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A novel Ku70 function in colorectal homeostasis separate from nonhomologous end joining

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Abstract

Ku70, a known nonhomologous end-joining (NHEJ) factor, also functions in tumor suppression, although this molecular mechanism remains uncharacterized. Previously, we showed that mice deficient for DNA ligase IV (Lig4), another key NHEJ factor, succumbed to aggressive lymphoma in the absence of tumor suppressor p53. However, the tumor phenotype is abrogated by the introduction of a hypomorphic mutant $p53^{R172P}$, which impaired p53-mediated apoptosis but not cell-cycle arrest. However, $Lig4^{-/-}p53^{R172P}$ mice succumbed to severe diabetes. To further elucidate the role of NHEJ and p53-mediated apoptosis *in vivo*, we bred $Ku70^{-/-}p53^{R172P}$ mice. Unexpectedly, these mice were free of diabetes, although 80% of the mutant mice had abnormally enlarged colons with pronounced inflammation. Remarkably, most of these mutant mice progressed to dysplasia, adenoma and adenocarcinoma; this is in contrast to the $Lig4^{-/-}p53^{R172P}$ phenotype, strongly suggesting an NHEJ-independent function of Ku70. Significantly, our analyses of $Ku70^{-/-}p53^{R172P}$ colonic epithelial cells show nuclear stabilization of β-catenin accompanied by higher expression of cyclin D1 and c-Myc in affected colon sections than in control samples. This is not due to the p53 mutation, as $Ku70^{-/-}$ mice share this phenotype. Our results not only unravel a novel function of Ku70 essential for colon homeostasis, but also establish an excellent *in vivo* model in which to study how chronic inflammation and abnormal cellular proliferation underlie tumorigenesis and tumor progression in the colon.

Keywords

nonhomologous end joining; Ku70; colorectal cancer; senescence; beta-catenin; Wnt signaling

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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INTRODUCTION

Colorectal cancer (CRC) is the third most common malignancy diagnosed in the United States, with ~140 000 new cases and 50 000 deaths in 2010.[1] Although the pathogenesis of CRC is not fully understood, it is well established that it arises from the chronic accumulation of genetic, epigenetic and cellular alterations that drive the normal colonic epithelial cells into neoplasia.[2–4] Evidence also indicates an increased incidence of CRC linked to chronic inflammation in the colon.[5,6] However, the detailed mechanism of this link is still unknown. Another major contributing factor in the development of CRC is the accumulation of DNA damage mainly owing to deficiency in repair pathways. For example, microsatellite instability, caused by deficiency in the mismatch repair pathway, directly results in CRC.[7] However, the roles of other types of DNA damage and the associated repair machineries in preventing tumorigenesis in the colon have not been well studied.

Among all types of DNA damage, double-strand breaks (DSBs) are the most devastating, directly threatening the stability of the genome. DSBs are repaired by one of the two major mechanisms: homologous recombination or the nonhomologous end joining (NHEJ). NHEJ is active mostly in the G0/G1 cell stage and is considered a major repair pathway for DSBs in mammalian cells.[8,9] Classic NHEJ first recognizes and binds to broken ends by the Ku70/Ku80 heterodimer,[10] which subsequently activates the DNA-dependent kinase complex.[11] This further recruits other factors, such as end-modifying enzymes, to prepare the DNA ends for ligation.[12] Finally, the NHEJ reaction is completed by the DNA ligation complex, composed of DNA ligase IV (Lig4) and its cofactors XRCC4 and XLF.[13] Mice deficient for Lig4 or XRCC4 have a late embryonic lethal phenotype owing to excessive neuronal apoptosis caused by spontaneous DNA breaks in a p53-dependent process.[14–16] In general, deficiency in classic NHEJ results in severe combined immunodeficiency (SCID) owing to the failure to repair programmed DSBs generated at the antigen-receptor genes during early lymphoid development.[17,18] In precursor lymphoid cells, the accumulation of these unrepaired DNA breaks activates p53-dependent apoptosis. In the absence of p53, NHEJ-deficient mice succumb to early lymphomas with chromosomal translocation and oncogenic amplification.[19,20] In this regard, $Lig4^{-/-}p53^{-/-}$ and $Ku70^{-/-}p53^{-/-}$ mice have similar phenotypes: proliferation of precursor lymphoid cells with unrepaired DNA damage drives these cells into tumorigenesis.

Previously, we crossed $Lig4^{-/-}$ mice with hypomorphic p53 mutant ($p53^{R172P}$) mice ($Lig4^{-/-}PP$).[21] The $p53^{R172P}$ mutant impairs the p53-mediated apoptosis but retains partial cell-cycle arrest function.[22] We showed that the $p53^{R172P}$ prevents embryonic lethality and lymphomagenesis.[23] Further analyses revealed that DSBs in precursor lymphoid cells activated cell-cycle arrest and cellular senescence, preventing genomic instability and tumorigenesis. In addition, the resultant cellular senescence owing to excessive DNA damage in pancreatic islets also suppressed the proliferation of pancreatic β-cells, leading to severe diabetes at an early age.[24] Remarkably, $Lig4^{-/-}PP$ mice, among the hundreds of mice we analyzed, were tumor free.

Ku70, another key component of classic NHEJ, is critical for detecting DSBs.[25,26] Unlike Lig4 and XRCC4, Ku70 deficiency does not produce an embryonic lethal phenotype;

however, Ku70$^{-/-}$ mice exhibit a leaky SCID phenotype and have a low rate of spontaneous lymphoma. Hence, it has been suggested that Ku70 is a tumor suppressor,[26] but this molecular mechanism has not been elucidated. Aside from its important role in NHEJ, Ku70 is also involved in other cellular events, such as DNA replication,[27] apoptosis,[28,29] telomere maintenance[30] and transcriptional regulation.[31] In this report, we studied Ku70 deficiency with the p53^{R172P} mutation (Ku70$^{-/-}$PP) mice and discovered that these mice develop colonic inflammation and CRC. Our study indicates that Ku70 has an essential role in the homeostasis of the colorectal epithelium and in the prevention of tumorigenesis.

RESULTS

Ku70$^{-/-}$PP mice were phenotypically different from Lig4$^{-/-}$PP mice

Ku70$^{-/-}$ mice are not embryonic lethal; therefore, it is possible to compare mice with p53$^{+/+}$ and p53$^{R172P/R172P}$ backgrounds. For this purpose, we crossed Ku70$^{-/-}$ and p53^{R172P} mice. The resultant Ku70$^{-/-}$PP mice were born at the normal Mendelian ratio. Similar to Ku70$^{-/-}$ mice, Ku70$^{-/-}$PP mice had poor growth, small bodies (Figure 1a) and an average life span of less than 6 months (Figure 1b). These features are reminiscent of the Lig4$^{-/-}$PP mice. To determine whether these mutant mice also developed diabetes, as did the Lig4$^{-/-}$PP mice, we measured their blood glucose and examined their pancreata. To our surprise, Ku70$^{-/-}$PP mice exhibited normal glucose levels and had normal pancreata.[32] In sharp contrast to the Lig4$^{-/-}$PP mice, Ku70$^{-/-}$PP mice were free of diabetes.

