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The armadillo repeat domain of *Apc* suppresses intestinal tumorigenesis

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Abstract

The adenomatous polyposis coli (*APC*) gene is known to act as a tumor suppressor gene in both sporadic and hereditary colorectal cancer by negatively regulating WNT signaling. Familial adenomatous polyposis (FAP) patients develop intestinal polyps due to the presence of a single germline mutation in *APC*. The severity of the FAP phenotype is a function of the position of the *APC* mutation, indicating a complex role for *APC* that extends beyond the canonical WNT pathway. *APC* encodes a large protein with multiple functional domains, including an armadillo repeat domain that has been linked to protein–protein interactions. To determine the effect of the armadillo repeat domain on intestinal tumorigenesis, we generated a congenic mouse line (*Apc*^{Δ242}) carrying a gene trap cassette between exons 7 and 8 of the murine *Apc* gene. *Apc*^{Δ242/+} mice express a truncated Apc product lacking the armadillo repeat domain as part of a fusion protein with β-geo. Expression of the fusion product was confirmed by X-gal staining, ensuring that *Apc*^{Δ242} is not a null allele. In contrast, *Apc*^{Min/+} mice produce a truncated Apc product that contains an intact armadillo repeat domain. On the C57BL/6J background, *Apc*^{Δ242/+} mice develop more polyps than do *Apc*^{Min/+} mice along the entire length of the small intestine; however, polyps were significantly smaller in *Apc*^{Δ242/+} mice. In addition, polyp multiplicity in *Apc*^{Δ242/+} mice is affected by polymorphisms between inbred strains. These data suggest that the armadillo repeat domain of the Apc protein suppresses tumor initiation in the murine intestine while also promoting tumor growth.

Introduction

Colorectal cancer has the third highest mortality rate for cancers among men and women in the United States, accounting for 9–10% of total cancer-related deaths (www.cancer.org). Genetic evidence suggests that the *APC* gene acts as a classical tumor suppressor gene in both sporadic and hereditary colorectal cancers, with inactivation of both alleles resulting in tumor growth (Miyaki et al. 1994; Powell et al. 1992). Inactivation of *APC* results in nuclear localization of β-catenin and subsequent activation of the WNT signaling pathway (MacDonald et al. 2009). Familial adenomatous polyposis (FAP) is a dominantly inherited disorder in which individuals inherit one defective *APC* allele. The presence of a mutated *APC* allele makes FAP patients susceptible to the development of colorectal adenomas following inactivation of the remaining wild-type *APC* allele through somatic mutation and/or loss of heterozygosity (LOH) (Grodin et al. 1991; Kinzler et al. 1991; Nishisho et al.

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1991). As a result, FAP patients are diagnosed by the development of hundreds to thousands of benign adenomatous polyps throughout the intestinal tract.

The study of human diseases, such as FAP, has been greatly facilitated by the use of animal model systems. Mouse models provide a fixed genetic background and controlled environment in which to analyze the cellular processes leading to neoplasia (Thompson et al. 2004). Several models of intestinal tumorigenesis have been generated by introducing specific mutations into the murine ortholog of the *APC* gene (*Apc*), allowing investigation of its role in both polyp initiation and carcinoma progression (reviewed in Siracusa et al. 2004; Uronis and Threadgill 2009). The most extensively studied of these models is the multiple intestinal neoplasia (*Apc^{Min}*) mouse, which was discovered through phenotypic screening following ENU mutagenesis (Moser et al. 1990). B6 mice carrying the *Apc^{Min}* mutation develop an average of 106 benign adenomas throughout the length of the intestinal tract. *Apc* is a large, multidomain protein and the position of a mutation can affect polyp number, location, and stage in mice (Heyer et al. 1999; Taketo and Edelmann 2009). For example, *Apc^{A716}* mice develop over three times as many polyps as *Apc^{Min/+}* mice, while a more distal mutation, *Apc^{I634N}*, results in greatly reduced tumor burden (Fodde et al. 1994; Oshima et al. 1995). These data recapitulate findings from FAP patients in which tumor multiplicity and secondary symptoms correlate with the location of the germline *APC* mutation (Burt and Jasperson 2008). Mouse lines carrying mutant *Apc* alleles have, therefore, provided valuable in vivo models for studying the diverse roles of *Apc* in intestinal tumorigenesis.

