Generation of an inducible colon-specific Cre enzyme mouse line for colon cancer research

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Current mouse models for colorectal cancer often differ significantly from human colon cancer, being largely restricted to the small intestine. Here, we aim to develop a colon-specific inducible mouse model that can faithfully recapitulate human colon cancer initiation and progression. Carbonic anhydrase I (*Car1*) is a gene expressed uniquely in colonic epithelial cells. We generated a colon-specific inducible *Car1*^{CreER} knock-in (KI) mouse with broad Cre activity in epithelial cells of the proximal colon and cecum. Deletion of the tumor suppressor gene *Apc* using the *Car1*^{CreER} KI caused tumor formation in the cecum but did not yield adenomas in the proximal colon. Mutation of both *Apc* and *Kras* yielded microadenomas in both the cecum and the proximal colon, which progressed to macroadenomas with significant morbidity. Aggressive carcinomas with some invasion into lymph nodes developed upon combined induction of oncogenic mutations of *Apc*, *Kras*, *p53*, and *Smad4*. Importantly, no adenomas were observed in the small intestine. Additionally, we observed tumors from differentiated *Car1*-expressing cells with *Apc/Kras* mutations, suggesting that a top-down model of intestinal tumorigenesis can occur with multiple mutations. Our results establish the *Car1*^{CreER} KI as a valuable mouse model to study colon-specific tumorigenesis and metastasis as well as cancer-cell-of-origin questions.

mouse model | gastrointestinal tract | colorectal cancer | Car1 | differentiated epithelial cells

The large intestine consists of the cecum, the colon, and rectum. Its simple columnar epithelium is organized into the crypts of Lieberkühn. Unlike the small intestine, the large intestine is bereft of villi. The colon epithelium is a constantly self-renewing tissue. The crypts of Lieberkühn contain actively cycling leucine-rich repeat-containing G-protein–coupled receptor 5 (*Lgr5*)-expressing stem cells at their base (1). These stem cells produce proliferative transit-amplifying cells that give rise to mature cells such as mucous-secreting goblet cells and short-lived absorptive enterocytes with apical microvilli (distinct from small intestine enterocytes) located at the luminal surface (1). Scattered in the large intestinal crypts are chromogranin A-positive enteroendocrine cells and tuft cells (2). M cells are also located in the Peyer's patches of the large intestine (3, 4).

Human colorectal cancer (CRC) is the second leading cause of cancer-related death in the Western world (5). Generally, disease progression from benign adenomas induced by Wnt pathway gene mutations [such as in *APC* (adenomatous polyposis coli)] in colonic epithelial cells to invasive carcinomas involves sequential accumulation of, for example, activating mutations in *RAS* oncogenes, inactivating activating mutations in the *SMAD* family of tumor suppressor genes, and inactivating *TP53* gene mutations (6). Rodent models with mutations that generate tumors in the colon, recapitulating many features of human CRC such as *Apc*$^{Pirc/+}$ (Pirc) (7), *Apc*-mutant Kyoto Alpha Delta (KAD) rats (8), and *Gstp*-null *Apc*^{Min} mice (9), have been informative in modeling cancers induced environmentally and sporadically. However, tumors are still observed in the small intestine of these models, albeit at a lower frequency, which is not seen in human CRC, limiting their ability to faithfully mimic colon-specific tumorigenesis.

Controlled in vivo studies in genetic mouse models based on the Cre–*loxP* system offer an important avenue to model the molecular etiology of CRC development via timed mutation of oncogenes and tumor suppressor genes, allowing testing of potential preventive and therapeutic interventions (10, 11). A preferred mouse model for CRC should involve *Cre* expression specifically in colonic epithelial cells to closely mimic the disease development observed in humans. The Carbonic anhydrase I (*Car1*) gene is expressed in normal colon epithelial cells as well as in colorectal tumors (12, 13). It encodes a metalloenzyme involved in hydration of carbon dioxide, pH balance, and anion exchange. Previously, a promoter/enhancer from the murine *Car1* gene was used to generate a *Cre*-expressing transgenic mouse (14). Although *Cre* expression was indeed restricted to the colon and absent from the small intestine, the usefulness of this model is limited because *Cre* is constitutively expressed from embryonic development onward. To overcome this limitation, we generated a mutant allele containing Cre recombinase fused to the mutated tamoxifen-inducible estrogen receptor (CreERT2) inserted into the 3′ untranslated region of the *Car1* gene, allowing for spatiotemporal control of Cre activity (15).

