compensatory proliferation were strongly dependent on IL-6 and that the absence of this tumor-promoting cytokine resulted in almost complete inhibition of DEN-induced hepatocarcinogenesis. IL-6 production by KCs was largely dependent on MyD88, an adaptor molecule that acts downstream of TLRs as well as IL-1 receptor (*26*). Because DEN is not a direct macrophage or KC activator (*27*), hepatocyte necrosis may be an intermediate in the pathway through which DEN or CCl_4 exposure results in IL-6 production. Various macromolecules released by necrotic cells activate macrophages through TLRs, the receptors which in turn activate MyD88 (*28*, *29*). TRIF, another TLR adaptor protein (*26*), is not required for DEN- or CCl_4-induced IL-6 production and liver injury (*27*). MyD88 signaling, but not TNFR1 signaling, was required for optimal DEN-induced hepatocarcinogenesis in male mice. DEN-induced hepatocarcinogenesis appears to depend on an inflammatory response, triggered by hepatocyte necrosis, that leads to production of IL-6. Estrogens, at concentrations present in females but not in males, suppress IL-6 production and therefore inhibit chemically induced liver carcinogenesis. A similar mechanism could account for the gender bias in liver cancer in humans. If so, estrogen-mimetic compounds capable of inhibiting excessive IL-6 production might be used to prevent progression of chronic liver disease to HCC in men.

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30. We thank J. Feramisco for help with image capture and analysis and E. Karin for help with the surgical models. Supported by National Institute of Diabetes and Digestive and Kidney Diseases grant DK007202 (W.E.N.); Japan Society for the Promotion of Science (T.S.); Human Frontier Science Program (S.K.); and NIH grants ES004151 and ES006376, CA118165, and the Superfund Basic Research Program (M.K.). M.K. is an American Cancer Society Professor.

Supporting Online Material
www.sciencemag.org/cgi/content/full/317/5834/121/DC1
Materials and Methods
Figs. S1 to S8
References

29 January 2007; accepted 4 June 2007
10.1126/science.1140485

Regulation of Spontaneous Intestinal Tumorigenesis Through the Adaptor Protein MyD88

Seth Rakoff-Nahoum and Ruslan Medzhitov*

Inflammation is increasingly recognized as an important component of tumorigenesis, although the mechanisms and pathways involved are not well understood. Tumor development is regulated by products of several modifier genes, but instructions for their tumor-specific expression are currently unknown. We show that the signaling through the adaptor protein MyD88 has a critical role in spontaneous tumor development in mice with heterozygous mutation in the adenomatous polyposis coli (APC) gene. We found that MyD88-dependent signaling controls the expression of several key modifier genes of intestinal tumorigenesis and has a critical role in both spontaneous and carcinogen-induced tumor development. This study thus reveals the important role of an innate immune signaling pathway in intestinal tumorigenesis.

Inflammatory responses contribute to carcinogenesis through multiple mechanisms (*1*–*3*). Activation of the transcription factor nuclear factor κB (NF-κB), a key mediator of inflammation, has a critical role in the regulation of tumor development resulting from chronic inflammation or exogenous mutagens (*4*, *5*). NF-κB is activated by multiple stimuli (*6*), and it is currently unknown which pathway is critically involved in cancer-associated inflammation and the tissue repair response (*7*). The role of inflammatory and tissue repair responses in spontaneous carcinogenesis, independent of chronic inflammation or administration of exogenous carcinogens, has not yet been characterized. However, signaling through Toll-like receptors (TLRs) of the innate immune system to MyD88 (a signaling adaptor of TLRs) has a critical role in the control of tissue renewal responses (*8*–*11*).

A link between intestinal tissue renewal and tumorigenesis was established when the genetic basis of familial associated polyposis (FAP) was mapped to the APC gene (*12*). Germline and sporadic mutations in *APC* occur in >85% of FAP and >80% of sporadic colorectal tumors (*12*). A mouse model of spontaneous intestinal tumorigenesis was discovered in a forward genetic screen (*13*). These mice, designated $Apc^{Min/+}$, have a mutation in the *APC* gene and develop 60 to 80 intestinal adenomas, mostly at the distal small intestine (*14*). Given the role of the MyD88 signaling in intestinal tissue renewal and repair, we investigated the role of this pathway in spontaneous intestinal carcinogenesis in $Apc^{Min/+}$ mice.