In this study we describe a new mouse model of FAP called *Apc^{A242}*, generated by insertion of a gene trap cassette between exons 7 and 8 of the *Apc* gene. Mice carrying the *Apc^{A242}* allele express a fusion product consisting of the N-terminus of *Apc* and a functional β -geo protein. C57BL/6J (B6) mice carrying the mutant *Apc^{A242}* allele develop significantly more tumors throughout the length of the small intestine than do B6 *Apc^{Min/+}* mice. The *Apc^{A242}* mouse strain provides a model system for dissecting the roles of domains within the *Apc* protein during tumor initiation, growth, and progression.

Materials and methods

Generation of the B6.129 *Apc^{A242/+}* colony

BayGenomics, Inc. (San Francisco, CA) generated gene trap clones by insertion of a β -geo cassette with a 5' splice acceptor site into 129P2/OlaHsd embryonic stem (ES) cells via the pGTOPfs gene trap vector (Stryke et al. 2003). Neomycin selection was used to identify clones carrying the gene trap allele. 5' RACE PCR was used to identify one clone, XA018, with an insertion between exons 7 and 8 of the *Apc* gene. XA018 ES cells were microinjected into C57BL/6J (B6) blastocysts by the KCC Transgenic/Knockout Mouse Facility to generate chimeric offspring. Genomic DNA was isolated from tail biopsies by DNeasy Kit (Qiagen), and mice were genotyped by PCR for the presence of the gene trap cassette (forward: GATGCCTGCTTGCCGAATATC; reverse: GAAGAACTCCAGCATGAGATCC). Offspring carrying the mutant allele were backcrossed to B6 mice from The Jackson Laboratory (Bar Harbor, ME) for ten generations to generate the congenic B6.129 *Apc^{Git(XA018)Byg}* line, hereafter referred to as *Apc^{A242}*. The colony was maintained in the AALAC-accredited TJU animal facility. B6.129 *Apc^{A242/+}* mice were mated to C3H/HeJ mice from The Jackson Laboratory to generate F₁ offspring.

Confirmation of β -galactosidase expression

Male *Apc^{A242/+}* mice at 120 days of age were euthanized by CO₂ asphyxiation followed by cervical dislocation. Distal small intestine and colon samples were washed in 1× PBS and fixed for 30 min in Formalde-Fresh 10% buffered formalin solution (Fisher Scientific).

Fixed tissues were incubated at 37°C in a 1:40 dilution of X-gal Solution (Novagen) in BetaBlue Reaction Buffer (Novagen). Staining was visualized on a Nikon SMZ-U dissection scope at 22.5× magnification. Pictures were taken with a Nikon DXM1200F digital camera.

Evaluation of intestinal tumorigenesis in *Apc*^{Δ242/+} mice

The intestinal tract was washed with 1× PBS and separated into four segments: proximal small intestine (SI), middle SI, distal SI, and colon. Polyp multiplicity and average size were determined for each segment with a Nikon SMZ-U dissection microscope at 22.5× magnification. The diameter was measured at the widest point of the tumor. Tumor volume was estimated using the equation for the volume of a sphere $[(4/3)\pi r^3]$. Tumor burden was calculated as the sum of the volumes of all tumors in an individual.

Histopathological analysis of tumors

Tissues were fixed in Formalde-Fresh 10% buffered formalin solution (Fisher Scientific) overnight at 4°C and stored in 70% EtOH. Fixed tissues were embedded in paraffin and tissues were cut and mounted on glass slides by the KCC Translational Research Core Facility. Tissues were stained with eosin and counterstained with hematoxylin. Staining was visualized on a Nikon Eclipse E600 microscope at either 4× or 20× magnification.