Significance

A major limitation of current mouse models of colorectal cancer (CRC) is that the cancer that develops is often significantly different from human colon cancer in terms of latency, intestinal location, or molecular signature. Carbonic anhydrase I (*Car1*) is a gene expressed by colonic epithelial cells. We generated an inducible *Car1*^{CreER} mouse model with Cre expression in the cecum and proximal colon. Mutations of genes that drive human CRC with our *Car1*^{CreER} mouse yielded tumors exclusively in the cecum and proximal colon. Differentiated colonic cells in the proximal colon with *Apc/Kras* mutations initiate tumors supporting a top-down model of intestinal tumorigenesis. The inducible *Car1*^{CreER} will be a useful model in studying human intestinal cancers originating from the proximal colon.

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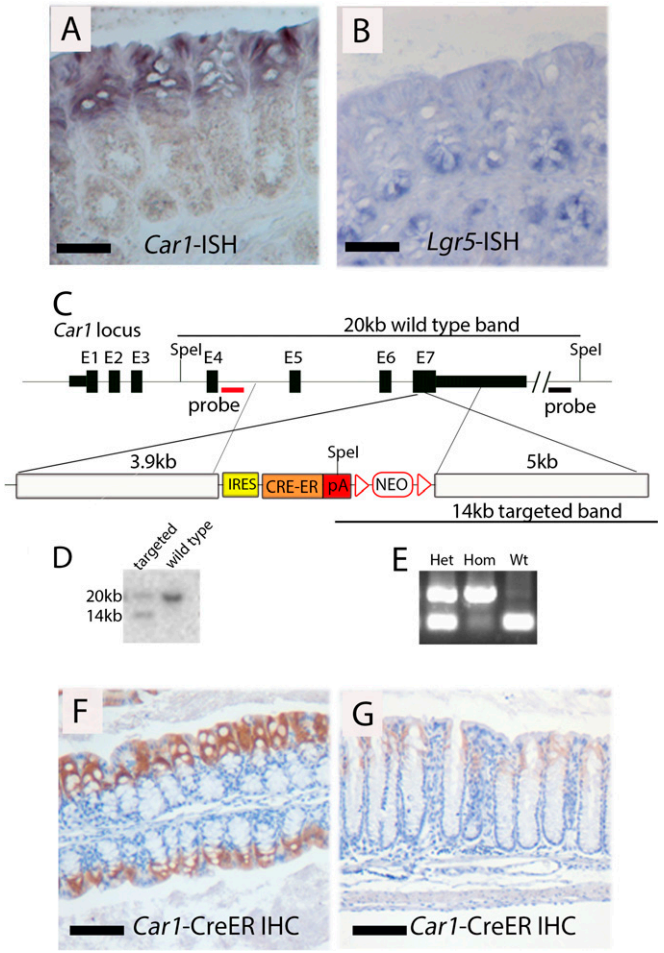

Fig. 1. *Car1* expression in colon and generation of *Car1*^{CreER} knock-in. Comparison of *Car1* mRNA expression (*A*) to stem cell marker gene *Lgr5* (*B*) in the proximal colon. *Car1* is localized to differentiated cells at the top of the colonic crypt whereas *Lgr5* is found in stem cells at the bottom of the crypt. (*C*) Targeting strategy. IRES–CreERT2 targeting construct used to target *Car1* locus. (*D*) Southern blotting showing successfully targeted embryonic stem cells. (*E*) Genotyping of *Car1*^{CreER} KI mice. (*F* and *G*) ER antibody staining showing expression of CreER fusion from the *Car1* promoter in the *Car1*^{CreER} KI. CreER expression is higher in the proximal colon (*F*) than in the distal colon (*G*). (Scale bars: *A* and *B*, 100 μm; *F* and *G*, 50 μm.)

Results

Generation of *Car1*^{CreER} Mice. Multiple studies have analyzed the organ-specific expression of *Car1* (12, 13, 16, 17). In the gastrointestinal tract, *Car1* is expressed in the colon but not in the small intestine, with highest expression in the proximal colon (16). We confirmed the expression of *Car1* by mRNA in situ hybridization with a probe specific for the *Car1* gene. Comparison of *Car1* mRNA expression with that of the colonic stem-cell marker gene *Lgr5* by in situ hybridization confirmed localization of *Car1* transcripts in differentiated colonic epithelial cells and *Lgr5* mRNA being detectable in the stem cells at the crypt bottom (Fig. 1 *A* and *B*).

To generate an inducible colon-specific Cre line, we inserted an internal ribosome entry site (IRES)–CreERT2 cassette at the stop codon located in the last exon of the *Car1* gene (*Car1*^{CreER}; Fig. 1 *C*–*E*). This strategy employs a poly(A) signal and the 3′ UTR of the *Car1* gene (Fig. 1*C*). Targeting arms and the IRES–CreERT2 cassette were subcloned, and the construct was linearized and electroporated into embryonic stem (ES) cells. Recombined ES clones expressing the neomycin gene were selected in G418 supplemented medium. Homologous recombination was confirmed by Southern blot analysis using a probe upstream of the targeted region (Fig. 1*D*). Accurate recombination efficiency in ES clones was 6%. Two independent positive clones were injected into C57BL/6 blastocysts according to standard procedures. The neomycin selection cassette flanked by *FRT* sites was excised in vivo by crossing the *Car1*^{CreER} knock-in (KI) mice with *FLP1* mice. Heterozygous and homozygous *Car1*^{CreER} mice (Fig. 1*E*) were retrieved at the expected Mendelian ratios at birth. No discernible abnormality was observed in adult homozygous transgenic animals, which had comparable life span and fertility as wild-type littermates.