To examine a potential role of the MyD88 signaling pathway in spontaneous intestinal tumorigenesis, we generated $Apc^{Min/+}$ mice on MyD88-sufficient and -deficient backgrounds and analyzed sex- and age-matched cohorts. On average, $Apc^{Min/+}$ mice die within 6 months of age from complications of intestinal tumors (*13*). In contrast, mortality of $Apc^{Min/+}$ $MyD88^{-/-}$ mice was dramatically reduced as compared with $Apc^{Min/+}$ littermate controls (Fig. 1A). We investigated the red blood cell (RBC) status of these mice, a marker of intestinal tumorigenesis, and found that the anemia observed in $Apc^{Min/+}$ mice was significantly ameliorated in $Apc^{Min/+}$ $MyD88^{-/-}$ mice (Fig. 1B). MyD88-dependent signaling therefore contributes substantially to the severe mortality and morbidity caused by inactivation of *APC*.

The number of visible polyps (≥0.5 mm in diameter) was next quantified by stereoscopic microscopy. The number of macroadenomas in the small intestines or colon of $Apc^{Min/+}$ $MyD88^{-/-}$ mice was reduced compared with that in $Apc^{Min/+}$ controls (Fig. 2A and fig. S1). Loss of MyD88 decreased the number of small intestinal tumors

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in all regions of the small intestine (Fig. 2B), most notably in the distal small intestine where the majority of tumor formation in $Apc^{Min/+}$ mice occurs (*13*) (Fig. 2B).

The number of polyps in $Apc^{Min/+}$ $MyD88^{-/-}$ mice was reduced, and the polyps that were present, both at the proximal and distal small intestine, were smaller in size than those present in age-matched $Apc^{Min/+}$ mice (Fig. 2B and fig. S1). This finding suggested that the MyD88-dependent signaling pathway regulated intestinal tumor growth in $Apc^{Min/+}$ mice. Because the size of tumors can be influenced by the balance of cell proliferation and death within the tumor mass, we investigated the proliferative and apoptotic rates within size-matched tumors from $Apc^{Min/+}$ and $Apc^{Min/+}$ $MyD88^{-/-}$ mice (*15*). Polyps from both genotypes showed similar proportions of proliferating cells (Fig. 3A). However, higher numbers of apoptotic cells were found in tumors from $Apc^{Min/+}$ $MyD88^{-/-}$ mice than in samples from $Apc^{Min/+}$ mice (Fig. 3B and fig. S2A). Thus, MyD88 may contribute to tumor progression through regulation of apoptosis.

Intestinal tumorigenesis is a stepwise process (*12*). At the first step, referred to as initiation, genetic changes in either oncogenes or tumor suppressors lead to the transformation of normal epithelium to dysplastic cells, resulting in the formation of microadenomas. Tumor progression results in the expansion of microadenomas and an increase in tumor size (*4*, *12*). The decrease in polyp frequency and size observed in $Apc^{Min/+}$ $MyD88^{-/-}$ compared with those in $Apc^{Min/+}$ mice indicated that MyD88 regulates initiation or the progression from microadenomas to macroadenomas. The cancer initiation step in both FAP patients and $Apc^{Min/+}$ mice involves the loss of heterozygosity of the *APC* gene and results in the formation of microadenomas (*12*). To investigate whether MyD88 affected early events in neoplasia, we analyzed $Apc^{Min/+}$ and $Apc^{Min/+}$ $MyD88^{-/-}$ mice for the presence of microadenomas. Four- to five-week-old $Apc^{Min/+}$ mice, an age at which the majority of microadenoma formation occurs (*13*), were observed to have similar frequencies [$Apc^{Min/+}$: 2.06 ± 1.63 (SEM); $Apc^{Min/+}$ $MyD88^{-/-}$: 2.19 ± 0.95; microadenomas per Swiss roll section of entire small intestine; $N = 4$ mice per genotype] and morphology of microadenomas regardless of the presence or absence of MyD88 (Fig. 3B). Thus, MyD88 appears not to influence the formation of early neoplastic lesions in intestinal tumorigenesis but rather contributes to tumor growth and progression. In addition, macroadenomas of both $Apc^{Min/+}$ and $Apc^{Min/+}$ $MyD88^{-/-}$ mice showed an equivalent increase in cytoplasmic and nuclear accumulation of β-catenin (Fig. 3D) and frequencies of cells with nuclear β-catenin (fig. S2B), suggesting that MyD88-dependent signaling does not affect β-catenin nuclear translocation in dysplastic epithelium.

