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CAMK2 γ in Intestinal Epithelial Cells Modulates Colitis-Associated Colorectal Carcinogenesis via Enhancing STAT3 Activation

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Abstract

Inflammation is one of the major risk factors for cancer. Here, we show that calcium/calmodulin-dependent protein kinase II gamma (CAMK2 γ) in intestinal epithelial cells (IECs) modulates inflammatory signals and promotes colitis-associated cancer (CAC) in mice. We have identified CAMK2 γ as a downstream target of colitis-induced WNT5a signaling. Furthermore, we have shown that CAMK2 γ protects against intestine tissue injury by increasing IEC survival and proliferation. CAMK2 γ knockout mice displayed reduced CAC. Furthermore, we used bone marrow transplantation to reveal that CAMK2 γ in IECs, but not immune cells, was crucial for its effect on CAC. Consistently, transgenic over-expression of CAMK2 γ in IECs accelerated CAC development. Mechanistically, CAMK2 γ in IECs enhanced epithelial STAT3 activation to promote survival and proliferation of colonic epithelial cells during CAC development. These results thus identify a new molecular mechanism mediated by CAMK2 γ in IECs during CAC development, thereby providing a potential new therapeutic target for CAC.

Keywords

Colitis-associated colorectal cancer; inflammation; CAMK2 γ ; STAT3; WNT5A

More than 20% of worldwide cancer cases are associated with chronic inflammation^{1, 2}. Chronic inflammation is now well recognized as an important risk factor for gastric cancer, lymphoma^{3, 4}, hepatocellular carcinoma (HCC)⁵, pancreatic cancer, esophageal cancer, cholangiocarcinoma and colorectal cancer (CRC)⁶. CRC is the third most common cancer type and the second leading cause of cancer deaths among all cancers in the United States⁷. Inflammatory bowel disease (IBD), which results from inflammatory disorders of the intestinal tract, shows a strong positive correlation with the occurrence of CRC⁸. Patients with various types of IBD, including ulcerative colitis (UC), display 3- to 7-fold greater CRC risk than healthy people⁹⁻¹³. Colitis-associated CRC (CAC) is the most severe consequence of IBD^{14, 15}. Patients with CAC are also frequently diagnosed at advanced stages, and have worse prognoses than other CRC patients¹⁵⁻¹⁸.

The immune cells in the intestine are key players to maintain tissue homeostasis^{14, 19}. However, defective innate immune responses and dysfunction of the intestinal epithelial cell (IEC) barrier can lead to IBD and enhance the intestinal susceptibility to bacterial invasion. The inflamed environment also promotes mutation accumulation, which can cause tumor development^{14, 19}. Recent studies show that IBD generates an inflammatory environment with extensive tissue regeneration signals, accompanied by activation of pro-oncogenic factors, such as STAT3 and NF- κ B, in pre-malignant cells. This inflammatory environment, in turn, suppresses cell death and promotes hyperplasia and malignancy of inflamed tissues^{14, 19}. Despite the recent progress in delineating the molecular mechanisms of CAC, the relative lack of knowledge in its pathogenesis still impedes the development of effective treatments for CAC and other inflammation-related cancers.

CAMK2 γ is a serine and threonine kinase that belongs to the calcium/calmodulin-dependent kinase II family²⁰. CAMK2 γ was previously reported to promote inflammation²¹⁻²⁴. CAMK2 γ is critical for T cell maturation, as well as the activation of mature T cells in

peripheral lymphoid organs²¹. It has also been reported that CAMK2 γ is the only CAMK2 family member expressed in macrophages²³, and that CAMK2 γ is required for inflammatory responses induced by a variety of factors^{22, 24, 25}. Recently, we and others found that CAMK2 γ is important in both hematopoietic malignancies and solid tumors^{26–29}. Therefore, we ask whether CAMK2 γ is involved in CAC.

RESULTS

DSS treatment activates colonic CAMK2 γ via a non-canonical WNT signal

Dextran sodium sulfate (DSS) challenge in mice causes intestinal inflammation and injury that resembles human colitis^{30, 31}. We first asked whether intestinal CAMK2 γ can be activated by DSS challenge. Wild-type (WT) mice received 3% DSS orally for five days to induce acute colitis^{31, 32}, followed by recovery for two days (Fig. 1A). We found that induction of colitis by DSS strongly increased the activity of CAMK2 γ in colon tissues, as measured by phosphorylation of CAMK2 γ at Thr287, whereas the total colonic CAMK2 γ protein level was not changed (Fig. 1B).

We then asked which signals might activate CAMK2 γ in colitis. Previous studies have indicated that activation of the non-canonical WNT pathway increases intracellular calcium flux, which could activate CAMK2 γ ^{33, 34}. Furthermore, colitis and activation of non-canonical WNT signaling can antagonize the canonical WNT signal and suppress the activity of β -catenin³⁵. Consistent with this prior work, we observed a reduction in β -catenin target gene expression in mouse colonic tissues after DSS treatment (Supplementary Fig. 1A). A decrease in WNT/ β -catenin activity may imply a balance shift from the canonical WNT/ β -catenin pathway to the non-canonical WNT pathway. Based on the activated CAMK2 γ and decreased canonical WNT signal observed in inflamed mouse colonic tissue (Fig. 1B and Supplementary Fig. 1A), we therefore asked whether CAMK2 γ was activated by non-canonical WNT ligand-induced signals in the DSS-induced mice. To address this question, we screened the expression of all 14 different WNT ligands in H₂O- and DSS-treated mouse colonic tissue samples. As expected, the expression levels of three canonical WNT ligands, *Wnt3*, *Wnt8b* and *Wnt9b*, were significantly decreased after DSS challenge, whereas the expression levels of two non-canonical WNT ligands, *Wnt5a* and *Wnt5b*, were robustly increased (Fig. 1C). Specifically, *Wnt5a* showed a nearly 10-fold induction. To test whether the changes observed in our mouse model are also present in human disease, we retrieved an ulcerative colitis (UC) patient microarray cohort dataset³⁶ from the NCBI Gene Expression Omnibus database. Analysis of the dataset indicated that *Wnt5A* was the most upregulated WNT ligand in UC patient samples (Fig. 1D).

To further confirm the activation of CAMK2 γ by WNT5A, we stably infected cell lines with retroviruses that permit doxycycline-inducible human WNT5A-HA-expression. Overexpression of WNT5A strongly increased CAMK2 γ phosphorylation (Fig. 1E). Overexpression of two known WNT5A receptors, FZD2 and FZD5, as well as the FZD co-receptor ROR2, also activated CAMK2 γ (Supplementary Fig. 1B). Moreover, treatment with the intracellular calcium chelator BAPTA/AM blunted the activation of CAMK2 γ by WNT5A in 293A cells (Fig. 1F). These results suggested that the colitis-induced WNT5A

signal is transduced through a non-canonical WNT pathway to activate CAMK2 γ in the colon.