We performed postmortem analysis and observed, unexpectedly, abnormal colon enlargement in 80% of the Ku70$^{-/-}$PP mice. We did not observe this abnormality in the colons of Lig4$^{-/-}$PP mice or Ku70$^{+/-}$p53^{R172P} littermates maintained in the same animal facility (Supplementary Figure S1a). Our results show that Ku70$^{-/-}$PP mice are phenotypically different from Lig4$^{-/-}$PP mice and demonstrate a possible role of Ku70 outside of NHEJ. Our results also reveal an important function of Ku70 in the homeostasis of the colonic mucosa.

Ku70$^{-/-}$PP mice succumbed to chronic inflammation, dysplasia and adenocarcinoma

The majority of the Ku70$^{-/-}$PP mice exhibited poor growth and impaction of feces, accompanied in some cases by rectal prolapse. We necropsied ~90 Ku70$^{-/-}$PP mice and found a pronounced enlargement of the colon in ~72 (80%) of them. We observed the highest incidence of enlarged colons in mice aged 60 days or older. On average, Ku70$^{-/-}$PP mice were approximately one-third smaller than their Ku70$^{+/-}$PP littermates; therefore, their colons were substantially shorter in the different age groups that we measured (Table 1). Ku70$^{-/-}$PP mice also had significantly thicker colons than did their littermates (Table 1 and Figure 1c).

We performed histopathological analysis of the affected colons and observed inflammation of the mucosa with leukocyte/lymphocyte infiltration (Figure 2a). We found milder inflammation in younger animals (<60 days old) that progressed to more severe inflammation in older mice (>100 days old). In addition, the affected colons showed loss of goblet cells (Figure 2b), unlike the colons of the Ku70$^{+/-}$PP controls. This condition is

frequently seen in patients with inflammatory bowel disease[33] and is associated with a defective colonic mucosa.[34] Importantly, we found low-grade dysplasia in the colonic epithelia of younger mice (as young as 64 days old) and high-grade dysplasia in older mice (>90 days old, Figure 2c). These lesions match the serrated phenotype typically formed by mass accumulation of cryptic epithelial cells that leads to internal enfolding.[35] Figure 2d shows a 139-day-old $Ku70^{-/-}$PP mouse with invasive CRC that caused penetration of the muscularis mucosa. We also analyzed more than 40 $Ku70^{-/-}p53^{+/+}$ mice. We detected various levels of colon inflammation in older mice (>6 months old). These mice also develop thymic lymphomas at a low rate. However, we did not detect dysplasia or CRC in $Ku70^{-/-}$ mice.

We did not observe gross histological abnormalities in the small intestine or in the other organs of $Ku70^{-/-}$PP mice. Neither did we find colonic abnormalities in $Ku70^{+/-}$PP littermates, nor in $Lig4^{-/-}$PP mice (Supplementary Figure S1b), which develop severe diabetes. In summary, $Ku70^{-/-}$PP mice uniquely developed colonic inflammation accompanied by mucosal hyperplasia, dysplasia and CRC. These mice can be used as a distinct *in vivo* model to study colitis-associated CRC.

T cells were present in the colons of $Ku70^{-/-}$PP mice

The risks of colitis and CRC rise sharply as a result of poorly regulated immune responses, especially those related to adaptive immunity.[36] Thus, we analyzed the status of the adaptive immunity of $Ku70^{-/-}$PP mice. Consistent with $Ku70^{-/-}$ mice, which typically exhibit a leaky SCID phenotype and early thymic involution,[25] the level of thymocytes from the $Ku70^{-/-}$PP mice at 30 days of age was less than 1% of that from the $Ku70^{+/-}$PP littermates (9.9×10^5 vs 6.4×10^7). However, we detected low levels of $CD4^+$ and $CD8^+$ double-positive thymocytes and low levels of single-positive T cells in the spleen, lymph nodes and blood of the $Ku70^{-/-}$PP mice (Supplementary Figure S2a). In the B-cell compartment, we detected very few or no immunoglobulinM (IgM)-positive cells and no B cells in the periphery (Supplementary Figure S2b). These results were consistent with the phenotypes that have been reported in $Ku70^{-/-}$ mice.[25,26] Therefore, the $p53^{R172P}$ mutation does not rescue the development of lymphocytes.

We suspected that an imbalance in the immune homeostasis from $Ku70^{-/-}$ mice might contribute to the inflammation.[37] To explore this hypothesis, we isolated lymphocytes from the lamina propria and colonic epithelia of mutant and control mice at different ages and analyzed them by flow cytometry. We found more $CD4^+$ T cells in the colons of $Ku70^{-/-}$PP mice than in the colons of $Ku70^{+/-}$PP littermates and wild-type controls at 22 days of age (22.8 vs 9.44%, Figure 2e) and at 139 days of age (43 vs 16.6%, data not shown). The increase in $CD4^+$ T cells in the older mice correlates well with the presence of progressive chronic colitis. This result is also consistent with increased $CD4^+$ T cells in their colonic lymph nodes compared with their littermates (Supplementary Figure S2a). These results indicate that few T cells in the thymus of $Ku70^{-/-}$ mice were able to migrate and expand in response to tissue stimulation, thus contributing to inflammation.

To determine whether $CD4^+$ T-cell expansion could be attributed to a lack of regulatory T cells in the colonic milieu, we isolated lymphocytes from the lamina propria and stained

them with $CD4^+$ T-cell $FoxP3^+$ markers. As shown in Figure 2f, no $FoxP3^+$ $CD4^+$ T cells were found. Thus, we concluded that effector $CD4^+$ T cells, and the lack of regulatory T cells, may be an important factor in colonic inflammation. We plan to conduct further detailed analyses of the T-cell repertoire in $Ku70^{-/-}$PP mice.

Cytokines interleukin-6 and tumor necrosis factor-α in the inflamed colons

To determine whether the inflamed colon hosted inflammatory cytokines, we performed immunohistochemical staining against interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α), two major proinflammatory cytokines. We were able to detect abundant TNF-α- and IL-6-producing cells in the inflamed colons of $Ku70^{-/-}$PP mice as compared with those of the $Ku70^{+/-}$PP controls (Figures 2g and h). These results indicated that a shift in the balance toward proinflammatory cytokines in $Ku70^{-/-}$PP mice with inflamed colons might promote a favorable environment for cancer development and progression.[38] Indeed, IL-6 has a direct role in activating oncogenic pathways.[39] Thus, we propose that the continuous influx of these cytokines could be a pivotal factor in transforming colonic tissue in $Ku70^{-/-}$PP mice.

DNA damage accumulated in the colonic epithelial cells of $Ku70^{-/-}$PP mice

Previously, we discovered the important role that NHEJ has in repairing spontaneous DNA damage in the lymphoid organs and pancreatic islets.[23,24] We ask here whether mutant mice with inflamed colons also had persistent DNA damage. We used anti-γH2AX antibodies to stain frozen colon sections from $Ku70^{+/-}$PP, $Ku70^{-/-}$PP and $Ku70^{-/-}$ mice at different ages. Our results showed many nuclear γH2AX foci in the colonic epithelia of $Ku70^{-/-}$PP and $Ku70^{-/-}$ mice (Figure 2i). We identified very few nuclear foci in the $Ku70^{+/-}$PP controls. Overall, these foci were restricted to the colonic epithelial cells. These results not only highlight the critical role of the NHEJ pathway in repairing spontaneous DNA damage in colonic epithelial cells, but also underscore the possible contribution of persistent DNA damage to the disruption of the epithelial integrity of the colon, which could lead to colitis or CRC.