To indirectly visualize expression of the Cre protein, we performed immunohistochemical staining on paraffin-embedded intestinal tissue slides with an antibody recognizing the mutant estrogen receptor domain (ERT2) fused to the Cre protein. This showed that Cre expression faithfully mirrored endogenous *Car1* expression (Fig. 1 *F* and *G*).

Characterization of Cre Activity of *Car1*^{CreER} KI. To further characterize Cre activity of the *Car1*^{CreER} allele and trace the behavior of *Car1*-expressing cells, mice were crossed to *R26R*$^{LSL–LacZ}$ reporter mice where the *LacZ* gene is under the control of the ubiquitous *Rosa26* locus (18). Eight- to 12-wk-old mice were injected with a single dose of 4OH-tamoxifen (TAM), killed at various time points (three mice per time point), and analyzed for β-galactosidase (β-gal) staining. In the proximal colon, β-gal–positive cells were detected in the upper crypt (Fig. 2 *A*–*D* and *G*). No β-gal–positive cells were observed after 28 d, suggesting that *Car1* labeling did not occur in colonic stem cells (Fig. 2*E*).

Analysis of Cre activity in the cecum of *Car1*$^{CreER/+}$;*R26R*$^{LSL–LacZ/+}$ mice showed β-gal–positive cells and estrogen receptor (ER) antibody-stained cells not only in differentiated cells but also at the crypt bottom, suggesting broad expression of *Car1* in both stem cells and differentiated cells of the cecum (Fig. S1). β-Gal–positive cells were also detected in long-lived hepatocytes in the liver, which have been reported to express *Car1* (Fig. 2*F*) (14).

Tumor Initiation from *Car1*-Expressing Cells. The adenoma-carcinoma model of colon cancer initiation posits that disease progression to the metastatic/invasive stage depends on sequential or combined mutations of *Apc*, *Kras*, *p53*, and *Smad4* genes (3, 19–21). To determine the utility of the *Car1*^{CreER} KI for colon cancer research, we crossed mice carrying mutant alleles for *Apc*$^{fl/fl}$, *Kras*$^{LSL–G12D/+}$, *p53*KO, and *Smad4*$^{fl/fl}$ with *Car1*^{CreER} KI mice and induced Cre recombination by TAM injection. The presence of tumors was confirmed by nuclear accumulation of β-catenin (Fig. 3 *A* and *B*), a hallmark of hyperactive Wnt signaling frequently found in human CRC (20). As expected, based on previous studies (20) in small intestine tumorigenesis, *Apc* mutation in differentiated *Car1*-expressing colonic cells yielded microadenomas that failed to progress to macroadenomas following TAM induction (Fig. S2). In the cecum, however, *Apc* deletion yielded much larger adenomas presumably because crypt bottom stem cells in the cecum express Cre (Fig. 3 *A*–*D*).

We next asked whether additional mutation in the *Kras* gene, another commonly mutated gene in CRC, can enhance the tumorigenic potential of differentiated *Car1*-expressing cells in the proximal colon. We observed pronounced microadenomas in *Car1*$^{CreER/+}$;*Apc*$^{fl/fl}$;*Kras*$^{LSL–G12D/+}$ colons 2 wk after TAM induction (Fig. 4 *A* and *B*). β-Catenin antibody staining of mice 56 d following TAM injection showed that these microadenomas had progressed into large adenomas originating from differentiated *Car1*-expressing cells in the proximal colon and growing toward the luminal side of the intestine (Fig. 4 *C*–*E*). Of note, no adenomas were ever observed in the small intestine, the distal colon, or the liver (Fig. S3).