CAMK2 γ deficiency increases DSS-induced colonic epithelial cell death and tissue injury

DSS disrupts the colonic epithelia by breaking down the integrity of the epithelial barrier and promoting the invasion of intestinal microbiota³⁷. The invaded microbiota activate innate immune cells and cause excessive inflammation, cell death and tissue damage, which leads to severe clinical symptoms of colitis, including body weight loss, colonic shortening, and rectal bleeding^{37, 38}. Because CAMK2 γ is activated in the intestine of DSS-treated mice, we next used CAMK2 γ knockout mice (KO) to determine the role of CAMK2 γ in DSS-induced colitis (Fig. 2A). We treated the mice as described in Fig. 1a and compared KO mice with their WT littermates. Surprisingly, quantitative real-time PCR (qRT-PCR) revealed that DSS treatment induced similar inflammatory cytokine and enzyme expression, including TNF α , IFN- γ , COX2 and IL-17A in colonic tissues from both WT and CAMK2 γ KO mice (Fig. 2A and 2B, Supplementary Fig. 2). Although there were trends toward increased expression of IL-6 and IL-1 β in the KO mice, the difference is not statistically significant. (Fig. 2B). The colitis-induced intestinal injury, however, was more severe in the CAMK2 γ KO mice than the WT controls (Fig. 2C–2G). At day 7 of the treatment regimen, the CAMK2 γ KO mice showed shorter colon lengths and higher rectal bleeding rates than the WT mice (Fig. 2C). Hematoxylin and eosin (H&E) staining of the colonic tissue showed that the KO mice had more inflammatory cell infiltration, ulceration and hyperplasia, as indicated by the epithelial damage score and infiltration score (Fig. 2D and 2E). CAMK2 γ KO mice also displayed decreased proliferation of colonic epithelial cells during the regeneration stage of colitis (Fig. 2F). CAMK2 γ KO mice not only had fewer proliferating crypts in the inflamed colonic tissue but also showed fewer BrdU-positive proliferating cells per crypt (Fig. 2D and 2F). TUNEL staining also indicated that there were more apoptotic epithelial cells after DSS challenge in the CAMK2 γ KO mice than the WT mice (Fig. 2D and 2E). Consistently, the CAMK2 γ KO mice showed significantly greater weight loss than the WT mice (Fig. 2G). Because enhanced epithelial damage could further lead to increased animal mortality, we then challenged the CAMK2 γ KO mice and their WT littermates with a longer DSS treatment (7 days) (Fig. 2H). In this model, 50% of the CAMK2 γ KO animals were dead by 10 days after the start of DSS treatment, whereas all of the WT animals survived (Fig. 2I). Taken together, these results indicate that CAMK2 γ protects mice from colitis-induced injury by increasing the survival and proliferation of colonic epithelial cells.

CAC is reduced by CAMK2 γ gene deletion in mice

Based on the above results, next we asked whether CAMK2 γ might promote colitis-associated tumorigenesis. Because combined delivery of DSS and azoxymethane (AOM) has been widely used as a model for CAC³⁹, we thus used this model to investigate the role of CAMK2 γ in inflammation-related tumorigenesis⁴⁰. WT and CAMK2 γ KO mice were injected with AOM once, followed by three rounds of oral administration of 3% DSS in water (Fig. 3A)⁴⁰.

At the end of the treatment, all of the mice developed CAC regardless of their genotypes, and tumors were mostly found in the distal colon, as previously described⁴⁰. Notably, the

CAMK2 γ KO mice developed much fewer tumors and the tumors were smaller than those in the WT mice (Fig. 3B and 3C). We further classified the tumors into three groups according to the tumor diameters. The CAMK2 γ KO mice not only showed lower maximum tumor sizes but also had fewer tumors in each of the tumor size categories (Fig. 3B and 3C). Moreover, histological analysis revealed a decreased high-grade tumor proportion in the CAMK2 γ KO mice (Fig. 3D, 3E and Supplementary Fig. 3). These results clearly demonstrated that CAMK2 γ KO mice had reduced CAC development in the AOM/DSS model.

CAMK2 γ KO in colonic tissue-resident cells, but not in immune cells, protects mice against CAC

CAMK2 γ is ubiquitously expressed in bone marrow-derived immune cells, as well as colonic tissue-resident cells, and both cell types are known to participate in CAC development^{14, 21, 22, 41, 42}. We first asked whether CAMK2 γ KO suppressed CAC development through directly regulating the function of inflammatory cells. To answer this question, we isolated bone marrow cells from both WT and CAMK2 γ KO CD45.2 donor mice, and then transplanted them into irradiated CD45.1 WT recipient mice (Fig. 4A). The bone marrow reconstitution efficiency was verified by flow cytometry analyses using antibodies targeting the mouse CD45.1 and CD45.2 antigens, as well as by transgenic allele-specific PCR on colonic tissue samples from the chimeric mice (Supplementary Fig. 4A). These two groups of chimeric mice were then subjected to CAC induction, as illustrated in Fig. 4B. Unexpectedly, we observed no difference in either the average tumor number or tumor size between these two groups of chimeric mice (Fig. 4C). Thus, specific deletion of CAMK2 γ in inflammatory cells failed to suppress the development of CAC.

We then asked whether CAMK2 γ KO in non-immune cells was responsible for the suppression of CAC. We generated two different groups of bone marrow chimeric mice by transplanting bone marrow cells from CD45.1 WT mice into CD45.2 WT and CD45.2 CAMK2 γ KO mice (Fig. 4D). The chimeric mice achieved more than 96% immune system reconstitution rates and showed expression of CAMK2 γ in peripheral immune cells before the mice were challenged with AOM/DSS, as illustrated in Fig. 4B (Supplementary Fig. 4B). As a result, the WT \rightarrow KO chimeric mice showed decreases in both the tumor numbers and tumor sizes (Fig. 4E), similar to the phenotype of the whole-body CAMK2 γ KO mice. Taken together, these results indicate that deletion of CAMK2 γ in tissue-resident cells, but not in bone marrow-derived immune cells, suppresses CAC development.

Constitutive CAMK2 γ over-expression in intestinal epithelial cells (IECs) protects mice from colitis-induced injury and promotes CAC development

Colonic epithelial cells are the major resident cells in the colonic tissue, and also the source of CRC cells⁴³. To further confirm the role of CAMK2 γ in tissue-resident cells during CAC development, we generated a new mouse line that conditionally over-expresses CAMK2 γ in the colonic epithelium. We inserted a conditional CAMK2 γ over-expression cassette, with a LoxP-flanked STOP cassette, under the control of the CAG promoter in the ROSA26a locus. A T-to-D mutation at Thr287 (T287D) was introduced into the *Camk2 γ* coding sequence (CDS) to allow constitutive activation of CAMK2 γ (CA-CAMK2 γ fl/fl) (Fig. 5A)²¹. By

crossing Villin-Cre mice with the CA-CAMK2 γ fl/fl homozygous mice, we generated Villin-tg (TG) mice with specific CA-CAMK2 γ over-expression in colonic epithelial cells (Fig. 5B). The TG mice showed normal gastro-intestinal development compared with CA-CAMK2 γ fl/wt (FW) littermates and had no signs of spontaneous colorectal tumor development up to the age of 8 months.