Results

Generation of a mouse strain carrying an *Apc* gene trap allele

The *APC* gene is mutated in both sporadic colorectal cancers and hereditary disorders such as FAP. To study the effects of the armadillo repeat domain of the Apc protein on intestinal tumorigenesis, a mouse model expressing a truncated Apc protein was generated. BayGenomics generated a library of ES cell clones using a randomly inserted gene trap vector (Stryke et al. 2003). The gene trap cassette carries a splice acceptor site, a sequence encoding the β -geo fusion protein, and a polyadenylation site (Fig. 1a). Insertion of the cassette into an intronic sequence results in the fusion of the N-terminus of the trapped gene to the β -geo protein. One clone (XA018) carried a cassette inserted between exons 7 and 8 of the *Apc* gene (Fig. 1a). The *Apc* gene consists of 16 exons, with the majority of the open reading frame (ORF) encoded by exon 16 (www.ensembl.org). Different *Apc* mutations result in different polyposis phenotypes in both FAP patients and mouse models (reviewed in Aoki and Taketo 2007). Therefore, we generated a congenic mouse strain carrying the *Apc*^{Git(XA018)Byg} allele (*Apc*^{Δ242}) to analyze the effect of this novel *Apc* mutation on intestinal tumorigenesis. XA018 ES cells were injected into C57BL/6J (B6) blastocysts. The resulting chimeric offspring were genotyped for the presence of the gene trap cassette (Fig. 1b) and mice carrying the *Apc*^{Δ242} allele were backcrossed to the B6 inbred strain for more than ten generations to generate a congenic line.

The *Apc*^{Δ242} allele generates an Apc fusion protein with β -galactosidase

Apc encodes a 2842-amino-acid protein containing multiple functional domains, including an N-terminal oligomerization domain and an armadillo repeat domain involved in protein–protein interactions (Fig. 2). The presence of the gene trap cassette in *Apc*^{Δ242/+} mice results in the splicing of the first seven exons of *Apc* to the β -geo gene. The *Apc*^{Δ242} allele generates a theoretical 172-kDa fusion product combining the first 241 residues of the Apc protein with the full-length β -geo protein (Fig. 2). The β -geo protein contains the functional domains of both the β -galactosidase enzyme and a neomycin resistant protein. To confirm expression of the mutant fusion protein, X-gal staining was performed on samples from the distal small intestine and distal colon of *Apc*^{Δ242/+} mice. Tissues from *Apc*^{Δ242/+} mice stained blue, while those of wild-type littermates did not stain blue, indicating the presence

of functional β -galactosidase activity in the $Apc^{\Delta242/+}$ intestinal tract and expression of the mutant allele (Fig. 3).

$Apc^{\Delta242/+}$ mice develop adenomas in the small intestine and colon

The most prominent phenotype resulting from *Apc* mutations is intestinal tumorigenesis. To identify how the $Apc^{\Delta242}$ mutation affects polyposis, the intestinal tracts of $Apc^{\Delta242/+}$ mice were evaluated for the presence of polyps at 120 days of age. These mutant mice had an average of 177 ± 30 polyps throughout the length of the intestinal tract. The polyp multiplicity of $Apc^{\Delta242/+}$ mice is significantly higher than the polyp multiplicity of B6 $Apc^{Min/+}$ mice (106 ± 28 , $P < 0.001$). Analysis of tumor distribution indicates that this increased tumorigenesis occurs along the entire length of the small intestine, but not in the colon (Fig. 4a). $Apc^{\Delta242/+}$ mice had significantly smaller polyps in all areas of the small intestine (Fig. 4b). Both polyp multiplicity and polyp size contribute to overall tumor burden, a measurement that negatively correlates with mortality in mouse models of intestinal tumorigenesis (Guillen-Ahlers et al. 2010; Korsisaari et al. 2007). Despite the decrease in polyp size, $Apc^{\Delta242/+}$ mice have a significantly increased tumor burden in the distal small intestine compared to B6 $Apc^{Min/+}$ controls (Fig. 4c).

$Apc^{\Delta242/+}$ tumors are pathologically similar to $Apc^{Min/+}$ tumors

While each polyp in individuals with FAP has only a small chance of progressing to adenocarcinoma, the large number of polyps in the intestinal tract guarantees the development of colorectal cancer in untreated patients. To determine if the increased intestinal tumorigenesis in $Apc^{\Delta242/+}$ mice results in the development of adenocarcinoma, tumors from 120-day-old $Apc^{\Delta242/+}$ mice were compared to tumors from age-matched $Apc^{Min/+}$ mice. Intestinal tissue samples from $Apc^{\Delta242/+}$ and $Apc^{Min/+}$ mice were stained with hematoxylin and eosin (H&E). H&E staining showed no invasion through the basement membrane in tumors from either model (Fig. 5a-d), further indicating that neither $Apc^{\Delta242/+}$ nor $Apc^{Min/+}$ mice progress to adenocarcinoma by 120 days of age.