Histopathological analysis of hematoxylin and eosin (H&E)-stained sections of tumors from *Car1*-expressing cells revealed that these resembled conventional human adenomas (Fig. 5*A* and Fig. S4). Proximal colon tumors from *Car1*$^{CreER/+}$;*Apc*$^{fl/fl}$;

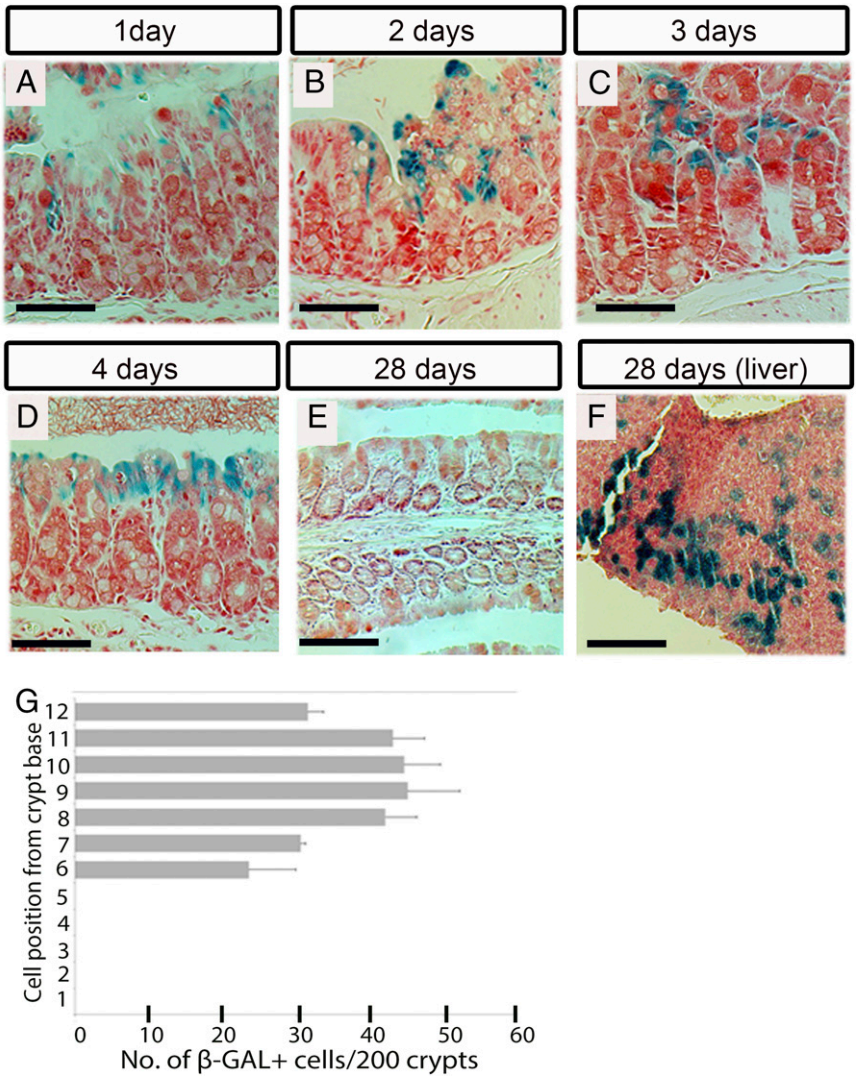

Fig. 2. Lineage tracing of *Car1*-expressing cells. *Car1*$^{CreER/+}$;*R26R*$^{LSL-LacZ/+}$ mice were induced with 5 mg/kg TAM and then killed after (*A*) 1 d, (*B*) 2 d, (*C*) 3 d, (*D*) 4 d, and 28 d (*E* and *F*). (*G*) Quantification of β-gal+ cells 24 h in proximal colon after TAM injection of *Car1*$^{CreER/+}$;*R26R*$^{LSL-LacZ/+}$ mice. β-Gal+ cells can be detected at cell position 6 from the crypt base and above, but not at the crypt bottom (n = 3; mean ± SD). Counts were made per 100 colonic crypts for each mouse. (Scale bars: 100 μm.)

Kras$^{LSL-G12D/+}$ mice had recognizable tubular structures and severely disturbed architecture. Glands within the tumors were lined with a stratified epithelium that showed an increased nuclear/cytoplasmic ratio, hyperchromatism, prominent nucleoli, and numerous abnormal mitotic figures. Tumors derived from *Car1*-expressing cells were also highly proliferative (Fig. 5*B*) and poorly differentiated (Fig. 5*C*). Analysis of stem-cell marker expression revealed high abundance of *Lgr5* mRNA by in situ hybridization and Musashi-1 (MSI1) and Eph receptor B2 (EPHB2) protein by antibody staining in tumors, interestingly originating from differentiated *Car1*-expressing cells (Fig. S5).