We first examined the effects of the IEC-specific constitutive CAMK2 γ over-expression in protecting mice from colitis-induced injury. This allowed us to analyze the role of CAMK2 γ in precancerous colonic disease. The mice were treated as illustrated in Fig. 1A. Over-expression of CA-CAMK2 γ in colonic epithelial cells, as expected, suppressed colitis-associated body weight loss (Fig. 5C) and colonic shortening, and decreased the rectal bleeding rate (Supplementary Fig. 5A). Histological analyses also showed that the TG mice maintained better epithelial crypt morphology with a lower epithelial damage score (Supplementary Fig. 5B). In agreement with this observation, the TG mice showed higher proliferation rates and decreased cell deaths in colonic epithelial tissue (Fig. 5D and 5E). To further exclude a possible effect of CAMK2 γ in innate immune cells, we crossed CA-CAMK2 γ fl/fl mice with Lyz2-Cre mice to over-express CA-CAMK2 γ in myeloid cells (Supplementary Fig. 6A). In contrast to the FW control mice, the Lyz2-TG mice showed slightly greater DSS-induced body weight loss but similar colonic shortening and splenic enlargement to the FW control mice (Supplementary Fig. 6B and 6C). Therefore, we conclude that activation of CAMK2 γ in colonic epithelial cells, but not that in immune cells, protects mice from colitis-induced tissue damage.

We then subjected TG mice and FW control mice to CAC induction, as illustrated in Fig. 3A. As expected, TG mice showed increased distal CAC compared to the FW control mice (Fig. 5F). The average tumor number per mouse was higher in the TG mice than that in the FW control littermates (Fig. 5G). The TG mice also showed an increased number of small tumors (Fig. 5G) as well as an increased proportion of high-grade tumors (Fig. 5G, supplementary Fig. 5C). Altogether, these results indicate that constitutive activation of CAMK2 γ in colonic epithelial cells restores the colonic epithelial morphology change caused by DSS challenge, improves epithelial cell survival and proliferation, protects mice from colitis-induced epithelial damage and promotes CAC development, further demonstrating the role of epithelial CAMK2 γ in modulating CAC development.

CAMK2 γ promotes IEC survival and proliferation via enhancing STAT3 activation

DSS is a non-genotoxic carcinogen, which requires AOM co-injection to induce CAC⁴⁴. Instead, administration of DSS leads to damage of the epithelial barrier and translocation of the microbiota. Bacterial infiltration-induced inflammation then leads to loss of the epithelial structure and extensive cell death. Next, tissue regeneration is initiated in response to the inflammation-induced damage signals. During this regeneration process, prolonged regeneration signals, including IL-6/IL-11, will activate anti-apoptotic pathways to antagonize cell death and further promote the compensatory proliferation of epithelial cells, which is critical for the development of CAC⁴⁵. Survival and proliferation modulators, including p38, ERK1/2, NF- κ B and STAT3, are reported to play essential roles in this process⁴⁶⁻⁴⁹. Because we observed enhancement of DSS-induced cell death and suppression

of DSS-induced colonic epithelial proliferation in CAMK2 γ KO mice (Fig. 1 and 2), we hypothesized that CAMK2 γ KO might suppress cell survival signaling pathways. To test this hypothesis, we measured the activity of known survival modulators in DSS-challenged WT and CAMK2 γ KO colonic tissue samples by western blotting (Fig. 6A). We did not observe any differences in the activities of p38 or ERK1/2, at day 7 after the start of DSS treatment. The protein level of I κ B α , the negative regulator of NF- κ B, was lower in KO colonic tissue, indicating a slightly higher NF- κ B activity in KO colon tissues. In contrast, phosphorylation of STAT3, a key regulator of colonic epithelial cell survival, was significantly reduced in CAMK2 γ KO mouse colitis samples (Fig. 6A). QRT-PCR results also indicated that the expression of several STAT3 target genes, which are involved in cell survival and proliferation, was decreased in colon tissues of KO mice (Fig. 6B). Similarly, decreased STAT3 phosphorylation and suppression of STAT3 target gene expression were also observed in CAMK2 γ KO tumor tissues from mice treated as described in Fig. 2B (Fig. 6C–E). To further determine whether inhibition of CAMK2 γ suppresses STAT3 phosphorylation, we treated human colon cancer cell lines with the CAMK2 inhibitor KN93, or its inactive analog KN92, together with IL-6. Indeed, KN93 suppressed IL-6-induced STAT3 phosphorylation (Fig. 6F). Knockdown of CAMK2 γ expression by shRNA in CRC cell lines also inhibited IL-6-induced STAT3 phosphorylation (Fig. 6F, Supplementary Fig. 7). These results indicate that CAMK2 γ KO suppresses the survival and proliferation of colonic epithelial cells, possibly through inhibiting IL-6-induced STAT3 activation.

To confirm the requirement for STAT3 in mediating the effects of CAMK2 γ *in vivo*, we injected the STAT3 inhibitor VII (LLL12) into both FW control and TG mice during DSS challenge (Fig. 7A). As expected, LLL12 suppressed STAT3 phosphorylation but did not affect the phosphorylation of CAMK2 γ or other signaling pathways (Fig. 7B). Treatment with LLL12 enhanced weight loss, colon length shortening in both FW and TG mice (Fig. 7C). However, although CA-CAMK2 γ protected mice treated with DSS and vehicle injection, there was no CA-CAMK2 γ -induced protection after LLL12 administration (Fig. 7C). H&E, Ki67 and TUNEL staining also indicated that LLL12-treated TG mice showed similar levels of epithelial damage, immune cell infiltration, epithelial cell death, and proliferation compared to FW mice that received LLL12 injection (Fig. 7D and 7E). These results further demonstrate that CAMK2 γ promotes colonic epithelial cell survival and proliferation *via* enhancing STAT3 activation.

DISCUSSION

In this study, we have identified a novel molecular pathway in a mouse model of inflammation-associated carcinogenesis. In this model, we show that the WNT signal induced by colitis activates CAMK2 γ in the IECs to enhance STAT3 activation, thereby promoting CAC. Analyses of the UC patient cohort indicated a positive correlation of the expression levels between WNT5A and a set of STAT3 target genes (Supplementary Fig. 8). Consistently, we showed that CAMK2 γ KO suppressed the development of CAC in mice. Interestingly, CAMK2 γ in colonic epithelial cells, rather than in inflammatory cells, was critical for the development of CAC, while over-expression of CAMK2 γ in IECs, but not in myeloid cells, ameliorated colitis-induced tissue injury and promoted tissue regeneration, resulting in enhanced CAC development. Although previous studies have established a role

for CAMK2 γ in immune cells to promote inflammation^{24, 25}, we observed no difference in overall inflammation between WT and CAMK2 γ KO mice. These results indicate an epithelial-specific role for CAMK2 γ in determining the outcome of colitis-induced CAC development by promoting IEC survival and proliferation. However, we could not exclude the possibility that CAMK2 γ might directly modulate immune responses in other types of cancers.

These results support the importance of non-canonical WNT signaling in colitis and CAC. In response to colitis, epithelial CAMK2 γ was activated by a non-canonical WNT5A signal. This result is consistent with several recent studies showing that WNT5A is one of the WNT ligands with the highest expression level in colonic myofibroblasts from IBD patients and mouse colitis models^{36, 50, 51}. Our results also uncovered an important role of non-canonical WNT/ CAMK2 γ signaling in CAC development. Moreover, a recent study of mutation landscape in human CAC tissues shows that mutations in non-canonical WNT associated pathways, such as the Rho and Rac GTPase network components, are observed in about 50% of CAC samples, but rarely observed in sporadic CRC samples⁵². This further supports a role for non-canonical WNT pathways in CAC development. Activation of WNT5A/ CAMK2 γ signaling could be one of the essential signal pathways that drives CAC development. This study indicates that in addition to the well-known canonical β -catenin oncogenic pathway, highly activated non-canonical WNT5A/CAMK2 γ signaling during colitis could also promote CAC development. Thus, maintenance of “WNT balance” is critical for colon cancer prevention and may also be important for other inflammation associated malignancies. Furthermore, up-regulation of non-canonical WNT signals, particularly WNT5A expression, is also observed in a variety of chronic inflammation diseases, including sarcoidosis and *Helicobacter pylori* infection, all of which are linked to increased cancer risk^{53, 54}. In this scenario, it is possible that WNT5A/ CAMK2 γ signaling also mediate other inflammation-associated malignancies.