We further investigated the role of MyD88 in intestinal tumorigenesis by comparing gene expression in size-matched polyps from $Apc^{Min/+}$ and $Apc^{Min/+}$ $MyD88^{-/-}$ mice. This analysis revealed two classes of genes that are overexpressed in tumors compared with normal tissue. One class of genes was MyD88-independent in that their expression was similarly upregulated in $Apc^{Min/+}$ and $Apc^{Min/+}$ $MyD88^{-/-}$ tumors (fig. S3). This class of genes included, for example, c-Myc, which is known to be a direct target of β-catenin (*16*). These tumor-upregulated, but MyD88-independent, genes serve as internal controls for tumor tissue sampling.

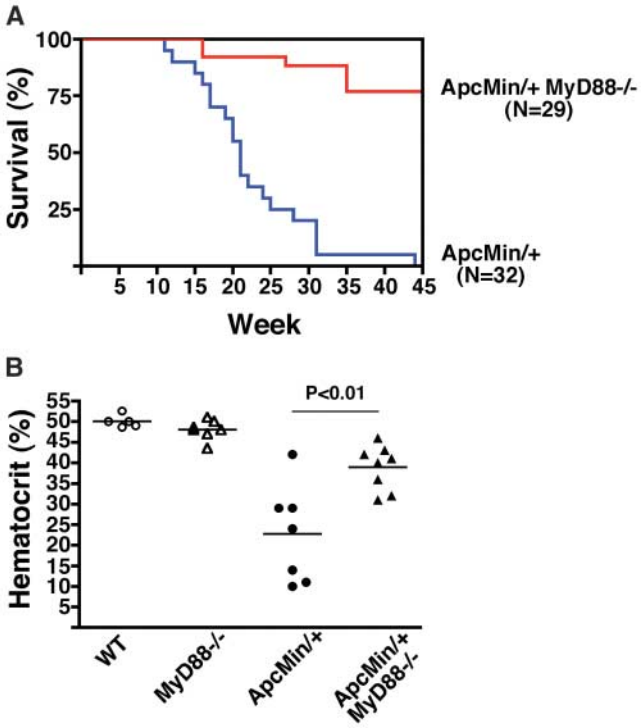

Fig. 1. Effects of MyD88-deficiency on mortality and blood loss in $Apc^{Min/+}$ mice. (**A**) $Apc^{Min/+}$ $MyD88^{+/+}$ or $MyD88^{+/-}$ ($Apc^{Min/+}$; N = 32) and $Apc^{Min/+}$ $MyD88^{-/-}$ ($Apc^{Min/+}$ $MyD88^{-/-}$; N = 29) mice were passively followed for long-term survival. Difference in survival was determined significant by the Mantel-Haenszel/Log-rank test. (**B**) At time of sacrifice, hematocrit of peripheral blood of age- and sex-matched mice (20 to 22 weeks old) was determined. Statistical analysis was performed using the Mann-Whitney U test.