$Apc^{\Delta242}$ -mediated tumorigenesis is altered by modifier genes on the C3H/HeJ genetic background

The $Apc^{Min/+}$ mouse has been an invaluable model system for identifying modifier genes that affect intestinal tumorigenesis (reviewed in Siracusa et al. 2004). As a closely related mutation with a different phenotype, $Apc^{\Delta242}$ may provide a complementary model system for identifying genes and pathways relevant to colorectal cancer. The C3H/HeJ (C3H) genetic background is known to contain modifier loci that make the strain resistant to Apc^{Min} -mediated polyposis (Korotkar et al. 2004). While B6 $Apc^{Min/+}$ mice develop 106 ± 28 polyps, B6C3 F₁ $Apc^{Min/+}$ offspring develop only 16 ± 6 polyps (Fig. 6). To determine if the $Apc^{\Delta242}$ phenotype is affected by genetic background, B6 $Apc^{\Delta242/+}$ mice were mated to C3H mice. B6C3 F₁ $Apc^{\Delta242/+}$ mice developed significantly fewer polyps (26 ± 13) than B6 $Apc^{\Delta242/+}$ mice in all sections of the small intestine, but not in the colon (Fig. 6). The percent decrease in polyp number due to the introduction of the C3H background was 86% in both $Apc^{\Delta242/+}$ and $Apc^{Min/+}$ mice. These data indicate that the modifiers of Apc^{Min} -mediated tumorigenesis found in the C3H inbred strain are also capable of modifying the phenotype of the $Apc^{\Delta242}$ allele.

Discussion

Numerous mouse models have been generated to study the role of the *Apc* gene in tumor initiation, growth, and progression in the intestinal tract. Many of these models carry *Apc* mutations that result in truncated proteins of varying sizes (Heyer et al. 1999; Taketo and Edelmann 2009). Comparison of polyp multiplicity, distribution, size, and stage between

these *Apc* mutant mice can be used to dissect the functions of the various domains contained in *Apc*. *Apc*^{Min/+} mice, a common model of familial adenomatous polyposis (FAP), carry a point mutation in codon 850 that results in a premature stop codon and a truncated protein product (Fig. 2). The *Apc*^{A474/+} and *Apc*^{A580/+} mouse models harbor mutations further upstream than the *Apc*^{Min} mutation, potentially elucidating the roles of the N-terminus of the *Apc* protein. *Apc*^{A580/+} mice, however, were generated on a mixed genetic background and were never made congenic on the B6 background (Kuraguchi et al. 2006). Different inbred backgrounds are known to harbor modifier genes that can alter the incidence, size, and stage of intestinal tumors (reviewed in Kwong and Dove 2009). Since *Apc*^{Min/+} mice are maintained on the B6 background, other mutant *Apc* alleles must also be present on the B6 background to provide a relevant comparison. *Apc*^{A474/+} mice are maintained on a B6 background and develop an average of 122 intestinal polyps; however, the truncated *Apc* protein expressed from the *Apc*^{A474} allele still carries a portion of the armadillo repeat domain (Fig. 2) (Sasai et al. 2000).

In this article we have described a new mouse model of intestinal tumorigenesis, *Apc*^{A242}, which is maintained on the B6 background. Although the location and stage of tumors did not differ between B6 *Apc*^{A242/+} mice and B6 *Apc*^{Min/+} mice, B6 *Apc*^{A242/+} mice develop 70% more polyps than B6 *Apc*^{Min/+} mice. These polyps are significantly smaller in all areas of the small intestine. The *Apc*^{A242/+} and *Apc*^{Min/+} alleles can be used to identify some of the mechanisms by which different *Apc* mutations in FAP patients can result in different phenotypes.

The majority of *APC* mutations identified in patients fall within a region between codons 1286 and 1513, known as the mutation cluster region (MCR) (Aoki and Taketo 2007; Fearhead et al. 2001; Miyoshi et al. 1992). MCR mutations result in C-terminal truncation proteins that are lacking the domains required for formation of the negative regulatory complex and degradation of β -catenin (Aoki and Taketo 2007). The presence of the MCR suggests a strong pressure selecting not only for loss of the C-terminus of *Apc*, but also retention of the N-terminus. Takacs et al. (2008) demonstrated that *Apc* proteins lacking the C-terminus upregulate signaling through the Wnt pathway in *Drosophila melanogaster*. Mutations in the MCR may, therefore, combine loss of the potent tumor suppressor function of *Apc* with the generation of an oncogenic truncated protein.