Finally, we show that CAMK2 γ may promote CAC development via enhancing STAT3 activity in IECs. It has been well established that the survival and expansion of pre-malignant epithelial cells, which rely on inflammation-related tissue repair signaling pathways during colitis, such as STAT3-mediated signaling pathway, is key for the development of CAC^{14, 16, 47}. When such signaling pathways are blocked, the development of CAC will be suppressed. For instance, IEC-specific STAT3 KO mice have more severe tissue injury in colitis than wild type mice, which is due to suppression of cell survival and G2/M phase transitions⁴⁷. Similar to CAMK2 γ KO mice, STAT3 KO mice are highly resistant to CAC development. Furthermore, genetic ablation of cytokines that activate STAT3 also generates phenotypes similar to those of the IEC-specific STAT3 KO mice^{47, 55, 56}. In contrast, mice with constitutively activated STAT3 are resistant to colitis-induced tissue damage, therefore are more susceptible to CAC development^{47, 55, 56}. In this study, we show that CAMK2 γ KO mice attenuate the activation of epithelial STAT3, thereby suppressing CAC development. The effect of CAMK2 γ on STAT3 could possibly rely on a direct interaction between CAMK2 γ and STAT3, as described in leukemia²⁶, or through several other indirect mechanisms as previously reported^{57, 58}. Interestingly, several studies indicate that CAMKII family members could also interact or directly activate other members of IL-6/STAT3 pathway, including JAK2^{59, 60}, implying the possibility that CAMK2 γ may

also function upstream of STAT3. However, further work is still needed to identify the specific molecular link between CAMK2 γ and STAT3 activation, particularly in CAC. Although a major role for STAT3 in promoting cancer is now well established, transcription factors such as STAT3 are notoriously difficult to directly target with small-molecule drugs that disrupt protein-protein or protein-DNA interactions⁶¹. In addition to our new targeting approaches such as CpG-Stat3 siRNA, developing inhibitors targeting upstream activators of STAT3 pathway is considered as an alternative strategy for STAT3 inhibition^{62, 63}. To this end, CAMK2 γ could be a potential target for future drug development to treat CAC and other inflammation-associated malignancies.

METHODS

Animals

CAMK2 γ KO mice (C57BL/6J background) were kindly provided by Johannes Backs at the University of Heidelberg⁶⁴. Mice expressing a conditional (LoxP-flanked STOP cassette) constitutively active CAMK2 γ (CA-CAMK2 γ fl/fl) (B6/129 mixed background) were generated at the Animal Resources Center of City of Hope National Medical Center, as described in the Results section. CA-CAMK2 γ fl/fl mice were crossed with Villin-Cre +/- mice to generate Cre⁻ CA-CAMK2 γ fl/wt (FW) mice and Cre⁺ CA-CAMK2 γ fl/wt (TG) littermates. CD45.1, Villin-Cre and Lyz2-Cre mice were purchased from the Jackson Laboratory. All the mice were maintained in a pathogen-free animal facility under standard 12:12 h light:dark cycle, and were fed with standard rodent chow and water *ad libitum*. All procedures followed the NIH guidelines for the care and use of laboratory animals, and were approved by the City of Hope Institutional Animal Care and Use Committee.

Induction of CAC and acute colitis

For CAC, 7-week-old male mice received intraperitoneal (i.p.) injection of azoxymethane (AOM, 10 mg/kg) on day -7, and received 3% DSS (MPbio, Cat #: 0216011080) in drinking water on days 0–5, 21–26 and 42–47. Then, the mice were euthanized at the indicated time points. For acute colitis, the mice received 3% DSS challenge on days 0–5, and then were allowed to recover for another two days before samples were collected. 5-Bromo-2'-Deoxyuridine (BrdU, Sigma, Cat#: B5002, 10mg/kg body weight) was i.p. injected to the mice two hours before they were euthanized.

Generation of bone marrow chimeric mice

Chimeric mice were generated as described elsewhere⁶⁵. Briefly, bone marrow cells were isolated from CAMK2 γ KO and WT donor mice. Isolated bone marrow cells (1×10^6) were transplanted into irradiated (10Gy) recipient mice through tail vein injection. Recipient mice were allowed access to autoclaved water containing antibiotics (sulfamethoxazole and trimethoprim oral suspension, Hi-Tech) for three weeks. The mice were then returned to antibiotic-free water, to allow repopulation of intestinal microbiota. The bone marrow reconstitution efficiency was tested by measuring the CD45.1/CD45.2 ratios by flow cytometry five weeks after transplantation.

Histology staining and analysis

Colonic specimens were prepared and analyzed by pathologists at the City of Hope Pathology Core Lab. Histology and immunohistochemical staining procedures were described previously²⁹. The antibodies used were as follows: BrdU (DAKO, Cat#: M0744), Ki67 (DAKO, Cat#: M7204), and P-Stat3 Tyr705 (Cell Signaling Technology, Cat#: 9145). TUNEL staining was performed using the Cell Death Detection Kit from Roche (Cat#: 11684795910), according to the manufacturer's instructions. Standards for tumor grading, epithelial damage score and inflammatory cell infiltration score are described in Tables S1, S2 and S3⁶⁶.

RNA isolation and quantitative real-time PCR

Total RNAs were isolated using the RNeasy kit (QIAGEN, Cat#:74104). Reverse transcription was performed using the Invitrogen RT III system (Cat#: 18080051). The qRT-PCR was performed using the SYBR green and ABI 7500 system (Applied Biosystems). The primers used for qRT-PCR were generated using Invitrogen perfect primer design tool or qPrimerDepot⁶⁷. Primer sequences were summarized in Table S4. The commercial kits were used according to the manufacturer's instructions.

Western blotting and quantification

Western blotting was performed as previously described²⁹. The antibodies used were described in Table S5. Quantification of the western blots was performed using the ImageJ software package, as described²⁷.

LLL12 treatment

The STAT inhibitor LLL12 (Calbiochem, Cat#: 573131) was suspended in vehicle (5% DMSO, 0.9% saline) and then i.p. injected into DSS-treated mice with a dosage of 5 mg/kg on days 0, 2, 4, 6 and 7. Control mice only received vehicle injection.

Plasmid and virus infection

HCT116 and HT29 human CRC cell lines were purchased from ATCC, and cultured using the ATCC-recommended methods. Wnt5A-HA over-expression viruses were generated using the tetracycline-inducible virus system from Clontech. Generation of CAMK2 γ knockdown cell lines was performed as described previously²⁹.

