pathway in CRC. Certainly, deletion of either *c-myb* or *c-myc* has a critical effect on colonic crypt development,[2,31,32] whereas c-Myb is additionally necessary for appropriate maintenance of adult crypt homeostasis.[2] As a corollary, *c-myb* or *c-myc* expression is highly elevated in $APC^{Min/+}$ adenomas in the SI and colon indicating involvement of these in the inappropriate expansion of epithelial cells. The more slowly forming adenomas in $APC^{Min/+} \times c\text{-}myb^{+/-}$ mice showed a correspondingly reduced level of both *c-myb* and *c-myc* expression. Whether this finding reflects the availability of a single *c-myb* allele for activation in adenoma-prone $APC^{Min/+}$ intestinal epithelia is currently unclear. However, when these observations are considered together, it is clear that *c-myb* and *c-myc* are required for normal crypt biology, but when overexpressed are associated with carcinogenesis.

Given that *MYC* is normally targeted by both the MYB and Wnt pathways, these data suggest a prospective mechanism for cooperation in which activated Wnt signaling leads to upregulation of *MYB*, making it available for cooperative activation of target genes such as *MYC*. Previous work by others highlighted *MYB* as a potential Wnt target gene.[20] However, we did not observe activation of either the *MYB* promoter or endogenous gene by an activated form of β-catenin. This discrepancy between our results and those of van de Wetering *et al*. may derive from the normal cellular biology of c-Myb/MYB. Expression of dominant-negative TCF4 in CRC cells causes cellular differentiation, a process that occurs in concert with decreased *MYB* expression. Nevertheless, we cannot at this stage rule out *MYB* as a bona fide Wnt target gene in CRC. It is possible that the variation in findings stems from the different cell types used in each study.

c-Myb requires cooperating factors in a number of circumstances to exert its full transactivation potential. For instance, c-Myb is known to work in partnership with a number of proteins, including CBP, the C/EBPβ family member NF-M, and p100, in regulating hemopoietic genes such as *mim-1* (reviewed in Ramsay and Gonda[1]). The present work extends this list of c-Myb partners to include β-catenin. We have confirmed previous reports that β-catenin alone activates the *MYC* promoter, but extended these observations by demonstrating that convergence with MYB leads to a more robust level of *MYC* promoter activity as well as expression from the endogenous gene locus. Our attempts to show direct interaction between the two factors were unsuccessful. The implication from this was that cooperative transactivation by the two factors is not achieved through physical association with each other, but rather through a mechanism that perhaps involves the independent binding of each factor to the target promoter, as shown in our ChIP assays. In further support of this assertion, ChIP assays against a histone motif correlated with transactivation showed increased acetylation at the *MYC*

promoter associated with the presence of both factors, consistent with the increased levels of endogenous *MYC* mRNA and protein.

The potential that c-Myb/MYB and activated β-catenin cooperate in regulating other genes central to CRC has also been considered. One gene of particular interest was cyclooxygenase 2 (*COX-2*). The *COX-2* promoter has both TCF4/LEF-1[33,34] and MYB binding sites within it promoter region, whereas its counterpart *COX-1* has none.[35] Expression of *Cox-2* is increased markedly in $APC^{Min/+}$ intestinal adenomas (Supplementary Figure 3a) as reported by others.[36,37] We observed cooperative induction of the human *COX-2* promoter in the presence of submaximal levels of MYB with activated β-catenin, recapitulating the data described here for *MYC* (Supplementary Figure 3b–d). This indicates that the cooperative interaction between MYB and activated β-catenin may extend beyond *MYC* alone to include the regulation of other genes important to adenoma formation and progression.

In conclusion, we have established a relationship between the two most commonly activated pathways in CRC in the generation of intestinal adenomas, a process that appears to function by cooperative regulation of CRC genes such as *MYC*. This cooperative mechanism of activation would be possible at the earliest stages of colonic crypt epithelial cell transformation where *APC* mutations are thought to initiate CRC, because *MYB* expression is a feature of normal colonocytes and thus available to work in cooperation with elevated levels of β-catenin. This work highlights the importance of discovering modes of therapeutic targeting of inappropriate *MYB* expression.[1]

Materials and Methods

Mice. C57Bl/6 $APC^{min/+}$ mice harboring an *Apc* allele mutation that encodes a truncated protein of 850 amino acids and $c\text{-}myb^{+/-}$ knockout mice were interbred to generate compound heterozygous mice. They were observed for any signs intestinal bleeding or ill health according the Peter MacCallum Cancer Centre Animal Experimentation Ethics Committee regulations. Sick mice were killed and examined for the presence of adenomas in the colon and SIs. Adenomas and histologically normal mucosa were collected and processed for total RNA using Trizol (Invitrogen).