Senescence was reduced in the colon sections from $Ku70^{-/-}$PP mice

We previously reported that persistent DNA damage leads to cellular senescence in $Lig4^{-/-}$PP lymphoid cells and pancreatic islets.[23,24] These results led us to ask here whether $Ku70^{-/-}$PP colonic epithelial cells affected by unrepaired DNA damage also underwent senescence. We used whole colons from $Ku70^{-/-}$PP and $Ku70^{+/-}$PP mice for senescence-associated beta-galactosidase staining and colons from $Lig4^{-/-}$PP mice as a positive control. Similar to the $Lig4^{-/-}$PP control, we observed positive senescence-associated beta-galactosidase staining in inflamed, non-neoplastic colon sections from $Ku70^{-/-}$PP mice. However, we noticed few positively stained cells at the bottom of the crypts and submucosa of the dysplastic or CRC colon sections. We found no positive staining in the colon sections from $Ku70^{+/-}$PP mice (Figure 3a and Supplementary Figure S1a). Our results suggest that the transformed epithelial cells need to overcome cellular senescence to proliferate. Thus, we propose that oncogenic activation in these cells promotes their escape from senescence to gain growth advantage, which is an important step in tumorigenesis.

The colonic epithelial cells of Ku70$^{-/-}$PP mice showed higher rates of proliferation

Colonic epithelial cells are defined by rapid tissue renewal that includes proliferation and differentiation, which are strictly regulated for tissue homeostasis.[40,41] We suspected a potential disruption of cell-cycle control in the colons of Ku70$^{-/-}$PP mice. To determine the rate of proliferation, we injected the mice with bromodeoxyuridine (BrdU) and detected incorporated BrdU in the colons of 30- and 60-day-old Ku70$^{-/-}$PP and Ku70$^{+/-}$PP littermates (Figure 3b). We noticed that, although BrdU-positive cells were confined to the basal region of the crypt in the Ku70$^{+/-}$PP controls, proliferating cells expanded from the basal region toward the lumen of the crypt in the Ku70$^{-/-}$PP mice.

A similar pattern and increased cell proliferation were confirmed by immunostaining for proliferating cell nuclear antigen (PCNA) on sections from Ku70$^{-/-}$PP, Ku70$^{+/-}$PP and age-matched wild-type controls at 30, 60, 90 and 150 days of age (Figure 3c). We counted 96 crypts from each age group ($n = 3$) and compared the number of positive cells of Ku70$^{-/-}$PP mice with those of the Ku70$^{+/-}$PP controls. We observed significantly ($P<0.001$) more PCNA-positive cells in Ku70$^{-/-}$PP mice than in Ku70$^{+/-}$PP mice or wild-type controls in all age groups (Figure 3d). Once again, whereas PCNA-positive cells were confined to the basal region of the crypt in Ku70$^{+/-}$PP mice, the proliferating cells expanded from the basal region toward the lumen of the crypt in Ku70$^{-/-}$PP mice. These results indicate an uncontrolled rate of proliferation in the colonic epithelial cells of the Ku70$^{-/-}$PP mice.

The p21 level decreased in transformed colon sections from Ku70$^{-/-}$ and Ku70$^{-/-}$PP mice

The increased rate of proliferation in the Ku70$^{-/-}$PP colons is rather surprising because (1) these colonic epithelial cells have high levels of DNA damage, and (2) our previous studies indicated that Lig4$^{-/-}$PP mice with increased DNA damage exhibited elevated levels of cyclin-dependent kinase inhibitor p21 and underwent senescence.[24] We therefore used immunohistochemical analysis to determine the cellular levels of p21 in colons from mutant and control mice at different ages. We observed very low levels of p21 in colon sections from Ku70$^{-/-}$PP mice that had dysplasia and CRC; however, p21 was clearly detected in surrounding sections that appeared normal, as well as in sections from Ku70$^{+/-}$PP mice and wild-type controls (Figure 4a). We confirmed this by western blot analysis: we found a lower level of p21 in dysplastic mucosa as compared with the normal portions from the same colon (Figure 4b). The lower level of p21 is consistent with the higher rate of proliferation that we observed in the colonic mucosa in Ku70$^{-/-}$PP mice as opposed to control mice.

The β-catenin/Wnt signaling pathway was dysregulated in Ku70$^{-/-}$PP mice

Regulation of the β-catenin/Wnt signaling pathway is important in the homeostasis of the colonic epithelium. Mutations in the components that regulate β-catenin lead to its uncontrolled activation, characterized by stabilization, cytoplasmic accumulation and nuclear translocation. In the nucleus, β-catenin interacts with transcription factors, such as T-cell factor/lymphoid enhancer factor, that control Wnt target genes, leading to oncogenic transformation.[42,43] As we found hyperproliferative epithelial cells in the colons, we next asked whether the level and location of β-catenin were affected. We used immunofluorescence to detect β-catenin in the colons from Ku70$^{-/-}$, wild-type, Ku70$^{-/-}$PP and Ku70$^{+/-}$PP mice. We observed an abnormal accumulation of β-catenin in the nuclei of

colonic epithelial cells from Ku70$^{-/-}$PP mice, but not in the nuclei of samples from the wild-type or littermate controls (Figure 4c). We detected nuclear localization of β-catenin, although at different levels, in most of the stained colon samples from the Ku70$^{-/-}$PP mice. Interestingly, we also observed a similar nuclear β-catenin pattern, but with a less severe outcome, in Ku70$^{-/-}$ mice. One extreme example is that of a 135-day-old Ku70$^{-/-}$ mouse that exhibited almost 100% nuclear β-catenin in the colon (Figure 4d).

To further confirm these results, we isolated colonic epithelial cells from Ku70$^{-/-}$, Ku70$^{-/-}$PP and control mice, and obtained cytoplasmic and nuclear fractions. Our western blot analyses clearly showed nuclear localization in samples from the Ku70$^{-/-}$ and Ku70$^{-/-}$PP mice, but not from their littermates or wild-type controls (Figure 4e). These results link Ku70 deficiency to the dysregulation of β-catenin, independently of the p53 mutation.

To assess whether this accumulation of β-catenin was due to adenomatous polyposis coli dysfunction, we immunostained colon samples from Ku70$^{-/-}$PP and Ku70$^{+/-}$PP controls against adenomatous polyposis coli, but observed no differences (Supplementary Figure S3). This result indicates that the Ku70 deficiency is linked to the dysregulation of β-catenin and likely the Wnt signaling pathway.

c-Myc and cyclin D1 were dysregulated in Ku70$^{-/-}$PP mice

Nuclear localization of β-catenin and activation of the Wnt signaling pathway are directly involved in tumorigenesis in CRC. We analyzed a known downstream target of Wnt signaling: cyclin D1.[44,45] We found increased levels of cyclin D1 in the colonic epithelium of Ku70$^{-/-}$PP mice (Figure 5a); conversely, a much lower level of cyclin D1 was detected in Ku70$^{+/-}$PP or age-matched wild-type control mice. This result was confirmed by western blot analysis: the dysplasic mucosa had highest expression of cyclin D1 as compared with normal nondysplasic ones (Figure 5b).