Statistical Analysis

The data are presented as mean \pm SEM. Student's two-tailed t test or one-way analysis of variance (ANOVA) with Dunn's post-test were used to determine significant differences among groups ($p < 0.05$).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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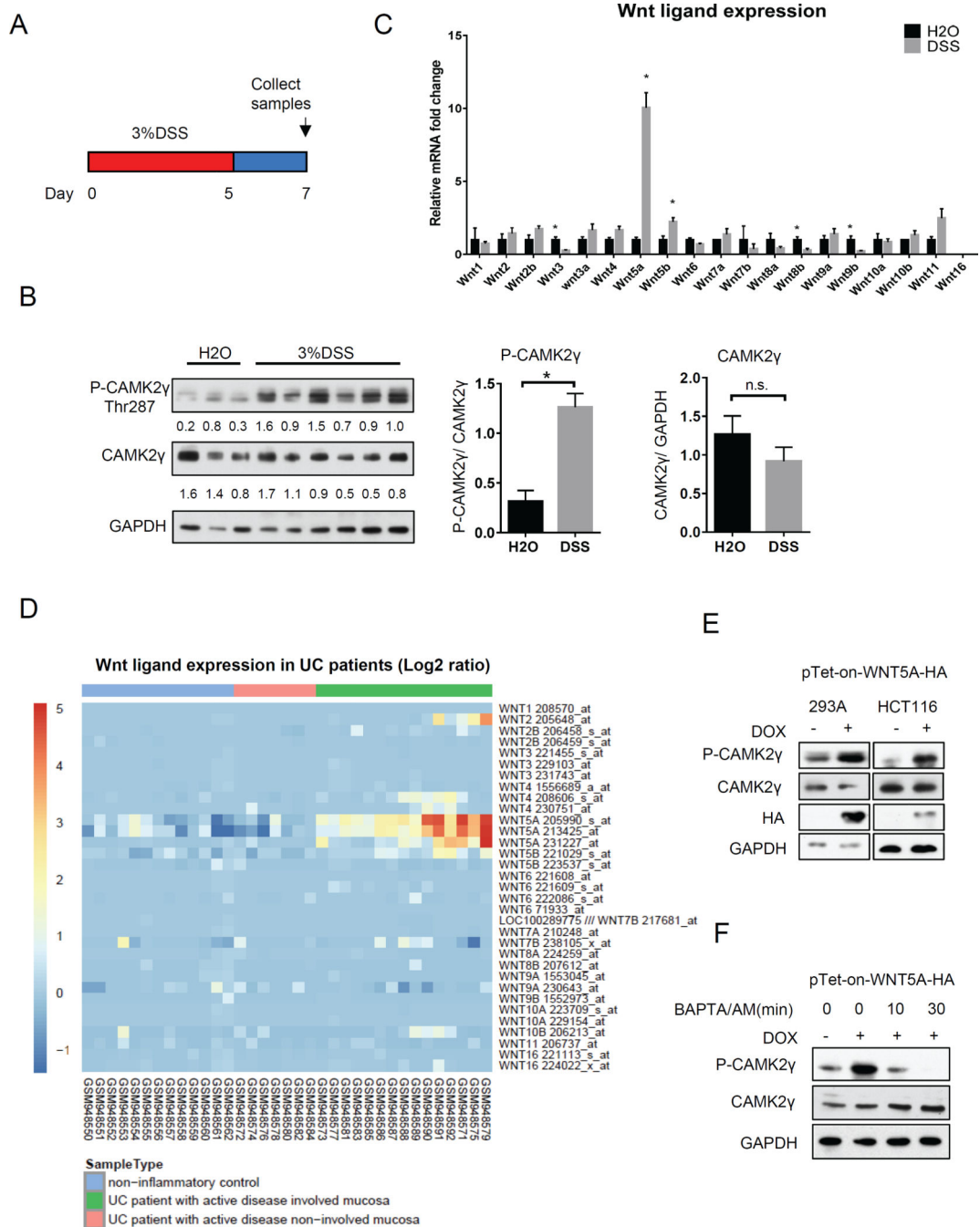


Figure 1. DSS induced colitis activates colonic CAMK2 γ via non-canonical WNT signaling
(A) Scheme for colitis induction. Red color indicates the duration of DSS treatment. **(B)** Protein expression and phosphorylation of CAMK2 γ in DSS-induced colitis. The relative expression levels normalized to GAPDH were shown under the blot. They were further quantified and shown as bar plots. **(C)** The relative mRNA expression profile of WNT ligands in the colonic tissue of H₂O- or DSS-treated mice. Expression levels were normalized to H₂O-treated mice. **(D)** Expression of WNT family ligands in a UC patient microarray cohort. **(E)** Effects of retroviral over-expression of WNT5A in cell lines on

CAMK2 γ phosphorylation. DOX = doxycycline. **(F)** Phosphorylation of CAMK2 γ by WNT5A in response to intracellular calcium chelator (BAPTA/AM) treatment. Results are presented as mean \pm s.e.m. of 3 independent experiments; *P<0.05. See also Supplementary Fig. 1.

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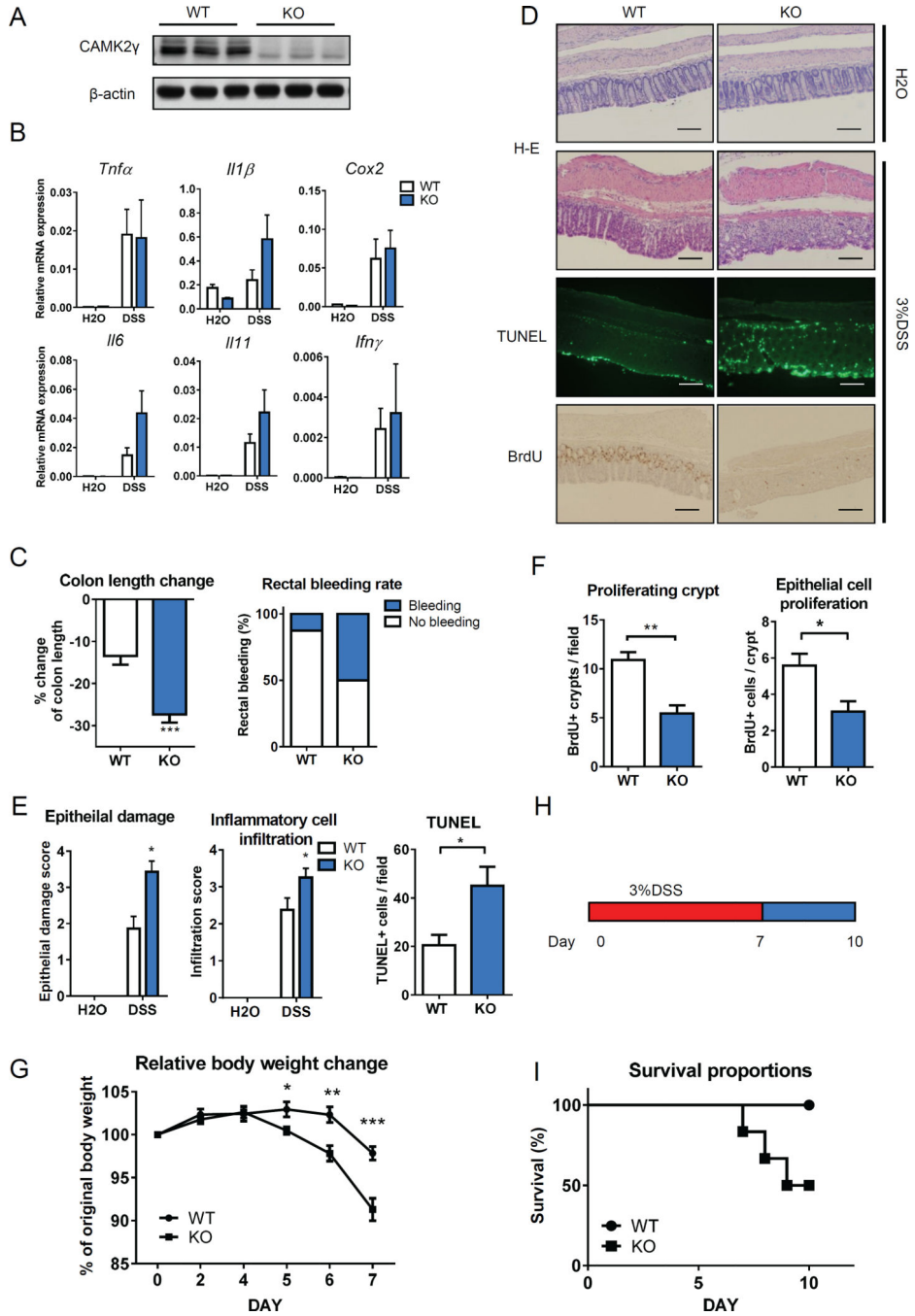