The disruption of the role of *APC* as a tumor suppressor in colorectal tumors has been extensively studied. The mechanism by which truncated *APC* may act as an oncogene, however, is still poorly understood. Truncated proteins resulting from MCR mutations retain the armadillo repeat and the oligomerization domains, both of which have potentially oncogenic functions. *Apc*^{Min/+} mice carry a mutation at codon 850 and produce a truncated *Apc* protein that also carries these two domains. The *Apc*^{A242} product, however, contains only the oligomerization domain (Fig. 2). *Apc*^{A242/+} mice develop significantly more polyps than *Apc*^{Min/+} mice and FAP patients with mutations similar to that of *Apc*^{A242} present with a severe form of the disease (Nugent et al. 1994). These data indicate that the *Apc*^{A242} mutation successfully mimics the human disease phenotype and suggest that loss of the armadillo repeats results in increased tumorigenesis. The molecular mechanism behind this increased tumorigenesis in both mice and humans remains unknown.

The oligomerization domain allows *Apc* proteins to interact with one another. Initial studies on the role of *Apc* as an oncogene focused on potential dominant negative effects of truncated *Apc* proteins. Studies have demonstrated that truncated *Apc* mutants can sequester full-length *Apc* proteins and this interaction could potentially block formation of the *Apc*–Axin–GSK3 β negative regulatory complex (reviewed in Aoki and Taketo 2007). In addition to preventing formation of the regulatory complex, sequestration of full-length *Apc* results

in improper formation of the mitotic spindle and errors in chromosomal segregation (Green et al. 2005). These data may explain the presence of *Apc* mutations at the earliest stages of adenoma development. The presence of the mutant *Apc* product results in chromosomal instability and increases the probability of the cell acquiring further genetic lesions (Fodde et al. 2001). The *Apc*^{Δ242} protein should still be capable of acting as a dominant negative due to the presence of the oligomerization domain. The observed increase in tumor multiplicity compared to *Apc*^{Min/+} mice could be the result of increased binding affinity between full-length *Apc* and truncated *Apc* mutants in which the armadillo repeats are absent.

The increased polyposis in *Apc*^{Δ242/+} mice compared to *Apc*^{Min/+}, *Apc*^{Δ474}, and *Apc*^{Δ580} mice more likely results from the loss of the armadillo repeat domain (Fig. 2). These repeats are involved in protein–protein interactions and there is conflicting evidence regarding the role of this domain in tumorigenesis (Barth et al. 2002). The human APC protein associates with the ends of microtubules during active cell migration, suggesting a role for APC in regulating migration (Mogensen et al. 2002). APC binds both the guanine nucleotide exchange factor ASEF and the GTPase effector IQGAP1, which in turn decrease cell adhesion and increase migration (Kawasaki et al. 2000; Watanabe et al. 2004). These protein–protein interactions occur via the armadillo repeat domain; truncated APC generated by MCR mutations is still able to activate ASEF, suggesting an oncogenic potential for the armadillo repeats (Kawasaki et al. 2000, 2003). In vivo data, however, demonstrate that B6 *Apc*^{Min/+} mice have decreased migration rates of intestinal cells along the crypt–villus axis compared to wild-type B6 mice (Javid et al. 2005). Other experiments have shown that the armadillo repeats are required for normal cell migration along the crypt–villus axis and indicate that the domain may have a tumor suppressive role (Kawasaki et al. 2003). Maintenance of the crypt–villus axis is essential for homeostasis in the intestinal tract. Cells of the gut are exposed to carcinogens present in the intestinal lumen and frequent turnover of intestinal epithelial cells serves as a protective mechanism. Dysregulation of cell migration along this axis can cause the accumulation of cells carrying genetic lesions and result in tumor initiation (Mahmoud et al. 1999). If the armadillo repeat domain of *Apc* is required for proper cell migration, loss of this domain in *Apc*^{Δ242/+} mice may result in increased tumorigenesis by disrupting cell turnover in intestinal crypts.