cDNA microarray expression analysis. A gene expression database representing 229 tumor samples belonging to 13 histological types has been developed for the classification of different cancers.[38] Following intensity-based (LOWESS) and per gene median normalization of the array data, fold-change ratios for the genes *MYB*, *MYC*, and β-catenin (*CTNNB1*) were visualized across ordered cancer types using the programs Cluster and Treeview. All gene probes were confirmed by DNA sequencing.

Reporter assays. HEK293 or NIH3T3 cells were transfected with a combination of either 0.2 μg of CAT or 1 μg of luciferase reporter plasmid (*pc-mycCAT*, *pTOPFlash*, or *pFOPFlash*, 0.5–3 μg of effector plasmid (*pACT-c-myb* or *pcDNA3.1-ΔN89-β-catenin*) and 0.33 μg of transfection efficiency plasmid (*pSV40-β*-galactosidase) using Metafectene (Biontex) according to the manufacturer's instructions. The *MYB* promoter construct has been described in detail elsewhere.[39]

Western blotting. Transfection of cells involved correspondingly higher amounts of plasmid effector DNAs per flask to recapitulate the ratios of effector DNAs to cell numbers used in the reporter studies. Proteins were partitioned into nuclear and cytoplasmic fractions by differential centrifugation in lysis buffer before separation on 10% polyacrylamide/SDS gels and transfer to PVDF membrane. Membranes were blocked in 10% non-fat skim milk powder and then probed with one of the following antibodies made up in 1% gelatin: mouse anti-c-Myb 1.1 (1 : 500), mouse anti-c-Myc tag, mouse anti-pan actin (1 : 2500; ICN Biomedical), and mouse anti-β-catenin. After washing, the membranes were probed with a goat anti-mouse secondary antibody conjugated to alkaline phosphatase (1 : 2500; Bio-Rad). Blots were developed using 5-bromo-4-chloro-3′-indolyphosphate *p*-toluidine salt and nitro-blue tetrazolium chloride reagents (Sigma).

***MYB* knockdown by RNA interference.** The doxycycline-inducible lentiviral shRNA vector *pLVTSH* will be fully described elsewhere (Barry *et al*., in preparation). In brief, vectors encoding an *MYB* shRNA 5′-**GAACCUCUUACA AUUUGCAGAAACACU**UUCAUGAGA**AGUGUUUCUGCAUUGUGUAAGAGGUU C**UU-3′ (with the bold sequences corresponding to sense and antisense *MYB* mRNA, respectively) and a scrambled control shRNA 5′-AUAAGAAU GUCCAUC AGUUACGCCAUAUUCAUGAGAUAUGGCGUAACUGAUGGACAUUCUUAUUU-3′ were used. Lentivirus was generated by co-transfection of HEK293T cells with packaging plasmids using Lipofectamine 2000 (Invitrogen) as directed by the manufacturer. The supernatant was collected after 48 h, filtered through a 0.45 μm filter before being used to transduce Colo201 cells. These cells were FACS-sorted based on expression of *pLVTSH*-encoded eGFP. Cell were allowed to expand and then treated with 1 μg/ml doxycycline (Sigma) for 3 days. A sample of each culture was collected, washed in PBS and placed in Trizol, and subsequently processed for total RNA. *MYB* and *MYC* knockdown efficiency was assessed by Q-RT-PCR.