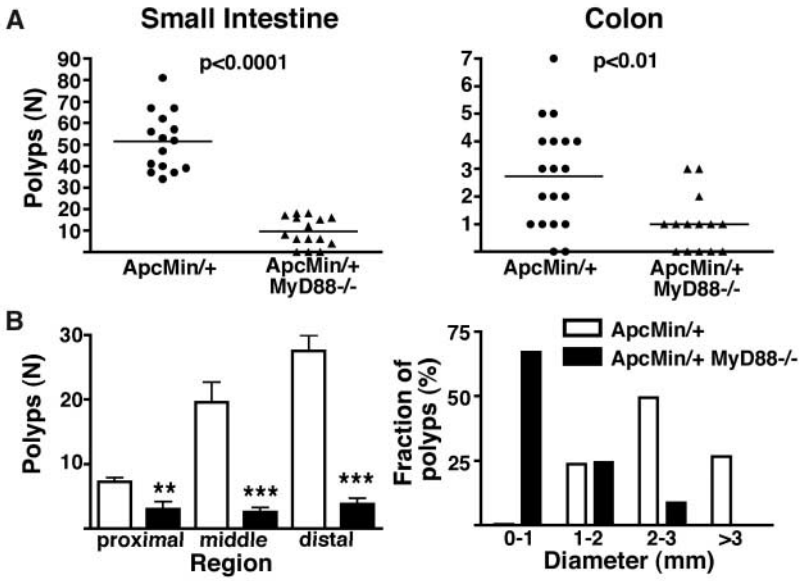

Fig. 2. Characterization of tumors in $Apc^{Min/+}$ mice in the presence or absence of MyD88. (**A**) (Left) The number of visible polyps in the small intestine (≥0.5 mm in diameter) was quantified by stereoscopic microscopy in age (20 to 33 weeks old)- and sex-matched $Apc^{Min/+}$ and $Apc^{Min/+}$ $MyD88^{-/-}$ mice. (Right) The number of visible polyps in the large intestine in age (20 to 28 weeks old)- and sex-matched $Apc^{Min/+}$ and $Apc^{Min/+}$ $MyD88^{-/-}$ mice. (**B**) (Left) Frequency of the polyps in $Apc^{Min/+}$ and $Apc^{Min/+}$ $MyD88^{-/-}$ mice stratified by small intestinal region. (Right) Size range of tumors from age- and sex-matched $Apc^{Min/+}$ and $Apc^{Min/+}$ $MyD88^{-/-}$ mice. Error bars, ±SEM. **, P < 0.01; ***, P < 0.001 (compared with $Apc^{Min/+}$); Mann-Whitney U test.

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However, in these same size-matched tumors, we found many genes that showed increased expression in a tumor-specific and MyD88-dependent manner (Fig. 4). These included genes encoding positive regulators of intestinal tumorigenesis, such as cyclooxygenase-2 (COX-2) (*17*, *18*), matrix metalloprotease (MMP) 7 (*19*), and cytosolic phospholipase A2 (cPLA2) (*20*, *21*) (Fig. 4). MyD88-dependent signaling was also required for the tumor-specific expression of genes encoding keratinocyte growth factor 2 (KGF2) [also called fibroblast growth factor 10 (FGF10)], CD44, MMP10, insulin-like growth factor binding protein (IGFBP) 5, insulin-like growth factor–1 (IGF1), regenerating gene product IIIβ (RegIIIβ), and various cytokines and chemokines such as keratinocyte derived chemokine (KC), interleukin (IL)–6, and IL-1β (Fig. 4 and fig. S4), all of which are components of the tissue repair response and have been either shown to be, or implicated as, positive regulators of tumorigenesis. Thus, MyD88-dependent signaling is critically involved in the tumor-specific induction of modifier genes of intestinal tumorigenesis, as well as genes involved in intestinal tissue repair. Interestingly, MyD88-dependent genes in $Apc^{Min/+}$ tumors included both classical NF-κB–regulated genes (KC and IL-6) and genes that are regulated by other signaling pathways (FGF10, RegIIIβ, IGF1, and IGFBP5).