Figure 2. CAMK2 γ KO increases DSS-induced colonic epithelial cell death and exacerbates tissue injury

(A) Verification of CAMK2 γ deficiency in the colons of CAMK2 γ knockout (KO) mice by western blotting. (B) Relative mRNA levels of inflammatory cytokines and enzymes in the colons of WT and KO mice (WT=9, KO=9). (C) The change in colon length and rectal bleeding rate of DSS-treated mice (n=9). (D) Representative images for H&E, TUNEL and BrdU staining of mouse colon sections. Scale bars indicate 200 μ m length (E) Colonic epithelial damage, inflammatory cell infiltration, and cell death calculated from the staining

shown in (d). **(F)** Average proliferating crypt number per field quantified from BrdU staining of (D). **(G)** DSS-induced changes in mouse body weight. **(H–I)** Survival rate of mice (I) in a more severe acute colitis induction model (H) (WT=6, KO=6). Results are presented as mean \pm s.e.m. of 3 independent experiments, except where otherwise noted. *P<0.05, **P<0.01, ***P<0.001. See also Supplementary Figure 2.

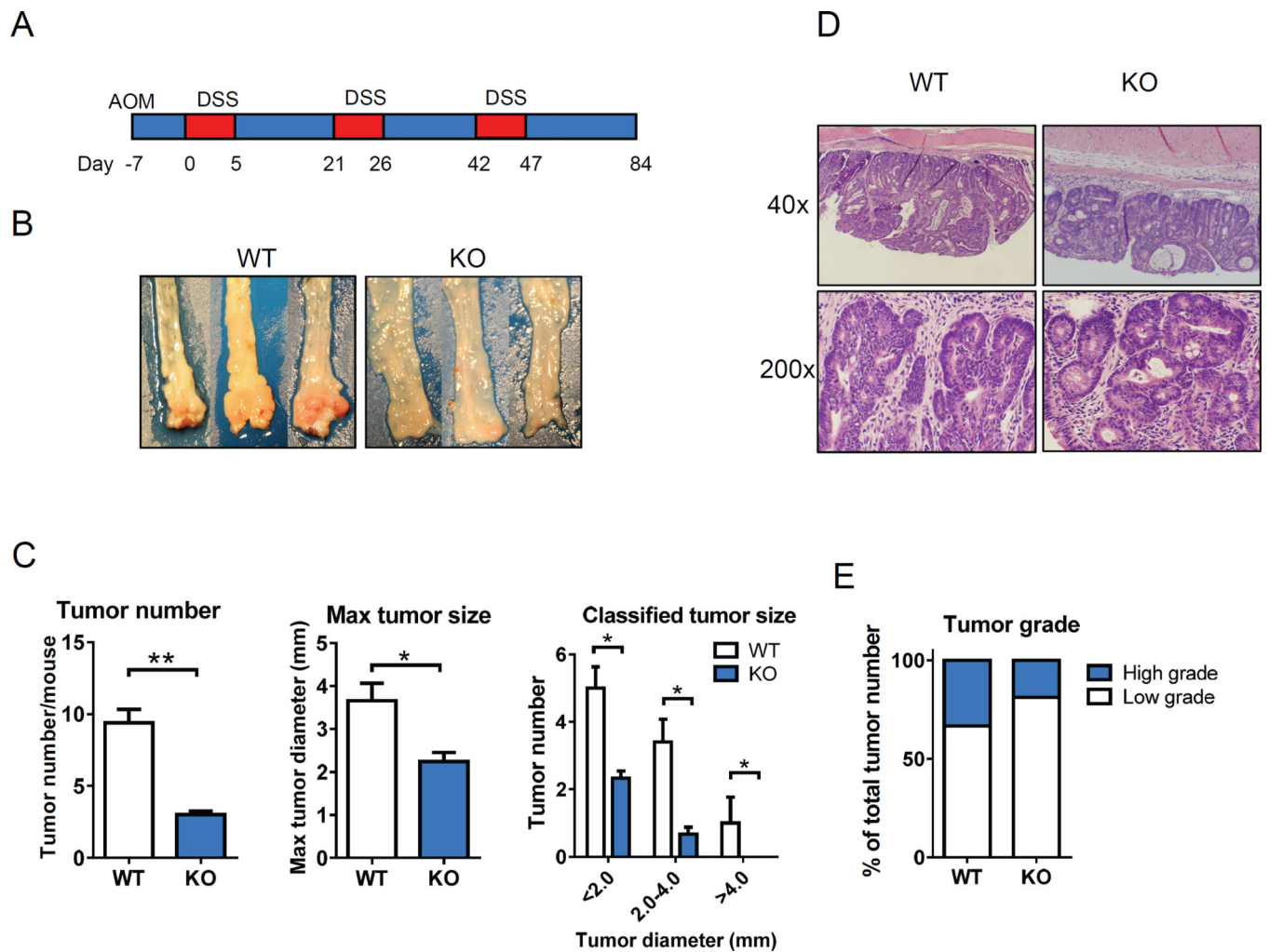


Figure 3. CAMK2 γ KO mice display reduced CAC development

(A) Scheme for AOM/DSS-induced CAC in mice. Intestinal tumors are analyzed on day 84.

(B) Representative photos of distal colon tissues from WT and CAMK2 γ KO mice. (C)

Tumor number, maximum tumor size and classified tumor size from the colon tissues of

mice. (WT=5, KO=6). (D) Representative H&E staining of colon sections from WT and KO

mice. (E) Quantification of tumor grade in WT and KO mice (WT=5, KO=6). Results are

presented as mean \pm s.e.m.; * P <0.05, ** P <0.01. See also Supplementary Figure 3.