Another known target of Wnt signaling is c-Myc, a key molecule responsible for oncogenic transformation in the colon.[46] Using immunohistochemical staining, we determined the level of c-Myc in the colon sections from Ku70$^{-/-}$PP mice. We observed more nuclear expression of c-Myc in the colonic epithelium of Ku70$^{-/-}$PP mice than in the samples from Ku70$^{+/-}$PP controls (Figure 5c). We also observed that c-Myc expression was more accentuated in samples with more advanced lesions Figure 5c, (right panel). Furthermore, our western blot analysis indicated that c-Myc levels were higher in Ku70$^{-/-}$ and Ku70$^{-/-}$PP colon samples than in their controls (Figure 5d). This result indicates that increased expression of c-Myc has a strong association with tumor progression in these mutant mice. In summary, our results showed dysregulation of both cyclin D1 and c-Myc, two of the known downstream targets of the Wnt signaling pathway, in the colons of Ku70$^{-/-}$PP mice.

DISCUSSION

Our results demonstrated that Ku70 has an essential role in the homeostasis of the colonic mucosa. In mice with a p53^{R172P} background, the absence of Ku70 led to DNA damage accumulation in colonic epithelial cells, chronic inflammation with infiltrating CD4^{+} T cells

and other innate immune cells, and increased IL-6- and TNF-α-producing cells. Significantly, most of the Ku70$^{-/-}$PP mice developed different degrees of colonic abnormalities, including loss of goblet cells, which indicates impaired differentiation and a compromised mucosal layer, hyperplasia, dysplasia and adenoma. In severe cases, we observed the onset of invasive CRC.

Unexpectedly, we found a higher rate of proliferation of colonic epithelial cells in Ku70$^{-/-}$PP mice, despite the presence of DNA damage, than in samples from the controls. This phenotype in Ku70$^{-/-}$PP mice is completely different from that of the Lig4$^{-/-}$PP mice, which exhibit intense senescence in the colonic epithelium and no tumor development. Remarkably, we found nuclear localization of β-catenin, a key marker for oncogenic transformation, in the colons of both Ku70$^{-/-}$ and Ku70$^{-/-}$PP mice, with elevated levels of c-Myc and cyclin D1 expression, which are known targets of the Wnt signaling pathway. These results lead us to conclude that Ku70 has a distinctive role, apart from classic NHEJ, and is likely linked to the nuclear stability of β-catenin and the activation of the Wnt signaling pathway.

The tumorigenesis in the colon we describe here is not entirely due to impaired p53-dependent apoptosis. First, neither p53$^{R172P/R172P}$ nor Ku70$^{+/-}$p53$^{R172P/R172P}$ mice exhibited any colonic abnormalities. Second, Lig4$^{-/-}$PP mice did not develop tumors in the colon or other tissues. Third, we found nuclear localization of β-catenin in the colons of Ku70$^{-/-}$ mice (Figure 4) with a p53$^{+/+}$ background, which suggests that Ku70 is intrinsically linked to this regulation. However, incidences of colonic abnormalities in Ku70$^{-/-}$ mice were delayed and less severe, indicating that p53-dependent apoptosis had an important role in eliminating irregular proliferating cells.

The tumorigenesis in the Ku70$^{-/-}$PP colons cannot be solely explained by the loss of genomic integrity owing to NHEJ deficiency. First, we did not observe any abnormalities in the colons of Lig4$^{-/-}$PP mice, even though these mice had the same genetic background and were maintained in the same facility. Lig4 is absolutely required for classic NHEJ. Second, our previous work, as well as that of others, revealed that NHEJ deficiency leads to lymphomagenesis in mice when combined with defective cell-cycle control. Although we did observe thymic lymphoma in some Ku70$^{-/-}$PP mice in the present study, the majority of the mutant mice did not develop lymphoma. Therefore, we believe NHEJ deficiency might contribute to genomic instability and tumorigenesis, but, by itself, NHEJ deficiency is not sufficient to cause tumorigenesis in the colon.

Our results seem to contradict the generally accepted phenotype of Ku70$^{-/-}$ mice, which exhibit retarded growth, premature senescence and aging. Ku70$^{-/-}$ mouse embryonic fibroblasts also senesced faster than did those of the littermate controls.[47] Although we observed accumulated DNA damage (Figure 2i) and senescence in some segments of the colonic mucosa of Ku70$^{-/-}$PP mice, we also observed higher cell proliferation and tumorigenesis than in the control samples. We believe many factors contribute to this proliferation. The leaky SCID phenotype in the Ku70-deficient mice led to an imbalance of the immune system, which resulted in inflammation, and provided a favorable condition for cell proliferation. One possible explanation of phenotypic differences between Lig4$^{-/-}$PP

and Ku70$^{-/-}$PP is that Lig4$^{-/-}$PP mice are devoid of adaptive immune cells, whereas the Ku70$^{-/-}$PP mice have a small population of T cells. We cannot completely rule out this possibility. However, we argue that leaky T cells may lead to inflammation, but this cannot explain the hyperproliferative phenotype in the colon and in the pancreas, as we demonstrated in a recent report.[32] Whereas Lig4$^{-/-}$PP mice succumbed to severe diabetes owing to increased cellular senescence, the Ku70$^{-/-}$PP mice were diabetes free and showed a higher rate of proliferation of pancreatic β-cells with increased levels of β-catenin.[32] This is not driven by inflammation, because we did not observe inflammation in the pancreata of Ku70$^{-/-}$PP mice. Second, we also observed higher levels of β-catenin (some in the nucleus) in the mouse embryonic fibroblasts of Ku70$^{-/-}$ mice.[32] These cells proliferated quickly in the early passages but underwent senescence in later passages, as DNA damage accumulated. Therefore, we propose that an intrinsic function of Ku70 is to help prevent abnormal cell proliferation in certain tissues. We hypothesize this function of Ku70 is linked to destabilizing β-catenin and preventing the activation of downstream target genes, which are associated with cell proliferation.

We suggest DNA Lig4 is a ‘pure’ NHEJ protein. Lig4 deficiency leads to the accumulation of DNA damage, p53 activation and apoptosis. In the p53^{R172P} background, DNA damage activates p21 and drives cells into senescence. This prevents oncogenesis by limiting genomic instability. Ku70$^{-/-}$ mice, in contrast, exhibit a different phenotype because Ku70 has additional functions. We propose that Ku70 has at least three roles in the homeostasis of the colon. First, Ku70 repairs spontaneous DNA damage in the epithelial cells, which prevents the impairment of barrier function caused by the accumulation of DNA damage. Second, Ku70 is important for building intact adaptive immunity (Supplementary Figure S2). The leaky SCID phenotype of the Ku70$^{-/-}$ mice[25] may lead to an imbalance of adaptive immunity, contributing substantially to chronic inflammation in the gut. Third, Ku70 also has a novel role in limiting cell proliferation in the presence of DNA damage, and thus Ku70 deficiency may result in abnormal cell proliferation. We further hypothesize that this function of Ku70 is linked to destabilizing β-catenin. Aberrant activation of the β-catenin/Wnt signaling pathway results in increased expression of cyclin D1 and c-Myc, which we observed in the colons of Ku70$^{-/-}$PP mice (Figure 5).