The tumor modifier MMP7 is expressed only in dysplastic epithelium (*19*), whereas another tumor modifier, COX-2, is expressed by multiple cell types in $Apc^{Min/+}$ tumors, including macrophages, fibroblasts, endothelial cells, and epithelium (*22*). Overexpression of both MMP7 and

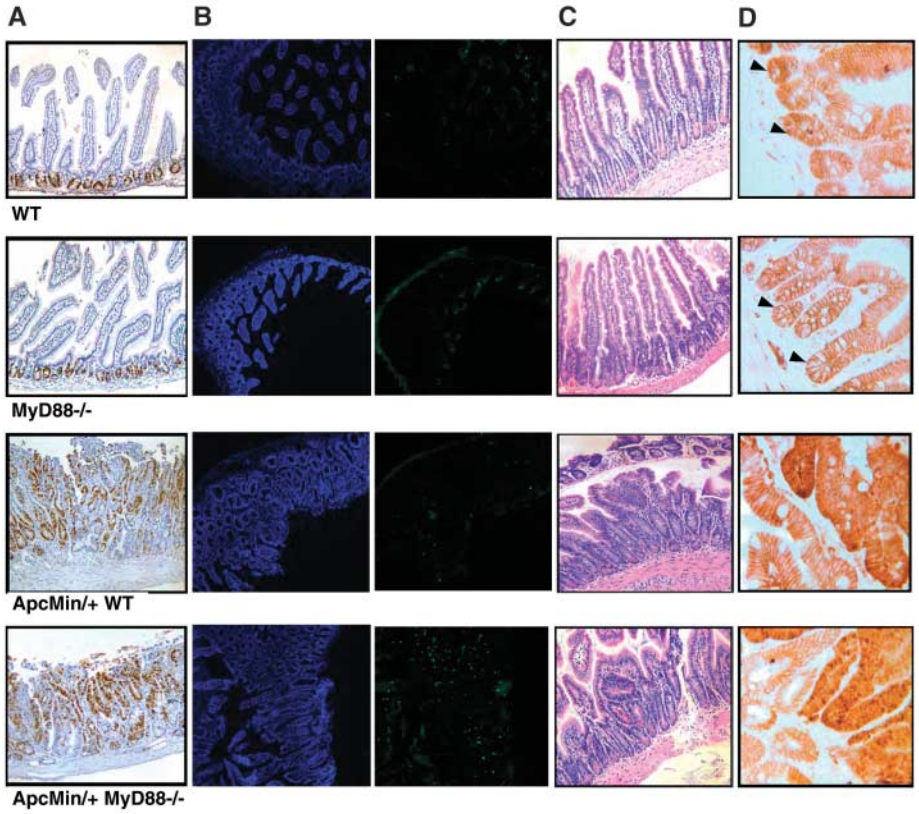

Fig. 3. MyD88-dependent and -independent epithelial homeostasis in intestinal tumors and formation of early neoplastic lesions. (**A**) 5-bromodeoxyuridine (BrDU), (**B**) (left panel) diamidino-phenylindole (DAPI), and (right panel) terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick end labeling (TUNEL) stain of intestinal tissue from WT and $MyD88^{-/-}$ mice and size-matched tumors from $Apc^{Min/+}$ and $Apc^{Min/+}$ $MyD88^{-/-}$ mice. (**C**) Representative histologic microphotos of distal small intestine from littermate mice at 4.5 weeks of age. Haemotoxylin and eosin stain; images are at 200x magnification. (**D**) Representative β-catenin staining of small intestine of age (20 weeks)- and sex-matched mice. Images are at 400x magnification. Arrowheads indicate nuclear localization of β-catenin at cells present at the crypt base in WT and $MyD88^{-/-}$ mice. Increased cytoplasmic and nuclear β-catenin staining can be seen in numerous cells in tumors of $Apc^{Min/+}$ and $Apc^{Min/+}$ $MyD88^{-/-}$ mice.