Tumor Progression upon Three or Four Oncogenic Mutations in *Car1*-Expressing Cells. We next generated *Car1*$^{CreER/+}$;*Apc*$^{fl/fl}$;*Kras*$^{LSL-G12D/+}$;*p53*KO triple-mutant mice, *Car1*$^{CreER/+}$;*Kras*$^{LSL-G12D/+}$;*p53*KO;*Smad4*$^{fl/fl}$ triple-mutant mice, and *Car1*$^{CreER/+}$;*Apc*$^{fl/fl}$;*Kras*$^{LSL-G12D/+}$;*p53*KO;*Smad4*$^{fl/fl}$ quadruple-mutant mice and injected TAM to mimic the effects of complex genotypes often associated with human CRC (21). Analysis after a single injection of TAM was limited to 1 mo as triple and quadruple mutations beyond this time point were lethal, mainly due to the extracolonic effects of the constitutively deleted *p53 gene*. Tumors in quadruple mutants (Fig. 6 *A* and *D*) showed significant Paneth cell metaplasia (Fig. 6 *B*, *E*, and *F*) and were more aggressive with some invasion into lymph nodes, as assessed by lymphatic vessel endothelial hyaluronan receptor 1 (LYVE1) immunostaining (Fig. 6*C*) (22). Due to high mortality only one animal carrying all four CRC mutations could be analyzed. Triple mutations of *Apc*, *Kras*, and *p53* led to aggressive tumors that developed into carcinomas in the cecum and proximal colon (Fig. S6 *A–C*). Combined mutations of *Kras*, *p53*, and *Smad4* gave rise to benign adenomas (Fig. S6 *D–F*).

Genetic mutations can foster the development of inflammation or recruitment of immune cells that promote tumorigenesis (23). In the gut, chronic inflammation has been shown to be a risk factor for the development and progression of colitis-associated CRC as well as sporadic CRC (24). We therefore performed immunohistochemical stainings for two prominent components of the inflammatory infiltrate present in these particular CRC types [T-cell marker CD3 (25) and macrophage marker F4/80 (26)] to exclude chronic inflammation as a cancer hallmark in mice carrying *Car1*-specific mutations of *Apc*, *Kras*, and *p53* with or without additional mutation of *Smad4*. Indeed, tumors arising from these mutations in *Car1*-expressing cells did not show significant infiltration with T cells or macrophages, suggesting absence of immunomodulation in this context (Fig. S7).

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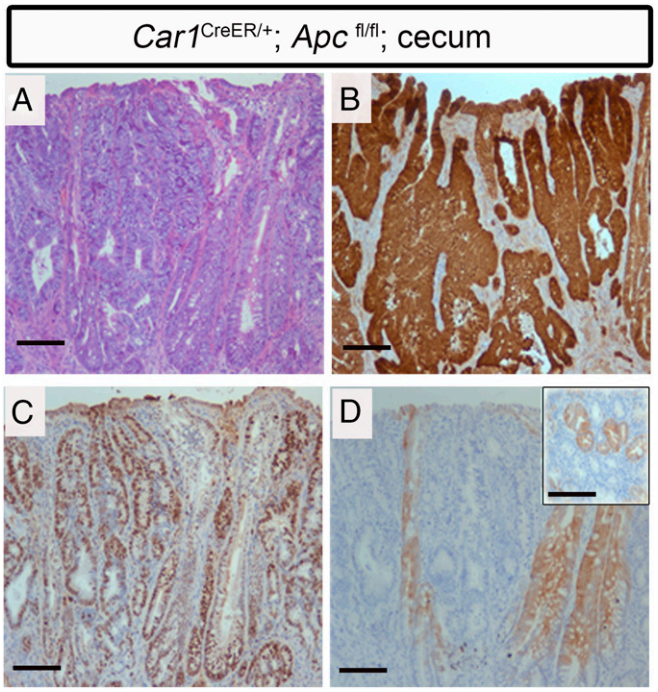

Fig. 3. Adenomas in cecum upon *Apc* deletion. *Car1*$^{CreER/+}$;*Apc*$^{fl/fl}$ mouse was injected with a single dose of TAM, killed after 2 mo, and analyzed. (*A*) H&E staining showing tubular adenomas in cecum. (*B*) Nuclear β-catenin staining showing Wnt hyperactivation in cecal adenomas. (*C*) KI67 staining showing highly proliferative cecal adenomas. (*D*) ER antibody staining showing Cre expression restricted in cecal adenomas, suggesting restricted expression in cancer cells fueling cancer progression (*Inset*). (Scale bars: 50 μm.)

No primary tumors were observed in the liver in triple and quadruple mutants, suggesting that *Car1*-expressing hepatocytes are resistant to neoplastic transformation by combined mutations of *Apc*, *Kras*, *p53*, and *Smad4* (Fig. S6 *G–I*).