In addition, the cell-cycle inhibitor p21 may be a key factor in regulating epithelial cell proliferation. The expression level of p21 is critical because it differentiates the hypoproliferative phenotype of Lig4$^{-/-}$PP mice from the hyperproliferative phenotype of Ku70$^{-/-}$PP mice. The aberrant activation of the Wnt signaling pathway may also be linked to the downregulation of p21.[48] Reports have suggested that the downregulation of p21 promotes the characteristic serrated aspect of dysplastic colons that results from uncontrolled cell replication, leading to enfolding of the epithelium.[35] Therefore, the phenotype that we observed in the Ku70$^{-/-}$PP mice correlates well with their reduced levels of p21, which, we propose, results from the aberrant activation of the Wnt signaling pathway.

In summary, our results indicate that Ku70 is essential for homeostasis in the colon. Ku70 not only helps repair damaged DNA in the epithelial layer to maintain its critical role as a barrier, but also has an intrinsic role, independent of NHEJ, in inhibiting the proliferation of

cells with damaged DNA. We report here a murine model in which mice spontaneously developed colonic inflammation and tumorigenesis with the dysregulation of the β-catenin/Wnt pathway.

MATERIALS AND METHODS

Mouse colony and maintenance

Ku70$^{-/-}$, PP and Ku70$^{-/-}$PP and wild-type mice were maintained in a specific pathogen-free barrier facility. All experiments were approved by the Institutional Animal Care and Use Committee at The University of Texas MD Anderson Cancer Center.

Gut histopathological analysis, immunohistochemistry and immunofluorescence

We removed colon samples, and washed and immersed them in optimal cutting temperature medium (Thermo Scientific, West Palm Beach, FL, USA) or in Bouin's fixative (Sigma-Aldrich, St Louis, MO, USA). We examined hematoxylin and eosin-stained sections under a light microscope (Olympus BX41, Center Valley, PA, USA). Antibodies for immunohistochemical or immunofluorescence analyses were as follows: anti-β-catenin and cyclin D1 (Cell Signaling, Danvers, MA, USA); γ-H2AX and adenomatous polyposis coli (Abcam, Cambridge, MA, USA); PCNA (Dako, Carpinteria, CA, USA); p21 (BD Biosciences, San Jose, CA, USA); IL-6, TNF-α and BrdU (Abd Serotec, Raleigh, NC, USA); and c-Myc (Santa Cruz Biotechnology, Santa Cruz, CA, USA). We used horseradish peroxidase-labeled antibodies and diaminobenzidine-chromogen substrate (Vector Scientific, Golden, CO, USA) for immunohistochemical detection. For immunofluorescence, we used fluorescently labeled (FITC or Alexa Fluor 594) antibodies (Molecular Probes, Eugene, OR, USA).

Isolation of immune cells from the colon

We isolated intraepithelial and lamina propria lymphocytes according to a published protocol.[49] Colons from three to four Ku70$^{-/-}$PP mice or their littermates were pooled. Intraepithelial and lamina propria lymphocytes were separated by Percoll gradient centrifugation. The resulting cells were counted and analyzed by flow cytometry.

In vivo BrdU incorporation assay and analysis of PCNA-positive cells

We injected mice intraperitoneally with BrdU (100 mg/kg, Sigma-Aldrich), euthanized them after 4 h and analyzed them by using rat anti-BrdU antibody (Abd Serotec) and anti-rat Alexa Fluor 594-labeled antibody (Molecular probes).

For PCNA, we obtained the average number of PCNA-positive cells by scoring crypts from six different regions of the colon with an average of 16 crypts per region, totaling 96 crypts per mouse. We analyzed Ku70$^{-/-}$PP and Ku70$^{+/-}$PP mice ($n = 3$) at the ages of 1, 2, 3 and 5 months. The results are presented as the mean ± the s.e. of the mean. Differences were determined by one-way analysis of variance, followed by Dunn's multiple comparison test ($P<0.001$).

Cellular senescence staining

We used a senescence-associated beta-galactosidase senescence detection kit (Cell Signaling) and followed the manufacturer's instructions. After staining, the tissues were embedded in paraffin, and the sections were counterstained using hematoxylin and eosin.

Flow cytometry

We stained lymphocytes with anti-CD4, CD8, CD3 and FoxP3, and B cells with anti-B220, CD43 and IgM (BD Biosciences), and analyzed them with an LSRII flow cytometer (BD Biosciences).

Western blot analyses

We extracted proteins from colonic epithelial cells according to the protocol described in literature.[50] We used the following antibodies: anti-β-catenin, anti-p21, anti-cyclin D1 and anti-c-Myc (Cell Signaling); anti-α-tubulin (Abcam); and anti-p84 (GeneTex, Irvine, CA, USA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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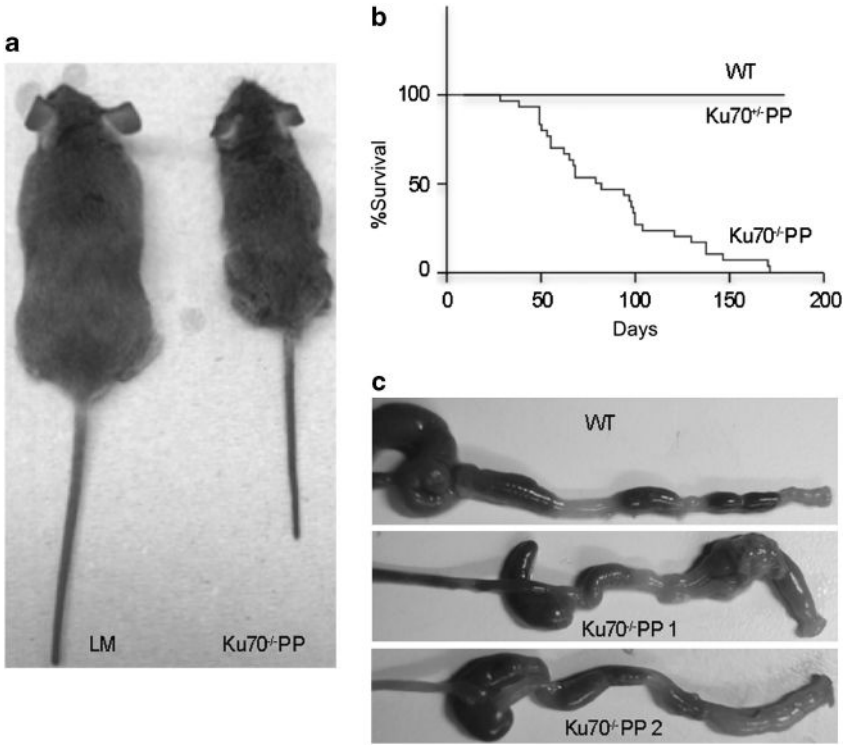

Figure 1.

Ku70$^{-/-}$PP mice died early, exhibited poor growth and had thicker colons. (**a**) Ku70$^{-/-}$PP mice showed poor growth and were smaller than the Ku70$^{+/-}$PP mice. The picture shows a pair of 43-day-old littermates. (**b**) Moribund mice were euthanized for analysis and annotated on the survival curve. Moribund condition was noticed as early as at 30 days of age. (**c**) Samples from two 45-day-old Ku70$^{-/-}$PP mice showed thicker colon areas spanning from the proximal to the distal portion. The top panel shows the colon of a 43-day-old wild-type mouse.