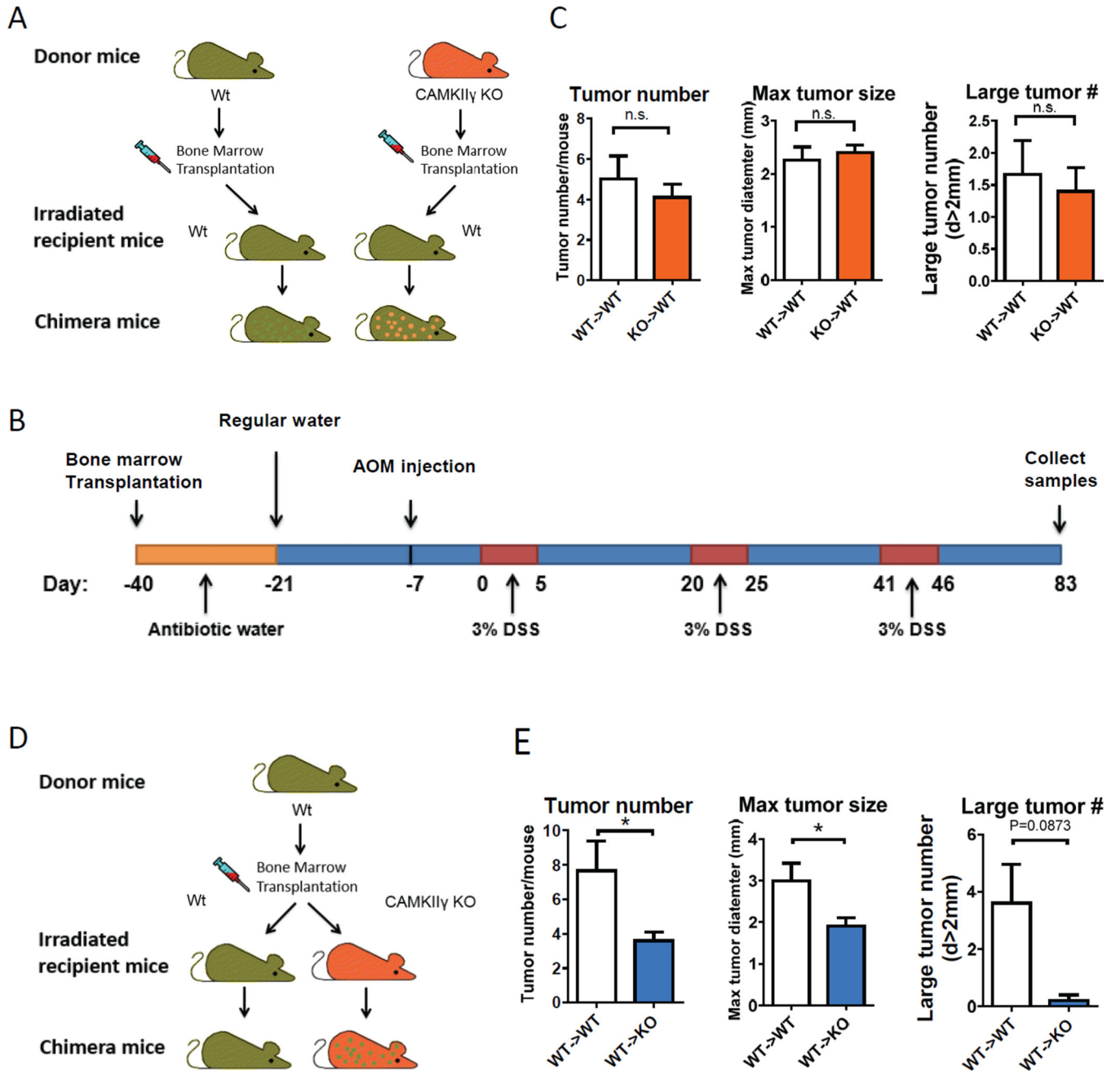


Figure 4. CAMK2 γ KO in colonic tissue-resident cells, but not in immune cells, results in reduced CAC

(A) Schematic overview of the generation of bone marrow chimeric mouse groups WT→WT and KO→WT. (B) Scheme for CAC induction in chimeric mice. (C) Tumor number, maximum tumor size, and the number of large tumors (diameter [d] > 2 mm) from WT→WT and KO→WT mice (n=10). (D) Schematic overview of the generation of bone marrow chimeric mouse groups WT→WT and WT→KO. (E) Tumor number, maximum tumor size, and the number of large tumors with diameter over 2 mm from WT→WT and

WT→KO mice (n=5). Results are presented as mean ± s.e.m.; *P<0.05, n.s., not significant. See also Supplementary Fig. 4.

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BrdU and TUNEL staining shown in (D). For C–E, the mice were treated as shown in Fig. 1A. (WT=6, KO=10) (F) Representative images of distal colon tissues and H&E staining of colon sections from FW and TG mice. (G) Tumor number, maximum tumor size, classified tumor size, and tumor grade of colon tumors from FW and TG mice. For F–G, the mice were treated as shown in Fig. 3A. Results are presented as mean \pm s.e.m.; *P<0.05, **P<0.01, n.s., not significant. See also Supplementary Fig. 5 and 6.

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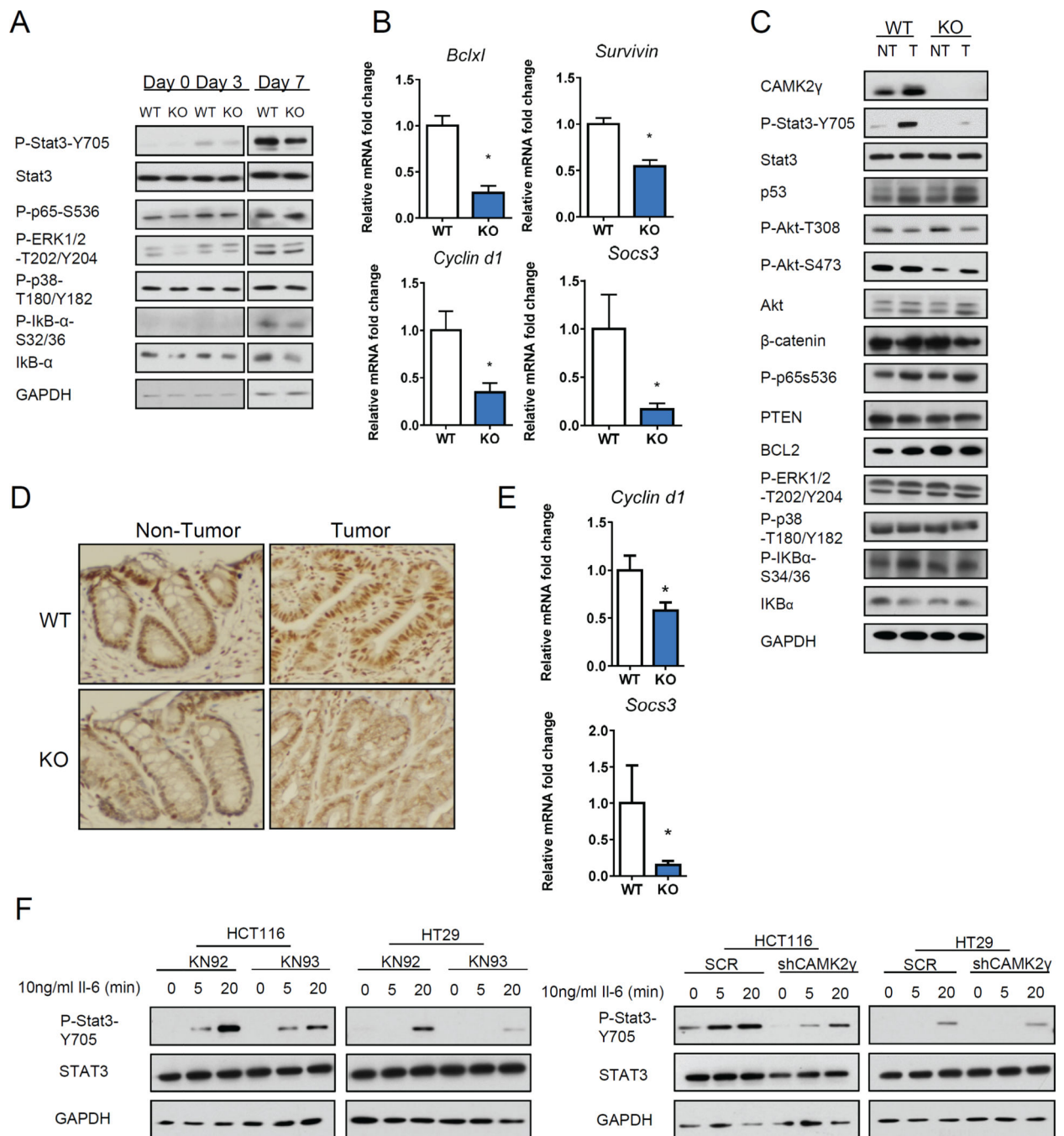