Discussion

In this study, we report the generation of an inducible proximal colon- and cecum-specific CreERT2 KI mouse line driven by the *Car1* gene allowing for spatiotemporal control of Cre activity. Intestinal Cre expression occurs broadly in epithelial cells in the cecum and is more restricted to differentiating colonocytes in the proximal colon. No Cre activity was detected in the small intestine. Recently, Xue and colleagues reported the generation of a *Car1*-Cre (CAC) transgenic line that drives Cre expression in the colon (14). The constitutive Cre activity in this model persists from embryonic stages into adulthood, making it unsuitable to study adult-onset biological processes such as CRC. Whereas Cre expression in the *Car1*^{CreER} KI mice was restricted to differentiated colonic cells of the proximal colon, Cre activity in the highly mosaic CAC transgene was observed in all epithelial cell types in the proximal, distal, and rectal sections of the colon (14). A reason for the apparent discrepancy of *Cre* expression in the two models may be due to the different targeting strategies used. In our *Car1*^{CreER} KI, the IRES–CreERT2 cassette is inserted at the 3′ UTR of the *Car1* gene without disrupting expression of the *Car1* gene; thus *Cre* expression follows endogenous expression of the *Car1* gene. In contrast, the targeting construct in the CAC transgene is inserted immediately downstream of the *Car1* promoter, which leads to random integration of the DNA-targeting construct; thus, each founder can show different *Cre* expression due to the chromosomal context of the integration (27).

Our model is also different from the previously generated colon-specific *CDX2P–CreERT2* transgene where Cre is expressed in both stem and differentiated epithelial cells of the ileum of the small intestine and entire colon. Inactivation of APC with this transgene induced adenomas in the ileum, cecum, and proximal and distal colon (28, 29). Cre protein delivered by an adenoviral vector has been used to conditionally delete a floxed Apc allele inducing tumors in the distal colon (30). In contrast, our inducible *Car1*^{CreER} model generates tumors exclusively in the proximal colon.

Identification of the tumor cell-of-origin is important for targeted interventions to combat cancer development and progression. We have previously reported that deletion of *Apc* in small intestinal crypt bottom *Lgr5*-positive stem cells induces macroadenoma formation (31). Loss of *Apc* in nonstem cells led to nuclear accumulation of β-catenin and formation of microadenomas that failed to progress into large adenomas, supporting a bottom-up model of intestinal cancer initiation. Alternatively, evidence

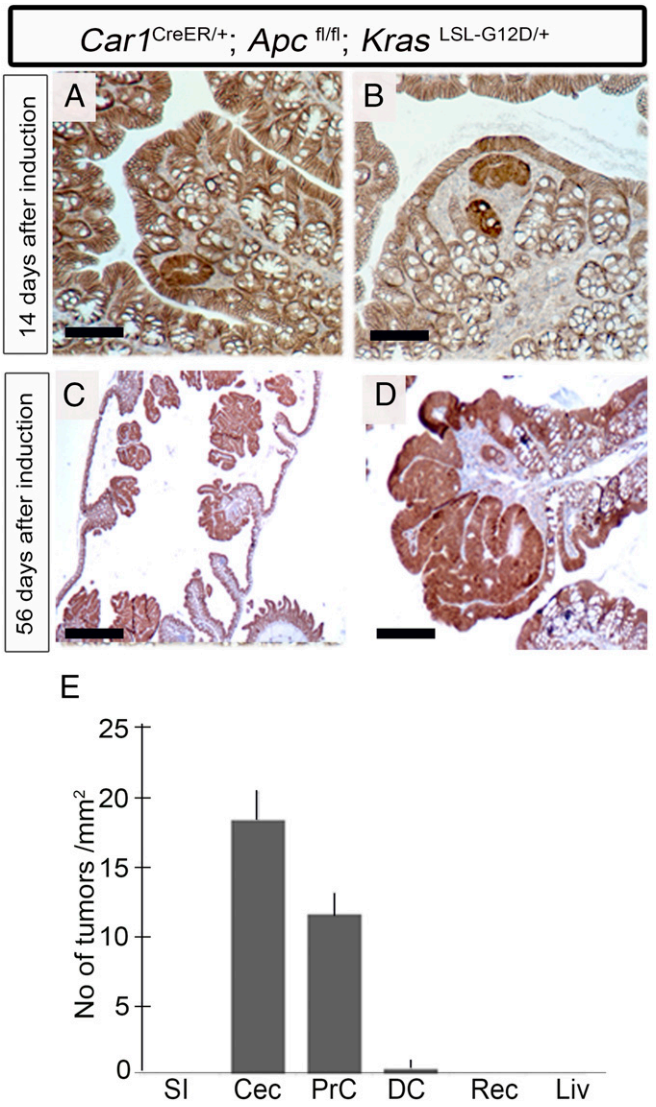

Fig. 4. Adenoma formation upon *Apc* and *Kras* mutations in *Car1*-expressing colonic epithelial cells. A single dose of TAM was injected into *Car1*$^{CreER/+}$;*Apc*$^{fl/fl}$;*Kras*$^{LSL-G12D/+}$ mice. β-Catenin nuclear staining was used to identify adenomas. (*A* and *B*) Microadenomas in proximal colon 14 d after TAM injection. (*C* and *D*) Macroadenomas in proximal colon 56 d after TAM injection. (*E*) Quantification of tumors formed per square millimeter (n = 3; mean ± SD). More tumors were observed in the cecum (Cec) than in the proximal colon (PrC). No tumors were observed in the small intestine (SI), distal colon (DC), rectum (R), and liver (Liv). (Scale bars: *A* and *B*, 100 μm; *C*, 800 μm; *D*, 200 μm.)