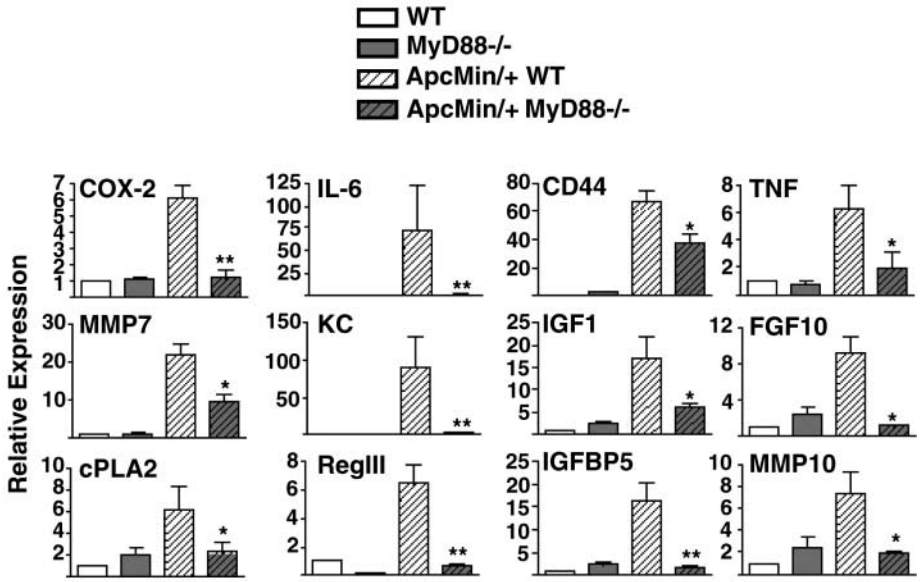

Fig. 4. MyD88 regulation of modifiers of tumor progression. RNA was prepared from the distal small intestine of normal intestine of WT and $MyD88^{-/-}$ mice and size-matched tumors of $Apc^{Min/+}$ and $Apc^{Min/+}$ $MyD88^{-/-}$ mice. cDNA was prepared, and quantitative polymerase chain reaction was performed. Expression of candidate genes was normalized to hypoxanthine-guanine phosphoribosyltransferase (HPRT) and relative induction compared with levels in the intestines of WT mice. Data are representative of five (tumor) tissue samples each isolated from different mice. Error bars, ± SEM. *, $P < 0.05$; **, $P < 0.01$ (compared to $Apc^{Min/+}$); Mann-Whitney U test.

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COX-2 is MyD88-dependent in tumors (Fig. 4 and fig. S5). This suggests that MyD88 regulates intestinal tumor progression by induction of tumor modifiers in both epithelial and stromal cells, similar to the role of IKKβ-dependent signaling in chronic inflammation-dependent intestinal tumorigenesis (*4*).

We observed similar frequencies of leukocytes, such as macrophages, both in normal mucosa and different sized tumors of $Apc^{Min/+}$ mice (*23*) (fig. S6), indicating that differences in gene expression observed in $Apc^{Min/+}$ and $Apc^{Min/+}$ $MyD88^{-/-}$ tumors did not result from differences in leukocyte recruitment.

In another model of intestinal (colonic) tumorigenesis [the administration of the carcinogen azoxymethane (AOM) (*24*)], incidence of tumor formation was again decreased in $MyD88^{-/-}$ mice compared with that in wild-type (WT) mice (fig. S7). Thus, the MyD88-dependent signaling pathway critically contributes to carcinogen-induced colonic tumorigenesis as well.

MyD88 functions in signaling downstream of both TLR and IL-1 receptor families. Although both types of receptors may contribute to the phenotype of $Apc^{Min/+}$ $MyD88^{-/-}$ mice, the function of IL-1 is likely to be downstream of TLRs because expression of IL-1 is dependent on TLR-MyD88 signaling (*25*) (fig. S4). The nature of the trigger for TLR signals in $Apc^{Min/+}$ tumors is currently unknown.