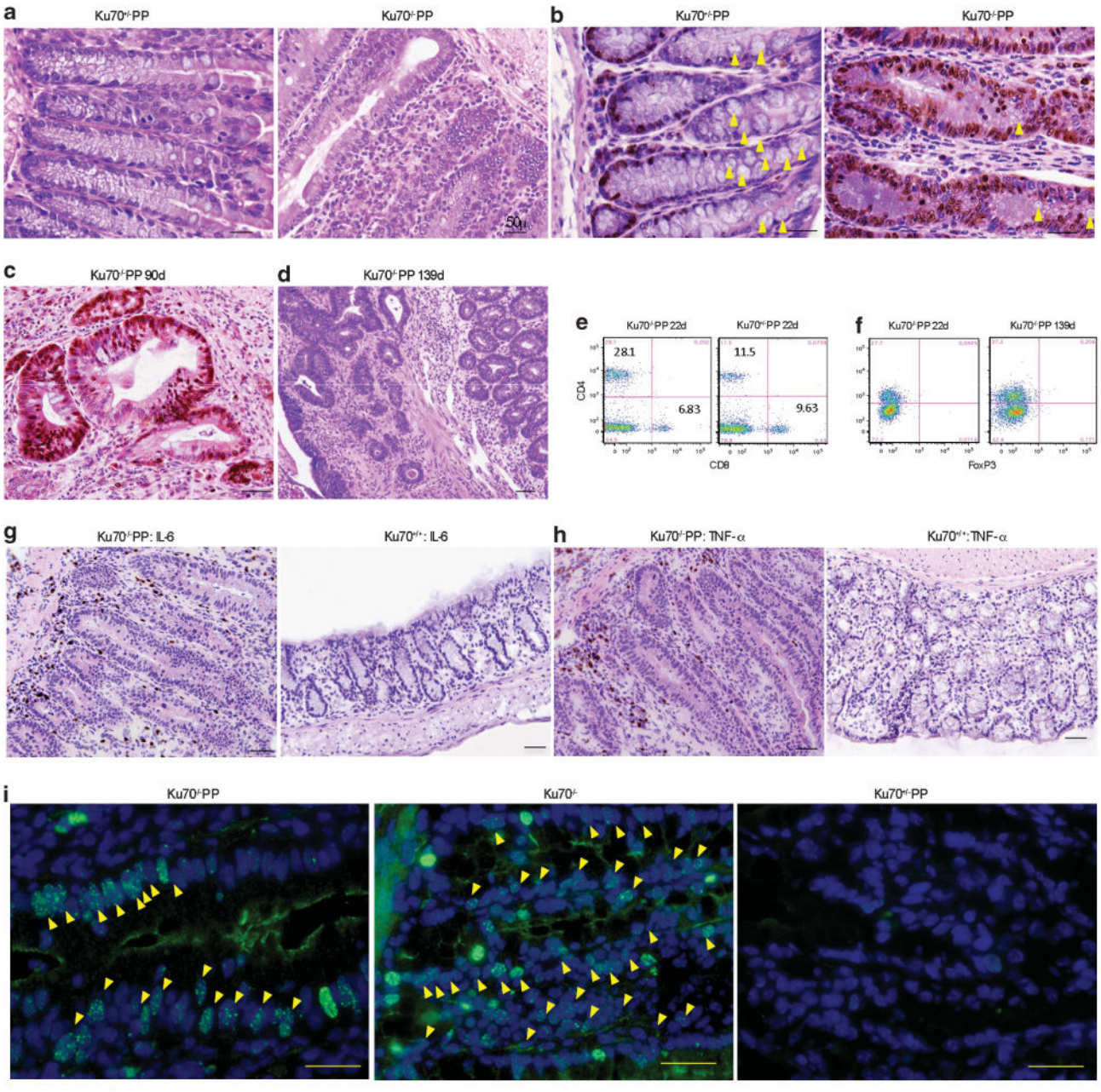

Figure 2.

$Ku70^{-/-}$PP mice developed spontaneous inflammation in the colonic mucosa, dysplasia and CRC. (**a**) Samples of the colonic mucosa from a 55-day-old $Ku70^{-/-}$PP mouse showed infiltrating inflammatory cells and hyperplasia, not present in the $Ku70^{+/-}$PP littermate (×400; ×40 objective and ×10 ocular). (**b**) Loss of goblet cells, indicated by yellow arrows, and low-grade dysplasia were observed in the colonic mucosa of a 30-day-old $Ku70^{-/-}$PP mouse, but not in the $Ku^{+/-}$PP littermate (×600). (**c**) High-grade dysplasia and the serrated crypt phenotype were observed in the colonic mucosa of $Ku70^{-/-}$PP mice. The brown color shows PCNA staining (×400). (**d**) Invasive CRC with severe inflammation in a 139-day-old mouse (×200). (**e**) Significant expansion of $CD4^{+}$ T cells in the intestinal epithelial

lymphocytes, as compared with the $Ku^{+/-}$PP controls. (**f**) The absence of regulatory T cells in the colonic lamina propria of a $Ku70^{-/-}$PP mouse without colonic inflammation at 22 days of age and with inflammation at 139 days of age. (**g**) Clear infiltration of IL-6-producing cells (**g**) and TNF-α-producing cells (**h**) in $Ku70^{-/-}$PP mice with colonic inflammation (brown cells) at 116 days of age as compared with the $Ku^{+/-}$PP controls (×200). (**i**) Increased staining of DNA damage marker γ-H2AX in the nuclei of epithelial cells from 44-day-old $Ku70^{-/-}$PP and $Ku70^{-/-}$ mice, but not in the $Ku^{+/-}$PP controls (×600). Scale bar: 50 μm.