The specific roles of the oligomerization and armadillo repeat domains in intestinal tumorigenesis have not been fully dissected in an in vivo system. Here we demonstrate in a novel mouse model that complete loss of the armadillo repeats results in increased tumorigenesis in all parts of the small intestine. Our data demonstrate that *Apc*^{Δ242/+} mice are a model of severe FAP, developing significantly more tumors than *Apc*^{Min/+} mice while showing no increases in average tumor size or stage. These data suggest that the loss of the armadillo repeats region results in increased tumor initiation, implying an unknown tumor suppressive function of the armadillo repeats. Further comparative analyses between *Apc*^{Min/+} and *Apc*^{Δ242/+} mice can dissect the respective roles of the oligomerization domain and the armadillo repeats. Future work must focus on integrating the in vivo and in vitro data to better understand the function of the armadillo repeats.

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Fig. 1. Generation of *Apc*^{Δ242/+} mice. **a** A gene trap cassette (BayGenomics) was inserted into mouse chromosome 18 between exons 7 and 8 of the *Apc* gene. The black numbered boxes indicate endogenous exons of murine *Apc*. Arrows represent the location of *Apc*^{Δ242} genotyping primers. **b** Genotyping of *Apc*^{Δ242/+} and *Apc*^{+/+} mice. A PCR product of ~300 bp is produced

Fig. 2. *Apc*^{Δ242/+} mice lack the armadillo repeat domain of the Apc protein. Diagram comparing wild-type Apc protein with the truncated proteins generated by the *Apc*^{Δ242}, *Apc*^{Δ474}, *Apc*^{Δ580}, and *Apc*^{Min} mutations. The domains of the Apc protein are labeled on the wild-type protein. The *Apc*^{Δ474} and *Apc*^{Δ580} proteins have novel C-terminal residues as a result of frameshift mutations

Fig. 3. *Apc*^{Δ242/+} mice express an *Apc* fusion product with functional β-galactosidase. Distal small intestine and colon from *Apc*^{+/+} and *Apc*^{Δ242/+} mice after incubation with X-gal solution. Blue tissues indicate the presence of a functional β-galactosidase protein. Western blots probing intestinal tissue lysates from *Apc*^{Δ242/+} mice with β-galactosidase antibody indicated the presence of a fusion protein at the expected size of 172 kDa (data not shown)

Fig. 4.

Comparison of polyp location, size, and tumor burden in *Apc^{Min/+}* and *Apc^{Δ242/+}* mice at 120 days of age. **a** Average polyp numbers detected in the proximal, middle, and distal portions of the small intestine and colon. **b** Average polyp sizes detected in the proximal, middle, and distal portions of the small intestine and colon. Diameters of polyps were measured and reported in mm. **c** Tumor burden in the proximal, middle, and distal portions of the small intestine and colon. Tumor burden was calculated using the formula $(4/3)\pi r^3$. * $P < 0.05$; ** $P < 0.005$; *** $P < 0.001$. $n = 15$ for *Apc^{Min/+}* mice and $n = 18$ for *Apc^{Δ242/+}* mice. Error bars represent standard deviation

Fig. 5. *Apc*^{Δ242/+} and *Apc*^{Min/+} polyps do not progress to adenocarcinoma. Intestinal polyp samples from 120-day-old *Apc*^{Δ242/+} and *Apc*^{Min/+} mice were stained with hematoxylin and eosin. Ten polyps were staged for each genotype. A representative distal small-intestine polyp from an *Apc*^{Δ242/+} mouse is shown at **a** 4× and **b** 20× magnification. A representative polyp from an *Apc*^{Min/+} mouse is also shown at **c** 4× and **d** 20× magnification. *Black boxes* indicate the areas shown in higher magnification

Fig. 6.

The C3H/HeJ background reduces polyp multiplicity in *Apc*^{Min/+} and *Apc*^{Δ242/+} mice. Average polyp numbers detected in the proximal, middle, and distal portions of the small intestine and colon of B6C3F1 *Apc*^{Min/+} mice and B6C3F1 *Apc*^{Δ242/+} mice at 120 days of age. **P* < 0.05. *n* = 35 for B6C3F1 *Apc*^{Min/+} mice and *n* = 4 for B6C3F1 *Apc*^{Δ242/+} mice. Error bars represent standard deviation