Chromatin Immunoprecipitation assays. Approximately 1×10^6 HEK293 cells were plated out in 100 mm dishes and transfected with 4 μg of one of the following constructs: *pcDNA3.1-ΔN89-β-catenin*, *pcDNA1-TCF4*, or *pACT-c-myb*. The same number of cells were also plated and left as untransfected controls. For examination of endogenous *MYC* promoter occupancy in CRC cells, approximately 1×10^6 Colo201 cells were plated in 100 mm dishes. ChIP assays were carried out 72 h after transfection for HEK293 cells or 72 h after plating for Colo201 cells. Briefly, cells were fixed with formaldehyde, washed, and subsequently lysed. The resulting lysates were sonicated on ice to yield DNA fragments of an appropriate size (300–1000 bp). After preclearing with Protein A Sepharose beads (Amersham Biosciences), samples were treated with one of the following antibodies to assess binding to the *MYC* promoter: mouse anti-Myb (clone 1.1, 1 : 500); TCF4 antibody (Santa Cruz Biotechnology); and mouse anti-β-catenin (Signal Transduction Laboratories). A mouse anti-pan actin antibody and rabbit serum (Zymed) served as isotype controls. Antibody–protein–DNA complexes were collected using Protein A Sepharose beads and washed to remove nonspecifically bound protein–DNA adducts. The formaldehyde cross-links were reversed before the immunoprecipitates were assessed by PCR. ChIP samples were used undiluted and subjected to 27 cycles of PCR at 60 °C. Approximately 12% of the original sample was taken as the input control and subjected to the same PCR thermocycling. The following primers were used for amplification: for transfected HEK293 and Colo201cell studies—forward, 5′-GCCTGCGATGATTTATACTCACAG-3′; reverse, 5′-CGGAGATTAGCGAGAGAGGATC-3′. These were designed using Primer Express software from Applied Biosystems.

RT–PCR analysis of endogenous *MYC* and *MYB* expression. The effect of ectopic c-Myb and activated β-catenin on the transcriptional regulation of genomic *MYC* and *MYB* was assessed by RT–PCR. Total RNA was extracted from HEK293 cells at various time points after transfection with the different expression constructs for MYB and activated β-catenin (see above) using Trizol (Invitrogen). Extracted RNA was DNAse-1-treated and subsequently used to synthesize cDNA before assessment of changes in expression by conventional PCR. The primers and annealing conditions used were—*MYC*: forward, 5′-CAGCTGCTTAGACGCTGG ATT-3′; reverse, 5′-GTAGAAATACGGCTGCACCGA-3′, 27 cycles of PCR at 60°C; *MYB*: forward, 5′-GCCAATTATCTCCCGAATCGA-3′; reverse, 5′-ACCAAC GTTTCGGACCGTA-3′; *GAPDH*: forward, 5′-AGCCTTCTCCATGGTGGTGAAG AC-3′; reverse, 5′-CGGAGTCAACGGATTTGGTCGTAT-3′, 22 cycles of PCR at 60 °C.

Expression of endogenous *c-myc*, *c-myb*, and *cox-2* in $APC^{min/+}$ mouse adenomas and normal tissue—*c-myc*: forward, 5′-AAGGCCCCCAAGGTAGTGA-3′; reverse, 5′-TGCTCGTCTGCTTGAATGGA-3′; *c-myb*: forward, 5′-AATTATCTGCC-CAACCGG-3′; reverse, 5′-AGACCAACGCTTCGGACC-3′; *cox-2*: forward, 5′-AGAA GGAAATGGCTGCAGAA-3′; reverse, 5′-CCCCAAAGATAGCATCTGGA-3′.

Chromatin immunoprecipitation assays. Q-RT-PCR was used to examine changes in acetylation levels at the endogenous *MYC* promoter associated with the binding of MYB and β-catenin. Approximately 1×10^6 HEK293 cells

were plated out in 100 mm dishes and transfected with 4 μg of either *pcDNA3.1-ΔN89-β-catenin* or *pACT-c-Myb* alone or in combination. The same number of cells were also plated and used as untransfected controls. ChIP assays were carried out 72 h after transfection. After fixation and sonication of the DNA, samples were precleared with Protein A Sepharose beads and treated with a rabbit acetylated-lysine27-specific antibody (Upstate Biotechnology). Rabbit serum (Zymed) served as an isotype control. Antibody–protein–DNA complexes were collected using Protein A Sepharose beads, washed, and the formaldehyde cross-links reversed before the immunoprecipitates were assessed by PCR. ChIP samples were diluted 15-fold in dH_2O before use. Reaction mixtures and thermocycling were performed using SYBR Green according to the manufacturer's instructions in a PerkinElmer real-time PCR machine, model 7000 (Applied Biosystems). Primer sets were used to generate 100 bp amplicons and designed using Primer Express software from Applied Biosystems. Primer set 1: forward, 5′-CTGGAAGGCAGCCAAATTTTA-3′; reverse, 5′-AAAATGCATCGATTCTGATCAAAG-3′. Primer set 2: forward, 5′-AGG ACAAGGATGCGGTTTGT-3′; reverse, 5′-CTACGCGCGCCTACCATTT-3′. Forty cycles of PCR were used at a temperature of 60 °C. Analysis of the primary data was undertaken as described.[40]

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (http://www.nature.com/cdd)