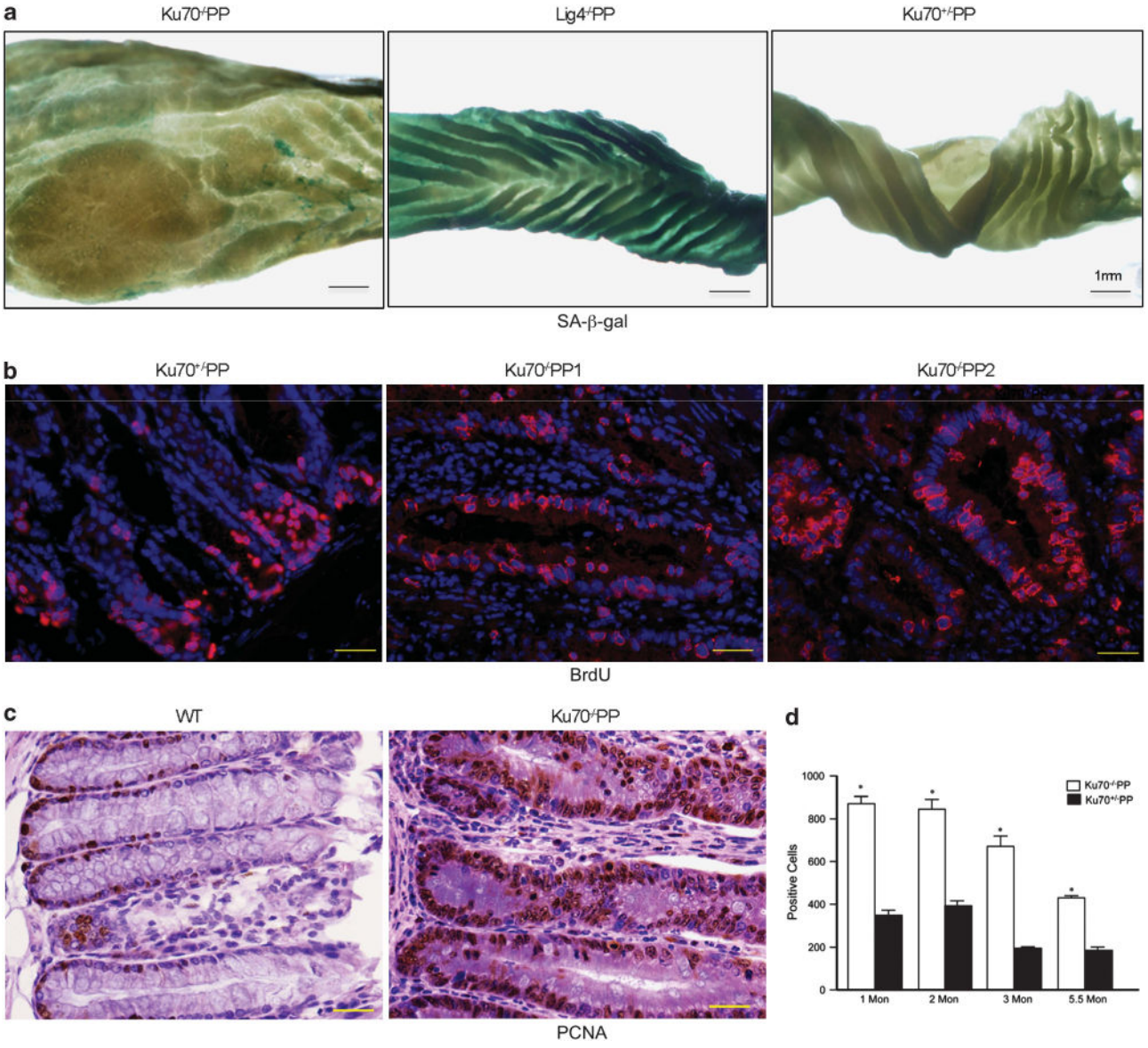

Figure 3.

Colons from Ku70$^{-/-}$PP mice exhibited a low level of senescence and a higher rate of proliferation than did controls. (**a**) Colons from Ku70$^{-/-}$PP, Lig4$^{-/-}$PP and Ku$^{+/-}$PP mice were stained with senescence-associated beta-galactosidase. (**b**) In vivo BrdU incorporation in the colon sections from Ku70$^{-/-}$PP mice showed a higher proliferation rate than did littermate controls. (**c**) Representative pictures of PCNA staining of colonic sections from Ku70$^{-/-}$PP mice (30-day-old) and wild-type mice (×400). (**d**) Colonic epithelial cells from Ku70$^{-/-}$PP mice showed a statistically higher rate of proliferation, assessed by PCNA staining, than did their littermates (*$P < 0.001$). Scale bar: 50 μm (**a**: 1 mm).

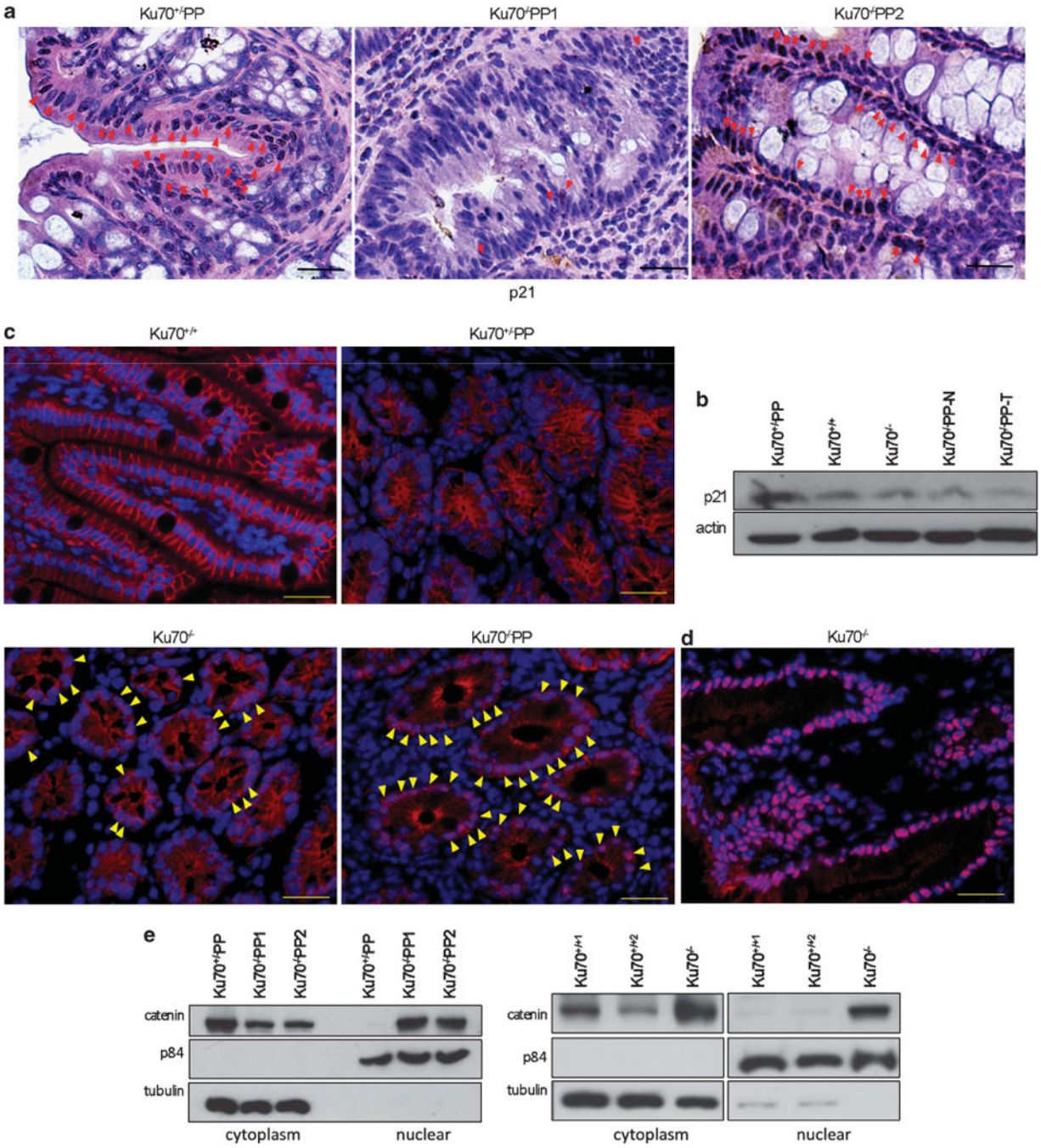

Figure 4.