The MyD88 signaling pathway triggered by the TLR/IL-1R family of receptors functions in the control of inflammation both in host defense from infection and in tissue repair (*8*, *9*, *11*, *26*). Many genes involved in tissue repair (*27*) are induced in a TLR and MyD88-dependent manner upon intestinal injury (*8*, *9*) (fig. S8). Thus, the requirement for the MyD88 signaling pathway in intestinal tumor progression could result from the essential role of this pathway in the induction of a tissue-repair program. This would support the notion that tumor growth is an abnormal form of a continuous and unregulated state of tissue repair (*12*, *28*, *29*). Indeed, unlike the self-limiting repair in normal tissues, the tumor-associated tissue-repair response both promotes and is triggered by the tumor growth, thus falling into the self-perpetuating and uncontrolled state of tissue repair.

Our findings establish that the MyD88 signaling pathway downstream of members of the TLR and IL-1R family has a critical role in intestinal tumorigenesis. Tumor-associated expression of key modifier genes, as well as many other positive regulators of intestinal tumor progression and tissue repair, are critically dependent on the MyD88 signaling pathway. It should be noted however, that there are substantial differences between human FAP and $Apc^{Min/+}$ tumorigenesis. In addition, the $Apc^{Min/+}$ model is sensitive to a variety of genetic alterations. Therefore, it will be important to determine in future studies whether the MyD88 pathway contributes to intestinal tumorigenesis in humans.

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30. This work was supported by the Howard Hughes Medical Institute and a grant from NIH. We thank M. Linehan for help with immunofluorescence and C. Annicelli and S. Holley for animal care.

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29 January 2007; accepted 7 June 2007
10.1126/science.1140488

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Yeast DNA Polymerase ε Participates in Leading-Strand DNA Replication

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Multiple DNA polymerases participate in replicating the leading and lagging strands of the eukaryotic nuclear genome. Although 50 years have passed since the first DNA polymerase was discovered, the identity of the major polymerase used for leading-strand replication is uncertain. We constructed a derivative of yeast DNA polymerase ε that retains high replication activity but has strongly reduced replication fidelity, particularly for thymine-deoxythymidine 5′-monophosphate (T-dTMP) but not adenine-deoxyadenosine 5′-monophosphate (A-dAMP) mismatches. Yeast strains with this DNA polymerase ε allele have elevated rates of T to A substitution mutations. The position and rate of these substitutions depend on the orientation of the mutational reporter and its location relative to origins of DNA replication and reveal a pattern indicating that DNA polymerase ε participates in leading-strand DNA replication.

Replication of the eukaryotic nuclear genome requires DNA polymerase α to initiate synthesis at origins and to initiate synthesis of Okazaki fragments on the lagging strand, allowing DNA polymerases δ (pol δ) and ε (pol ε) to then perform the bulk of chain elongation (*1*, *2*). Pol δ is implicated in lagging-strand replication (*1*), but the identity of the polymerase(s) that replicates the leading strand is unknown (*1*, *2*). Null alleles of the *POL2* (pol ε) and *POL3* (pol δ) genes are uninformative for identifying the leading-strand polymerase, because both genes are essential for normal replication. To retain replication activity while generating a distinct mutational signature in vivo that allows assignment of pol ε to leading- and/or lagging-strand replication in yeast cells, we substituted glycine for Met^{644} at the *Saccharomyces cerevisiae* pol ε active site. Yeast pol ε with the $Met^{644}Gly$ change retains 44% of wild-type polymerase activity (Fig. 1A) and retains full 3′ exonuclease activity (Fig. 1B). A haploid *pol2-M644G* yeast strain grows at a rate similar to a *POL2* strain (Fig. 1C), indicating that $M^{644}G$ pol ε retains substantial replicative capacity. In both its exonuclease-proficient (Fig. 1D) and exonuclease-deficient forms (Fig. 1E), $M^{644}G$ pol ε synthesizes DNA in vitro with reduced fidelity in comparison with wild-type (i.e., Met^{644}) pol ε (Fig. 1 and table S1) (*3*), i.e., it is defective in discriminating against deoxynucleotide triphosphate (dNTP) misinsertion. Even the exonuclease-

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Regulation of Spontaneous Intestinal Tumorigenesis Through the Adaptor Protein MyD88

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Science **317** (5834), 124-127. [doi: 10.1126/science.1140488]

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