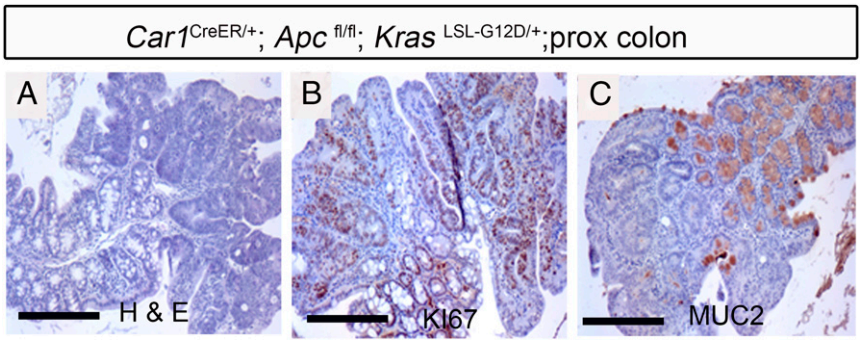

Fig. 5. Histological analysis of *Car1*$^{CreER/+}$;*Apc*$^{fl/fl}$;*Kras*$^{LSL-G12D/+}$ proximal colon tumors. (*A*) H&E staining showing tubular adenomas in *Apc*/*Kras* tumors. (*B*) Tumors were highly proliferative as shown by antibody staining of the proliferation marker KI67. (*C*) Proximal colon tumors were poorly differentiated as shown by the absence of differentiated MUC2-positive goblet cells. (Scale bars: 300 μm.)

exists for a top-down mode of tumor initiation from nonstem cells, although the differentiation status of these nonstem cells is unclear (whether fully differentiated or committed progenitors, short-lived or long-lived cells) (32). In our colon model, nuclear accumulation of β-catenin was observed upon Apc deletion in short-lived Car1-positive differentiated cells in the proximal colon (Fig. S4). However, these cells do not progress to form macroadenomas, similar to what has been previously reported in the small intestine (31).

Cre-mediated mutations of *Apc* and *Kras* in intestinal stem cells as well as xenograft studies with human cancer cells bearing *APC* and *KRAS* mutations transplanted into mice have shown that oncogenic *KRAS* enhances tumor progression in CRC harboring *APC* mutations (20, 33). Intriguingly, simultaneous *Apc* deletion and oncogenic *Kras* hyperactivation transformed differentiated *Car1*-expressing cells into tumor-initiating cells with cancer-stem-cell characteristics, arguing that cancer can also be generated in a top-down model, where differentiated cells acquire multiple mutations. Of note, these tumors developed in the colon without the induction of an inflammatory stimulus.

Whereas only *Apc* mutation is sufficient to transform *Lgr5*-positive stem cells into cancerous cells, multiple mutations are necessary to transform differentiated *Car1*-positive cells into cancer stem cells. *Apc*/*Kras* mutant cells in the distal colon have been demonstrated to metastasize to the liver (30). However, we did not observe any metastasis into lymph nodes and liver upon *Apc*/*Kras* or *Apc*/*Kras*/*p53* mutations in proximal colon epithelial cells. The minimal invasion of lymph nodes by tumors from quadruple-mutant mice suggests that metastasis is enhanced by multiple mutations of *Apc*, *Kras*, *p53*, and *Smad4* (Table S1). It is likely that tumor location affects the metastatic potential of transformed cells. In support, a recent study reported that proximal colon cancer in humans has lower metastasis rates compared with distal colon cancer (34).

Paneth cells can provide niche factors to small intestinal stem cells (35). Colorectal Paneth cell metaplasia is commonly associated with idiopathic inflammatory bowel disease and thought to be a preneoplastic sign for tumorigenesis, although the causal mechanism is not fully understood (36). In our study we did not