Transformed Ku70$^{-/-}$PP colonic epithelial cells showed lower levels of p21 than did control samples, and aberrant localization of β-catenin was detected in both Ku70$^{-/-}$ and Ku70$^{-/-}$PP colonic epithelial cells. (**a**) Changes in the colonic mucosa of Ku70$^{-/-}$PP mice included decreased levels of p21 in the epithelium with dysplasia as compared with normal, nearby tissue (large goblet cells, right) and tissue from wild-type, age-matched controls (×400X). (**b**) Western blot detection of p21 from colonic epithelium of Ku70$^{+/-}$PP littermate, wild-type, Ku70$^{-/-}$ and Ku70$^{-/-}$PP mice (N: sample from normal nearby tissue; and T: sample

from enlarged dysplastic colon). Samples from Ku70$^{-/-}$PP dysplastic colon (Ku70$^{+/-}$PP-T) showed lower level of p21 than normal adjacent colon and controls. (**c**) Stabilization and aberrant nuclear accumulation of β-catenin was observed in the nuclei of colonic epithelial cells from 114-day-old Ku70$^{-/-}$PP mice and 80-day-old Ku70$^{-/-}$ mice as compared with Ku$^{-/-}$PP littermates (×400). (**d**) An extreme case of a 135-day-old Ku70$^{-/-}$ mouse showed almost exclusive nuclear localization of β-catenin. (**e**) Western blotting was performed on the cytoplasmic and nuclear lysates from colonic epithelial cells. Cytoplasmic and high-nuclear β-catenin was present in Ku70$^{-/-}$PP (left panel) and Ku70$^{-/-}$ colonic epithelium samples (right panel) as opposed to the cytoplasmic localization only observed in the wild-type or littermate control samples. Nuclear samples from the wild-type colons had minor contaminations of cytoplasmic proteins, as demonstrated by the faint bands of α-tubulin.

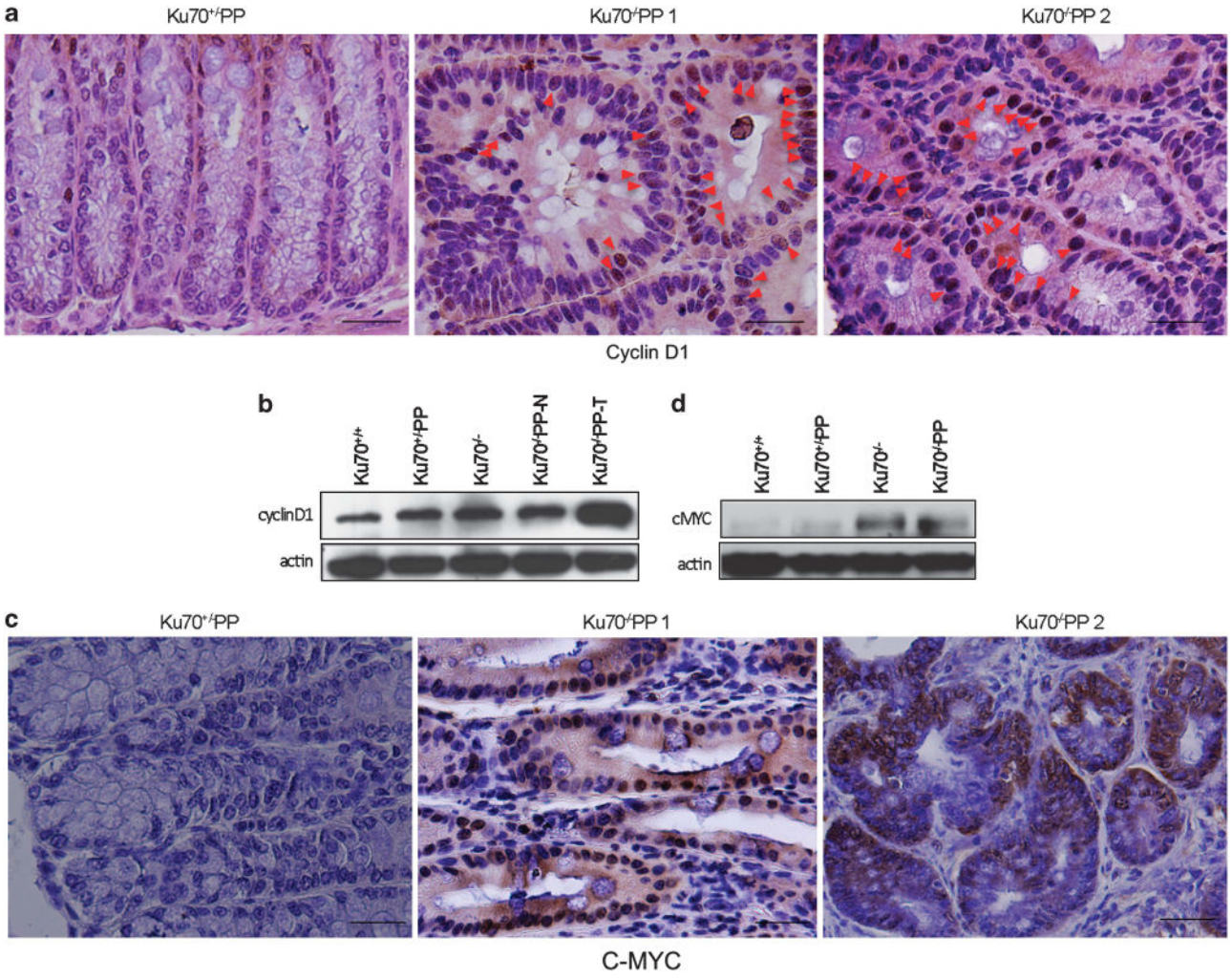

Figure 5.

Ku70$^{-/-}$PP colonic epithelial cells showed higher expression of cyclin D1 and c-Myc than did control samples. (**a**) An increased level of cyclin D1 was also observed in the nuclei of colonic epithelial cells from 79-day-old Ku70$^{-/-}$PP mice, predominantly in areas with visibly serrated dysplastic crypts (×400). (**b**) Western blotting for cyclin D1 detection was performed on purified colonic epithelial cells from Ku70$^{-/-}$, wild-type, Ku70$^{-/-}$PP and littermates. Increased concentration of cyclin D1 is observed in dysplastic Ku70$^{-/-}$PP colon as compared with normal adjacent and control colon samples. (**c**) Higher levels of nuclear localization of c-Myc were observed in the colon of a 79-day-old Ku70$^{-/-}$PP mouse (center panel); dysplastic colon crypts also showed strong staining in the colon of a 91-day-old Ku70$^{-/-}$PP mouse (right panel) as compared with its Ku$^{+/-}$PP littermate (far left panel) (×400). Scale bar: 50 μm. (**d**) Western blotting for c-Myc detection was performed on purified colonic epithelial cells from Ku70$^{-/-}$, wild-type, Ku70$^{-/-}$PP and littermates.

Table 1

Length and thickness measurements of colons taken from Ku70$^{+/-}$PP and Ku70$^{-/-}$PP mice aged 30, 60 and 90 days

	30-day-old		60-day-old		90-day-old	
	Length	**Thickness**	**Length**	**Thickness**	**Length**	**Thickness**
Ku70$^{+/-}$PP	82.7±1.1	2.5±0.1	82.2±0.5	2.5±0.1	95.7±0.1	2.0±0.1
Ku70$^{-/-}$PP	59.1±0.5	3.4±0.1*	67.5±0.5	3.1±0.13*	69.8±4.9	2.64±0.4*

Three mice were analyzed for each age group. Unit = mm;

*statistically significant $P < 0.05$ by two-tailed t-test.