Figure 6. CAMK2 γ promotes intestinal epithelial cell survival and proliferation via enhancing STAT3 activation

(A) Representative western blot analysis of the molecules involved in survival and proliferation signaling pathways in pooled colon tissues from mice with acute colitis. (For Day0 and Day3, n=3; For Day7, n=8) (B) Relative mRNA expression of STAT3 target genes in the colon tissues of DSS-challenged WT and KO mice (n=8). (C) Western blot analysis of molecules involved in survival and proliferation signaling pathways in pooled colonic lysate samples from AOM/DSS treated WT and KO mice. T, tumor tissues in colon; NT, paired

adjacent non-tumor colon tissues; **(D)** Representative IHC staining of P-STAT3 in tumor and non-tumor tissues. **(E)** Expression of STAT3 target genes in WT and CAMK2 γ KO CAC tissues. **(F)** Suppression of CAMK2 γ activity by a specific inhibitor (KN93), but not the inactive analog KN92, decreased IL-6-induced STAT3 activation in human colorectal cancer cell lines. Knockdown of CAMK2 γ by shRNAs in human colorectal cancer cell lines also decreased IL-6-induced STAT3 activation *in vitro*. See also Supplementary Fig. 7.

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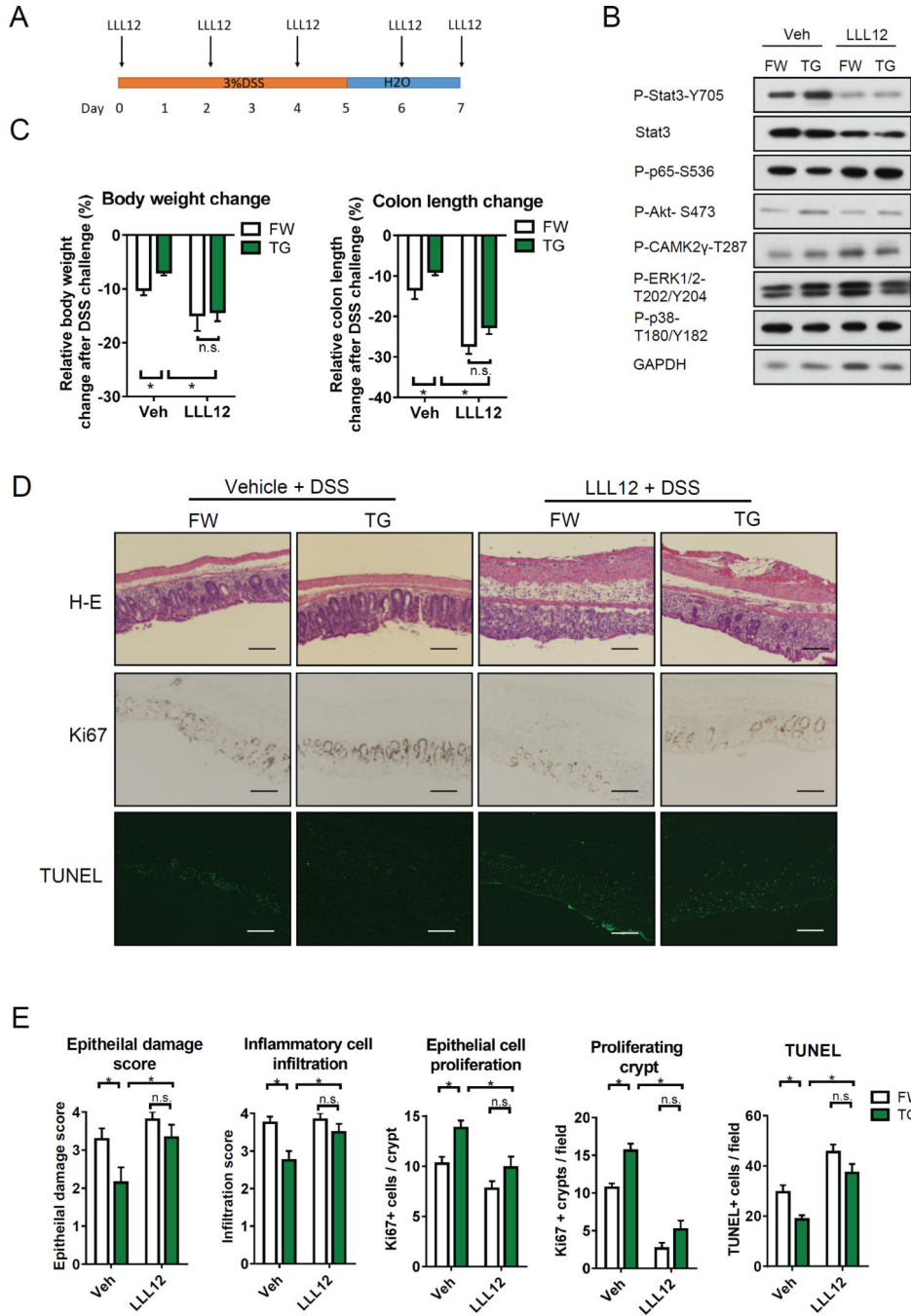


Figure 7. Inhibition of STAT3 blocks the protective effects of CA-CAMK2γ on epithelial cell survival and proliferation in DSS treated mice
(A) Scheme for LLL12 treatment in DSS-induced colitis on FW and TG mice. The colon tissues were analyzed on day 7. **(B)** Western blot analysis of molecules involved in survival and proliferation signaling pathways in pooled mouse colonic lysate samples. **(C)** DSS-induced changes in mouse body weight and colon length. **(D)** Representative images of H&E, Ki67 and TUNEL staining of mouse colon tissues. Scale bar indicate 200μm length. **(E)** epithelial damage score and inflammatory cell infiltration were calculated from the H&E

staining shown in (D); the number of proliferating epithelial cells and crypts proliferation was calculated from the Ki67 staining shown in (D); Epithelial cell death was calculated from the TUNEL staining shown in (D). n=8. Results are presented as mean \pm s.e.m.; *P<0.05, n.s., not significant.

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