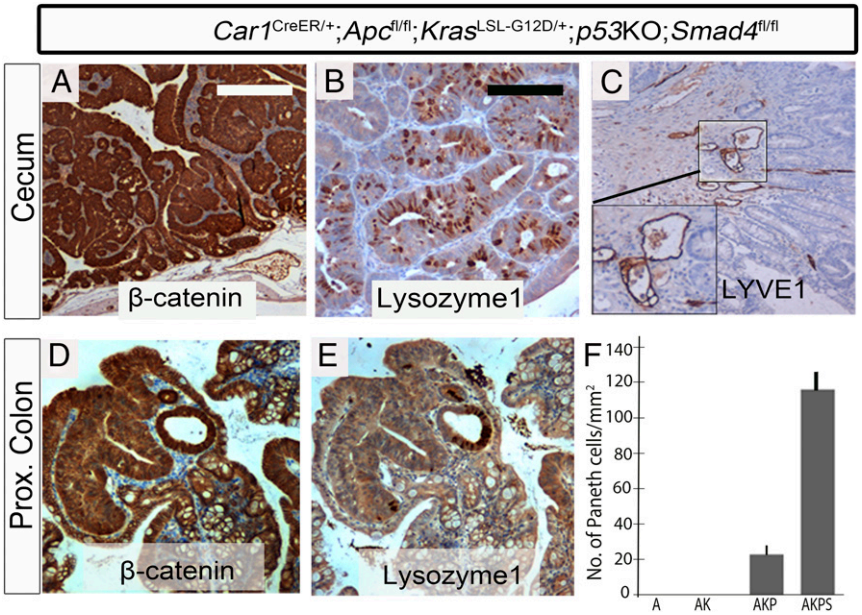

Fig. 6. Histological analysis of *Apc*/*Kras*/*p53*/*Smad4* deletion. *Car1*^{CreER};*Apc*$^{fl/fl}$;*Kras*$^{LSL-G12D/+}$;*p53*KO;*Smad4*$^{fl/fl}$ mice were injected with TAM and killed after 1 mo. Histological analysis of nuclear β-catenin indicating tumors: lysozyme 1 to observe Paneth cell metaplasia and lymphangiogenic marker LYVE1 to indicate invasiveness into lymph nodes. Aggressive carcinomas in cecum (*A*) and proximal colon (*D*). Paneth cell metaplasia in both cecum (*B*) and proximal colon (*E*). (*C*) LYVE1 staining. (*Large Inset*) Example of blood vessel with infiltrating tumor cells. (Scale bars: 100 μm.) (*F*) Quantification of Paneth cell metaplasia in cecum: *Apc* (A) and *Apc*/*Kras* (AK) mutations did not give rise to adenoma Paneth cells. *Apc*/*Kras*/*p53*/*Smad4* mutants (AKPS) had fivefold more lysozyme-positive Paneth cells than *Apc*/*Kras*/*p53* mutants (AKP) (n = 3, unless AKPS condition, where n = 1 due to high mortality; mean ± SD).

observe this phenomenon with *Apc* only or with *Apc*/*Kras* double mutations. However, Paneth cell metaplasia was observed in *Apc*/*Kras*/*p53* triple mutants and quadruple mutants, being strongest in quadruple mutants (Table S1). Human CRC is a heterogeneous disease with proximal (ascending and transverse sections) and distal (descending and sigmoid sections) exhibiting multiple biological and clinical differences with distinct pathological outcomes (13). Another level of heterogeneity is the cell of origin of CRC, which can be initiated by either stem cells or nonstem cells. Our data illustrate that the *Car1*^{CreER} KI mouse can be used to model genetic aberrations contributing to proximal colon tumorigenesis. Additionally, this model will be useful in deciphering the mechanisms of tumor initiation from differentiated cells and therapeutic interventions to mitigate proximal cancers.

Materials and Methods

A detailed description of materials and methods can be found in *SI Materials and Methods*.

Animal Procedures. *Car1*^{CreER} KI mice were crossed with *R26R*$^{LSL-LacZ}$ reporter mice mice (18) and used for lineage-tracing experiments. For tumor studies, *Car1-CreERT2* KI mice were bred to mice carrying a mutant allele of *Apc*$^{fl/fl}$ (37), *Kras*$^{LSL-G12D/+}$ (38), *p53*KO (39), and/or *Smad4*$^{fl/fl}$ (40) to generate various genotypes (double-, triple-, or quadruple-mutant mice). A total of three mice were used for each time point analyzed for each experimental group, unless stated otherwise. All animal procedures were performed in accordance with national animal welfare laws under a project license obtained from the Dutch Government and were reviewed by the Animal Ethics Committee of the Royal Netherlands Academy of Arts and Sciences (KNAW).

Human Material. Human tissue was obtained from CRC resections performed at the University Medical Center Utrecht, The Netherlands, according to the guidelines of the UMC Utrecht's Research Ethics Committee. We used anonymous archival leftover pathology material. Therefore, no ethical approval is required according to Dutch legislation as this use of redundant tissue for research purposes is part of the standard treatment agreement with patients in our hospital (41).

β-Galactosidase Staining. Isolated tissue was flushed, fixed in paraformaldehyde (PFA) solution, and further processed for β-gal staining as detailed in *SI Materials and Methods*.

Immunohistochemistry. Isolated tissue was flushed, fixed in formalin, and embedded in paraffin wax. Paraffin sections were processed for stainings as described in *SI Materials and Methods*.

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